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# The Basis of Freezing Tolerance Between and Within Species Across Environmental Gradients with a Focus on Arctic, Alpine and Moorland Plants

Alice Victoria Rowland



Department of Biosciences

Submitted for the Degree of Doctor of Philosophy by Research

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# Abstract

Freezing events have devastating impacts on crops around the world. Climate change is resulting in more extreme freezing events as well as an increase in winter warm periods and shorter winters which can alter the process of acclimation and deacclimation leading to greater freezing susceptibility. Genes involved in freezing tolerance therefore need to be targeted by crop breeders to improve crop resistance to these events. The *CBF* family is one of these potential targets due to their presence across the Spermatophyta, including crop species, and their role in acclimation as transcription factors which activate cold response (*COR*) genes, thereby increasing freezing tolerance. Plants adapted to environments with frequent and very low temperature freezing events, such as arctic and alpine locations may, therefore, already possess modifications to these genes which improve freezing tolerance. The ability of native, dominant cover species to endure and adapt to these climatic changes can also be investigated via the study of variation within *CBF* over a species range.

*CBF* sequences were isolated from numerous arctic and alpine species. Several common polymorphisms in key *CBF* regions were identified and applied to *Arabidopsis thaliana CBF1*. The effect upon freezing tolerance and CRT/DRE activation of these modified *A. thaliana CBF1* sequences were then tested. No definitive conclusions could be drawn, however potential routes of further investigation are highlighted and discussed.

*CBF* sequences of *Empetrum nigrum* samples from a wide distribution and both high and low altitude were compared, no differences between sequences which correlated with sample location, were found. However preliminary expression studies indicated a difference in the kinetics of *CBF* expression between samples from different locations. Further study of *CBF* expression kinetics within this species is highly recommended.

Routes of further exploration leading to potential targets for crops are discussed, alongside suggested routes of further investigation for *Empetrum nigrum* and *Calluna vulgaris*.

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# List of Abbreviations

#### CBF - C-Repeat Binding Factor

- COR Cold Responsive
- CRT/DRE C-Repeat/Dehydration Response Element
- EDTA Ethylenediaminetetraacetic acid
- ICE1 Inducer of CBF Expression 1
- LTI78 Low Temperature Protein 78
- MATK Maturase K
- PCR Polymerase Chain Reaction
- PEX4 Peroxin 4
- pp Primer Pair
- qPCR Quantitative Polymerase Chain Reaction
- TBE Tris/Borate/EDTA

#### Nucleotides

- A Adenine
- T-Thymine
- C-Cytosine
- G-Guanine

### Amino Acids

- A Alanine
- R Arginine
- N Asparagine
- D-Aspartic acid
- C Cysteine
- E-Glutamic acid
- Q Glutamine
- G Glycine
- H-Histidine
- I Isoleucine
- L Leucine
- K Lysine
- M Methionine
- F Phenylalanine
- P Proline
- S Serine
- T Threonine

W-Tryptophan

Y – Tyrosine

V – Valine

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### **1.1 General Background**

### 1.1.1 Freezing

Freezing events can be problematic for plants. When exposed to temperatures below their tolerance level severe damage and death can occur. The level of tolerance of plants varies greatly: some plants cannot tolerate temperatures below 0 °C, whilst others have been shown as being capable of surviving temperatures below -196 °C (Stushnoff and Junttila, 1986). -196 °C is colder than any temperature ever recorded upon Earth; the lowest ever recorded temperature being -92 °C in Antarctica (Phillips, 2013), although plants do not grow at this location due to other limiting factors (and temperatures this low incur other problems aside from freezing; such as the transition of CO<sub>2</sub> from gaseous to solid phase). It is therefore possible for some plants to tolerate the minimum temperature of any earthly environment where other requirements for growth are met. However, other plants are incapable of surviving temperatures below freezing point and a wide spectrum of tolerances between these two extremes exist.

The freezing tolerance of a species limits its distribution. This is demonstrated by hardiness zone maps on which locations are scored given their annual average extreme minimum temperature and then plants are categorised to a zone hardiness based on the minimum temperatures they can tolerate. The most commonly used is The United States Department of Agriculture (USDA) plant hardiness zone system (Unites States Department of Agriculture, 2018). This system is heavily used worldwide to identify where plants can be grown without dying due to low/freezing temperatures. Species cannot expand into (or be cultivated in) environments which may be otherwise favourable if they are incapable of surviving the minimum temperature of that environment. This

also limits available crop species that can be grown in certain environments. Therefore, understanding the mechanisms of freezing tolerance and how species respond to freezing is key to understanding, and modifying, their distribution as well as predicting distribution changes in response to climate change.

### **1.1.2 Acclimation**

The ability to tolerate freezing is not a static state but is induced (or increased) via the process of cold acclimation. Not all plants are capable of cold acclimating and are therefore incapable of surviving freezing. One of the stimuli known to induce acclimation is exposure to low, but non-freezing, temperatures. This results in a change in the transcriptome (Fowler and Thomashow, 2002, Wang et al., 2013, Winfield et al., 2010, Hannah et al., 2005, Svensson et al., 2006, Lee et al., 2005) with the regulation of an estimated >10,000 genes altering in response to cold in Arabidopsis thaliana (Hannah et al., 2005). This leads to a variety of biochemical changes which increases freezing tolerance (Cook et al., 2004, Gilmour et al., 2000). One cold response pathway, that leads to acclimation is the CBF (C-repeat Binding Factor) pathway (simplified in Fig. 1.1.2.1). The basic premise of the response is that upon sensing low (non freezing) temperatures a signalling cascade leads to the increase in the transcription factor ICE1 (Inducer of CBF Expression 1) which upregulates the CBF transcription factors, which, in turn, alter expression of a large number (414 In Arabidopsis thalaiana (Zhao et al., 2016)) of COR genes leading to acclimation. This pathway responds rapidly, with the time between exposure to low (non freezing) temperatures and increased CBF expression taking approximately ~15 minutes in Arabidopsis thaliana and peaking at around 2 hours (Gilmour et al., 1998, Medina et al., 1999). Evidence exists for other pathways which also lead to acclimation (such as the change in gene regulation due to cold estimated to be >10,000 genes (Hannah et al., 2005) as opposed to 414 genes by CBF transcription factors

(Zhao et al., 2016)) but are not as highly studied as the *CBF* pathway (which, itself, is still not fully understood). Reduction or loss of freezing tolerance occurs via deacclimation, one of the stimuli which is associated with this process is prolonged warm temperatures (Kalberer et al., 2006).



Figure 1.1.2.1 Depicting current proposed model for the CBF pathway of cold acclimation, as based upon Arabidopsis thaliana. S denotes SUMO and U denotes ubiquitin. CBF stands for the cold responsive members of the CBF transcription family (CBF1-3 *in Arabidopsis thaliana*).

### **1.2 The Effect of Climate Change**

# **1.2.1** The Effect of Climate Change upon Acclimation and Native Plants

Climate change is affecting when plants acclimate and deacclimate due to altering the timing of stimuli which naturally induce acclimation or deacclimation as well as altering winter temperatures. Shortened winters lead to later acclimation and earlier deacclimation putting plants at greater risk of sudden frosts occurring outside of their acclimated period. More extreme winter freezing temperatures are also predicted to occur (Petoukhov and Semenov, 2010) which could kill plants incapable of tolerating those temperatures. Winter warm periods are also increasing in frequency and length (Shabbar and Bonsal, 2003, Beniston, 2005, IPCC Working Group I, 2013) therefore increasing the likelihood of midwinter deacclimation. These warm periods, coupled with increased rainfall can also result in the loss of insulating snow cover (Räisänen and Eklund, 2012, IPCC Working Group II, 2014) which, upon a decrease in temperatures again, exposes plants to colder than usual temperatures. Loss of snow cover has been shown to lead to the death of more sensitive plants which rely upon snow cover in alpine environments (Wheeler et al., 2014, Rixen et al., 2012) and severe damage to plants in arctic environments (Bokhorst et al., 2009). It is unlikely that this damage is exclusively due to the loss of insulating snow cover but rather deacclimation (or a reduction in the degree of acclimation) for example Empetrum nigrum has been recorded as capable of tolerating -80 °C upon acclimation (with a 70 % survival rate) (Yamori et al., 2005) yet severe damage of this species after the loss of snow-cover has been observed in subarctic (Bokhorst et al., 2009) and alpine (Rixen et al., 2012) environments. It is unlikely that temperatures as low as or lower than -80 °C were experienced after the snow melt, however *Empetrum nigrum* is only capable

of tolerating temperatures around -15 °C (~80 % survival rate) prior to acclimation (Yamori et al., 2005). It therefore seems likely that this warm period also resulted in full or partial deacclimation therefore leaving *Empetrum nigrum* susceptible to low temperature damage. It should be noted, however, that the freezing tolerance study was performed upon samples from the Japanese Mt Iwo whereas the survival studies were performed in the Swiss Alps and Northern Scandinavia. They are therefore not directly comparable, as it is possible there is also natural variation in the populations effecting freezing tolerance.

Empetrum nigrum is a dominant understory species in the subarctic and is also common throughout moorlands. Calluna vulgaris is a dominant moorland species in the UK. Both of these environments are key carbon stores. Loss or severe damage to these species due to winter warming would have a large impact upon these environments. For example; loss of these cover species would result in areas of exposed peat or tundra. Exposed areas, now lacking the stabilising plant cover, are subject to erosion of surface soils. Reestablishment of plants on these easily eroded surfaces can prove difficult as exemplified by the necessity of peatland restoration programs in areas where peatland plant cover has been lost due to other environmental and anthropological factors. This is especially difficult where further weathering has resulted in steep slopes of peak tussocks which must be flattened prior to reintroduction to enable establishment of plants (Moors For The Future Partnership, 2019). This erosion combined with oxidation and cessation of carbon uptake due to loss of species results in carbon storage loss, increasing the problems of climate change (Evans et al., 2006). Heavily damaged areas of bare environment lose the "cultural service value" i.e. the effects of landscape beauty such as health and wellbeing, tourism, recreation etc (North Pennines AONB Partnership, 2019). Loss of these species can also impact upon the commercial and cultural value, for example Empetrum nigrum is a minor crop in its own right and is also grazed by reindeer herds (Inga and Öje, 2012) which are vital for the livelihoods of indigenous reindeer herders such as the Sami (Heikkilä, 2002). Change in the dominant species cover also disrupts the ecosystem, altering plant diversity and therefore the animal and fungal life which relies on these species. Even if the loss of cover of these species occurred gradually allowing for changes in vegetation rather than sudden loss (which would depend upon the natural variation in tolerance within the population – which this study, in part, intends to investigate) Changes in vegetation distribution may also alter how much of the incident solar ration is reflected back or absorbed via altering the albedo (i.e. altering the reflectivity of the earth's surface), changing carbon storage and uptake, soil-vegetation interactions and impacting upon other ecological processes (ACIA, 2005). Therefore, understanding the molecular mechanisms and timings of freezing tolerance acquisition and maintenance in these species is of high importance to both predict how this species will be affected by climate change and also determine if variation exists within the species which is capable of tolerating these changes. If so this information can be utilised to influence reintroduction and maintenance programs. Cost of restoring greater than that of maintaining (North Pennines AONB Partnership, 2019) therefore prioritising maintenance via knowledge of susceptibility of different systems is also economically advisable.

### 1.2.2 The Effect of Climate Change upon Crops

Changes in climate, especially early and late frosts (relative to the acclimated period) are also a significant problem for crops, resulting in large scale crop loss worldwide e.g. (Brammer, 2018, News Staff, 2012, Ewing, 2013, The Local, 2017, Park, 2018) there is therefore great impetus to breed crops capable of surviving these events. Increased hardiness is also desirable to increase viable locations for crops to be grown and prepare for predicted increases in winter extreme freezing events (Petoukhov and Semenov, 2010). Genes involved in cold acclimation and freezing resistance therefore need to be targeted by crop breeders to improve crop resistance to these climatic events. Plants already adapted to environments which experience extreme freezing events plus year-round frosts, such as those in arctic and alpine locations, are likely to already possess genetic differences which account for improved freezing tolerance and hence it is logical to investigate the potential of these genes as targets for crop breeding.

# **1.3 Potential Gene Sources For use in Crops**

Arctic and alpine plants are generally, by necessity, very freezing tolerant. For example, on a yearly basis, weather stations within a 120 mile radius of one of our Swedish collection sites, Abisko (68.3500°N, 18.8167 E°) record winter extreme lows of -35 °C to -50 °C (Norwegian Meteorological Institute and Norwegian Broadcasting Corporation, 2007-2014). Plants exposed to these temperatures, especially those above the snow-lie, therefore need to be capable of surviving these, and potentially lower, temperatures. In their 1986 paper Stushnoff & Junttilla identified the LST66 (lowest survival temperature at which 66 % of samples showed no damage) of 17 species from nearby northern Norway, four of which included crop genera - namely: Rubus, Ribes and Prunus. The LST66 of these species ranged from -15 °C (where the plants probably rely on sheltered spots or deep snow lie to insulate them from the coldest temperatures) to -50 °C for the crop genera and from -15 °C to below -196 °C for all other species (Stushnoff and Junttila, 1986). Yamori et al., (2005) studied a further nine species from alpine locations in Japan, which after acclimation, showed over a 60 % survival rate at -80 °C. Prior to acclimation these species were "only" freezing tolerant (with an LT60) to between -10 °C and -20 °C (Yamori et al., 2005). These plants are therefore able to tolerate freezing events (to between -10 and -20 °C) pre-acclimation. The majority of crop plants however are killed at much warmer subzero temperatures outside of the acclimated winter period therefore an understanding of how arctic and alpine plants tolerate freezing could prove highly advantageous to the breeding of crops.

Likely targets of altered molecular function to improve freezing tolerance are genes involved in acclimation; influencing the degree of freezing tolerance, when acclimation occurs and at what rate. A large number of genes are upregulated in response to cold (Fowler and Thomashow, 2002, Winfield et al., 2010, Wang et al., 2013, Hannah et al., 2005, Svensson et al., 2006, Lee et al., 2005), therefore large scale modification of numerous genes would be required to alter freezing tolerance at the end of the pathway. Genes activated early in the process of acclimation which encode transcription factors which influence a large number of other genes are, therefore, more likely targets of selection. A likely candidate gene family encoding transcription factors which influence freezing tolerance is the *CBF* (C-repeat Binding Factor) family.

# **1.4 C-Repeat Binding Factor (CBF)**

# **1.4.1 The Importance of CBF in Cold Acclimation and Freezing** Tolerance

Some members of the *CBF* family are rapidly upregulated in response to cold (Gilmour et al., 2004, Gilmour et al., 1998) and/or a shortened photoperiod (Lee and Thomashow, 2012), as part of the acclimation pathway (Fig. 1.1.2.1). CBFs bind to the C-Repeat/Dehydration Response Element (CRT/DRE) found in promoters of cold regulated (*COR*) genes (Canella et al., 2010, Stockinger et al., 1997, Liu et al., 1998) and alter the activation of these *COR* genes ultimately resulting in increased freezing tolerance (Thomashow et al., 2001, Gilmour et al., 2000, Gilmour et al., 1998, Gilmour et al., 2004, Jaglo-Ottosen et al., 1998). *CBF* genes are found even in plants that cannot tolerate freezing (Zhang et al., 2004, Ito et al., 2006). Introduction of *CBF* genes from a species

which can cold acclimate to tolerate freezing into one that is freezing sensitive has been shown to confer freezing tolerance to the freezing sensitive species such as A. thaliana CBFs in Solanum tuberosum (Pino et al., 2007) and Triticum spp. CBFs in Nicotiana benthamiana (Takumi et al., 2008). However introduction of non-native CBF genes does not always confer freezing tolerance to all freezing sensitive species such as seen in A. thaliana CBF genes in Solanum lycopersicum (Zhang et al., 2004). Constitutive overexpression of native CBF genes in freezing tolerant species confers constitutive freezing tolerance (Jaglo-Ottosen et al., 1998, Gilmour et al., 2000). However, this often results in reduced yields and a dwarf phenotype (Achard et al., 2008, Gilmour et al., 2000, Liu et al., 1998, Suo et al., 2012). As with previous overexpression studies constitutive overexpression of A. thaliana CBF genes in Solanum tuberosum also resulted in smaller leaves, stunted plants, delayed flowering, and reduced, or lack of, tuber production (Pino et al., 2007). However, addition of these CBF under a stress-inducible promoter (as opposed to a constitutive promoter), provided the ability for that species to acclimate and tolerate mild freezing with no change in yield (Pino et al., 2007). Interestingly overexpression of Triticum spp. CBF into tobacco did not result in reduced growth but did increase freezing tolerance (Takumi et al., 2008). It is therefore possible that mutations may exist in CBF genes that do not result in the associated stunted growth and reduced yield. This stunting may also be overcome by use of a cold-inducible CBF promoter, as seen in Solanum tuberoum (Pino et al., 2007). Since CBF genes are capable of altering freezing tolerance, either within a species via upregulation or via insertion of CBF genes from more freezing tolerant species into less freezing tolerant species and are present in numerous species, across the Spermatophyta, including crop and arctic species it seems likely mutations may exist naturally that confer greater freezing tolerance. This family of transcription factors are therefore a likely candidate for investigation for understanding differences in freezing tolerance between and within species and as a target for crop breeding.

### **1.4.2 Key Domains within the CBF Protein**

Two main protein sequence domains have been identified as important in the activity of CBF: The *COR* binding domain, consisting of the AP2/ERF domain (Sakuma et al., 2002) and the flanking PKKP/RAGRxKFxETRHP (PKKPAGR) and DSAWR sequences (Canella et al., 2010), and the COOH terminus (Wang et al., 2005) (Fig. 1.4.2.1).

Within the RKKFR motif (a region predicted to form an  $\alpha$ -helix) of the PKKPAGR sequence several key amino acids related to binding have been identified, namely; R(7)and F(10) (Canella et al., 2010). Substitution of these amino acids affects the ability of CBF to bind to the CRT/DRE element. Conserved and non-conserved substitution of R(7)significantly impaired binding. Non-conserved substitution of F(10) with A or P prevented binding however a conserved substitution of F(10) to Y greatly enhanced binding (Canella et al., 2010). Other substitutions in this region namely K(8) to R (conserved) and A (non conserved), K(9) to R (conserved) and A (non conserved) and R(11) to S (non conserved); all had no significant effect on binding (Canella et al., 2010). Another amino acid that was found to be key to binding is V(14) in the AP2/ERF domain; a conserved substitution of which with A prevented binding (Sakuma et al., 2002). It is therefore possible that substitutions in these regions, altering the binding ability of CBF to the CRT/DRE element may therefore result in a different degree of expression of the target genes. A series of hydrophobic loops in the COOH domain have been shown to affect the ability of CBF to activate target genes (Wang et al., 2005). The authors found this trend of groups of hydrophobic clusters to be conserved across several plant taxa.

However there was a high degree of redundancy in the system whereby multiple loops could be disrupted before there was any effect upon activity (Wang et al., 2005).



Figure 2.4.2.1 Schematic of CBF protein sequence based upon *Arabidopsis thaliana* CBF1-4, showing relative positions domains discussed: The COOH terminus (yellow) and the COR binding domain (green) consisting of the the AP2/ERF domain (dark green) and CBF signature sequences PKKP/RAGRxKFxETRHP and DSAWR (pale green). \*NOT TO SCALE\*

### **1.5 Scope of the Thesis**

The aforementioned information about the structure of CBF proteins and their influence on binding and activation activity can therefore be used to predict the activity of proteins from sequenced *CBF* genes from arctic and alpine plants. Protein sequences of the same species from different locations which experience a different degree of freezing stress can also be compared, which may indicate key polymorphisms which may correlate with greater freezing tolerance. This information can then be used to assess the potential of these polymorphisms as a target for crop breeding. Data from the same species from different locations can also be used to assess the genetic plasticity of these species and their robustness against upcoming climatic change.

### **1.5.1 Thesis Aims & Rationales**

There are two main overarching rationales for the studies undertaken in this project:

- 1. To protect the world's crop food sources against freezing damage and loss *via* an attempt to identify, if possible, a way to alter freezing tolerance or freezing response without impacting upon yield.
- To identify methods to predict key plant species responses to climate change and the genetic variation in responses to climate change in order to influence restoration and conservation projects.

Therefore, two approaches were used:

- A. Plants from environments which experience extreme winter temperatures and regular risk of frosts may contain mutations in their *CBF* genes which confer greater freezing tolerance. By comparing *CBF* sequences from these species it may be possible to identify polymorphisms which confer greater freezing tolerance which could then ultimately be screened for in crop species to identify potentially more freezing resistant strains, without the need for genetic modification. (Aims 1-3)
- B. A species that occupies a wide environmental range, therefore experiencing differing maxima and timing of freezing events, may therefore, exhibit differing freezing responses across this environmental range. If a difference is present, knowledge of this and the genetic basis, would prove useful to restoration and conservation projects. Introduction of plants from areas with certain genetic responses to freezing which would prove the most favourable for successful establishment and growth in the environment (or predicted future environment) undergoing restoration would be advantageous. Knowledge of the genetic

variability would also be useful in conservation projects to prioritise conservation efforts, to avoid loss of this genetic variability. Any genetic differences identified could also potentially prove useful in informing further studies for crop breeding as in point A. (Aims 4-8)

To this end the specific aims and rationale for experiments undertaken in this thesis were:

- 1. To isolate novel *CBF* sequences from a wide variety of arctic, alpine and moorland plants (Chapter 3);
  - In order to provide a large collection of *CBF* sequences from a wide range of species that naturally experience extreme freezing and/or early late frosts in order to provide a basis from which to address aim 2.
- 2. To identify polymorphisms common across multiple arctic and alpine species which may be of interest for altering freezing tolerance (Chapter 3);
  - In order to identify the most likely polymorphisms that alter CBF binding or activity and therefore freezing tolerance which could then be tested empirically for inducing changes to freezing tolerance (aim 3).
- To test the effect of these polymorphisms upon freezing tolerance and the ability to activate *COR* genes when introduced into *Arabidopsis thaliana* CBF1 (Chapter 4);
  - In order to empirically test the polymorphisms identified in aim 2 for changes in binding and activation, downstream gene expression and overall freezing tolerance. Any advantageous polymorphisms identified could then, (outside the scope of this thesis) be tested in crops with the aim of informing crop breeding.
- To identify and assess the potential for specific arctic, alpine or moorland species to be experimental models (Chapter 5);

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- In order to identify suitable species and methods of growth and propagation to address aims 5-8.
- 5. To compare and identify differences between *CBF* sequences from *Empetrum nigrum* samples taken from across a wide environmental range (Chapter 5);
  - In order to identify if polymorphisms exist in the sequence of *Empetrum nigrum CBF* across the environmental range which could be responsible for different freezing tolerances or responses. If present, these polymorphisms could be tested as per aim 3.
- 6. To establish methods to assess *CBF* expression kinetics and freezing tolerance in *Empetrum nigrum* (Chapter 5);
  - In order to identify if these methods can be successfully adapted to this species in order to address aim 7.
- To identify if there is any difference in *CBF* expression kinetics between *Empetum* nigrum plants grown in common garden conditions from cuttings taken from differing environmental locations (Chapter 5);
  - In order to see if the same species from different environments has different *CBF* expression kinetics, indicating local selection of different freezing responses. If so, this could influence the survival chances of plants from certain populations when introduced elsewhere during restoration programs and could be used to predict probable responses of those populations to environmental changes. If a difference is present this could also form the basis of further exploration into the possible cause and effect and provide potential gene targets for crop breeding.
- 8. To assess if there is any correlation between observed morphology of *Empetrum nigrum* and *Calluna vulgaris* plants grown in common garden conditions from

cuttings taken from differing environmental locations and the environment of origin (Chapter 5).

• To identify if there is a genetic or epigenetic influence of origin upon morphology which may indicate population differences. This would be useful knowledge for predicting responses to environmental change, and to influence reintroduction & conservation programs.

# 2.1 Sample Collection

### 2.1.1 Collection

Seventy-seven plant samples were collected from various sites across Abisko, Northern Sweden (Table 2.1.5) between 17/07/14 & 19/07/14. One *Fragaria vesca* sample was collected on 21/07/14 in the Northern Pennines, UK (Table 2.1.5) by Professor Marc Knight and Dr. Heather Knight. A further fifty samples were collected from the sites across the Northern Pennines, UK on 23/07/14 & 24/7/14 (Table 2.1.5).

Forty samples were collected by Dr. Adrian Brennan on 18/06/2015 from various sites across Abisko, Northern Sweden. In August 2016 nine samples from Abisko, Northern Sweden were collected by Dr. Johan Olofsson and ten samples from near Trondheim, Norway were collected by Dr. Annika Hofgaard (Table 2.1.5).

*Calluna vulgaris var. Con Brio* and *Calluna vulgaris var. kinlochruel* plants rooted in 21 pots were purchased from Poplar Tree Garden Centre, Durham in April 2016.

Cuttings from six plants were collected from various sites across Nags Head, North Pennines, UK on 13/11/15. A further six were collected from sites across the North Pennines (Table 2.1.5) on 16/11/2015. Numerous samples were taken for both DNA and propagation across the UK: Fifty samples were taken from various sites across the Cairngorms, Highland Scotland between 12-14/07/2016, twenty-one samples were taken from sites across Exmoor, Southern England between 27-28/07/2016, twenty samples from Galloway, Southeast Scotland between 23-24/08/2016, thirty-one samples from the Yorkshire Moors, Northeast England between 30-31/08/2016, twenty-five samples from

south Wales between 13-14/09/2016 and twenty Seven from Derbyshire, Midlands, England on 19-20/09/2016 (Table 2.1.5).

### 2.1.2 Transport and Storage

Samples collected from Abisko, Northern Sweden in 2014 were kept cool and damp (for a maximum of 3 Days) until return to Durham University, UK where they were then stored at -80 °C. After collection, samples from the Northern Pennines, UK were immediately transported to Durham University, UK and stored at -80 °C.

Samples from sites across the UK were stored in cool bags with damp tissue. For a maximum of 3 days before transport back to Durham University, UK. Tissue for cuttings were treated as per section 2.2.2 and (where samples were sufficiently large) some tissue was stored at -80 °C whilst the remainder was dried on Sigma-aldrich Honeywell Silica Gel Orange in a drying oven, before storing in vacuum desiccators or boxes containing Sigma-aldrich Honeywell Silica Gel Orange.

Samples collected by Dr. Annika Hofgaard and Dr. Johan Olofsson were dried prior to postage to Durham University, UK and upon arrival they were stored in vacuum desiccators. Samples collected by Dr. Adrian Brennan, were bagged before travelling back to Durham University, UK where they were then dried on Sigma-aldrich Honeywell Silica Gel Orange.

#### 2.1.3 Identification

Photos for species identification after 10/2/14 were taken at the time of collection using a combination of Nikon D80 DSLR, Olympus OMD EM10II and a Pentax WG1 compact camera.

Species identification was performed using a combination of identification guides: British Bryological Society (2010), Cope and Gray (2009), Poland and Clement (2009), Stenberg and Mossberg (2006), Rose (2006), Blamey et al. (2003), Fitter et al. (1992), Rose (1989), Jermy et al. (1982).

# 2.1.4 Soil pH Calculation

Soil samples were taken at sites where *Empetrum nigrum* and *Calluna vulgaris* samples for cuttings were collected, where possible, using a soil auger. This soil was stored at 4  $^{\circ}$ C for a maximum of 4 months until measured. 50 % soil was mixed with 50 % H<sub>2</sub>O swirled and left to settle for 15 minutes. The pH measurement was then taken using a Milwaukee Mi 180 Bench Meter.
Table 2.1.5a. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
		Deschampsia cespitosa??				N 68°	E 018°					
1	Alice Rowland	Avena??	1	Sweden; Abisko	576	19.131′	50.560'	68.31885	18.842667		N/A	
						N 68°	E 018°					
2	Alice Rowland	Diapensia lapponica	2 (north)	Sweden; Abisko	590	19.044'	50.751'				N/A	
						N 68°	E 018°					
3	Alice Rowland	Arctostaphylos alpina	2 (north)	Sweden; Abisko	590	19.044'	50.751'				N/A	
						N 68°	E 018°					
4	Alice Rowland	Betula nana	2 (north)	Sweden; Abisko	590	19.044'	50.751'				N/A	
						N 68°	E 018°					
5	Alice Rowland	Empetrum nigrum	2 (north)	Sweden; Abisko	590	19.044′	50.751'				N/A	
						N 68°	E 018°					
6	Alice Rowland	Loiseleuria procumbens	2 (north)	Sweden; Abisko	590	19.044'	50.751'				N/A	
						N 68°	E 018°					
7	Alice Rowland	Polytrichum piliferum?	2 (north)	Sweden; Abisko	590	19.044'	50.751'				N/A	
						N 68°	E 018°					
8	Alice Rowland	Dryas octopetala	2 (west)	Sweden; Abisko	589	19.038'	50.753'				N/A	
						N 68°	E 018°					
9	Alice Rowland	Silene acaulis	2 (west)	Sweden; Abisko	589	19.038'	50.753'				N/A	
						N 68°	E 018°					
10	Alice Rowland	Vaccinium vitis-idaea	2 (S/E)	Sweden; Abisko	590	19.037′	50.776′				N/A	
						N 68°	E 018°					
11	Alice Rowland	Deschampsia sp?	2 (S/E)	Sweden; Abisko	590	19.037'	50.776'				N/A	
		_				N 68°	E 018°					
12	Alice Rowland	Poaceae sp.	2 (S/E)	Sweden; Abisko	590	19.037′	50.776′				N/A	
						N 68°	E 018°					
13	Alice Rowland	Juniperus communis	2 (S/E)	Sweden; Abisko	590	19.037′	50.776′				N/A	
			2 (2 (5)			N 68°	E 018°					
14	Alice Rowland	Linnaea borealis	2 (S/E)	Sweden; Abisko	590	19.037	50.776′				N/A	
			2 (2 (5)			N 68°	E 018°					
15	Alice Rowland	Polytrichum sp. Commune?	2 (S/E)	Sweden; Abisko	590	19.037′	50.776′				N/A	
			2 (2 (5)			N 68°	E 018°					
16	Alice Rowland	Pleurozium schreberi	2 (S/E)	Sweden; Abisko	590	19.037	50.776				N/A	
			2 (2 (5)			N 68°	E 018°					
17	Alice Rowland	Peltigera aphthosa	2 (S/E)	Sweden; Abisko	590	19.037	50.776				N/A	
			_			N 68°	E 018°					
18	Alice Rowland	Andromeda polifolia	3	Sweden; Abisko	576	19.057′	50.754′				N/A	

Table 2.1.5b. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
						N 68°	E 018°					
19	Alice Rowland	Lycopodium annotinum	4	Sweden; Abisko	475	20.458'	49.660'				N/A	
		Parmelia olivacea (colour				N 68°	E 018°					
20	Alice Rowland	1)	4	Sweden; Abisko	475	20.458'	49.660'				N/A	
		Parmelia olivacea (colour				N 68°	E 018°					
21	Alice Rowland	2)	4	Sweden; Abisko	475	20.458'	49.660'				N/A	
		Cladonia uncialis (prob				N 68°	E 018°					
22	Alice Rowland	subsp biuncialis)	4	Sweden; Abisko	475	20.458'	49.660'				N/A	
		Diphasiastrum				N 68°	E 018°					
23	Alice Rowland	complanatum	4	Sweden; Abisko	475	20.458'	49.660'				N/A	
						N 68°	E 018°					
24	Alice Rowland	Cetrelia olivetorum?	4	Sweden; Abisko	475	20.458'	49.660'				N/A	
						N 68°	E 018°					
25	Alice Rowland	Equisetum palustre	4	Sweden; Abisko	475	20.458'	49.660'				N/A	
						N 68°	E 018°					
26	Alice Rowland	Cornus suecica	5	Sweden; Abisko	430	25.924'	31.018'				N/A	
						N 68°	E 018°					Exposed ground near
27	Alice Rowland	Juncus trifidus	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
28	Alice Rowland	Carex cabescens?	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
29	Alice Rowland	Luzula pilosa?	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
30	Alice Rowland	Icmadophila ericetorum?	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
31	Alice Rowland	Cladonia coccifera	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
		Stereocaulon sp.										
		(pileatum, dactylophyllum,				N 68°	E 018°					Exposed ground near
32	Alice Rowland	nanodes or vesuvianum)	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
33	Alice Rowland	Eriphorum scheuchzeri	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
34	Alice Rowland	Carex nigra var juncea	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway

Table 2.1.5c. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
		Physcia caesia (several				N 68°	E 018°					Exposed ground near
35	Alice Rowland	other possibilities)	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
36	Alice Rowland	Huperzia selago	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
37	Alice Rowland	Kiaeria falcata?	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
38	Alice Rowland	Gymnomitrion coninnatum	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
39	Alice Rowland	Tetralophozia setiformis	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
40	Alice Rowland	Vaccinium vitis-idaea	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
41	Alice Rowland	Sphagnum compactum	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
42	Alice Rowland	Dicranum scoparium??	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
43	Alice Rowland	Andromeda polifolia	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
44	Alice Rowland	Xanthoria candelaria	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
		several possibilities inc.										
		Carbonea vitellinaria,										
		Caloplaca xantholyta,				N 68°	E 018°					Exposed ground near
45	Alice Rowland	Candelariella vitellina	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
		Ophioparma ventosa				N 68°	E 018°					Exposed ground near
46	Alice Rowland	(poss. Sub. Festiva)	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
47	Alice Rowland	Calluna domestic escapee?	6	Sweden; Abisko	507	26.091'	06.740′	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
48	Alice Rowland	Ochrolechia frigida	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
		Solidago virgaurea prob.				N 68°	E 018°					Exposed ground near
49	Alice Rowland	ssp alpestris	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway

Table 2.1.5d. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
						N 68°	E 018°					Exposed ground near
50	Alice Rowland	Athyrium sp.	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
51	Alice Rowland	Salix herbacea	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
52	Alice Rowland	Parnassia palustris	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
		Cladonia sulphurina?										
		(check which is 53 and				N 68°	E 018°					Exposed ground near
53	Alice Rowland	which is 54)	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
		Cladonia coccifera,				N 68°	E 018°					Exposed ground near
54	Alice Rowland	polydacta?	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
55	Alice Rowland	-	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					
56	Alice Rowland	Saussurea alpina	7	Sweden; Abisko	369	21.356'	46.214'				N/A	Near River
		Antennaria alpina Or				N 68°	E 018°					
57	Alice Rowland	dioica	8	Sweden; Abisko	397	21.340'	46.044'				N/A	
						N 68° 21'						
58	Alice Rowland	Trifolium repens	9	Sweden; Abisko	370	16''	E 018° 14' 57''				N/A	Research Station
						N 68°	E 018°					
59	Alice Rowland	equisetum fluviatile	10	Sweden; Abisko	482	20.357'	49.626'				N/A	Boggy board-walk
						N 68°	E 018°					
60	Alice Rowland	Pseudobryum cinclidioides	10	Sweden; Abisko	482	20.357'	49.626'				N/A	Boggy board-walk
						N 68°	E 018°					
61	Alice Rowland	Sphagnum subnitens	10	Sweden; Abisko	482	20.357'	49.626'				N/A	Boggy board-walk
						N 68°	E 018°					
62	Alice Rowland	ID not poss.	10	Sweden; Abisko	482	20.357'	49.626'				N/A	Boggy board-walk
		matricaria recutita? (not										Disturbed ground
		that far north, but				N 68°	E 018°					near helipad and
63	Alice Rowland	invaded?)	11	Sweden; Abisko	425	20.737′	49.870'				N/A	carpark
												Disturbed ground
						N 68°	E 018°					near helipad and
64	Alice Rowland	Barbaea vulgaris?	11	Sweden; Abisko	425	20.737′	49.870'				N/A	carpark

Table 2.1.5e. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
												Disturbed ground
						N 68°	E 018°					near helipad and
65	Alice Rowland	Hesperis matronalis?	11	Sweden; Abisko	425	20.737'	49.870'				N/A	carpark
												Disturbed ground
						N 68°	E 018°					near helipad and
66	Alice Rowland	Cardaminopsis arenosa	11	Sweden; Abisko	425	20.737'	49.870'				N/A	carpark
		Anthriscus sylvestris??										Disturbed ground
		Really not sure carrot				N 68°	E 018°					near helipad and
67	Alice Rowland	family like? (NOT A FERN!)	11	Sweden; Abisko	425	20.737′	49.870'				N/A	carpark
						N 68°	E 018°					Near Tourist-station
68	Alice Rowland	Capsella bursa-pastoris?	12	Sweden; Abisko	405	20.843'	49.868'				N/A	and road
						N 68°	E 018°					Near Tourist-station
69	Alice Rowland	Arabis alpina???	12	Sweden; Abisko	405	20.843'	49.868'				N/A	and road
		·				N 68°	E 018°					Near Tourist-station
70	Alice Rowland	Rumex acetosa?	12	Sweden; Abisko	405	20.843'	49.868'				N/A	and road
				· · ·		N 68°	E 018°					Near Tourist-station
71	Alice Rowland	Arabis hirsuta?	12	Sweden; Abisko	405	20.843'	49.868'				N/A	and road
72	Alice Rowland	Pinus sylvestris	13	Sweden; Abisko	N/A	N/A	N/A	N/A	N/A		N/A	
73	Alice Rowland	Pinus sylvestris	13	Sweden; Abisko	N/A	N/A	N/A	N/A	N/A		N/A	
	Prof. Marc											
	Knight & Dr.											
	Heather			England; North Pennines;		N 54°	W 002°					
74	Knight	Fragaria vesca (Heather)	14	Weardale	70	44.582'	08.839′				N/A	
				England; North Pennines;		N 54°	W 002°					
75	Alice Rowland	Picea abies	15	Weardale	293	44.285'	08.797′				N/A	
		Juncus (inflexus? More										
		resembles maritimus but		England; North Pennines;		N 54°	W 002°					
76	Alice Rowland	not brackish)	16	Weardale	308	44.508'	08.828′				N/A	
				England; North Pennines;		N 54°	W 002°					
77	Alice Rowland	Trifolium repens	16	Weardale	308	44.508'	08.828'				N/A	
				England; North Pennines;		N 54°	W 002°					
78	Alice Rowland	Fragaria vesca	16	Weardale	308	44.508'	08.828′				N/A	

Table 2.1.5f. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
				England; North Pennines;		N 54°	W 002°					
79	Alice Rowland	Ranunculus flammula	16	Weardale	308	44.508'	08.828'				N/A	
				England; North Pennines;		N 54°	W 002°					
80	Alice Rowland	Juncus castaneus	16	Weardale	308	44.508'	08.828′				N/A	
				England; North Pennines;		N 54°	W 002°					
81	Alice Rowland	Ranunculus acaris	16	Weardale	308	44.508'	08.828′				N/A	
				England; North Pennines;		N 54°	W 002°					
82	Alice Rowland	Rumex sanguineus	16	Weardale	308	44.508'	08.828'				N/A	
		Thuidium tamariscinum,										
		Delicatulum, assimile (only										
		distinguishable via		England; North Pennines;		N 54°	W 002°					
83	Alice Rowland	microscopic features)	17	Weardale	321	44.503'	08.833′				N/A	
				England; North Pennines;		N 54°	W 002°					
84	Alice Rowland	Dryopteris sp. (cristata?)	17	Weardale	321	44.503'	08.833′				N/A	
				England; North Pennines;		N 54°	W 002°					
85	Alice Rowland	Carex sylvatica	17	Weardale	321	44.503'	08.833′				N/A	
				England; North Pennines;		N 54°	W 002°					
86	Alice Rowland	Luzula sylvatica	17	Weardale	321	44.503'	08.833′				N/A	
				England; North Pennines;		N 54°	W 002°					
87	Alice Rowland	Rhizomnium punctatum	18	Weardale	290	44.747'	08.834'				N/A	
				England; North Pennines;		N 54°	W 002°					
88	Alice Rowland	plagiomnium undulatum	18	Weardale	290	44.747'	08.834′				N/A	
		Anthriscus sylvestris??										
		Really not sure carrot		England; North Pennines;		N 54°	W 002°					
89	Alice Rowland	family like? (NOT A FERN!)	18	Weardale	290	44.747'	08.834'				N/A	
		Leucanthemum vulgare		England; North Pennines;		N 54°	W 002°					
90	Alice Rowland	(note hairs so poss. not)	19	Weardale	295	44.366'	08.762'				N/A	
				England; North Pennines;		N 54°	W 002°					
91	Alice Rowland	Xanthoria parietina?	20	Weardale	290	44.281'	08.874′				N/A	
				England; North Pennines;		N 54°	W 002°					
92	Alice Rowland	Caloplaca sp??	20	Weardale	290	44.281'	08.874′				N/A	
				England; North Pennines;		N 54°	W 002°					
93	Alice Rowland	Equisetum arvense	21	Weardale	293	44.250'	09.845'				N/A	

Table 2.1.5g. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
				England; North Pennines;		N 54°	W 002°					
94	Alice Rowland	Trifolium repens	22	Weardale	497	40.363'	17.902'				N/A	
				England; North Pennines;		N 54°	W 002°					
95	Alice Rowland	Juncus conglomeratus	22	Weardale	497	40.363'	17.902'				N/A	
		Yellow sedge, Carex flava,		England; North Pennines;		N 54°	W 002°					
96	Alice Rowland	demissa or serotina	22	Weardale	497	40.363'	17.902'				N/A	
				England; North Pennines;		N 54°	W 002°					
97	Alice Rowland	Pleurozium schreberi	22	Weardale	497	40.363'	17.902'				N/A	
				England; North Pennines;		N 54°	W 002°					
98	Alice Rowland	Juncus squarrosus	22	Weardale	497	40.363'	17.902'				N/A	
				England; North Pennines;		N 54°	W 002°					
99	Alice Rowland	Ranunculus repens	22	Weardale	497	40.363'	17.902'				N/A	
				England; North Pennines;		N 54°	W 002°					
100	Alice Rowland	Deschampsia cespitosa	22	Weardale	497	40.363'	17.902'				N/A	
				England; North Pennines;		N 54°	W 002°					
101	Alice Rowland	Equisetum palustre	22	Weardale	497	40.363'	17.902'				N/A	
				England; North Pennines;		N 54°	W 002°					
102	Alice Rowland	Climacium dendroides	22	Weardale	497	40.363'	17.902'				N/A	
				England; North Pennines;		N 54°	W 002°					
103	Alice Rowland	Equisetum palustre?	22	Weardale	497	40.363'	17.902'				N/A	
				England; North Pennines;		N 54°	W 002°					
104	Alice Rowland	Didymodon insulanus?	22	Weardale	497	40.363'	17.902'				N/A	
				England; North Pennines;		N 54°	W 002°					
105	Alice Rowland	Thymus polytrichus	22	Weardale	497	40.363'	17.902'				N/A	
		Cladonia uncialis (prob		England; North Pennines;		N 54°	W 002°					
106	Alice Rowland	subsp biuncialis)	22	Weardale	497	40.363'	17.902'				N/A	
				England; North Pennines;		N 54°	W 002°					
107	Alice Rowland	Minuartia verna	22	Weardale	497	40.363'	17.902'				N/A	
		Cladonia sp. (probably a		England; North Pennines;		N 54°	W 002°					
108	Alice Rowland	more unusual one)	22	Weardale	497	40.363'	17.902'				N/A	
		Trentepohlia sp. (Green		England; North Pennines;		N 54°	W 002°					
109	Alice Rowland	alga!)	22	Weardale	497	40.363'	17.902'				N/A	
				England; North Pennines;		N 54°	W 002°					
110	Alice Rowland	Rumex acetosa	23	Weardale	522	40.450'	17.830′				N/A	

Table 2.1.5h. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
		Rhytidiadelphus		England; North Pennines;		N 54°	W 002°					
111	Alice Rowland	squarrosus	23	Weardale	522	40.450'	17.830'				N/A	
				England; North Pennines;		N 54°	W 002°					
112	Alice Rowland	Aulacomnium palustre?	24	Weardale	519	40.483'	17.756'				N/A	
				England; North Pennines;		N 54°	W 002°					
113	Alice Rowland	Juncus castaneus	24	Weardale	519	40.483'	17.756'				N/A	
				England; North Pennines;		N 54°	W 002°					
114	Alice Rowland	Vaccinium myrtillus	25	Weardale	626	47.436'	13.022'				N/A	
		Eriophorum angustifolium		England; North Pennines;		N 54°	W 002°					
115	Alice Rowland	(possibly others)	25	Weardale	626	47.436'	13.022'				N/A	
				England; North Pennines;		N 54°	W 002°					
116	Alice Rowland	Calluna vulgaris	25	Weardale	626	47.436'	13.022'				N/A	
		Sphagnum sp.										
		Angustifolium?? - check										
		freezer sample more		England; North Pennines;		N 54°	W 002°					
117	Alice Rowland	closely	25	Weardale	626	47.436'	13.022'				N/A	
				England; North Pennines;		N 54°	W 002°					
118	Alice Rowland	Vaccinium vitis idea	25	Weardale	626	47.436'	13.022'				N/A	
				England; North Pennines;		N 54°	W 002°					
119	Alice Rowland	Anaptychia ciliaris??	25	Weardale	626	47.436'	13.022'				N/A	
				England; North Pennines;		N 54°	W 002°					
120	Alice Rowland	Polytrichum juniperinum	25	Weardale	626	47.436'	13.022'				N/A	
				England; North Pennines;		N 54°	W 002°					
121	Alice Rowland	Huperzia selago?	26	Weardale	629	42.051'	12.863'				N/A	
		Cladonia coccifera (poss.		England; North Pennines;		N 54°	W 002°					
122	Alice Rowland	polydactyla)	26	Weardale	629	42.051'	12.863'				N/A	
				England; North Pennines;		N 54°	W 002°					
123	Alice Rowland	Lycopodium clavatum	27	Weardale	629	41.968'	12.889'				N/A	
		Diphasiastrum/lycopodium		England; North Pennines;		N 54°	W 002°					
124	Alice Rowland	alpinum	27	Weardale	629	41.968'	12.889'				N/A	
						N 68°	E 018°					Exposed ground near
125	Alice Rowland	Lecidea lapicida?	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
						N 68°	E 018°					Exposed ground near
126	Alice Rowland	Lecanora polytropa??	6	Sweden; Abisko	507	26.091'	06.740′	68.43485	18.112333		N/A	Norway

Table 2.1.5i. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
		Buellia aethalea?				N 68°	E 018°					Exposed ground near
127	Alice Rowland	Rhizocarpon	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
		Toninia aromatica??? -				N 68°	E 018°					Exposed ground near
128	Alice Rowland	really not sure	6	Sweden; Abisko	507	26.091'	06.740'	68.43485	18.112333		N/A	Norway
	Dr. Adrian					N	E 018°					
130	Brennan	Dryas octopetala	28	Sweden; Abisko	520	68°19'32.3"	50'47.6"			NE	N/A	Vegetated Hummocks
	Dr. Adrian					N	E 018°					
131	Brennan	Dryas octopetala	28	Sweden; Abisko	520	68°19'32.3"	50'47.6"			NE	N/A	Vegetated Hummocks
	Dr. Adrian					N	E 018°					
132	Brennan	Dryas octopetala	28	Sweden; Abisko	520	68°19'32.3"	50'47.6"			NE	N/A	Vegetated Hummocks
	Dr. Adrian					N	E 018°					
133	Brennan	Empetrum nigrum	28	Sweden; Abisko	520	68°19'32.3"	50'47.6"			NE	N/A	Vegetated Hummocks
	Dr. Adrian					N	E 018°					
134	Brennan	Empetrum nigrum	28	Sweden; Abisko	520	68°19'32.3"	50'47.6"			NE	N/A	Vegetated Hummocks
	Dr. Adrian					N	E 018°					
135	Brennan	Empetrum nigrum	28	Sweden; Abisko	520	68°19'32.3"	50'47.6"			NE	N/A	Vegetated Hummocks
	Dr. Adrian					N	E 018°					
136	Brennan	Empetrum nigrum	28	Sweden; Abisko	520	68°19'32.3"	50'47.6"			NE	N/A	Vegetated Hummocks
	Dr. Adrian					N	E 018°					
137	Brennan	Saxifraga azoides	28	Sweden; Abisko	520	68°19'32.3"	50'47.6"			NE	N/A	Vegetated Hummocks
	Dr. Adrian					N	E 018°					
138	Brennan	Saxifraga azoides	28	Sweden; Abisko	520	68°19'32.3"	50'47.6"			NE	N/A	Vegetated Hummocks
	Dr. Adrian					N	E 018°					
139	Brennan	Saxifraga azoides	28	Sweden; Abisko	520	68°19'32.3"	50'47.6"			NE	N/A	Vegetated Hummocks
	Dr. Adrian					N	E 018°					Vegetated Hummocks
140	Brennan	Dryas octopetala	29	Sweden; Abisko	530	68°19'25.5"	50'15.7"			None	N/A	with Wet Depressions
	Dr. Adrian					N	E 018°					Vegetated Hummocks
141	Brennan	Dryas octopetala	29	Sweden; Abisko	530	68°19'25.5"	50'15.7"			None	N/A	with Wet Depressions
	Dr. Adrian					N	E 018°					Vegetated Hummocks
142	Brennan	Dryas octopetala	29	Sweden; Abisko	530	68°19'25.5"	50'15.7"			None	N/A	with Wet Depressions
	Dr. Adrian					N	E 018°					Vegetated Hummocks
143	Brennan	Empetrum nigrum	29	Sweden; Abisko	530	68°19'25.5"	50'15.7"			None	N/A	with Wet Depressions
	Dr. Adrian					N	E 018°					Vegetated Hummocks
144	Brennan	Empetrum nigrum	29	Sweden; Abisko	530	68°19'25.5"	50'15.7"			None	N/A	with Wet Depressions

Table 2.1.5j. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
	Dr. Adrian					N	E 018°					Vegetated Hummocks
145	Brennan	Empetrum nigrum	29	Sweden; Abisko	530	68°19'25.5"	50'15.7"			None	N/A	with Wet Depressions
	Dr. Adrian					N	E 018°					Vegetated Hummocks
146	Brennan	Saxifraga azoides	29	Sweden; Abisko	530	68°19'25.5"	50'15.7"			None	N/A	with Wet Depressions
	Dr. Adrian					N	E 018°					Vegetated Hummocks
147	Brennan	Saxifraga azoides	29	Sweden; Abisko	530	68°19'25.5"	50'15.7"			None	N/A	with Wet Depressions
	Dr. Adrian					N	E 018°					Vegetated Hummocks
148	Brennan	Saxifraga oppositifolia	29	Sweden; Abisko	530	68°19'25.5"	50'15.7"			None	N/A	with Wet Depressions
	Dr. Adrian					N	E 018°					Vegetated Hummocks
149	Brennan	Saxifraga oppositifolia	29	Sweden; Abisko	530	68°19'25.5"	50'15.7"			None	N/A	with Wet Depressions
	Dr. Adrian					N	E 018°					Vegetated Hummocks
150	Brennan	Saxifraga oppositifolia	29	Sweden; Abisko	530	68°19'25.5"	50'15.7"			None	N/A	with Wet Depressions
	Dr. Adrian					N	E 018°					Vegetated Hummocks
151	Brennan	Saxifraga oppositifolia	29	Sweden; Abisko	530	68°19'25.5"	50'15.7"			None	N/A	with Wet Depressions
												Birch Woodland with
	Dr. Adrian					N	E 018°					Empetrum
152	Brennan	Empetrum nigrum	30	Sweden; Abisko	510	68°19'49.8"	50'00.0"			S	N/A	understorey
												Birch Woodland with
	Dr. Adrian					N	E 018°					Empetrum
153	Brennan	Empetrum nigrum	30	Sweden; Abisko	510	68°19'49.8"	50'00.0"			S	N/A	understorey
												Birch Woodland with
	Dr. Adrian					N	E 018°					Empetrum
154	Brennan	Empetrum nigrum	30	Sweden; Abisko	510	68°19'49.8"	50'00.0"			S	N/A	understorey
												Birch Woodland with
	Dr. Adrian					N	E 018°					Empetrum
155	Brennan	Empetrum nigrum	31	Sweden; Abisko	510	68°19'46.9"	50'02.6"			Ν	N/A	understorey
												Birch Woodland with
	Dr. Adrian					N	E 018°					Empetrum
156	Brennan	Empetrum nigrum	31	Sweden; Abisko	510	68°19'46.9"	50'02.6"			Ν	N/A	understorey
												Birch Woodland with
	Dr. Adrian					N	E 018°					Empetrum
157	Brennan	Empetrum nigrum	31	Sweden; Abisko	510	68°19'46.9"	50'02.6"			Ν	N/A	understorey
	Dr. Adrian					N	E 017°					
158	Brennan	Empetrum nigrum	32	Sweden; Abisko	250	68°29'23.3"	45'42.6"			W	N/A	Distrubed gravelly soil

Table 2.1.5k. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
	Dr. Adrian					N	E 017°					
159	Brennan	Saxifraga oppositifolia	32	Sweden; Abisko	250	68°29'23.3"	45'42.6"			W	N/A	Distrubed gravelly soil
	Dr. Adrian					N	E 018°					Low Vegetation with
160	Brennan	Dryas octopetala	33	Sweden; Abisko	1030	68°17'09.6"	51'52.8"			NE	N/A	Bare Rock
	Dr. Adrian					N	E 018°					Low Vegetation with
161	Brennan	Dryas octopetala	33	Sweden; Abisko	1030	68°17'09.6"	51'52.8"			NE	N/A	Bare Rock
	Dr. Adrian					N	E 018°					Low Vegetation with
162	Brennan	Dryas octopetala	33	Sweden; Abisko	1030	68°17'09.6"	51'52.8"			NE	N/A	Bare Rock
	Dr. Adrian					N	E 018°					Low Vegetation with
163	Brennan	Empetrum nigrum	33	Sweden; Abisko	1030	68°17'09.6"	51'52.8"			NE	N/A	Bare Rock
	Dr. Adrian					N	E 018°					Low Vegetation with
164	Brennan	Empetrum nigrum	33	Sweden; Abisko	1030	68°17'09.6"	51'52.8"			NE	N/A	Bare Rock
	Dr. Adrian					N	E 018°					Low Vegetation with
165	Brennan	Empetrum nigrum	33	Sweden; Abisko	1030	68°17'09.6"	51'52.8"			NE	N/A	Bare Rock
	Dr. Adrian					N	E 018°					Low Vegetation with
166	Brennan	Saxifraga oppositifolia	33	Sweden; Abisko	1030	68°17'09.6"	51'52.8"			NE	N/A	Bare Rock
	Dr. Adrian					N	E 018°					Low Vegetation with
167	Brennan	Saxifraga oppositifolia	33	Sweden; Abisko	1030	68°17'09.6"	51'52.8"			NE	N/A	Bare Rock
	Dr. Adrian					N	E 018°					Low Vegetation with
168	Brennan	Saxifraga oppositifolia	33	Sweden; Abisko	1030	68°17'09.6"	51'52.8"			NE	N/A	Bare Rock
				England; North Pennines;		N 54° 46'	W 002° 18'					
169	Alice Rowland	Empetrum nigrum	34	Nags Head	645	33.3"	19.0''			NEE	N/A	
				England; North Pennines;		N 54° 46'	W 002° 18'					
170	Alice Rowland	Vaccinium vitis idea	34	Nags Head	645	33.3"	19.0''			NEE	N/A	
				England; North Pennines;		N 54° 46'	W 002° 18'					
171	Alice Rowland	Rubus chamaemorus	35	Nags Head	653	37.8"	18.9''			S	N/A	
				England; North Pennines;		N 54° 46'	W 002° 18'					
172	Alice Rowland	Rubus chamaemorus	35	Nags Head	653	37.8"	18.9''			S	N/A	
				England; North Pennines;		N 54° 46'	W 002° 18'					
173	Alice Rowland	Calluna vulgaris	35	Nags Head	653	37.8"	18.9"			S	N/A	
				England; North Pennines;		N 54° 46'	W 002° 18'					
174	Alice Rowland	Empetrum nigrum	35	Nags Head	653	37.8"	18.9''			S	N/A	
				England; North Pennines;		N 54° 40'	W 002° 00'					
175	Alice Rowland	Empetrum nigrum	36	Long man	533	54.1"	20.6"			NE	N/A	Mostly Bare Patch

Table 2.1.5I. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
				England; North Pennines;		N 54° 40'	W 002° 00'					
176	Alice Rowland	Vaccinium myrtillus	36	Long man	533	54.1"	20.6"			NE	N/A	Mostly Bare Patch
				England; North Pennines;		N 54° 40'	W 002° 00'					
177	Alice Rowland	Calluna vulgaris	36	Long man	533	54.1"	20.6"			NE	N/A	Mostly Bare Patch
						N	W 004°					
178	Alice Rowland	Calluna vulgaris	38	England; Dartmoor	288	50°29'12.5"	00'48.9"	50.486806	-4.013583		N/A	
				England; North Pennines;		N	W 002°					
179	Alice Rowland	Calluna vulgaris	39	Teesdale	414	54°39'02.1"	15'52.8"	54.650594	-2.264654		N/A	
				England; North Pennines;		N 54° 46'	W 001° 46'					
180	Alice Rowland	Calluna vulgaris	40	Hedleyhope Fell	192	12.5"	23.3"	54.770139	-1.7731389	NWW	N/A	Steep slope
												Large overhang,
												Calluna and
				England; North Pennines;		N 54° 46'	W 001° 46'					Empetrum dangling
181	Alice Rowland	Empetrum nigrum	41	Hedleyhope Fell	200	09.5"	28.0"	54.769306	-1.7744444	NWW	N/A	through
												Large overhang,
												Calluna and
				England; North Pennines;		N 54° 46'	W 001° 46'					Empetrum dangling
182	Alice Rowland	Calluna vulgaris	41	Hedleyhope Fell	200	09.5"	28.0''	54.769306	-1.7744444	NWW	N/A	through
				England; North Pennines;		N 54° 46'	W 001° 46'					slight slope, bank of
183	Alice Rowland	Empetrum nigrum	42	Hedleyhope Fell	215	01.2"	37.8"	54.767	-1.7771667	Ν	N/A	boggy area
				England; North Pennines;		N 54° 46'	W 001° 46'					Slight slope, bank of
184	Alice Rowland	Calluna vulgaris	42	Hedleyhope Fell	215	01.2"	37.8''	54.767	-1.7771667	Ν	N/A	boggy area
				England; North Pennines;		N 54° 47'	W 002° 20'					N/E slope near
185	Alice Rowland	Calluna vulgaris	43	Nenthead Mines	451	02.4"	17.9''	54.784	-2.3383056	NE	N/A	stream
				Scotland; Cairngorms;								
186	Alice Rowland	Empetrum nigrum	44	Braemar	493.18			56.995007	-3.412244	Ν	N/A	Rock
				Scotland; Cairngorms;								
187	Alice Rowland	Calluna vulgaris	44	Braemar	493.18			56.995007	-3.412244	Ν	N/A	Rock
				Scotland; Cairngorms;								
188	Alice Rowland	Empetrum nigrum	45	Braemar; Morrone	497.86			56.994921	-3.412361	W	N/A	Sheltered ledge
				Scotland; Cairngorms;								
189	Alice Rowland	Calluna vulgaris	45	Braemar; Morrone	497.86			56.994921	-3.412361	W	N/A	Sheltered ledge

Table 2.1.5m. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
												Exposed rock top
												(slight E slant for
				Scotland; Cairngorms;								E.nigrum slight W
190	Alice Rowland	Empetrum nigrum	46	Braemar; Morrone	637.26			56.991598	-3.419635		N/A	slant for C. vulgaris)
												Exposed rock top
												(slight E slant for
				Scotland; Cairngorms;								E.nigrum slight W
191	Alice Rowland	Calluna vulgaris	46	Braemar; Morrone	637.26			56.991598	-3.419635		N/A	slant for C. vulgaris)
400				Scotland; Cairngorms;	774.05			56 005000				
192	Alice Rowland	Calluna vulgaris	47	Braemar; Morrone	774.05			56.985393	-3.422861	Flattish	N/A	N side
				Scotland; Cairngorms;								
193	Alice Rowland	Empetrum nigrum	47	Braemar; Morrone	774.05			56.985393	-3.422861	Flattish	N/A	N side
				Scotland; Cairngorms;	050.44			56 004000	2 4224			
194	Alice Rowland	Calluna vulgaris	48	Braemar; Morrone	850.14			56.981033	-3.4301		N/A	Top of Morrone
4.05				Scotland; Cairngorms;	050.44			56 004000	2 4224			
195	Alice Rowland	Empetrum nigrum	48	Braemar; Morrone	850.14			56.981033	-3.4301		N/A	Top of Morrone
100			10	Scotland; Cairngorms;	054.00			56 000660	2 420420			T
196	Alice Rowland	Calluna vulgaris	49	Braemar; Morrone	851.88			56.980669	-3.430129		N/A	Top of Morrone
407		E	10	Scotland; Cairngorms;	054.00			56 000660	2 420420			T
197	Alice Rowland	Empetrum nigrum	49	Braemar; Morrone	851.88			56.980669	-3.430129		N/A	Top of Morrone
100		College and a serie	50	Scotland; Cairngorms;	045 40			56 00000	2 420012	C) A /	N1/A	
198	Alice Rowland		50	Sectland: Coimporter	845.48			50.98008	-3.430013	310	N/A	
100	Alico Powland	Empotrum pigrum	50	Scotland; Calingonis;	01E 10				2 120012	C/W/	NI/A	
199	Alle Kowialiu	Empetrum myrum	50	Sectland, Coimport	045.40			50.96006	-3.430013	300	N/A	
200	Alico Powland	Calluna vulgaric	E1	Scotland; Calingonis;	000.20			EC 070740	2 420565	CE.	NI/A	
200	Alice Rowland		51	Scotland: Cairngorms:	809.38			50.578748	-3.429505	JL	N/A	
201	Alice Rowland	Empetrum nigrum	51	Braemar: Morrone	809 38			56 978748	-3 129565	SE	NI/A	
201	Alice Nowland	Empetrannigrann	51	braemar, worrone	805.58			50.578748	-3.425505	JL	N/A	Shaltarad larga
												"crater" warmer and
												damper environemt
				Scotland: Cairngorms:								(diff. vegetation
202	Alice Rowland	Calluna vulgaris	52	Braemar; Morrone	706.61			56.975483	-3.426292	E	N/A	obvious)

Table 2.1.5n. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
												Sheltered large
												"crater" warmer and
												damper environemt
				Scotland; Cairngorms;								(diff. vegetation
203	Alice Rowland	Empetrum nigrum	52	Braemar; Morrone	706.61			56.975483	-3.426292	E	N/A	obvious)
				Scotland; Cairngorms;								
204	Alice Rowland	Calluna vulgaris	53	Creag Dubh	408.32			57.037646	-4.18528	S	N/A	Rocky outcrop
				Scotland; Cairngorms;								
205	Alice Rowland		53	Creag Dubh	408.32			57.037646	-4.18528	S	N/A	Rocky outcrop
				Scotland; Cairngorms;								
206	Alice Rowland	Calluna vulgaris	54	Creag Dubh	479.49			57.036996	-4.187389		4.04	Boggy flatland
				Scotland; Cairngorms;								
207	Alice Rowland	Empetrum nigrum	55	Creag Dubh	N/A	N/A	N/A	N/A	N/A	Ν	N/A	Rock
				Scotland; Cairngorms;								
208	Alice Rowland	Calluna vulgaris	55	Creag Dubh	N/A	N/A	N/A	N/A	N/A	Ν	N/A	Rock
				Scotland; Cairngorms;								
209	Alice Rowland	Calluna vulgaris	56	Creag Dubh	606.05			57.040812	-4.186332	W	N/A	Rock
				Scotland; Cairngorms;								
210	Alice Rowland	Empetrum nigrum	56	Creag Dubh	606.05			57.040812	-4.186332	W	N/A	Rock
				Scotland; Cairngorms;								
211	Alice Rowland	Calluna vulgaris	57	Creag Dubh	663.65			57.043354	-4.18419		4.22	Top near path
				Scotland; Cairngorms;								
212	Alice Rowland	Empetrum nigrum	57	Creag Dubh	663.65			57.043354	-4.18419		4.22	Top near path
				Scotland; Cairngorms; Ben								
213	Alice Rowland	Calluna vulgaris	58	Macdui	643.55			57.13212	-3.674385	nw	4.08	
				Scotland; Cairngorms; Ben								
214	Alice Rowland	Empetrum nigrum	58	Macdui	643.55			57.13212	-3.674385	nw	4.08	
				Scotland; Cairngorms; Ben						Slightly		
215	Alice Rowland	Calluna vulgaris	59	Macdui	692.29			57.124714	-3.679581	N	5.21	
				Scotland; Cairngorms; Ben						Slightly		
216	Alice Rowland	Empetrum nigrum	59	Macdui	692.29			57.124714	-3.679581	Ν	5.21	
				Scotland; Cairngorms; Ben						v.slight		
217	Alice Rowland	Calluna vulgaris	60	Macdui	757.32			57.120221	-3.683789	ly N	4.22	wet

Table 2.1.5o. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
										v.		
				Scotland; Cairngorms; Ben						slightly		
218	Alice Rowland	Empetrum nigrum	60	Macdui	757.32			57.120221	-3.683789	N	4.22	wet
				Scotland; Cairngorms; Ben								
219	Alice Rowland	Calluna vulgaris	61	Macdui	832.84			57.115853	-3.685988	slight N	N/A	
				Scotland; Cairngorms; Ben								
220	Alice Rowland	Empetrum nigrum	61	Macdui	832.84			57.115853	-3.685988	slight N	N/A	
				Scotland; Cairngorms; Ben								
221	Alice Rowland	Calluna vulgaris	62	Macdui	913.42			57.112778	-3.689726	slight N	4.47	
				Scotland; Cairngorms; Ben								
222	Alice Rowland	Empetrum nigrum	62	Macdui	913.42			57.112778	-3.689726	slight N	4.47	
				Scotland; Cairngorms; Ben								
223	Alice Rowland	Empetrum nigrum	63	Macdui	1030.3			57.108391	-3.691205		4.46	Rocky
				Scotland; Cairngorms; Ben								
224	Alice Rowland	Empetrum nigrum	64	Macdui	1089.6			57.09834	-3.690388		4.62	
				Scotland; Cairngorms; Ben								
225	Alice Rowland	Silene acaulis	65	Macdui	1154			57.08474	-3.678577		N/A	Rock top
				Scotland; Cairngorms; Ben								
226	Alice Rowland	Silene acaulis	65	Macdui	1154			57.08474	-3.678577		N/A	Rock top
				Scotland; Cairngorms; Ben								
227	Alice Rowland	Silene acaulis	65	Macdui	1154			57.08474	-3.678577		N/A	Rock top
				Scotland; Cairngorms; Ben								
228	Alice Rowland	Silene acaulis	65	Macdui	1154			57.08474	-3.678577		N/A	Rock top
			65	Scotland; Cairngorms; Ben				57.00474	0.000000			
229	Alice Rowland	Silene acaulis	65	Macdui	1154			57.08474	-3.6/85//		N/A	Rock top
				Scotland; Cairngorms; Ben	1000 0			57.0750.47	2 6 6 6 6 7 7			
230	Alice Rowland	Silene acaulis	66	Macdui	1262.2			57.075247	-3.669697		N/A	Rock top
224		Cite and a second in	67	Scotland; Cairngorms; Ben	1202.1			57.070454	2 6 6 9 4 5 9		4.2	and the
231	Alice Rowland	Silene acaulis	67		1303.1			57.070451	-3.669158		4.2	госк тор
222		E		Scotland; Cairngorms; Ben	1100 1			57 44 2222	2 65 4276			ale all and all a second
232	Alice Rowland	Empetrum nigrum	68		1100.1			57.112233	-3.654376		N/A	sheltered dip on rock
222		Cara atauna alianna		Scotiand; Cairngorms; Ben	1102.0			F7 112217	2 65 4602		NI / A	liebth, choltoned
233	Alice Rowland	Empetrum nigrum	69		1103.8			57.112217	-3.654602	مانمامة	N/A	lightly sheltered
22.4		E		Scotiand; Cairngorms; Ben	042.25			57 424040	2 660265	slight	4.00	
234	Alice Rowland	Empetrum nigrum	70	Iviacdul	912.25			57.121848	-3.660385	N/W	4.98	

Table 2.1.5p. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
				Scotland; Cairngorms; Ben						slight		
235	Alice Rowland	Calluna vulgaris	70	Macdui	912.25			57.121848	-3.660385	N/W	4.98	
				England; Exmoor; near								
236	Alice Rowland	Calluna vulgaris	71	Minehead	338.36			51.047209	-3.485905	flattish	3.9	
				England; Exmoor; near								
237	Alice Rowland	Erica	71	Minehead	338.36			51.047209	-3.485905	flattish	3.9	
				England; Exmoor; near								
238	Alice Rowland	Calluna vulgaris	72	Minehead	350.8			51.047342	-3.482884	flattish	4.04	
				England; Exmoor; near								
239	Alice Rowland	Calluna vulgaris	73	Minehead	341.06			51.048369	-3.481563	slight N	3.9	
				England; Exmoor; near								
240	Alice Rowland	Calluna vulgaris	74	Minehead	N/A	N/A	N/A	N/A	N/A	Ν	4.17	
				England; Exmoor; near						slight N		
241	Alice Rowland	Calluna vulgaris	75	Minehead	301.01			51.049423	-3.474745	slope	4.13	
				England; Exmoor; near								more gorse and other
242	Alice Rowland	Calluna vulgaris	76	Minehead	322.7			51.046746	-3.475084	flattish	4.32	shrubs present
				England; Exmoor; near								
243	Alice Rowland	Calluna vulgaris	77	Minehead	329.64			51.046761	-3.478684		4.09	
				England; Exmoor; Dunkery								
244	Alice Rowland	Calluna vulgaris	78	Beacon	491.71			51.160928	-3.59412	flattish	4.18	
				England; Exmoor; Dunkery								
245	Alice Rowland	Empetrum nigrum	78	Beacon	491.71			51.160928	-3.59412	flattish	4.18	
				England; Exmoor; Dunkery								
246	Alice Rowland	Calluna vulgaris	79	Beacon	498.85			51.161835	-3.590186	flattish	4.14	
				England; Exmoor; Dunkery								
247	Alice Rowland	Empetrum nigrum	79	Beacon	498.85			51.161835	-3.590186	flattish	4.14	
				England; Exmoor; Dunkery								
248	Alice Rowland	Calluna vulgaris	80	Beacon	517.36			51.162548	-3.586045	flattish	4.15	shallow soil
				England; Exmoor; Dunkery								
249	Alice Rowland	Empetrum nigrum	80	Beacon	517.36			51.162548	-3.586045	flattish	4.15	shallow soil
				England; Exmoor; Dunkery								
250	Alice Rowland	Calluna vulgaris	81	Beacon	476.06			51.163464	-3.578501		4.24	dip near path
				England; Exmoor; Dunkery								
251	Alice Rowland	Empetrum nigrum	81	Beacon	476.06			51.163464	-3.578501		4.24	dip near path

Table 2.1.5q. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
				England; Exmoor; Dunkery								
252	Alice Rowland	Calluna vulgaris	82	Beacon	394.06			51.169812	-3.560574	SE/S	4.22	
				England; Exmoor; Dunkery								
253	Alice Rowland	Calluna vulgaris	83	Beacon	350.83			51.170979	-3.553356	E	4.2	
				England; Exmoor; Dunkery								
254	Alice Rowland	Calluna vulgaris	84	Beacon	336.59			51.172035	-3.55249	NE	4.35	
				England; Exmoor; Dunkery								hard ground pH
255	Alice Rowland	Calluna vulgaris	85	Beacon	356.26			51.174008	-3.555352	NNE	4.02	unreliable
				England; Exmoor; Dunkery								
256	Alice Rowland	Calluna vulgaris	86	Beacon	383.3			51.174429	-3.559254	NNE	3.97	
	Prof. Annika	Calluna vulaaris										
257	Hofgaard		87	Norway; Grødalen	1000	N/A	N/A	N/A	N/A		N/A	treeline
	Prof. Annika	Empetrum niarum										
258	Hofgaard		88	Norway; Kongsvoll	1000	N/A	N/A	N/A	N/A		N/A	Forest line
	Prof. Annika	Phyllodoce caerulea										
259	Hofgaard	,	88	Norway; Kongsvoll	1000	N/A	N/A	N/A	N/A		N/A	Forest line
	Prof. Annika	Diapensia lapponica										
260	Hofgaard		87	Norway; Grødalen	1000	N/A	N/A	N/A	N/A		N/A	treeline
	Prof. Annika	Loiseleuria procumbens										
261	Hofgaard	,	88	Norway; Kongsvoll	1000	N/A	N/A	N/A	N/A		N/A	Forest line
	Prof. Annika	Arctostaphylos uv-ursi										
262	Hofgaard	. ,	88	Norway; Kongsvoll	1000	N/A	N/A	N/A	N/A		N/A	Forest line
2.52	Prof. Annika	Arctostaphylos alpina			1000							<b>F</b> 1.11
263	Hofgaard		88	Norway; Kongsvoll	1000	N/A	N/A	N/A	N/A		N/A	Forest line
264	Prof. Annika	Vaccinium myrtillus			1000							
264	Hofgaard		88	Norway; Kongsvoll	1000	N/A	N/A	N/A	N/A		N/A	Forest line
265	Prof. Annika	Vaccinium uliginosum		No Konstantill	1000				N1 / A			E constalla c
265	Hotgaard	-	88	Norway; Kongsvoli	1000	IN/A	IN/A	N/A	N/A		N/A	Forest line
200	Prot. Annika	Vaccinium vitis-idaea			1000	N1/A	N1/A	N1/A	N1/A		NI / A	Forest line
200	norgaard		88	Norway; Kongsvoll	1000	IN/A	IN/A	IN/A	IN/A	Clintral	N/A	Forest line
267		Callura a un la ania		Scotland; Dumtries and	6 75 44			F4 005224	F 4 4F705	Slightly	F 70	and fasting subta
267	Alice Rowland	Calluna Vulgaris	89	Galloway; Portpatrick	6.7541			54.865224	-5.145795	NNW	5.78	rock facing out to sea
200		Callura a un la ania		Scotland; Dumfries and	C 75 44			54.005224	F 4 4F 70F	Slightly	F 70	unal, factor autor
268	Alice Rowland	calluna vulgaris	89	Galloway; Portpatrick	6.7541			54.865224	-5.145795	ININVV	5.78	FOCK TACING OUT TO SEA

Table 2.1.5r. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
												SW facing rock
				Scotland; Dumfries and								sheltered from sea by
269	Alice Rowland	Calluna vulgaris	90	Galloway; Portpatrick	3.7428			54.866653	-5.144319	SW	N/A	larger rock
				Scotland; Dumfries and						slight		
270	Alice Rowland	Calluna vulgaris	91	Galloway; Portpatrick	6.7122			54.867877	-5.144627	SW	4.92	rock facing out to sea
				Scotland; Dumfries and								
271	Alice Rowland	Calluna vulgaris	92	Galloway; Portpatrick	5.7			54.868093	-5.144428	NNW	N/A	rock
				Scotland; Dumfries and								
272	Alice Rowland	Calluna vulgaris	93	Galloway; Portpatrick	22.91			54.862023	-5.14468	W	4.92	
				Scotland; Dumfries and								
273	Alice Rowland	Calluna vulgaris	94	Galloway; Portpatrick	23.511			54.857729	-5.141591	SW	3.99	
				Scotland; Dumfries and								
274	Alice Rowland	Calluna vulgaris	95	Galloway; Portpatrick	50.827			54.853987	-5.137544	NE	3.98	
				Scotland; Dumfries and								
275	Alice Rowland	Calluna vulgaris	96	Galloway; Portpatrick	44.381			54.853513	-5.137316	NW	N/A	rock
				Scotland; Dumfries and								sheltered area with
276	Alice Rowland	Calluna vulgaris	97	Galloway; Portpatrick	22.305			54.851351	-5.133886	NNE	5.19	overhang
				Scotland; Dumfries and								sheltered area with
277	Alice Rowland	Empetrum nigrum	97	Galloway; Portpatrick	22.305			54.851351	-5.133886	NNE	5.19	overhang
				Scotland; Dumfries and								
278	Alice Rowland	Calluna vulgaris	98	Galloway; Portpatrick	5.7649			54.851304	-5.130704	W	4.48	
				Scotland; Dumfries and								
279	Alice Rowland	Calluna vulgaris	99	Galloway; Portpatrick	8.8097			54.850459	-5.128882	NW	5.49	
				Scotland; Galloway; Glen								
280	Alice Rowland	Calluna vulgaris	100	Trool	136.37			55.080224	-4.525737	SW	3.82	iron rich rock
												slope birch under
				Scotland; Galloway; Glen								story by path - tall
281	Alice Rowland	Calluna vulgaris	101	Trool	105.64			55.081918	-4.519367		4.84	plant
				Scotland; Galloway; Glen								
282	Alice Rowland	Calluna vulgaris	102	Trool	92.189			55.073593	-4.512777	W	N/A	rock
				Scotland; Galloway; Glen								ditch near path,
283	Alice Rowland	Calluna vulgaris	103	Trool	64.669			55.0762	-4.516981		3.79	birrch understory
				Scotland; Galloway; Glen								
284	Alice Rowland	Calluna vulgaris	104	Trool	65.885			55.075843	-4.518165		4.63	near black pond

Table 2.1.5s. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
				Scotland; Galloway; Glen								
285	Alice Rowland	Calluna vulgaris	105	Trool	N/A	N/A	N/A	N/A	N/A		N/A	under pine
				Scotland; Galloway; Glen								
286	Alice Rowland	Calluna vulgaris	106	Trool	67.07			55.071041	-4.541832		4.42	near river
				England; Yorkshire; Moors								
287	Alice Rowland	Calluna vulgaris	107	near Fylindales	212.78			54.430163	-0.570075		N/A	
				England; Yorkshire; Moors								
288	Alice Rowland	Empetrum nigrum	108	near Fylindales	212.69			54.430226	-0.570282		N/A	
				England; Yorkshire; Moors								
289	Alice Rowland	Calluna vulgaris	109	near Fylindales	210.44			54.431404	-0.571897		N/A	
				England; Yorkshire; Moors								
290	Alice Rowland	Empetrum nigrum	109	near Fylindales	210.44			54.431404	-0.571897		N/A	
				England; Yorkshire; Moors								
291	Alice Rowland	Calluna vulgaris	110	near Fylindales	209.45			54.43299	-0.572405		N/A	regenerating burn
				England; Yorkshire; Moors								
292	Alice Rowland	Empetrum nigrum	110	near Fylindales	209.45			54.43299	-0.572405		N/A	regenerating burn
				England; Yorkshire;								
				Eskdaleside cum								
293	Alice Rowland	Calluna vulgaris	111	Ugglebarnby	191.9			54.440159	-0.667083		3.98	
				England; Yorkshire;								
				Eskdaleside cum								
294	Alice Rowland	Empetrum nigrum	111	Ugglebarnby	191.9			54.440159	-0.667083		3.98	
				England; Yorkshire;								
				Eskdaleside cum								
295	Alice Rowland	Empetrum nigrum	112	Ugglebarnby	194.37			54.441189	-0.66966	NNW	3.62	hollow
				England; Yorkshire;								
				Eskdaleside cum								
296	Alice Rowland	Calluna vulgaris	112	Ugglebarnby	194.37			54.441189	-0.66966	NNW	3.62	hollow
				England; Yorkshire;								
				Eskdaleside cum						slightly		
297	Alice Rowland	Empetrum nigrum	113	Ugglebarnby	212.33			54.439667	-0.676668	Ν	3.72	
				England; Yorkshire;								
				Eskdaleside cum						slightly		
298	Alice Rowland	Calluna vulgaris	113	Ugglebarnby	212.33			54.439667	-0.676668	Ν	3.72	

Table 2.1.5t. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
				England; Yorkshire;								
				Eskdaleside cum								
299	Alice Rowland	Calluna vulgaris	114	Ugglebarnby	250.61			54.439241	-0.686467		3.94	
				England; Yorkshire;								
				Eskdaleside cum								
300	Alice Rowland	Calluna vulgaris	114	Ugglebarnby	250.61			54.439241	-0.686467		3.94	
				England; Yorkshire; Moors								
301	Alice Rowland	Calluna vulgaris	115	near Hutton-le-Hole	197.54			54.316042	-0.930714		4.01	
				England; Yorkshire; Moors								
302	Alice Rowland	Empetrum nigrum	115	near Hutton-le-Hole	197.54			54.316042	-0.930714		4.01	
				England; Yorkshire; Moors								
303	Alice Rowland	Calluna vulgaris	116	near Hutton-le-Hole	196.14			54.315984	-0.930004		3.91	regenerating burn
				England; Yorkshire; Moors								
304	Alice Rowland	Empetrum nigrum	116	near Hutton-le-Hole	196.14			54.315984	-0.930004		3.91	regenerating burn
				England; Yorkshire; Moors								
305	Alice Rowland	Calluna vulgaris	117	near Hutton-le-Hole	144.85			54.315566	-0.923258	W	3.91	near stream
				England; Yorkshire; Moors								
306	Alice Rowland	Calluna vulgaris	118	near Hutton-le-Hole	144.76			54.315442	-0.923008	W	N/A	further from stream
				England; Yorkshire; Moors								
307	Alice Rowland	Empetrum nigrum	118	near Hutton-le-Hole	144.76			54.315442	-0.923008	W	N/A	further from stream
				England; Yorkshire; Moors								
308	Alice Rowland	Calluna vulgaris	119	near Hutton-le-Hole	151.66			54.315586	-0.921248		4.08	
				England; Yorkshire; Moors								
309	Alice Rowland	Calluna vulgaris	120	near Hutton-le-Hole	158.99			54.315765	-0.919813		N/A	thick billberry patch
				England; Yorkshire; Moors								
310	Alice Rowland	Empetrum nigrum	120	near Hutton-le-Hole	158.99			54.315765	-0.919813		N/A	thick billberry patch
				England; Yorkshire; Moors								
311	Alice Rowland	Calluna vulgaris	121	near Hutton-le-Hole	216.56			54.323427	-0.916739		3.63	
				England; Yorkshire; Moors								
312	Alice Rowland	Calluna vulgaris	122	near Hutton-le-Hole	219.13			54.32382	-0.916636		3.67	
				England; Yorkshire; Moors								
313	Alice Rowland	Empetrum nigrum	122	near Hutton-le-Hole	219.13			54.32382	-0.916636		3.67	
				England; Yorkshire; Moors								
314	Alice Rowland	Calluna vulgaris	123	near Hutton-le-Hole	264.18			54.331656	-0.917853		4.04	

Table 2.1.5u. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
				England; Yorkshire; Moors								
315	Alice Rowland	Calluna vulgaris	124	near Hutton-le-Hole	270.95			54.334102	-0.918089		3.82	over knee high plant
				England; Yorkshire; Moors								
316	Alice Rowland	Calluna vulgaris	125	near Hutton-le-Hole	314.56			54.341785	-0.921009		3.75	Boggy
				England; Yorkshire; Moors								
317	Alice Rowland	Empetrum nigrum	126	near Hutton-le-Hole	316.78			54.341925	-0.921397		N/A	
	Dr. Johan											
318	Olofsson	Calluna vulgaris	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
	Dr. Johan											
319	Olofsson	Rhododendron lapponum	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
	Dr. Johan											
320	Olofsson	Andromeda polifolia	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
	Dr. Johan	Empetrum										
321	Olofsson	hermaphroditum	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
	Dr. Johan											
322	Olofsson	Arctostaphylus alpinus	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
	Dr. Johan											
323	Olofsson	Phyllodoce caerulea	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
224	Dr. Johan			21/2	N1/A							
324	Diofsson	Cassiope tetragona	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
225	Dr. Jonan		NI / A	N1/A	NI / A	NI/A	N1/A	N1/A	N1 / A	N1/A	NI / A	
325	Diofsson	Loiseleuria procumbens	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
276	Dr. Jonan Olofsson	Durula minor	NI/A	N/A	NI/A	NI/A			NI / A	NI/A		
520	UDISSUI	Fyruiu minor	IN/A	N/A	IN/A	N/A	N/A	N/A	N/A		N/A	
227	Alico Powland	Calluna vulgaris	129	Wales: Brocon Boacons	108 22			51 85/06	2 468621	slopo	5 5 2	Poadsidod
527	Alice Rowland	Cullulla Valgaris	120	Wales, Brecon Beacons	408.32			51.85450	-3.408021	slope	5.55	Nodusiueu
328	Alice Rowland	Calluna vulgaris	129	Wales; Brecon Beacons	468.7			51.868522	-3.467467	W	4.1	
329	Alice Rowland	Calluna vulgaris	130	Wales; Brecon Beacons	496.06			51.869194	-3.464969	W	4.44	Near path
330	Alice Rowland	Calluna vulgaris	131	Wales; Brecon Beacons	593.08			51.878613	-3.457791	N	N/A	cliff edge by stream
										Slight		
331	Alice Rowland	Calluna vulgaris	132	Wales; Brecon Beacons	667.81			52.021619	-3.104595	W	3.74	
										Slight		
332	Alice Rowland	Empetrum nigrum	132	Wales; Brecon Beacons	667.81			52.021619	-3.104595	W	3.74	

Table 2.1.5v. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
333	Alice Rowland	Calluna vulgaris	133	Wales; Brecon Beacons	670.4			52.022082	-3.103914		3.65	
334	Alice Rowland	Empetrum nigrum	133	Wales; Brecon Beacons	670.4			52.022082	-3.103914		3.65	
335	Alice Rowland	Calluna vulgaris	134	Wales; Brecon Beacons	672.82			52.022882	-3.101938		3.58	
336	Alice Rowland	Empetrum nigrum	134	Wales; Brecon Beacons	672.82			52.022882	-3.101938		3.58	
337	Alice Rowland	Calluna vulgaris	135	Wales; Brecon Beacons	663.79			52.019552	-3.096422		3.54	
338	Alice Rowland	Calluna vulgaris	136	Wales; Brecon Beacons	662.18			52.018906	-3.095584		3.88	
339	Alice Rowland	Empetrum nigrum	136	Wales; Brecon Beacons	662.18			52.018906	-3.095584		3.88	
340	Alice Rowland	Calluna vulgaris	137	Wales; Brecon Beacons	692.49			52.015125	-3.088687		3.54	
341	Alice Rowland	Empetrum nigrum	137	Wales; Brecon Beacons	692.49			52.015125	-3.088687		3.54	
342	Alice Rowland	Calluna vulgaris	138	Wales; Brecon Beacons	700.32			52.00855	-3.08601		3.66	patchy landscape
343	Alice Rowland	Empetrum nigrum	138	Wales; Brecon Beacons	700.32			52.00855	-3.08601		3.66	patchy landscape
344	Alice Rowland	Calluna vulgaris	139	Wales; Brecon Beacons	663.27			51.997474	-3.076552		3.92	
345	Alice Rowland	Empetrum nigrum	139	Wales; Brecon Beacons	663.27			51.997474	-3.076552		3.92	
346	Alice Rowland	Calluna vulgaris	140	Wales; Brecon Beacons	623.05			51.989496	-3.070013		3.63	
347	Alice Rowland	Empetrum nigrum	140	Wales; Brecon Beacons	623.05			51.989496	-3.070013		3.63	
348	Alice Rowland	Calluna vulgaris	141	Wales; Brecon Beacons	482.81			51.982589	-3.093898		N/A	
349	Alice Rowland	Calluna vulgaris	132	Wales; Brecon Beacons	667.81			52.021619	-3.104595	Slight W	3.74	
350	Alice Rowland	Empetrum nigrum	132	Wales; Brecon Beacons	667.81			52.021619	-3.104595	Slight W	3.74	
351	Alice Rowland	Calluna vulgaris	143	Wales; Brecon Beacons	661.66			51.997794	-3.109974		3.69	
352	Alice Rowland	Empetrum nigrum	143	Wales; Brecon Beacons	661.66			51.997794	-3.109974		3.69	
353	Alice Rowland	Empetrum nigrum	144	England; Derbyshire; Bealey Moor	N/A	N/A	N/A	N/A	N/A		3.91	woodland near road

Table 2.1.5w. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
				England; Derbyshire;								
354	Alice Rowland	Calluna vulgaris	144	Bealey Moor	N/A	N/A	N/A	N/A	N/A		3.91	woodland near road
				England; Derbyshire;								
355	Alice Rowland	Calluna vulgaris	145	Bealey Moor	322.91			53.216389	-1.538042		3.98	flattish
				England; Derbyshire;								
356	Alice Rowland	Calluna vulgaris	146	Bealey Moor	323.07			53.216359	-1.538876		3.58	on tussock
				England; Derbyshire;								
357	Alice Rowland	Empetrum nigrum	146	Bealey Moor	323.07			53.216359	-1.538876		3.58	on tussock
										mild		
				England; Derbyshire;						SSW		
358	Alice Rowland	Calluna vulgaris	147	Bealey Moor	N/A	N/A	N/A	N/A	N/A	slope	3.98	knee high
				England; Derbyshire;						slight		
359	Alice Rowland	Calluna vulgaris	148	Bealey Moor	318.55			53.215186	-1.541475	NNE	3.85	
				England; Derbyshire;								
360	Alice Rowland	Empetrum nigrum	149	Bealey Moor	327.33			53.214914	-1.544077		4	Possible burn site
				England; Derbyshire;								
361	Alice Rowland	Calluna vulgaris	149	Bealey Moor	327.33			53.214914	-1.544077		4	Possible burn site
				England; Derbyshire;								
362	Alice Rowland	Calluna vulgaris	150	Bealey Moor	333.97			53.214604	-1.549234		3.91	
				England; Derbyshire;								
363	Alice Rowland	Empetrum nigrum	150	Bealey Moor	333.97			53.214604	-1.549234		3.91	
				England; Derbyshire;								
364	Alice Rowland	Calluna vulgaris	151	Bealey Moor	358.79			53.213578	-1.555351		3.83	
				England; Derbyshire;								
365	Alice Rowland	Calluna vulgaris	152	Bealey Moor	294.3			53.212275	-1.580897		N/A	little hillock
				England; Derbyshire;								
366	Alice Rowland	Calluna vulgaris	153	Bealey Moor	292.24			53.213007	-1.581507		3.85	centre of stone circle
				England; Derbyshire;								
367	Alice Rowland	Calluna vulgaris	154	Bealey Moor	513.44			53.434909	-1.867804		3.48	
				England; Derbyshire;								
368	Alice Rowland	Empetrum nigrum	154	Bealey Moor	513.44			53.434909	-1.867804		3.48	
				England; Derbyshire;								
369	Alice Rowland	Empetrum nigrum	155	Bealey Moor	556.51			53.44463	-1.854526		3.56	

Table 2.1.5x. Table listing details for samples collected as per section 2.1.1. Showing the individual sample code, name of the collector of the sample, species identification (N.B. "?" indicates uncertainty with identification either due to being outside of the expected range or due to altered morphology under extreme environments often combined with limitations of vegetative identification), Site code, general location collected from, altitude (m above sea level), latitude and longitude of the collection site (either in the degrees, minutes, seconds or decimal degrees format) slope and aspect (where present), Soil pH (where recorded) and any additional details recorded.

Sample					Altitude			decimal	decimal	Slope/		
code	Collector	Species	Site	Location	(m)	Latitude	Longitude	latitude	longitude	aspect	рН	Additional Details
				England; Derbyshire;								
370	Alice Rowland	Empetrum nigrum	156	Bealey Moor	560.7			53.448028	-1.862098		3.44	
				England; Derbyshire;								
371	Alice Rowland	Calluna vulgaris	157	Bealey Moor	605.36			53.448856	-1.86733		4.09	
				England; Derbyshire;								
372	Alice Rowland	Empetrum nigrum	158	Bealey Moor	606.73			53.450676	-1.86459		3.38	
				England; Derbyshire;								
373	Alice Rowland	Calluna vulgaris	158	Bealey Moor	606.73			53.450676	-1.86459		3.38	
				England; Derbyshire;								
374	Alice Rowland	Calluna vulgaris	159	Bealey Moor	601.63			53.452622	-1.862828		3.59	
				England; Derbyshire;								
375	Alice Rowland	Empetrum nigrum	159	Bealey Moor	601.63			53.452622	-1.862828		3.59	
				England; Derbyshire;								
376	Alice Rowland	Calluna vulgaris	160	Bealey Moor	613.78			53.458049	-1.859905		3.67	
				England; Derbyshire;								
377	Alice Rowland	Empetrum nigrum	160	Bealey Moor	613.78			53.458049	-1.859905		3.67	
				England; Derbyshire;								
378	Alice Rowland	Empetrum nigrum	161	Bealey Moor	625.59			53.461137	-1.860756		4.75	
				England; Derbyshire;								
379	Alice Rowland	Calluna vulgaris	161	Bealey Moor	625.59			53.461137	-1.860756		4.75	

# 2.2 Live Test Species Selection and Growth Conditions2.2.1 Selection and Growth of Alpine Model Plants

#### 2.2.1.1 Selection Criteria

Different geographic locations experience different seasonal day/night cycles, freezing extremes and frequency. Therefore genetic, molecular and physiological study of different populations of the same species in different geographic locations may aid in elucidating the mechanisms involved in freezing tolerance, especially where differences are seen. Hence a study species which could be grown in controlled conditions from seed collected from different locations was required. A requirements shortlist was constructed to aid in selection of such a species (see below) and species distributions were checked in Encyclopaedia of Life (EOL, 2014) and Global Biodiversity Information Facility (Global Biodiversity Information Facility). The shortlist of species which met these requirements to be taken for further tests consisted of *Dianthus superbus*, *Dryas octopetala*, *Gentiana verna*, *Minuartia verna*, *Saxifraga azoides* and *Saxifraga oppositifolia*. Initially horticultural seed was used to assess germination ease and rate, growth rate, ability to force under glass and, in some cases such as *Dianthus superbus*, freezing tolerance.

Required criteria:

- Overwinters above ground
- Good freezing tolerance
- Hermaphroditic or monoecious
- No incompatibility systems
- Wide distribution covering different temperatures and day/night cycles

#### Preferred criteria:

- Rapid growth rate
- Rapid seed set
- Able to force, under glass
- Fast germination rate possible to induce
- Vegetative propagation possible
- Molecular literature available
- Horticultural seed for initial tests available

#### 2.2.1.2 Germination and Growth Media

Each species had a specific soil requirement which was kept consistent for both germination and growth: *Dianthus superbus* - 4 parts John Innes seed compost to 1 parts horticultural sand. *Dryas octopetala* - 1 parts John Innes seed compost to 2 parts horticultural sand, *Gentiana verna* - 5 parts John Innes seed compost to 1 part horticultural sand, *Minuartia verna* - John Innes seed compost only and *Saxifraga azoides & Saxifraga oppositifolia* - equal parts John Innes seed compost to horticultural sand. These substrate requirements were estimated to provide the desired degree on drainage using descriptions of preferred growth media from the following online-sources for each species: *Dianthus superbus* (Seedaholic.com), *Dryas octopetala* (GardenersHQ.com, Alpiner, 2005), *Gentiana verna* (Upward, RHS) *Minuartia verna* (BluestonePerennials), *Saxifraga* (McGregor).

#### 2.2.1.3 Germination Conditions

Seeds for preliminary tests were ordered from Jelitto Perennial Seeds, St Ives, Huntingdon, Cambridgeshire. Initially all seeds were soaked in 1 mM GA<sub>3</sub> overnight prior to sowing. This was then switched to 0.3 mM GA4+7 for all seeds except *Dianthus*  *superbus* which was switched to sowing directly, due to previously displayed rapid germination. Seeds were sown in half seed trays in the appropriate media (see 2.7.2) with a basal gravel filling. Trays were then placed in large bags to keep humidity high. After the first attempt *Dryas octopetalla* seeds were sown in tall pots instead of trays due to the observed very early development of long roots for which seed trays were not sufficiently deep. Once sown seed trays/pots were placed in a growth chamber (Weiss Gallenkamp, Loughborough, UK) set at 20 °C, 70 % humidity, 18h day length with 600 µmol m<sup>-2</sup> s<sup>-1</sup> light with a one hour sunrise and one hour sunset of 300µmol m<sup>-2</sup>s<sup>-1</sup> and supplemental red light from tungsten lamps during programmed daylight hours.

#### 2.2.1.4 Growth Conditions of Seedlings

Once germinated, the growth temperature was altered to 16 °C during daylight hours, 15 °C during simulated sunrise and sunset and 14 °C during the night. All other chamber settings remained the same as during germination. Upon reaching first true leaves plants were potted on and pots placed in plunge beds made from horticultural sand to keep roots cool, humidity high and provide a consistent supply of moisture.

# 2.2.2 Growth of *Empetrum nigrum* and *Calluna vulgaris* From Cuttings

#### **2.2.2.1 Conditions**

Cuttings were, when possible, grown in a growth chamber (Weiss Gallenkamp, Loughborough, UK) set at 16 °C during simulated daylight hours dropping to 14 °C over night, 70 % humidity, 16h day length with 600  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> light with a one hour sunrise and one hour sunset of 300 $\mu$ mol m<sup>-2</sup>s<sup>-1</sup> and supplemental red light from tungsten lamps during programmed daylight hours.

During frequent multiple mechanical failures of the growth chambers and long periods of downtime, plants from rooted cuttings experienced a variety of temperatures, a maximum of around 35 °C during some failures which resulted in the loss of a number of samples. Whilst awaiting repair to these facilities, plants went into a variety of non-controlled conditions including outdoor conditions (spring/summer) and greenhouse conditions (with seasonal dependant lighting). A datalogger tracked the temperature changes.

#### 2.2.2.2 Rooting of Cuttings

The best method of rooting of cuttings of the selected species of interest, *Empetrum nigrum* and *Calluna vulgaris*, was investigated. Numerous cuttings from Nags Head, North Pennines, UK were collected. These cuttings included a mix of sizes and tissue ages including cuttings consisting of only this season's growth and those with older, more woody tissue. Several rooting methods were then attempted. Both species from a mixture of locations and cutting types were used for each rooting method (unless otherwise stated) and all cuttings had several leaves stripped back leaving just a few leaves remaining which remained above the substrate. The methods attempted were as follows:

- Planting in peat-plugs (Jiffy Products, International AS, Norway);
- Planting in sand;
- Planting in John Innes seed compost;
- Immersion in de-ionised water in 50 ml falcon tubes;
- Placed horizontally on damp filter paper in petri-dishes (only cuttings of woody tissue that was found growing horizontally in the wild were used in these tests).

After initial tests, rooting in falcon tubes with de-ionised water was chosen as the optimal rooting method and used for all further rooting of cuttings.

Upon the visible development of a root system with greater than two roots or branches and at a length greater than 250 mm rooted cuttings were potted up into black sphagnum peat in 90 mm diameter pots, the wet roots were dipped into Empathy Rootgrow Ericoid Mychorrhizal fungi which was also sprinkled into the planting hole prior to the addition of further peat. Pots were then well watered and returned to standard growing conditions (Section 2.2.2.1).

Due to limited availability of space, the maximum size pot for plants was 90 mm diameter, therefore upon testing, all samples were heavily pot-bound. However, this was consistent across all samples.

### 2.2.3 Nicotiana benthamiana Growth Conditions

*Nicotiana benthamiana* plants were grown in temperature controlled rooms at 25 °C with 12 hours daylight 12 hours night cycle with a light intensity of 250-300  $\mu$ mol m<sup>-2</sup> sec<sup>-1</sup>.

# 2.2.4 Growth of Arabidopsis thaliana

Wildtype line Col-0 of *Arabidopsis thaliana* was used for all transformations and wildtype controls. All *Arabidopsis thaliana* were grown at 20 °C with a light intensity of 150  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> with varying day/night cycles.

#### 2.2.4.1 MS Media

Murashige and Skoog (MS) media was added at a ratio of 4.3 g per litre to MilliQ water. This was increased to a pH of 5.8 using KOH, then added to Sigma plant agar at a ratio of 8 g L<sup>-1</sup>. This was then autoclaved for 20 minutes at 121 °C at 105 Pa. Once hand-hot, the antibiotics Timentin at 200  $\mu$ g ml<sup>-1</sup> and Hygromycin at 50  $\mu$ g ml<sup>-1</sup> were added if required and then poured into petri dishes (of varying size, dependant upon experiment).

#### 2.2.4.2 Seed Sterilisation

Two methods of seed surface sterilisation were used for growth of *Arabidopsis thaliana* seeds: short seed sterilisation for wildtype and established lines and long seed sterilisation for seeds collected from transformed plants.

**Short seed sterilisation:** Seeds (max. volume of approx. 150  $\mu$ l) were shaken in a 1.5 ml Eppendorf filled with 70 % ethanol for 5 minutes, before being left to dry on filter paper in a Microflow Horizontal Laminar flowhood. Once dried, seeds were either stored or plated out.

**Long seed sterilisation:** 50 ml Falcon tubes containing seeds to be sterilised were filled with 70 % ethanol and shaken for 5 minutes. The ethanol was then decanted and a mixture containing 0.25 % (w/v) sodium dodecyl sulphate (SDS) and 10 % (v/v) sodium hypochlorite in sterile (autoclaved, reverse osmosis filtered) water was added. Tubes were then shaken for 10 minutes. This mixture was then decanted and the seeds washed in sterile water. This washing step was repeated seven times. Seeds were then resuspended in a volume of water approximately twice the volume of the seeds, and then plated out.

Upon surface sterilisation, seeds were sprinkled (short sterilisation) or pipetted (long sterilisation) onto MS Agar plates (section 2.2.4.1). For selection of seeds from transformed plants, plates containing the antibiotics 200  $\mu$ g ml<sup>-1</sup> Timentin and 50  $\mu$ g ml<sup>-1</sup> Hygromycin were used. These plates were then placed in the dark at 4 °C for a minimum of 48 hours before transferal to germination conditions (Section 2.2.4.3)

#### 2.2.4.3 Germination and Growth

Seeds were germinated on MS agar plates in a 16 h day 8 h night cycle in a Plant Climatics Percival CU-36l5D. Once seedlings had reached an appropriate size on plates (approx. at 7-10 days for wild type) or were showing distinction from non-transformed seeds on selection plates, seedlings were transferred to re-hydrated sterile 44 mm peat plugs (Jiffy Products, International AS, Norway) at a ratio of 1 seedling per plug for experiments and bulking of seed and 3 seedlings per plug for dipping.

Seedlings were then transferred to a 12 hour day 12 hour night cycle at 100 % humidity (by covering with plastic) for 24 hours, humidity was then reduced (by partial removal of plastic covering) for a further 24 hours before complete removal of the plastic covering. Upon approaching mature plant size, or displaying evidence of the formation of flower primordia, plants were transferred to a 16 h day 8 h night cycle.

10 days prior to dipping, plants for dipping had the flower bolts trimmed to a few millimetres above the surface of the rosette.

#### **2.2.4.4 GA<sub>3</sub> stock for topical application**

Some *Arabidopsis thaliana* plants transformed to overexpress synthetic *CBF* genes (Section 2.8.11) showed extreme dwarfism, a topical application of  $GA_3$  was applied to these plants to assist growth. A 20 mM stock of  $GA_3$  (in ethanol) was produced and then diluted in water to a produce a 100  $\mu$ M solution. This solution was then applied to plants displaying extreme dwarfism via a soft bristled paintbrush.

# 2.3 Freezing and Chilling Treatments for Live Samples 2.3.1 *Empetrum nigrum* Chilling Time-course for qPCR

*Empetrum nigrum* plants to undergo chilling for tissue collection for RNA extraction for qPCR, after growth as per section 2.2.2.1, were subjected to a period of chilling at 5 °C in growth chambers (Weiss Gallenkamp, Loughborough, UK). Tissue was sampled at 0,

2, 4, 8, 14 and 24 hours as per Figure 2.3.1.1 then frozen in liquid nitrogen and stored at -80  $^{\circ}$ C.

5 °C was chosen as a low, but non-freezing, temperature to attempt induce acclimation and *CBF* expression. This is the standard chilling temperature used in the Knight lab for inducing *CBF* expression in *Arabidopsis thaliana*. Since there is no knowledge on the temperature required to induce *CBF* expression in *Empetrum nigrum*, and a temperature above freezing was desired for initial tests, 5 °C was selected.



Figure 3.3.1.1. Showing changes in light intensities (blue, primary axis) and temperature (red, secondary axis) across the timecourse in *which Empetrum nigrum* tissue samples were collected for CBF expression analysis. The time of day at which each timepoint at which tissue was collected are marked with a \*. Light cycle (blue) was kept consistent from previous growing conditions; temperature was dropped from previous growing conditions (16°C) to 5°C.

### 2.3.2 Arabidopsis thaliana Visual Freezing Assay

Plants were grown as per methods 2.2.4 and members of each line were given a colour coded marker. At an age of 4 weeks, plants were reorganised into repeating patterns to evenly distribute members of the same line, to avoid potential edge effects by having members of the same line clustered together, and were photographed. They were then placed at -7 °C for 24 h followed by 24h at 5 °C, then placed back in growing conditions (methods 2.2.4) and subsequently photographed after 3 days.

-7 °C was selected as the freezing temperature because previous studies found the LT50 for wildtype Col-0 plants was -5 °C for non-acclimated and -10 °C for acclimated plants (when grown in 16-h photoperiod with a day/night temperature of 20 °C/18 °C – similar to our growth conditions) (Hannah et al., 2006). Therefore, since the *CBF* constructs are under a constitutive promoter (simulating activation during acclimation), -7 °C was predicted to be cold enough to isolate non-functional CBF constructs from functional ones, with a predicted shift in survival percentages reflecting construct potency.

-7 °C freezing shock was followed by 24 h at 5 °C to gently thaw and return the plants to growth temperatures and reduce sudden heat shock.

# **2.4 Measuring Indicators of Plant Health**

## 2.4.1 Chlorophyll Content

#### 2.4.1.1 Freezing Tests for Chlorophyll Extractions

Samples for testing were clipped from plants and wrapped in aluminium foil and kept at either; 16 °C, -20 °C or -80 °C for 24 hours then left to defrost at 16 °C for 1 hour before photographing (fresh samples were immediately frozen and stored at -80 °C before being taken onto the extraction step). Samples were then placed in darkened petri dishes at

16 °C and photographed at 24, 51 and 78 hours after removal from freezing. Samples were then frozen in liquid nitrogen and transferred to -80 °C for storage prior to freeze drying.

-20 °C and -80 °C were selected as the preliminary test freezing temperatures to assess the feasibility of this method primarily due to pragmatic reasons; namely the easy availability of freezers at these temperatures (maintaining a steady -15 °C, for example, not being possible with the available laboratory equipment). Had the chlorophyll extraction method been further explored, so would avenues of achieving other freezing temperatures. The main purpose of the initial study was to identify if it was possible to easily perform chlorophyll extractions and clearly identify between living and dead tissue. However, there was also some basis in biology for selecting these temperatures: Previous studies found that ~-15°C is a temperature at which *Calluna vulgaris* is freezing tolerant during months in which it is likely to be acclimated (Sæbø et al., 2001, Caporn et al., 1994), however the sample selected for chlorophyll extraction was likely to experience temperatures below -15 °C in its natural environment (WeatherOnline, 1999-2018, Met-Office, 1959-2018). Therefore, -20°C was deemed an appropriate test temperature. Empetrum nigrum has been shown to withstand ~-15 °C prior to acclimation (Yamori et al., 2005) and survive at -80 °C (Yamori et al., 2005) post acclimation, therefore -80 °C was chosen as a second freezing temperature.

#### 2.4.1.2 Chlorophyll Extraction

Chlorophyll content of *Empetrum nigrum* and *Calluna vulgaris* samples was calculated using a modified method from Monni et al. (2001). Samples for chlorophyll extraction were frozen in liquid nitrogen and stored in a -80 °C freezer prior to freeze drying. Freeze dried material was then ground and separated into 10 mg aliquots and 3 ml of 80 % acetone was added and stored overnight at 4 °C. The solution was transferred to a 5 ml Eppendorf and spun at 3900 G for 3 minutes in a Beckman Coulter Allegra X-22R Centrifuge. Supernatant was transferred into cuvettes and the absorbance measured on a Boeco S-20 spectrophotometer at 647 and 664 nm. If absorbance was too high samples were diluted in 80 % acetone and the equations adjusted accordingly. Chlorophyll *a* and *b* (µmol  $1^{-1}$ ) were calculated using the equations, based on MacKinney's coefficient (Graan and Ort, 1984); Chlorophyll *a* (µmol  $1^{-1}$ ) = 13.19×A<sub>664</sub>-2.57×A<sub>647</sub>, and chlorophyll *b* (µmol  $1^{-1}$ ) = 22.10×A<sub>647</sub>-5.26×A<sub>664</sub>. The results were then expressed as chlorophyll content per gram of tissue dry weight.

# 2.5 Primers & Gene Blocks

# 2.5.1 Primer Design

A full list of primers designed and used in this project can be found in table 2.5.1a-g. Standard pre-existing primers were used for qPCR from *Arabidopsis thaliana* and are therefore not included in the table.
Table 2.5.1a. List of primers designed and used in this project. Showing Primer number code, full primer name (containing species, genus or family designed for), primer sequence and the direction in which the primer primes.

Primer			
Number	Primer Name	Primer Sequence	Direction
5	Fv-CBF-F	ATGGATGGTGGTTTCTCAGCTTTGT	Forward
6	Fv-CBF-R	CTAAATGGAGTAACTCCATAGTGACACATCA	Reverse
7	Rumex-CBF-F	ATGGAAGAATTTTCAGCTGATATCTACAAGCC	Forward
8	Rumex-CBF-Rev	TCAGAAGCTCCAGAGTGGTAAGTCC	Reverse
9	Ranu-CBF-F	ATGGATTATTCGCAGTACGGTTATCCTTCT	Forward
10	Ranu-CBF-R	TTAATCGTTCCACAAGGACAACTCCATG	Reverse
		ATGTTTACTATGAATCAATTTTCGGAGTCACATGA	
11	Trifolieae-SB-CBF-F	Т	Forward
	Lamiaceae-	ATGAACAATAAAGACCCTTTATTAGATAGGGATTC	
12	KC776910.1-CBF-F	ATTAACT	Forward
13	Trifolieae-SC-CBF-F	ATGAATCAATTTTCGGAGTCACATGATCCA	Forward
		ATGGATATGTTTACAAATAATAACTCCTATTCACAC	
14	Trifolieae-GA-CBF-F	ССС	Forward
	Asteraceae-GD-	ATGAACATGAATGAATCTTATAACTCTTTTCCGTCT	
15	CBF-F	TC	Forward
16	Asteraceae-GA-	ATGAACATGRATAWGWACGCYTCCWACR	Forward
10		ΔΤGGACKTGTCTSAACTTTCCGAC	Forward
10			Forward
10			Forward
19	Rosaceae-SA-CBF-F	AIGGAIGGIGGAIIIICAGCIIICCC	Forward
20	Caryophynales-GB- CBF-F	ATGGCCTCTTTCGATTACGACAAAGC	Forward
21	Asteraceae-GE-	ATGRCTACTTTTATCCAATTTAATGCTGGGAATATT	Forward
		ATGGATTTCTTCATGTCATCATTTAGTGATTACTCA	Torward
22	Trifolieae-SA-CBF-F	GA	Forward
	Asteraceae-GC-	ATGRATAYCTTTATCGAAWCMYATAWCCCATTTC	
23	CBF-F	CW	Forward
	Lamiaceae-	ATGGATTCATTAGGTTCATGTTCTGATCCAAATC	
24	HQ443516.1-CBF-F		Forward
25	Asteraceae-SA-CBF-	ATGGATATGTACGCCTCCAACAAATCTTATAAC	<b>F</b>
25			Forward
26	Trifolieae-GC-CBF-F		Forward
27	Caryophyliales-GA- CBF-F	ATGGCTGCTGCAATAGATACATATAGTAGCT	Forward
28	Caryophyllales-SA- CBF-F	ATGGCTACAGCTATTGATATTTACAGTGGT	Forward
29	Ericaceae-CBF-F	ATGGAATATWACTCWAGTCCACATTCCGGT	Forward
30	Trifolieae-GB-CBF-F	ATGTTTMCWWCWAASAACYCTTCCTMTTCACA	Forward
31	Rosaceae-SC-CBF-F	ATGAATACGATCTTCAGTCAAATCTCCGA	Forward
	Lamiaceae-		
32	KC776908.1-CBF-F	ATGGACATGTTCTCAAACTCGTCTGATTC	Forward
33	Rosaceae-GF-CBF-F	ATGAACAGGTTCTTCTCTCATTTTTCTGACT	Forward
34	Rosaceae-SB-CBF-F	ATGAATACGATCTTCAGTACTCAACTCTCCG	Forward

Table 2.5.1b. List of primers designed and used in this project. Showing Primer number code, full primer name (containing species, genus or family designed for), primer sequence and the direction in which the primer primes.

Primer			
Number	Primer Name	Primer Sequence	Direction
35	Lamiaceae- AY536056.1-CBF-F	ATGGCAGTTTATGATCAGAGTGGAGATAGA	Forward
36	Rosaceae-GE-CBF-F	ATGGATWTGATCTACAGCCAGGTCTCTG	Forward
37	Rosaceae-GA-CBF-F	ATGGATGSTTKCTCTMATTACTACGTGGA	Forward
38	asteraceae-GB- CBF-F	ATGGCTACCCTTATCCMATTCAACACT	Forward
39	Rosaceae-GB-CBF-F	ATGGATGGTTTTTGTCCTTACTACGTCG	Forward
40	Rosaceae-GG-CBF-F	ATGRACACRATCTCCAGACAACTCT	Forward
41	Trifolieae-GD-CBF-F	ATGTWTACWARTAACAAYGGGGAGGGG	Forward
42	Caryophyllales -SB- CBF-F	ATGTGTGGAGGAGCAGTAATCTCC	Forward
43	Poeae-FpB-CBF-F	ATGTGTCCCATCAAGAAAGAGATGAGC	Forward
44	Caryophyllales-SD- CBF-F	ATGAGCTACTCATACCCACCTCCAC	Forward
45	Rosaceae-GD-CBF-F	ATGGACATGTTCTYCKCTCARC	Forward
46	Rosaceae-SD-CBF-F	ATGCTTGAATCGTCGTCGCA	Forward
47	Poeae-SaA-CBF-F	ATGTGCGGGATCAAGCGAGA	Forward
48	Poeae-As-CBF-F	ATGGACATGASCGGCTCSG	Forward
49	asteraceae-SB-CBF- F	ATGGATATCGAATCACACTACCAACATCAAGA	Forward
50	Lamiaceae- KC776909.1-CBF-F	ATGGCGTTCACTGGCTGC	Forward
51	Caryophyllales-SD- CBF-R	TTAAGATGATGATGATAATATAGCATCCCAATCAA TCTCA	Reverse
52	Poeae-SaFp-CBF-R	CTAGCTCTGGTAGCTCCAGAGC	Reverse
53	Rosaceae-GF-CBF-R	TYAMTTTGAGAAGCTCCACAGAYTGAC	Reverse
54	Rosaceae-GA-CBF- R	CTAAATGGARWAACTCCAYARTGRCACGT	Reverse
55	Rosaceae-GE-CBF-R	TTAAWYSGAAWAACTCCATARKGSCACGT	Reverse
56	Trifolieae-GD-CBF- R	TCAAACTGAAAAACTCCATAGTGACACCTC	Reverse
57	Lamiaceae- KC776909.1+KC776 908.1-CBF-R	TCARAWTGAGTAACTCCAWARTGACACGTC	Reverse
58	Rosaceae-GB-CBF-R	TCAGATAGAGAARCTCCAYARBTTGGVAT	Reverse
59	Lamiaceae- HQ443516.1-CBF-R	TCAAATAGAATAGCTCCAAAGCTCCATCTCA	Reverse
60	Rosaceae-GD-CBF- R	TTAAWTGGAGAAACTCCACAAKYTGAYATCAG	Reverse
61	Rosaceae-GC-CBF-R	TCAAATAGWAAAACTCCACAGTTTCAAATCATCAT CA	Reverse
		TCAAATAGAGAAGTTCCAAAGTGAAACTAATTGA	
62	Trifolieae-SB-CBF-R	GTATCA	Reverse

Table 2.5.1c. List of primers designed and used in this project. Showing Primer number code, full primer name (containing species, genus or family designed for), primer sequence and the direction in which the primer primes.

Primer			
Number	Primer Name	Primer Sequence	Direction
		TTAAAATGAGTAACTCCACAATGAAACTTCAGTTT	
63	Trifolieae-GB-CBF-R	CA	Reverse
	Caryophyllales-GA-	ΤΤΛΛΛΑΘΑΛΑΤΟΟΛΑΤΟΛΑΤΤΤΟΛΑΘΟΘΛΑΘ	
64	CBF-R		Reverse
65	Rosaceae-SA-CBF-R	TTATGAGAAGCTCCACAGACTGACGT	Reverse
	Caryophyllales-SB-	TCACAACACTCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	
66	CBF-R		Reverse
67	Poeae-As2-CBF-R	TCAGTAGCTCCAGAGCGCG	Reverse
68	Poeae-As1-CBF-R	TCAGCTCCTCCACCGTCTC	Reverse
	Lamiaceae-		
69	KC776910.1-CBF-R		Reverse
	Asteraceae-SaSA-		
70	CBF-R		Reverse
	Asteraceae-SA-CBF-		
71	R		Reverse
72	Trifolieae-SA-CBF-R	TCAGAAGTTCCATAGCGATACCTCTTCA	Reverse
	Asteraceae-GA-		
73	CBF-R		Reverse
	Caryophyllales-GB-	TTACATACCCACTTCATCAACATCCACAAA	
74	CBF-R		Reverse
	Lamiaceae-		
75	AY536056.1-CBF-R	TTAGTTCTCCAGATCCAAGTAACTCAAGTCG	Reverse
	Asteraceae-SaGB-	ΤΥΛΡΛΛΛΓΤΟΓΛΤΛΛΤΩΛΝΑΡΟΡΤΟΛΛΟΛΝΑΝΛΛΛ	
76 CBF-R			Reverse
	Caryophyllales-SA-		
77	CBF-R	TCAATCGATCTCCATAGACGGAAAATTATCCA	Reverse
78	Trifolieae-GC-CBF-R	TTAGAAGTTCCATAGKGATACCTCWTCATCTTGA	Reverse
	Asteraceae-GB-	TCAAAAACTCCATAATGACACRTCAACACTAAATT	
79	CBF-R	C	Reverse
	Asteraceae-SaSB-	CTAGAAACTCCATAAAGGTAAGTCAACACAGAATT	
80	CBF-R	С	Reverse
	Asteraceae-SvGB-	TTAGAAACTCCATAATGAAGCGTCAACAAAAAATT	
81	CBF-R	C	Reverse
	Trifolieae-GA-CBF-	TTAAAAAYTCCAYAGTRATACYTCYTCATCTTGWA	_
82	R	ARTCT	Reverse
83	Ericaceae-CBF-R	CTAACTCCACAACGAGACCTCAACATC	Reverse
	Salicaceae-GA-CBF-		
84	F	ATGGATGTTTTCTGTAGTTATTCTGATCAGAACC	Forward
	Salicaceae-GA-CBF-		
85	R	TYAAACAGAAAAACTCCATAATGACATGTCDGTG	Reverse
	Salicaceae-SA-CBF-	ATGGGTACTCTTGATCAATATTCTAAAGCTACTTCT	_ ·
86		AIG	Forward
	Salicaceae-SA-CBF-		
87	K		Reverse
	Salicaceae-GB-CBF-		
88	F	AIGGICHIGGSAGGAICAAAIICSI	Forward

Table 2.5.1d. List of primers designed and used in this project. Showing Primer number code, full primer name (containing species, genus or family designed for), primer sequence and the direction in which the primer primes.

Primer			
Number	Primer Name	Primer Sequence	Direction
	Salicaceae-GB-CBF-		
89	R	TTAACTACTCCACAACGACCAATCAGC	Reverse
90	Salicaceae-SB-CB-F	ATGGAATTTGAGAATCACTCACCATCAATATCTTC	Forward
91	Salicaceae-SB-CB-R	CTAGTCCTCCCCATAATGTCAAGTCC	Reverse
	Salicaceae-SC-CBF-		
92	F	ATGCCCAATGACAGGCAAGAAG	Forward
	Salicaceae-SC-CBF-	CTAATAACTCCATAACGACAAATCAGAACCATTCT	
93	R	С	Reverse
	Salicaceae-SD-CBF-		
94	F	ATGTGTTTATTGTGTCCAACATATGTAACTTCATGT	Forward
	Salicaceae-SD-CBF-		
95	R	TCACTGAGAATCTAAGTAGTCGTCCTCC	Reverse
96	Salicaceae-SE-CB-F	ATGCTAGCTTCGAGGAATCCGAAG	Forward
		CTATATAGAAAAACTCCATAATGGCATATCAGCGT	
97	Salicaceae-SE-CB-R	С	Reverse
98	Corylus-CBF-F	ATGGATATCTTCGCCCGATACTCAGA	Forward
		TTAGATTGAGAAACTCCATAAAGAAACTTCATCAG	
99	Corylus-CBF-R	С	Reverse
100	Betula-CBF-F	ATGGATGTTTTCTCYCAATATTCGTSGGA	Forward
101	Betula-CBF-R	TCAAATTGAGTAACTCCACAATGAAACKTCG	Reverse
	Brassicaceae-GA-		
102	CBF-F	ATGGATCCATTTTACACTTCTTTCTCAGACT	Forward
	Brassicaceae-SA-		
103	CBF-F	ATGAACTCACTTTCTACTATGTTTGGCTCC	Forward
	Brassicaceae-GB-		
104	CBF-F	ATGASCTCATTYTCTRCYTTYTCTGAAMTGW	Forward
	Brassicaceae-GC-		
105	CBF-F		Forward
100	Brassicaceae-GD-		Formerad
106	CBF-F		Forward
107		ΑΤΩΑΛΟΤΟΑΤΩΑΤΩΤΟΤΟΤΩΟΤΤΟΤΟΤΩΑ	Forward
107	CDF-F		FOIWAIU
109		ΑΤΩΑΛΟΤΟΛΩΤΟΤΟΤΑΟΤΟΤΤΙΟΤΩΑΛΩΤΤΟΤΩ	Forward
100	Brassicaceae-SD-		TOTWARd
109	CRF-F	ATGGTTGGCTCCGAGAACGAG	Forward
105	Brassicaceae-GF-		
110	CBF-F	ATGGATAACWAYGATGRKAYTAWKCTGGCG	Forward
	Brassicaceae-GE-		
111	CBF-F	ATGGAAAACRACRACAWTKYCYTGGC	Forward
	Brassicaceae-SA-		
112	CBF-R	TTAATAGTTGTAACTCCACAGTGACATATCTTGGT	Reverse
	Brassicaceae-GA-		
113	CBF-R	TTARTAACKGTAACTCCASAGTGAYRTRTCTTCA	Reverse

Table 2.5.1e. List of primers designed and used in this project. Showing Primer number code, full primer name (containing species, genus or family designed for), primer sequence and the direction in which the primer primes.

Primer			
Number	Primer Name	Primer Sequence	Direction
	Brassicaceae-SB-		
114	CBF-R	TTAGTAACTCCAAAGTGACAGCTCTTCGT	Reverse
	Brassicaceae-SC-		
115	CBF-R	TTAATAGCTCCATAAGGACACGTCATCATCA	Reverse
	Brassicaceae-SD-		
116	CBF-R	TCAGATTCTCCAAAGGGACACGT	Reverse
	Brassicaceae-GB-		_
117	CBF-R	CTARWAACTCCARAGKGACACGTCAG	Reverse
	Brassicaceae-GC-		_
118	CBF-R		Reverse
110	Brassicaceae-GD-	TTAATACCTCCATAACCACACCTCATCATCT	Daviana
119			Reverse
120		TTAATABCTCCATAASCAVACCTCDTCATCIAW	Boyorco
120	CDF-R Brassicacoao GE		Reverse
121	CRE-R	ΤΤΛΕΤΛΛΟΤΟΟΛΛΕΟΕΛΟΛΟΕΤΟΛΟ	Roverse
121	Brassicaceae-GG-		Neverse
122	CBF-R	TTAATAAYTCCAWAGCGACAVRTCATCWYCR	Reverse
	Brassicaceae-GH-		neverse
123	CBF-R	TTAATAACTCCAWAGGGRCAYGTCRGC	Reverse
	Brassicaceae-SE-		
124	CBF-R	CTATTCTTCGTAACACCTTTCTTCTTCCACG	Reverse
	Rosaceae CBF		
125	central region fwd1	GCBGGDMGGARRRDDTT	Forward
	Rosaceae CBF		
126	central region fwd2	GCBGGDMGGARRRDDTTYMR	Forward
	Rosaceae CBF		
127	central region Rev	NGAGTCNGSRAARTTNADVCA	Reverse
128	SAH control 1 (fwd)	TGC AAC ATM TTC TCM ACY AGG	Forward
129	SAH control 2 (rev)	TTR TCA AAC TTG CTC TTG GTR AC	Reverse
130	Ribosomal_S11b_F	ATGGCNGAVCARACNGARARRKC	Forward
	Ribosomal_S11b_O		
131	L06G01490_F	ATGGCTGCCGAGCAACAC	Forward
	Ribosomal_S11b_O		
132	T06G01700_F	ATGCGCGACGCGCGAAACC	Forward
	Ribosomal_S11b_		
133	Micromonas_F	ATGGCGGCTGAGCAGACT	Forward
134	Ribosomal_S11b_R	GGCCTGCAYTGNCCRATRVT	Reverse
	Ribosomal_S11b_al		
135	gal_R	TTGAADCGRACRGTCTTSGASARV	Reverse
		GCGCGCGGCCGCATAACAATGAACTCATTTTCTGC	
136	ATCBF2_TOPO_F		Forward
4.0-		GCGCGGCGCGCCTTAATAGCTCCATAAGGACACG	Datast
137	AICBEZ_TOPO_R		Keverse
138	LUC-F	GCGCCATTCTATCCGCTAGAGGA	Forward

Table 2.5.1f. List of primers designed and used in this project. Showing Primer number code, full primer name (containing species, genus or family designed for), primer sequence and the direction in which the primer primes.

Primer			
Number	Primer Name	Primer Sequence	Direction
139	LUC-R	GCTGCGAAATGTTCATACTG	Reverse
140	VrCBF4-F	GATTCCCTGAATTTGCCTGA	Forward
141	VrCBF4-R	ATGTCATCTCCACCGTAGCC	Reverse
142	GUSPLUS-F	CCGTCCCAAGCAGTTACAAT	Forward
143	GUSPLUS-R	TTCGGAATCTCCACGTTACC	Reverse
	FiLUC outward p-		
144	check Rev	GCCTTATGCAGTTGCTCTCC	Reverse
	GUS+ outward p-		
145	check Rev	AGTGTCGGTCAGCTTGCTTT	Reverse
	RiLUC outward p-		
146	check Fwd	ACCACTGCGGACCAGTTATC	Forward
	vrCBF4 outward p-		
14/	check Fwd		Forward
1.40	VrCBF4 outward		Devenue
148	sequencing rev		Reverse
140	RILUC Outward		Boyorco
149	Sequencing rev		Reverse
150	sequencing fwd		Forward
150	hpoCPE1EW/D		Forward
151			Forward
152	bpeCBF2FWD		Polivaru
153			Reverse
154			Forward
155	At CBF1 REV		Reverse
156	VrCBF4 fwd	ATGAATACTTCTCCACCATATTCCGACC	Forward
157	VrCBF4 Rev	CTAAATAGAGTAACTCCATAACGACATGTCAGC	Reverse
	26. Trifolieae-GC-	GCGCGCGGCCGCATAACAATGTATCCAWCTAMM	
26T	CBF-F-TOPO		Forward
207	30. Trifolieae-GB-		E a museu al
301	LBF-F-TUPU		Forward
1/1			Forward
141	15 Asteraceae-GD-		FUIWalu
15T	CBE-E-TOPO		Forward
1.51	56 Trifolieae-GD-		TOTWATA
56T	CBF-R-TOPO	GACACCTC	Reverse
	78. Trifolieae-GC-	GCGCGGCGCGCCTTAGAAGTTCCATAGKGATACCT	
78T	CBF-R-TOPO	CWTCATCTTGA	Reverse
	82. Trifolieae-GA-	GCGCGGCGCGCCTTAAAAAYTCCAYAGTRATACYT	
82T	CBF-R-TOPO	CYTCATCTTGWAARTCT	Reverse
	73. Asteraceae-GA-	GCGCGGCGCGCCCTARAAGTTCCATARVGACATG	
73T	CBF-R-TOPO	TYAGCACA	Reverse

Table 2.5.1g. List of primers designed and used in this project. Showing Primer number code, full primer name (containing species, genus or family designed for), primer sequence and the direction in which the primer primes.

Primer			
Number	Primer Name	Primer Sequence	Direction
	29. Ericaceae-CBF-	GCGCGCGGCCGCATAACAATGGAATATWACTCW	
29T	F-TOPO	AGTCCACATTCCGGT	Forward
	83. Ericaceae-CBF-	GCGCGGCGCGCCCTAACTCCACAACGAGACCTCA	
83T	R-TOPO	ACATC	Reverse
	22. Trifolieae-SA-	GCGCGCGGCCGCATAACAATGGATTTCTTCATGTC	
22T	CBF-F-TOPO	ATCATTTAGTGATTACTCAGA	Forward
	62. Trifolieae-SB-	GCGCGGCGCGCCTCAAATAGAGAAGTTCCAAAGT	
62T	CBF-R-TOPO	GAAACTAATTGAGTATCA	Reverse
158	CV EN MATK FWD	KAATTTASGATCAATTCATTCAATATTTCC	Forward
159	CV EN MATK REV	AAKGATCCGCTATGATAATGAGAAAKATTT	Reverse
	FWD ERICALES		
160	INTERNAL (3)	CACCCVRTDTACMGVGGWGT	Forward
	FWD ERICACEAE		
161	INTERNAL (3)	CACCCVRTYTACAGAGGMGT	Forward
	REV ERICALES		
162	INTERNAL (8)	CAMRCYGARTCNGCRAA	Reverse
	REV ERICACEAE		
163	INTERNAL (8)	CACACYGAGTCKGCRAA	Reverse
	fwd Empetrum		
164	specific	GAGACGATGATAACACTGGCTTC	Forward
	Rev Empetrum		_
165	specific		Reverse
166	E.nigrumCBFRTa_F	AAGCCAGCCGGAAGAAAGAA	Forward
167	E.nigrumCBFRTa_R	CCTCGACTTCTTGTTCGGCT	Reverse
168	E.nigrumCBFRT7_F	AGCGTTATGGTTTCCCCTGG	Forward
169	E.nigrumCBFRT7_R	CCCTTCCGCCAATGTCAAAA	Reverse
170	RPB2Ia_F	CAACAGGTTGCGCTTGATTA	Forward
171	RPB2Ia_R	TCTTTGGCATACCTGATCCTC	Reverse
172	RPB2Ib_F	GTTGACAACGGGAAGGATGT	Forward
173	RPB2Ib_R	TTGTCCCCAGTTCCCAGTAG	Reverse
174	35S FWD	ACGTAAGGGATGACGCACAATCCCAC	Forward

#### **2.5.1.1 Standard Primers**

The Encyclopaedia of Life (EOL, 2014) was used to find the full NCBI taxonomic classification for each species collected and each classification was recorded in MS Excel. These data were then used to perform NCBI nucleotide searches (Geer et al., 2010) to find the nearest relative(s) of these samples with already sequenced CBF genes. Initial searches were performed using the actual species then progressively higher taxa with the search terms \*CBF\*, DREB1, \*DREB\* and C-Repeat Binding Factor. A BLASTn (Basic Local Alignment Tool) (Altschul et al., 1990) search was then performed on the nucleotide sequences of any results to identify further relatives with sequenced CBF genes within the database. These sequences were then recorded in a Microsoft word 2010 document along with, where present, the protein translation. From these sequences, only those sequences with full coding regions and that were identified as *CBF* genes, rather than other similar sequences, were selected. For each sample the coding sequences of the nearest relatives were imported into Bioedit Sequence Alignment Editor version 7.1.7 (Hall, 1999). Separately the 5' and 3' ends of the sequences were manually aligned and then grouped to create a number of consensus sequences. Groups were based on similarity so that primers designed from the consensus sequence would not contain more than 7 degenerate bases (example: Fig. 2.5.1.1.1). SnapGene version 2.4, using default settings, was used to create primers from these consensus sequences. Since the sequences which were grouped to form a particular forward primer did not necessarily group together to form a reverse primer a list of primer pairings had to be produced. This was performed by recording which forward and reverse primers went together for each sequence. Therefore, only those primer pairs which could have amplified some of the sequences they were designed from were run together with the collected samples. The following is a list of primer pair codes and the two primers which make up that pair (see table 2.5.1a-g) that were used for attempted isolation of *CBF* from members of the named taxon:

Primer pair 1: 11. Trifolieae-SB-CBF-F + 63. Trifolieae-GB-CBF-R

Primer pair 2: 12. Lamiaceae- KC776910.1-CBF-F + 69. Lamiaceae-KC776910.1-CBF-R

Primer pair 3: 13. Trifolieae-SC-CBF-F + 63. Trifolieae-GB-CBF-R

Primer pair 4: 14. Trifolieae-GA-CBF-F + 82. Trifolieae-GA-CBF-R

Primer pair 5: 15. Asteraceae-GD-CBF-F + 73. Asteraceae-GA-CBF-R

Primer pair 6: 16. Asteraceae-GA-CBF-F + 73. Asteraceae-GA-CBF-R

Primer pair 7: 17. Rosaceae-GH-CBF-F + 60. Rosaceae-GD-CBF-R

Primer pair 8: 18. Rosaceae-GC-CBF-F + 58. Rosaceae-GB-CBF-R

Primer pair 9: 19. Rosaceae-SA-CBF-F + 54. Rosaceae-GA-CBF-R

Primer pair 10: 20. Caryophyllales-GB-CBF-F + 74. Caryophyllales-GB-CBF-R

Primer pair 11: 21. Asteraceae-GE-CBF-F + 76. Asteraceae-SaGB-CBF-R

Primer pair 12: 21. Asteraceae-GE-CBF-F + 79. Asteraceae-GB-CBF-R

Primer pair 13: 22. Trifolieae-SA-CBF-F + 62. Trifolieae-SB-CBF-R

Primer pair 14: 23. Asteraceae-GC-CBF-F + 70. Asteraceae-SaSA-CBF-R

Primer pair 15: 23. Asteraceae-GC-CBF-F + 80. Asteraceae-SaSB-CBF-R

Primer pair 16: 24. Lamiaceae- HQ443516.1-CBF-F + 59. Lamiaceae-HQ443516.1-

CBF-R

Primer pair 17: 25. Asteraceae-SA-CBF-F + 73. Asteraceae-GA-CBF-R

Primer pair 18: 7. Rumex-CBF-F + 8. Rumex-CBF-Rev

Primer pair 19: 26. Trifolieae-GC-CBF-F + 82. Trifolieae-GA-CBF-R

Primer pair 20: 27. Caryophyllales-GA-CBF-F + 64. Caryophyllales-GA-CBF-R

Primer pair 21: 28. Caryophyllales-SA-CBF-F + 77. Caryophyllales-SA-CBF-R

Primer pair 22: 29. Ericaceae-CBF-F + 83. ericaceae-CBF-R

Primer pair 23: 30. Trifolieae-GB-CBF-F + 56. Trifolieae-GD-CBF-R

Primer pair 24: 30. Trifolieae-GB-CBF-F + 78. Trifolieae-GC-CBF-R

Primer pair 25: 31. Rosaceae-SC-CBF-F + 65. Rosaceae-SA-CBF-R

Primer pair 26: 32. Lamiaceae- KC776908.1-CBF-F + 57.Lamiaceae-

KC776909.1+KC776908.1-CBF-R

Primer pair 27: 33. Rosaceae-GF-CBF-F + 60. Rosaceae-GD-CBF-R

Primer pair 28: 34. Rosaceae-SB-CBF-F + 73. Rosaceae-GF-CBF-R

Primer pair 29: 35. Lamiaceae- AY536056.1-CBF-F + 75. Lamiaceae-AY536056.1-CBF-R

Primer pair 30: 36. Rosaceae-GE-CBF-F + 61. Rosaceae-GC-CBF-R

Primer pair 31: 9. Ranu-CBF-F + 10.Ranu-CBF-R

Primer pair 32: 37.Rosaceae-GA-CBF-F + 55. Rosaceae-GE-CBF-R

Primer pair 33: 37.Rosaceae-GA-CBF-F + 54. Rosaceae-GA-CBF-R

Primer pair 34: 38. asteraceae-GB-CBF-F + 76. Asteraceae-SaGB-CBF-R

Primer pair 35: 38. asteraceae-GB-CBF-F + 81. Asteraceae-SvGB-CBF-R

Primer pair 36: 39. Rosaceae-GB-CBF-F + 55. Rosaceae-GE-CBF-R

Primer pair 37: 40. Rosaceae-GG-CBF-F + 73. Rosaceae-GF-CBF-R

Primer pair 38: 41.Trifolieae-GD-CBF-F + 72. Trifolieae-SA-CBF-R

Primer pair 39: 41.Trifolieae-GD-CBF-F + 78. Trifolieae-GC-CBF-R

Primer pair 40: 42. Caryophyllales -SB-CBF-F + 66. Caryophyllales-SB-CBF-R

Primer pair 41: 43.Poeae-FpB-CBF-F + 52. Poeae-SaFp-CBF-R

Primer pair 42: 44. Caryophyllales-SD-CBF-F + 51. Caryophyllales-SD-CBF-R

Primer pair 43: 45. Rosaceae-GD-CBF-F + 58. Rosaceae-GB-CBF-R

Primer pair 44: 46. Rosaceae-SD-CBF-F + 60. Rosaceae-GD-CBF-R

Primer pair 45: 47.Poeae-SaA-CBF-F + 52. Poeae-SaFp-CBF-R

Primer pair 46: 48.Poeae-As-CBF-F + 67.Poeae-As2-CBF-R

Primer pair 47: 48.Poeae-As-CBF-F + 68. Poeae-As1-CBF-R

Primer pair 48: 49. asteraceae-SB-CBF-F + 71. Asteraceae-SA-CBF-R

Primer pair 49: 50. Lamiaceae- KC776909.1-CBF-F + 57. Lamiaceae-

C776909.1+KC776908.1-CBF-R

Primer pair 50: 5. Fv-CBF-F + 6. Fv-CBF-R

Primer pair 51: 84. Salicaceae-GA-CBF-F + 85. Salicaceae-GA-CBF-R

Primer pair 52: 86. Salicaceae-SA-CBF-F + 87. Salicaceae-SA-CBF-R

Primer pair 53: 88. Salicaceae-GB-CBF-F + 89. Salicaceae-GB-CBF-R

Primer pair 54: 90. Salicaceae-SB-CB-F + 91. Salicaceae-SB-CB-R

Primer pair 55: 92. Salicaceae-SC-CBF-F + 93. Salicaceae- SC-CBF-R

Primer pair 56: 94. Salicaceae-SD-CBF-F + 95. Salicaceae-SD-CBF-R

Primer pair 57: 96. Salicaceae-SE-CB-F + 97. Salicaceae-SE-CB-R

Primer pair 58: 98. Corylus-CBF-F + 99. Corylus-CBF-R

Primer pair 59: 100. Betula-CBF-F + 101. Betula-CBF-R

Primer pair 60: 102. Brassicaceae-GA-CBF-F + 117. Brassicaceae-GB-CBF-R

Primer pair 61: 103. Brassicaceae-SA-CBF-F + 122. Brassicaceae-GG-CBF-R

Primer pair 62: 104. Brassicaceae-GB-CBF-F + 117. Brassicaceae-GB-CBF-R

Primer pair 63: 104. Brassicaceae-GB-CBF-F + 116. Brassicaceae-SD-CBF-R

Primer pair 64: 104. Brassicaceae-GB-CBF-F + 123. Brassicaceae-GH-CBF-R

Primer pair 65: 105. Brassicaceae-GC-CBF-F + 122. Brassicaceae-GG-CBF-R

Primer pair 66: 106. Brassicaceae-GD-CBF-F + 119. Brassicaceae-GD-CBF-R

Primer pair 67: 106. Brassicaceae-GD-CBF-F + 120. Brassicaceae-GE-CBF-R

Primer pair 68: 106. Brassicaceae-GD-CBF-F + 123. Brassicaceae-GH-CBF-R

Primer pair 69: 106. Brassicaceae-GD-CBF-F + 115. Brassicaceae-SC-CBF-R

Primer pair 70: 106. Brassicaceae-GD-CBF-F + 121. Brassicaceae-GF-CBF-R

Primer pair 71: 107. Brassicaceae-SB-CBF-F + 122. Brassicaceae-GG-CBF-R

Primer pair 72: 108. Brassicaceae-SC-CBF-F + 118. Brassicaceae-GC-CBF-R

Primer pair 73: 109. Brassicaceae-SD-CBF-F + 118. Brassicaceae-GC-CBF-R

Primer pair 74: 110. Brassicaceae-GE-CBF-F + 112. Brassicaceae-SA-CBF-R Primer pair 75: 110. Brassicaceae-GE-CBF-F + 113. Brassicaceae-GA-CBF-R Primer pair 76: 111. Brassicaceae-GF-CBF-F + 114. Brassicaceae-SB-CBF-R Primer pair 77: 111. Brassicaceae-GF-CBF-F + 124. Brassicaceae-SE-CBF-R Primer pair bpeCBF1: bpeCBF1FW + bpeCBF1,2REV

Primer pair bpeCBF2: bpeCBF2FWD + bpeCBF1,2REV

	-		1				1		1				1	
	•	10	20	30	40	50	60	70	80	90	100	110	120	130
gi	161897786 gb E	ATGGATATGTTTAC	AAATAATAACT	CCTATT	-CACACCCCTTTI	CTCCAA	CTTG	CTCCGAGAG	TTCATTCC	-CGAATTCAC	GAGGGCTCGCA	AGGGATGTCA	ATTTCCAAC	GAGGAGGTGAG
ği	525031623 gb K	ATGGATATGTTTAC	AAATAATAACT	CCTATT	-CACACCCCTTTT	CTCCAA	CTTG	CTCCGAGAG	TTCATTCC	-CGAATTCAC	SAGGGCTCGCA	AGGGATGTCA	ATTTCCAAC	GAGGAGGTGAG
Gr	oup A Consensus	ATGGATATGTTTAC	AAATAATAACT	CCTATT	-CACACCCC <mark>TTTT</mark>	CTCCAA	CTTG	CTCCGAGAG	TTCATTCC	-CGAATTCAC	SAGGGCTCGCA	AGGGATGTCA	ATTTCCAACO	GAGGAGGTGAG
gi	525031602 gb K	ATGTTTCC	ATCTAACAACC	CTTCCTCT	TCACATCCCTCTI	CCTCAAAA	CCTTCATCTTC	CTCACACAC	AATATCACTT	CCAAATTTAC	SAGGTCTCTCG	IGGGATGTTI	GTTACCAAC	GACGAGGTCCG
gi	525031604 gb K	ATGTTTCC	ATCAAAGAACC	CTTCCTCT	TCACAGCCCAGTI	CCTCAAAA	CCTTCATCTTC	CTCACACAC	AATATCACTT	CCAAATTTAC	SAGGTCTCTCG	TGGGATGTTI	GTTACCAAC	GACGAGGTCCG
gi	110559315 gb D	ATGTTTAC	TACTAACAACT	CTTCCTAT	TCACACTCCATTI	CCTCAGAA	GCATCTTCTTC	CTACTATAA	CTCATTAC	-CGGAATC				GGAGATACG.
Gr	oup B Consensus	ATGTTTMC	WWCWAASAACY	CTTCCTMT	TCACABYCCWBTI	CCTCARAA	SCWTCWTCTTC	CTMMYAYAM	MWYATYACTT	CCRRAWTYAC	SAGGTCTCTCG	IGGGATGTTI	GTTACCAAC	GASGAGRTMCG
gi	161897788 gb E		ATGTAT	CCAACTA-	-CAAACTCTGTCI	CTTCCT	CTTC	CTCCGACAT	GTCGCTGC	-CGAATTCAC	SAGGGCTCTCA	TTGGATGTCA	ATTTGCAACO	GAGGAGATGCG.
gi	82400105 gb DQ		ATGTAT	CCAACTA-	-CAAACTCTGTCT	CTTCCT	CTTC	CTCCGACAT	GTCGCTGC	-CGAATTCAC	SAGGGCTCTCA	TTGGATGTCA	ATTTGCAACO	GAGGAGATGCG.
đi	525031611 gb K		ATGTAT	CCAACTA-	-CAAACTCTGTCT	CTTCCT	CTTC	CTCCGACAT	GTCGCTGC	-CGAATTCAC	SAGGGCTCTCA	TTGGATGTCA	ATTTGCAACO	GAGGAGATGCG.
đi	525031609 gb K		ATGTAT	CCAACTA-	-CAAACTCTGTCT	CTTCCT	CTTC	CTCCGACAT	GTCGCTGC	-CGAATTCAC	SAGGGCTCTCA	TTGGATGTCA	ATTTGCAACO	GAGGAGATGCG.
đi	525031607 gb K		ATGTAT	CCAACTA-	-CAAACTCTGTCT	CTTCCT	CTTC	CTCCGACAT	GTCGCTGC	-CGAATTCAC	SAGGGCTCTCA	TTGGATGTCA	ATTTGCAACO	GAGGAGATGCG.
дı	525031615 gb K		ATGTAT	CCATCTA-	ACAACTCTGTCT	CTTCTT	CTTC	CTCCGACAT	GTCGCTGC	-CGAATTCAC	SAGGGCTCTCA	TTGGATGTCA	ATTTGCAACO	GAGGAGATGCG
Gr	oup C Consensus		ATGTAT	CCAWCTA-	AMCCAMMMOMMOZ	CTTCIT	mmma cmcamma	CTCCGACAT	MMCMMCAM	-CGAATTCAC	SAGGGCTCTCA	TTGGATGTCA		GAGGAGATGCG.
gı	35/481/44 ret				ATGGATTTCTTCA	TGTCATCA	TTTAGTGATTA	CTCAGACAC	TTCTTCAT	CAGAAACAC	CTTCATC	AAACCG1		TCAGAAGTGAT
se	quence A				ATGGATTTCTTCA	TGTCATCA	ATTAGTGATTA		AMCAAMMMM-		CTTCATC		ACAAGTTCT	CAGAAGTGAT
gı	302952542 gb H						AIGI	TIACIAIGA	ADCAAIIII	-CCCACTCAC	CATGATCC	>mcmmc	ATCATCITCA	ACACACCOMM-
se	quence B						AIGI	TIACIAIGA	ATCAATITI	-CCCACTCAC	CATGATCC	>mcmmc	ATCATCITCA	ACACACCOULT
gı	35/444088 rei							AUGA	ATCAATITI	-CGGAGICAC	CATGATCC		ATCATCITC	ACACACCTTT-
se	quence C							A10A	AIGAAIIII	COORGICA	SAIGAICC		ACTAICIICA	CCCCACCCCCC
gi	15250316291gb1K												AGIAACAAC(	CCCCACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
91	15250316331gb K												AATAACAAT	
gi	1222021833 gb K											ATCTINC	ARTAACAAI	CCCCACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
GI	oup D consensus											ATOTWIAC	ANTAGCART(	000080000000

Figure 2.5.1.1. Example of forward primer design for members of the Fabaceae from NCBI sequences in Bioedit. Consensus sequences from which primers were designed highlighted in green.

#### 2.5.1.2 Primers for Cloning

Primers that successfully amplified a product that we wished to take forward for cloning were re-ordered with a 5' sequence addition to add NOT1 (highlighted yellow) and ASC1 (highlighted pink) restriction sites and a translational initiation consensus sequence (green) to the PCR products. These additional sequences were GCGCGCGCGCGCATAACA and GCGCGCGCGCCC for the forward and reverse primer respectively.

#### 2.5.1.3 Primers for qPCR

Primers for qPCR from *Empetrum nigrum* RNA were designed using Primer3 to amplify a fragment of between 80 and 1200 base pairs in length. The *CBF* sequence isolated as per sections 2.5.1.1 and 2.7 was used as the template for the test gene. The two best sequences were then ordered, tested and the one that showed best results chosen for future qPCR reactions. The housekeeping gene for *Empetrum nigrum* was selected by searching all sequenced genes of *Empetrum nigrum* in NCBI. Only RPB2I was found to be a suitable housekeeping gene. Primers were then designed to exons in this sequence and tested; the one that worked reliably was then used in further experiments.

Existing qPCR primers for *Arabidopsis thaliana CBF*, *KIN2* and *LTI78* were used to investigate gene expression in *A. thaliana* plants.

## 2.5.2 Synthetic Gene Design

The CBF signature sequences PKKP/RAGRxKFxETRHP, identified as crucial for binding of CBF1 to the CRT/DRE element in *A. thaliana* by Canella et al., (2010), and DSAWR were compared between *A. thaliana* CBF 1-4 and sequenced samples. Any amino acid differences in collected sequences within these regions, relative to *A. thaliana* 

CBF sequences, were identified and noted. Single amino acid changes were then performed in *A. thaliana* CBF1 to match the amino acid sequence differences within these regions from collected sequences (See Table 2.5.2.1 for list of synthetic genes created and amino acid changes relative to *A. thaliana* CBF1). For sequences with several amino acid changes multiple modified *A. thaliana* CBF1 sequences were produced; sequences with just the singular version of each differing amino acid, then pairs (no more than two amino acid differences within each sequence were observed). A codon usage table was used to select the most appropriate codon for the new amino acid, namely a codon with the most similar usage to the native *A. thaliana* codon. Flanking sequences containing a translational initiation sequence, BAMHI, NOTI, SACI and ASCI cut sites were then added.

These modified *CBF* sequences under a 35S promoter were then ordered from Integrated DNA Technologies Inc. (Glasgow, UK). Gene blocks were supplied dehydrated and were then rehydrated in  $H_2O$  to form a 100  $\mu$ M stock

Table 2.5.2.1. The CBF signature sequences of the synthetic CBF genes created. Changes to *Arabidopsis thaliana* wild type CBF1 highlighted in yellow. No further changes to the CBF1 sequence were made. Short hand names for synthetic genes also shown. CBF WT is a synthetic copy of native *Arabidopsis thaliana* CBF1.

CBF WT	Ρ	к	К	Ρ	А	G	R	К	К	F	R	Е	Т	R	Н	Ρ	 D	S	А	w	R
РКК	Ρ	К	К	Ρ	А	G	R	К	К	F	К	Е	Т	R	н	Ρ	 D	S	А	w	R
DSAWQ	Ρ	к	К	Ρ	А	G	R	К	К	F	R	Е	Т	R	Н	Ρ	 D	S	А	w	Q
DSLWR	Ρ	К	К	Ρ	А	G	R	К	К	F	R	Е	Т	R	Н	Ρ	 D	S	L	w	R
DSVWQ	Ρ	к	К	Ρ	А	G	R	К	К	F	R	Е	Т	R	н	Ρ	 D	S	V	w	Q
DSVWR	Ρ	К	К	Ρ	А	G	R	К	К	F	R	Е	Т	R	Н	Ρ	 D	S	V	w	R

### **2.5.3 Primer Sequences**

Primers were ordered from FisherScientific (Loughborough, Leicestershire, UK) and Integrated DNA Technologies Inc. (Glasgow, UK). Primers were supplied dehydrated and were then rehydrated in  $H_2O$  to form a 100µM stock.

## 2.6 DNA and RNA Extraction

## **2.6.1 DNA Extraction**

#### **2.6.1.1 Edwards Extraction**

(From Edwards, Johnstone and Thompson, 1991)

Edwards Buffer (All chemicals used were supplied by Sigma-Aldrich Ltd. (Dorset, UK))

for 5 ml buffer:

1 ml of 1 M pH 7.5 Tris-HCl,

1.25 ml of 1 M NaCl,

0.25 ml of 0.5 M pH 8.0 EDTA,

0.25 ml of 10 % SDS,

 $2.25 \text{ ml of H}_2\text{O}.$ 

DNA was extracted from *Arabidopsis thaliana* using the Edwards extraction method. Samples were frozen in liquid nitrogen and then ground with a plastic micropestle in a 1.5 ml eppendorf tube. Samples were then vortexed using a Labnet vortex mixer VX100 for 5 seconds after the addition of 400  $\mu$ l of Edwards extraction buffer. The samples were then spun down in a microcentrifuge (eppendorf AG centrifuge 5415D) at full speed for one minute. 300  $\mu$ l of the supernatant was then transferred to a fresh 1.5 ml eppendorf and 300  $\mu$ l of isopropanol added and left at room temperature for 2 minutes. The samples were then spun at full speed in a microcentrifuge (eppendorf AG centrifuge 5415D) for 5 minutes and the supernatant removed, the remaining sample was respun for one minute and re-aspirated. The remaining pellet was then spun to dryness using a spin vacuum desiccator (Eppendorf concentrator 5301). The pellet was then resuspended in 30  $\mu$ l H<sub>2</sub>O a left to dissolve at 4 °C overnight.

#### 2.6.1.2 Powerplant® Pro DNA Isolation

DNA was extracted from all other samples using a modified version of the protocol supplied with PowerPlant ® Pro DNA Isolation Kit from Mo Bio Laboratories Inc, USA.

To labelled bead beating tubes 410  $\mu$ l of solution PD1 and 40  $\mu$ l of Phenolic separation solution were added. Frozen tissue samples were ground in a liquid nitrogen chilled mortar and pestle and added to the bead beating tubes (to a maximum amount of ground tissue of ~1 ml) along with 50  $\mu$ l of solution PD2 and 3  $\mu$ l of RNase A. Each tube was briefly mixed on a Labnet vortex mixer VX100 then heated at 65 °C for 10 minutes and shaken on a flat-bed vortex (Labnet VX100) at maximum speed for a further 10 minutes. Tubes were then spun at 13000 G for 2 minutes and the resulting supernatant removed to 1.5 ml Eppendorf tubes. To the supernatant 250 µl of solution PD3 was added, mixed for 5 seconds and then incubated at 4 °C for 5 minutes. After incubation the samples were spun at 13000 G for 2 minutes and a maximum of 600 µl supernatant, from each sample, was removed to new 1.5 ml Eppendorf tubes. To this supernatant 600 µl of solution PD4 and 600  $\mu$ l of solution PD6 were added and mixed for 5 seconds then ~600  $\mu$ l of the mix was loaded onto a spin filter and spun at 10,000 G for 30 seconds, flow through was discarded and  $\sim 600 \,\mu$ l more of the mix added to the spin filter and spun and so forth until all the mix was used. To each spin filter 500  $\mu$ l of solution PD5 was then added and they were spun at 10,000 G for 30 seconds and the flow through discarded. Then 500 µl of solution PD6 was added to each spin filter and they were spun at 10,000 G for 30 seconds

and the flow through discarded. Filters were spun again at 16,000 G for 2 minutes, to remove residual solution PD6 and then placed into new collection tubes. To elute the DNA, 50  $\mu$ l of 37 °C Nuclease free H<sub>2</sub>O was then added to the centre of each spin filter and incubated at room temperature for 2 minutes. After incubation spin filters were spun at 10,000 G for 30 seconds. The spin filter was then placed into another collection tube and the same elution procedure repeated. To check for DNA presence 2  $\mu$ l of the elution, mixed with 6  $\mu$ l H2O and 2  $\mu$ l loading dye was then run on an 8 % agarose gel. The first DNA elution was then stored at 4 °C and the second at -20 °C.

## 2.6.2 RNA Extraction

#### 2.6.2.1 RNA Extraction from Arabidopsis thaliana

RNA was extracted from using the Promega ReliaPrep<sup>TM</sup> RNA Tissue Miniprep system as per the protocol using the volumes for less than 5 mg of tissue (even when greater than 5 mg of tissue was used). RNA was then stored at -80 °C prior to cDNA synthesis.

#### 2.6.2.2 RNA Extraction from *Empetrum nigrum*

Numerous methods and kits were tried to extract RNA from *Empetrum nigrum* with various modifications to the protocols. This included QuiAgen RNeasy mini kit, Mo Bio Powerplant® RNA isolation kit and CTAB which all met with limited to no success. Ultimately RNA was extracted from *Empetrum nigrum* samples using a modified GeneAll Ribospin<sup>TM</sup> Plant protocol. Numerous adaptations were tried until the following modified protocol was selected:

Plant tissue was frozen in liquid nitrogen then ground in a liquid nitrogen chilled mortar. 100 mg was then weighed out into a 1.5 ml Eppendorf. 700  $\mu$ l of buffer RPL was added and the sample vortexed vigorously prior to incubation at room temperature for 3 minutes.

The sample was then transferred to the EzPure<sup>TM</sup> filter and centrifuged at 10,000 G for 30 s, the filter was then removed and discarded and the flow through centrifuged at 16,000 G for 2 minutes. The supernatant was then transferred to a new 1.5 ml Eppendorf, an equal volume of 70 % ethanol added, mixed via inversion the applied to a type W spin column (any clots seen in the solution were not transferred). The column was then spun at 10,000 G for 30 s and the flow through discarded. 500 µl of buffer RBW was added to the column, spun again at 10,000 G for 30 s and flow through discarded. 70 µl of DNase I mixture  $(2 \mu I DNase I + 70 \mu I buffer DRB)$  was then applied to the column and the column incubated at room temperature for 10 minutes. 500 µl of buffer RBW was then added and the column stood for a further 2 minutes before centrifugation at 10,000 G for 30s. Flow through was discarded and 500  $\mu$ l of buffer RNW was added prior to centrifugation at 10,000 G for 30 s this step was then repeated. A final spin at 10,000 G for 1 minute was then performed before transferal to a new collection tube. 30 µl RNase free water was then added to column and incubated at room temperature for 5 minutes before elution at 16,000 G for 1 minute. The elution step was then repeated for a second or third elution. RNA was then stored at -80 °C prior to cDNA Synthesis.

## 2.6.3 cDNA Synthesis

cDNA was synthesised from RNA using Applied Biosystems<sup>™</sup> High Capacity cDNA Synthesis Kit.

For each CDNA synthesis from *Arabidopsis thaliana* 1  $\mu$ g of RNA was used. For each CDNA synthesis (for different qPCR experiments) from *Empetrum nigrum* the amount of RNA in 10  $\mu$ l of the lowest concentration sample was used (0.4297  $\mu$ g for the timecourse, other concentrations were used for the various qPCR optimisation steps and testing of newly designed qPCR primers).

Per reaction to 10 µl of RNA and water (to desired amount of RNA) 4.2 µl of nuclease free water, 2 µl 10X RT buffer, 2 µl random primers, 0.8 µl 25X 100 mM DNTP mix and 1 µl multiscribe reverse transcriptase were added. For the no reverse transcriptase and no template controls the reverse transcriptase and template RNA, respectively, were replaced with an equal volume of nuclease free water. The samples were then placed in a Hybaid PCRExpress machine set at the following program: 25 °C 10 minutes, 37 °C 120 minutes, 85 °C 5 seconds, 4 °C Hold. cDNA was then stored at -20 °C.

## **2.7 PCR Amplification and Product Purification**

## **2.7.1 PCR Amplification**

#### 2.7.1.1 PCR Mixes

PCR mix 1

Per 50 µl rection:

39.5 µl, PCR H<sub>2</sub>O

5 µl, Bioline 10X buffer

1.5, 50 µM MgCl<sub>2</sub>

 $0.5 \ \mu$ l, 100  $\mu$ M fwd primer

 $0.5 \ \mu l$ ,  $100 \ \mu M$  rev primer

1 µl, 10 µM DNTPs

1 μl, DNA

1 µl, TaQ

#### PCR mix 2

Per 50 µl reaction:

25 µl KAPA plant PCR buffer + DNTps (2X) 1.5 mM Mg at 1X

 $1.5~\mu l~10\mu M$  Forward primer

1.5 µl 10µM Reverse primer

1 µl DNA

0.4 µl KAPA3G plant DNA polymerase

#### MYTAQ PCR

Per 50 μl reaction: 10 μl MyTaq Buffer (red) 0.2 μl 100 μM Forward primer 0.2 μl 100 μM Reverse primer 1 μl DNA

1 µl MyTaq DNA polymerase

## 2.7.1.2 PCR Conditions

Several different annealing step temperatures were used in the following PCR order to find the optimal temperature for the designed primers to anneal. A Thermo PX2 Thermal Cycler and a Hybaid PCR Express were used for PCR.

#### **PCR1-3**

1X:

94 °C, 2 minutes

40X:

94 °C, 30 seconds

X °C, 30 s (PCR1: 60-65 °C, PCR2: 48-58 °C, PCR3: 53 °C)

72 °C 45 seconds

1X:

72 °C 10 minutes

4 °C Hold

PCR 4 (KAPA3G)

1X:

95 °C 5 minutes

40X

95 °C 30 seconds

55 °C 15 seconds

72 °C 45 seconds

1X

72 °C 45 seconds

 $4 \,^{\circ}C$  hold

#### MY TAQ PCR

X1:

95 °C 1 minute

30X

95 °C 15 seconds

58 °C 15 seconds

72 °C 10 seconds

4 °C hold

## 2.7.2 Gels

## 2.7.2.1 TBE Gels

All chemicals used were supplied by Sigma-Aldrich Ltd. (Dorset, UK)

For 5X TBE stock:

54 g l<sup>-1</sup> Tris base,

27.5 g l<sup>-1</sup> Boric acid,

20 ml l<sup>-1</sup> 0.5M pH8 EDTA,

H<sub>2</sub>O to desired volume.

In a 500 ml conical flask, 0.5 g agarose (for PCR products) or 0.4 g agarose (for genomic DNA) was dissolved in 50 ml of 0.5X TBE buffer using a Kenwood 850W microwave on full power. Once cooled 2.5  $\mu$ l Midori Green Direct was mixed into the solution and then poured into an 8cm long gel tank and left to set. Gels were run on a Consort EV243 powerpack at 100V and 35 mAmps for 1 h.

For testing for the presence of DNA after extraction a mixture of 2  $\mu$ l DNA, 6  $\mu$ l water and 2  $\mu$ l 5X orange loading dye was run for each sample on a TBE gel.

For testing for the presence of PCR products a mixture of 8  $\mu$ l DNA and 2  $\mu$ l 5X orange loading dye was used and run on a TBE gel.

### 2.7.2.2 Imaging Gels

Gels were imaged using a Fotodyne Inc. (Wisonsin, USA) UV transilluminator.

## 2.7.3 PCR Product Purification

#### 2.7.3.1 Gel Extraction

For PCR products with multiple bands, PCR products were purified using gel extraction.

PCR products for gel extraction were run on 0.8 % agarose gels. After sufficient time to allow easy distinction between the bands to be cut out, ~2 hours, gels were dabbed dry with tissue to remove excess TBE. Bands were cut out on a Fotodyne UV transilluminator using scalpel blades. The weight of each gel slice was calculated by deducting the weight

of the 1.5 ml Eppendorf tube before the addition on the gel slice from the weight of the 1.5 ml Eppendorf with the gel slice.

The DNA was extracted from the gel using either a modified version of the protocol from the QIAquick Gel Extraction kit for PCR products to be sequenced (Qiagen, Sussex, UK) (see below) or Monarch® DNA Gel Extraction Kit Protocol (New England Biolabs, UK) as per supplied protocol.

Modified QIAquick Gel Extration Protocol; To one volume of gel 3 1/2 volumes of buffer QG were added and then placed on a Stuart roller mixer SRT6 at room temperature for 15 minutes until the gel had dissolved. One gel volume of ice cold IPA was then added and mixed using a Labnet vortex mixer VX100 for 5 seconds. The sample was then applied to a Promega (Wisconsin, USA) spin column and spun at full speed in a Progen GenFuge 24D centrifuge for 30 seconds. The flow through was then reapplied and respun at full speed for 30 seconds. Flow through was discarded and 500  $\mu$ l of QG buffer added to column and spun at full speed for 1 minute. Flow through was discarded again and 750  $\mu$ l of buffer PE was then added to the column, incubated at room temperature for 2-5 minutes then spun at 13000RPM for 1 minute. Flow through was then placed in a clean 1.5 ml Eppendorf, and DNA was eluted using 50  $\mu$ l of warm (~37 °C) H<sub>2</sub>O applied to the column membrane. The column and Eppendorf were left to stand for 2 minutes, then spun at 13000RPM for 1 minute and then the elution procedure was repeated.

DNA concentration was then calculated by running 2  $\mu$ l of flow through with 6  $\mu$ l H<sub>2</sub>O and 2  $\mu$ l orange loading dye on an 0.8 % gel and calculating concentration from Hyperladder I relative to band brightness. If necessary elutions were then dried down to

at least 25 ng  $\mu$ l<sup>-1</sup> using a spin vacuum desiccator (Eppendorf concentrator 5301) in order to be sent for sequencing.

#### 2.7.3.2 Direct From Product Band Extraction

For PCR products showing only a single clearly defined band, PCR products were purified using either the EXO-SAP method or Omega Bio-tek E.Z.N.A cycle pure kit as per protocol

EXO-SAP method: To 16  $\mu$ l of PCR product 2  $\mu$ l of EXO-SAP mix (0.2  $\mu$ l EXO1, 1  $\mu$ l SAP and 2.8  $\mu$ l) was added and briefly vortexed on a Labnet vortex mixer VX100. Samples were heated to 37 °C for 15 minutes then 80 °C for a further 15 minutes. Product was then stored at 4 °C prior to being sent to sequencing.

## 2.7.4 Sequencing and Analysis of Sequence Results

Sanger DNA sequencing was performed by DBS Genomics, Durham University.

#### 2.7.4.1 Quality Control and Cleaning of Sequences

Raw sequences were entered into BLASTX (Altschul et al., 1990) to check for similarity to *CBF* and the results recorded in MS Excel. Chromas LITE version 2.1 was then used to visually inspect sequences for read out accuracy, sequence purity and clarity. Where peak quality or clarity was consistently low sequences were not used for further analysis. Sequences that passed this quality control were again checked for correct base calling, edited manually if necessary, and then exported as a FASTA file. Forward and reverse sequences of the same sample were imported into either BioEdit Sequence Alignment Editor version 7.1.7 (Hall, 1999) and pairwise aligned (with ends allowed to slide) or Jalview Desktop (Waterhouse et al., 2009) and manually aligned. These aligned sequences, where possible, were combined to make one complete sequence. Any

sequences which contained primer sequences had the primer sequences removed from the sequence; this is because we cannot be certain that the primer sequence used was an exact match to the true native sequence. These consensus sequences were then exported as a FASTA file.

#### 2.7.4.2 Protein Translation and Comparison

Nucleotide sequences were then translated into protein sequences using SnapGene version 2.4, Jalview Desktop (Waterhouse et al., 2009) or ExPASy (Gasteiger et al., 2003). The correct reading frame was found via combination of BlastX (Altschul et al., 1990) searches, identification of primer fragments where possible in longer sequences, and using SnapGene-which highlights the ORF with the longest sequence without a stop codon. A search was then performed using BLASTp (Altschul et al., 1990) using these protein sequences to confirm that the correct ORF was chosen via similarity to other CBF protein sequences.

Protein sequences were viewed in Jalview Desktop (Waterhouse et al., 2009) and aligned using Clustal (McWilliam et al., 2013).

## 2.7.5 Quantitative PCR

A 1:50 dilution of cDNA was used for all qPCR experiments (after initial testing of *Empetrum nigrum* cDNA at 1:10, 1:20, 1:50, 1:100 and 1:200). To 96 wells plates 5  $\mu$ l of diluted cDNA was added to 10  $\mu$ l of mastermix consisting of 7.5  $\mu$ l Promega GoTaq® qPCR 2X SYBR mix with added CXR reference dye, 0.9  $\mu$ l forward primer, 0.9  $\mu$ l reverse primer and 0.7  $\mu$ l nuclease free water per reaction. Three technical replicates were performed for each test sample or control. Reactions with primers for housekeeping genes (*PEX4* for *Arabidopsis thaliana*, and RPB2I for *Empetrum nigrum*) were also run on the same plate as the reactions for the test gene with the same cDNA. The plate was then spun

down qPCR performed using the Applied biosystems 7300 qPCR machine. The  $\Delta\Delta$  Ct method (Livak and Schmittgen, 2001) was used to calculate gene expression levels and further analysis of the qPCR data was performed using algorithms described in Relative Quantitation (RQ) algorithms, Applied Biosystems Real-Time PCR Systems Software, July 2007 and implemented by (Moffat et al., 2012) namely results were normalised to their respective housekeeping genes via subtraction of Log2 and converted to fold difference relative to WT or time 0.

## 2.8 Cloning

## 2.8.1 Media

All chemicals were supplied by Melford laboratories Ltd., Suffolk.

#### LB agar

20 g l<sup>-1</sup> Granulated LB broth Low Salt

15 g l<sup>-1</sup> agar

De-ionised water

### LB

20 g l<sup>-1</sup> Melford Granulated LB broth low salt

De-ionised water

## 2.8.2 Selection of *Escherichia coli* Containing the pEntr-D-TOPO Vector

*Escherichia coli* cells containing the pEntr-D-TOPO entry vector plasmid encoding kanamycin resistance and containing Not1 and Asc1 cleavage sites were isolated from a dormant glycerol stock on 10 % concentration kanamycin LB agar plates. Individual

colonies were lifted and re-suspended in 5 ml of LB containing 10 % Kanamycin. Cells were then grown on in an N-Biotek NB-205 shaking incubator at 37 °C shaking at a speed of ~220RPM for approximately 16 hours.

## 2.8.3 Plasmid Extraction

Plasmids were extracted from the E.coli cells using Promega winzard® plus SV minipreps DNA purification system kit with a modified protocol: The LB/E. coli mix was transferred into 15 ml Eppendorfs and spun and 10,000 G for 5 minutes in a Beckman Coulter Allegra X-22R Centrifuge. The supernatant was then removed and discarded. The pellet was re-suspended in 250 µl of Cell Resuspension Solution and transferred to a 1.5 ml Eppendorf. To the re-suspended pellet, 250 µl of cell lysis solution was added and mixed by inversion until the solution became transparent. The solution was then incubated at room temperature for 3 minutes followed by the addition of 10 µl of alkaline protease solution, mixed by inversion and incubated at room temperature for a further 3 minutes. After incubation 350 µl of neutralization solution was added, mixed by inversion and spun at 11000 G for 10 minutes in a Progen GenFuge 24D centrifuge. The supernatant was then transferred to a spin column and spun at 11,000 G for 1 minute. Flow through was discarded and 750 µl of wash solution was added prior to spinning at 11,000 G for 1 minute. Again, flow through was discarded and the column spun at 11,000 G for another 2 minutes before being placed in a new tube. The plasmids were then eluted from the spin column by applying 50 µl nuclease free water to the column membrane and spinning the column are 11,000 G for 1 minute. Concentration was then calculated using a Labtech NanoDrop ND-1000 Spectrophotometer and plasmids stored and -20 °C.

## 2.8.4 Digestion

New England BioLabs' online double digest finder tool (New England BioLabs Inc.) was used to select the buffer and concentrations required for the enzymes. Reactions were performed in a total of 40  $\mu$ l. Digests were mixed using a Labnet vortex mixer VX100 and Left in 37 °C Water bath overnight to digest. The desired digested fragments were isolated via gel extraction (See section: 2.7.3.1)

#### **Plasmid Digestion**

X µl Plasmid\*

1 µl Not1HF or BAMHI-HF

1 µl Asc1 or SACI-HF

4 µl 10X CutSmart buffer

To a total of 40 µl with Water

\*Where X is the amount of a known concentration of the plasmid to make an overall concentration of 25 ng  $\mu$ l<sup>-1</sup> of plasmid.

#### **Insert Digestion**

34 µl insert DNA containing NOT1 and Asc1 restriction sites.

1 µl NOT1HF or BAMHI-HF

1 µl Asc1 or SACI-HF

4 μl cut smart buffer

## 2.8.5 Ligation

A 3:1 molar ratio of vector to insert was used for ligation. Using between 20-200 ng  $\mu$ l<sup>-1</sup> of vector per reaction and a maximum concentration of 10 ng  $\mu$ l<sup>-1</sup> of insert DNA in the whole reaction. Of the total reaction volume, 0.5 % of T4 DNA ligase and 10 % of 10X

T4 DNA Ligase buffer were also added and the remainder of the volume was made up with water. A control for each reaction was also run where the insert was replaced by water. Ligations were performed in a total volume of 20  $\mu$ l and incubated at 16 °C overnight. The ligation was then heat inactivated at 65 °C for 10 minutes.

## 2.8.6 Transformation Into Escherichia coli

Ligation reactions were deactivated via heat shock at 70 °C for 15 minutes using a Thermo PX2 Thermal Cycler or a Hybaid PCR Express. To 40  $\mu$ l of  $\alpha$  silver competent *E. coli* cells, 4  $\mu$ l of the deactivated ligation were added, mixed and placed on ice for 30 minutes. The *E.coli*/ligation mix was then heat shocked to 42 °C using a water-bath in a 42 °C oven for 40 seconds, then placed upon ice again for 2 minutes. To this mixture, 1 ml of 37 °C LB was added and then shaken horizontally for 1 hour. Onto 37 °C pre-warmed 10 % kanamycin LB agar plates, 200  $\mu$ l of the transformant was then spread. The remaining mixture was then spun at full speed in a Beckman Coulter Allegra X-22R Centrifuge for 1 minute and the resulting pellet re-suspended in 200  $\mu$ l of the supernatant, discarding excess supernatant. This more concentrated transformant solution was then also spread onto 37 °C pre-warmed 10 % kanamycin LB agar plates. All plates were then incubated at 37 °C overnight.

## 2.8.7 Gibson Assembly and Transformation of Gene Blocks

Synthetic gene blocks were inserted into pENTR-D-TOPO vectors using Gibson assembly as per the protocol (New England Biolabs, Gibson Assembly® Cloning Kit Manual V3.2). Plasmid constructs were then transformed into competent *E. coli* cells either via the cells and protocol provided in the kit or as per 2.8.6 using DH $\alpha$  silver competent cells minus the initial deactivation step. Cells were then spread onto 37 °C pre-warmed 10 % kanamycin LB agar plates and incubated at 37 °C overnight. Single

colonies were then selected, grown in 5 ml LB agar overnight and used to create glycerol stocks (section 2.8.12.1)

For transient expression in *Nicotiana benthamiana* these transformed cells were grown up in 5 ml LB agar at 37 °C overnight then the plasmids extracted as per section 2.8.3 these plasmids were then cut with BAMHI-HF and SACI-HF alongside PCambia 1305.1 effector plasmid (Nassuth et al., 2014; Fig. 2.8.7.1), run on a gel to isolate the Geneblock insert and the PCambia effector plasmid from their respective digests, gel extracted as per section 2.7.3.1 and ligated and transformed as per sections 2.8.5 and 2.8.6.



Figure 2.8.7.1 Simplified schematic representation of control, effector and reporter plasmids showing only relevant features. Based on diagram from Nassuth et al.,2014. 35S promoter is a constitutive promotor. The CRT motif is activated by CBF binding. For transient expression studies a combination of either control + reporter or effector + reporter were infiltrated into the leaf.

## 2.8.8 Transformation into Agrobacterium tumefaciens

Two *Agrobacterium tumefaciens* strains were used; GV3101 for transient expression in *Nicotiana benthamiana* or C58C1 for stable transformation into *Arabidopsis thaliana*. 100  $\mu$ l of cells were used per reaction, to which 1 $\mu$ g plasmid DNA was added and mixed gently via inversion. Cells and plasmids were then heat shocked at 37 °C for 5 minutes then added to 1 ml of LB which was shaken at 20 °C at 150RPM for 2-4hours. This was then transferred to a 1.5 ml Eppendorf and spun in an eppendorf AG centrifuge 5415D for 30s. The majority of the supernatant was then removed and the pellet resuspended in approximately 200  $\mu$ l supernatant then plated onto LB plates containing 100  $\mu$ g ml<sup>-1</sup> rifampicin and 100  $\mu$ g ml<sup>-1</sup> kanamycin. Then incubated at 28 °C for 3 days.

## 2.8.9 Plasmid Quality Control

At various points cells were checked to ensure they contained the correct plasmid and geneblock insert. This was done via plasmid extraction (section 2.8.3) followed by PCR amplification (Section 2.7.1) using primers 138-157 (table 2.5.1e-f) dependant on what was being checked.

# 2.8.10 Transient Expression by Infiltration in Nicotiana benthamiana

A day prior to infiltration 4 week old *Nicotiana benthamiana* plants were watered and placed in an autoclave bag to increase humidity and open stomata.

Glycerol stocks of *Agrobacteria tumefaciens* containing the binary vectors to be studied; the effector vectors now containing the *CBF* gene blocks, the dual luciferase reporter and pCAMBIA1305.1 control vectors (Nassuth et al., 2014; Fig. 2.8.7.1) were streaked out onto LB-agar plates containing 100  $\mu$ g ml<sup>-1</sup> rifampicin and 100  $\mu$ g ml<sup>-1</sup> kanamycin and grown at 29 °C for 3 days. Single colonies were then used to inoculate 5 ml bottles of LB containing the same antibiotics and grown for 24 h at 29 °C shaking at 150RPM in a Jeio Tech SI-300R incubated shaker. The culture was then spun down at 3000 G using a Beckman Coulter Allegra X-22R Centrifuge, supernatant was then discarded and the pellet resuspended in 1 ml 10 mM MgCl<sub>2</sub>. 20  $\mu$ l of this solution was added to 980  $\mu$ l H<sub>2</sub>O and the absorbance measured using a Boeco S-20 spectrophotometer at 600nm wavelength. 10µM MgCl<sub>2</sub> was then added to reach an optical density of 0.3 using the equation (ODx50)/Desired OD = ml of 10  $\mu$ M MgCl<sub>2</sub> to add to 1 ml of construct. *Nicotiana benthamiana* leaves were then infiltrated using a 1 ml syringe (without needle) applied with pressure to the underside of the leaf. One half of the leaf contained the effector and reporter plasmid (Fig. 2.8.7.1), the other contained the control (the same plasmid as the effector but without the added CBF gene or 35S promoter) and reporter plasmid for comparison (Fig. 2.8.7.1). The edge of the infiltrated area was then marked with black marker. Three leaves, per construct, per experiment, on different individual plants were infiltrated. Plants were then transferred back to previous growth conditions for 48 hours. Three leaf disks, per infiltration, of area 64mm<sup>2</sup> were then taken and individually floated abaxial side up in aluminium wells filled with 500 µl H<sub>2</sub>O. 500 µl of substrate solution (20µM coelenterazine for RiLUC, 2 mM D-Luciferin for FiLUC) was then added and disks were imaged using a Photek photon counting camera and analysed using Photek Image 32.

Photons emitted from the leaf disks were counted over a set period of time (1200 seconds for Fig 4.1.1.3 & 4.1.1.4 and 879 seconds for Fig. 4.1.1.2) and the emission per unit area across the three repeats was calculated, the background reading was then deducted.

FiLUC activation was indicative of the amount of expression due to the constitutive 35S promoter whilst RiLUC was indicative of the degree of activation by *CBF* of the CRT motif (See Fig. 2.8.7.1). Therefore, photon count values for RiLUC (CRT motif) were

divided by corresponding values for FilUC (35S promoter) to normalise for the amount of reporter plasmid. This result for the reporter affected by the control (an effector plasmid lacking the 35S promotor and *CBF* sequence (Fig. 2.8.7.1), was then deducted from the result for the matching reporter affected by the effector plasmid leaf disks (infiltrated on the other half of the leaf to the control) to account for any CRT activation by native *CBF*s or by the introduction of the plasmid.

For figure 4.1.1.1 data were compared across all infiltration events via further normalisation by setting the wildtype overexpressor for each plate as an arbitrary value of 1 and expressing the other constructs as fold changes in CRT expression relative to this.

## 2.8.11 Stable Transformation of Arabidopsis thaliana

Stable transformations were achieved via floral dipping (Clough and Bent, 1998). Agrobacteria from previously prepared glycerols were streaked onto LB plates containing 100  $\mu$ g ml<sup>-1</sup> rifampicin and 100  $\mu$ g ml<sup>-1</sup> kanamycin and grown at 29 °C for 3 days, single colonies were transferred to a 5 ml LB containing 100  $\mu$ g ml<sup>-1</sup> rifampycin and 100  $\mu$ g ml<sup>-1</sup> kanamycin and grown at 29 °C for 48 h in a Jeio Tech SI-300R incubated shaker at 150RPM. These cultures were then used to inoculate a 200 ml LB flask containing the same concentration of antibiotics. This was grown at 29 °C for 24 h in a shaking incubator Jeio Tech SI-300R incubated shaker at 150RPM. The cultures were then centrifuged at 3500 G for 15 minutes using a Beckman Coulter Allegra X-22R Centrifuge and the supernatant discarded. The pellet was then gently resuspended in 200 ml of 5 % sucrose solution plus 0.5  $\mu$ l ml<sup>-1</sup> of Lehle Vac-In-Stuff silwet L-77. Plants prepared for dipping (section 2.2.4) were then physically dipped head first in a beaker of this solution and swilled round. Plants were then lain on their side in a tissue lined plastic tray and covered with clingfilm to maintain high humidity for 24 h. Clingfilm and tissue was then removed and plants righted and watered and grown in long day conditions (section 2.2.4) until ready for seed harvesting.

## 2.8.12 Stocks and Storage

#### 2.8.12.1 Glycerol Stocks

Glycerol stocks were made from 0.525 ml culture added to 0.25 ml 100 % glycerol. The stock was then flash frozen in liquid nitrogen and stored at -80 °C.

## 2.9 Phenotypic Scoring

All Empetrum nigrum and Calluna vulgaris plants grown from cuttings were photographed using an Olympus OM-D EM-5 on 13/08/18 and 20/08/18 respectively. Two photographs per plant were taken, one top-down view and one side view with the pots laid on their side to accommodate the sprawling nature of several specimens. All photographs were then added to a excel spreadsheet documenting various details of each specimen including, code, location harvested from (including GPS location and altitude) (see supplementary disk Excel documents "Morphology Calluna vulgaris" and "Morphology Empetrum nigrum"). All specimens were given a random number code then just the code and associated photographs were transferred to a separate sheet and sorted by random number to maintain anonymity of sample details and avoid unconscious bias. All samples were then given a rank code by eye based on the morphology of the sample; ranging from 1 for very leggy samples with very little/no branching, long stems, widely spaced leaves and thin stems, to 10 for highly compact samples with frequent branching, short branches, closely bunched leaves and thick stems. This was repeated three times to avoid potential influence of previous image(s) morphology on the score of the following image. The mean of these scores was then used.

Results were then visualised using Microsoft Excel 2016 to produce scatter graphs of the data; each series then being colour-coded based on locale. The data were then imported into R (R Core Team, 2014) and Spearman's Rank Correlation tests performed for each comparison.
# Chapter 3: Isolating and Analysing C-Repeat Binding Factor (CBF) Sequences from Arctic, Alpine and Moorland Plant Species

## Aims and Objectives

The aims of this chapter were to isolate full *CBF* coding sequences from a wide variety of Arctic, alpine and moorland species and to compare these *CBF* sequences, in order to identify any polymorphisms which could affect *CBF* activity, particularly any polymorphisms common between distantly related species. The majority of species collected had no previous *CBF* sequence data available, therefore primers were designed from pre-existing sequences of the closest known relatives of the species collected (methods section: 2.5.1.1). In order to isolate the full coding sequences these primers were designed to the 3'and 5' end of the sequence, which contain a high degree of sequence variation. Therefore, it was unknown if these primers would be sufficiently specific to isolate corresponding *CBF* sequences from related species. The primary objective of this chapter was therefore to identify if primers designed in this manner could amplify *CBF* sequences from other species and, if so, for each collected species, which of these primers could be used to isolate *CBF* sequences from that particular species.

## **Hypotheses**

• It is possible to isolate full *CBF* sequences from a wide range of species using primers designed to the 3' and 5' ends of pre-existing *CBF* sequences of closely related species.

- *CBF* sequences from Arctic, alpine and moorland species will contain polymorphisms in regions previously identified as influencing CBF binding and activity.
- Common polymorphisms will be observed across distantly related Arctic, alpine and moorland species.

# **3.1 Results 3.1.1** *CBF* **Nucleotide Sequences**

*CBF* sequences could not be successfully isolated from a large number of species. Of those that were successfully isolated Clustal (Larkin et al., 2007) alignments of *CBF* nucleotide sequences were split into three groups for ease of analysis. Brassicaceae (Fig. 3.1.1.1.1 a-b), Ericaceae (Fig. 3.1.1.2.1 a-b) and all other remaining genera (Fig. 3.1.1.3.1 a-c). All were compared to *Arabidopsis thaliana CBF1-4* sequences. When sorted by Clustal *Arabidopsis thaliana CBF1-4* sequences grouped together except in the case of the Brassicaceae alignment (of which *Arabidopsis* is itself a member) (Fig. 3.1.1.1.1 a-b).

# 3.1.1.1 Brassicaceae

Sample 68 *Capsella bursa-pastoris* consisted of four distinct sequences. The sequences amplified by primer pair 61 and 71 (Table 2.5.1a-g) were very similar to one another except for 1 nucleotide difference near the start of the primer pair 61 sequence (T to G) (Fig. 3.1.1.1 a). The sequences were otherwise identical except for some degenerate bases and that the readable sequence amplified by primer pair 61 was shorter (Fig. 3.1.1.1 a-b). Another set of sequences which were identical to one another (except that a shorter section of sequence was readable for the sequence amplified by primer pair 64) were amplified primer pairs 64 and 68 (Table 2.5.1a-g) (Fig. 3.1.1.1.1 a-b). A unique

sequence was amplified by primer pair 75 (Table 2.5.1a-g) (Fig. 3.1.1.1.1 a-b). Another distinct sequence was amplified by primer pair 76 (Table 2.5.1a-g) (Fig. 3.1.1.1.1 a-b).

Two distinct sequences were amplified from sample 66 *Cardaminopsis arenosa*. One sequence, amplified by primer pair 77 (Table 2.5.1a-g) was identical to that amplified from sample 68 *Capsella bursa-pastoris* using primer pair 76 (Table 2.5.1a-g) (Fig. 3.1.1.1 a-b). Three sequences identical to one another (except for one degenerate base) but distinct from other sequences were amplified using primer pair 66, 69 and 67 (Table 2.5.1a-g) (Fig. 3.1.1.1.1 a-b).

One sequence was amplified from sample 69 *Arabis alpina* using primer pair 77 (Table 2.5.1a-g). This sequence was very similar to the sequence amplified from sample 68 *Capsella bursa-pastoris* using primer pair 76 and sample 66 *Cardaminopsis arenosa* primer pair 77 (Fig. 3.1.1.1.1 a-b).

One sequence was amplified from sample 64 *Barbarea vulgaris* using primer pair 60 (Table 2.5.1a-g). This sequence displayed a large number of differences when compared to all other *Brassicaceae CBF* sequences except for *Arabidopsis thaliana CBF4* to which it showed greater similarity and grouped with during Clustal sorting (Fig. 3.1.1.1.1 a-b).

64pp60\_Barbaea\_vulgaris?\_S11\_Abisko Arabidopsis thaliana CBF4 AT5G51990.1 68pp61 Capsella hursa-pastoris? S12 Abisko REVonly 68PP71\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 64\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 68 Capsella bursa-pastoris? S12 Abisko Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 66pp 66\_Cardaminopsis\_arenosa\_S11\_Abisko 66pp 69\_Cardaminopsis\_arenosa\_S11\_Abisko 66pp67 Cardaminopsis arenosa S11 Abisko Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis thaliana CBF3 AT4G25480.1 66pp77\_Cardaminopsis\_arenosa\_S11\_Abisko 68PP76\_Capsella\_bursa-pastoris?\_S12\_Abisko 69pp77 Arabis alpina? S12 Abisko 68pp 75\_Capsella\_bursa-pastoris?\_S12\_Abisko

64pp60\_Barbaea\_vulgaris?\_S11\_Abisko Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 68pp61 Capsella bursa-pastoris? S12 Abisko REVonly 68PP71\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 64\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 68\_Capsella\_bursa-pastoris?\_S12\_Abisko Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 66pp66\_Cardaminopsis\_arenosa\_S11\_Abisko 66pp69 Cardaminopsis arenosa S11 Abisko 66pp 67\_Cardaminopsis\_arenosa\_S11\_Abisko Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis thaliana CBF3 AT4G25480.1 66pp77\_Cardaminopsis\_arenosa\_S11\_Abisko 68PP76\_Capsella\_bursa-pastoris?\_S12\_Abisko 69pp77\_Arabis\_alpina?\_S12\_Abisko 68pp 75\_Capsella\_bursa-pastoris?\_S12\_Abisko

64pp 60\_Barbaea\_vulgaris?\_S11\_Abisko Arabidopsis thaliana CBF4 AT5G51990.1 68pp61\_Capsella\_bursa-pastoris?\_S12\_Abisko\_REVonly 68PP71\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 64\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 68 Capsella bursa-pastoris? S12 Abisko Arabidopsis thaliana CBF1 AT4G25490.1 66pp 66\_Cardaminopsis\_arenosa\_S11\_Abisko 66pp69 Cardaminopsis arenosa S11 Abisko 66pp 67\_Cardaminopsis\_arenosa\_S11\_Abisko Arabidopsis thaliana CBF2 AT4G25470.1 Arabidopsis\_thaliana\_CBF3\_AT4G25480.1 66pp77\_Cardaminopsis\_arenosa\_S11\_Abisko 68PP76 Capsella bursa-pastoris? S12 Abisko 69pp77\_Arabis\_alpina?\_S12\_Abisko 68pp 75\_Capsella\_bursa-pastoris?\_S12\_Abisko



Figure 4.1.1.1.1.a Clustal alignment of *CBF* nucleotide sequences isolated from members of the *Brassicaceae* compared *to Arabidopsis thaliana CBF1-4* genbank sequences. Sequences are ordered by Clustal sorting. Names are in the form "Species code pp primer pair code\_Genus\_Species\_Site code\_Location". Genbank gene names are in the form "Genus Species Gene Genbank code". 64pp60 Barbaea vulgaris? S11 Abisko Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 68pp61 Capsella bursa-pastoris? S12 Abisko REVonly 68PP71\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 64\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 68\_Capsella\_bursa-pastoris?\_S12\_Abisko Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 66pp66 Cardaminopsis arenosa S11 Abisko 66pp 69\_Cardaminopsis\_arenosa\_S11\_Abisko 66pp67\_Cardaminopsis\_arenosa\_S11\_Abisko Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis thaliana CBF3 AT4G25480.1 66pp77\_Cardaminopsis\_arenosa\_S11\_Abisko 68PP76\_Capsella\_bursa-pastoris?\_S12\_Abisko 69pp77 Arabis alpina? S12 Abisko 68pp 75\_Capsella\_bursa-pastoris?\_S12\_Abisko

64pp60\_Barbaea\_vulgaris?\_S11\_Abisko Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 68pp 61\_Capsella\_bursa-pastoris?\_S12\_Abisko\_REVonly 68PP71\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 64\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 68\_Capsella\_bursa-pastoris?\_S12\_Abisko Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 66pp 66\_Card a minopsis\_arenosa\_S11\_Abisko 66pp 69\_Cardaminopsis\_arenosa\_S11\_Abisko 66pp 67\_Cardaminopsis\_arenosa\_S11\_Abisko Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF3\_AT4G25480.1 66pp77\_Cardaminopsis\_arenosa\_S11\_Abisko 68PP76\_Capsella\_bursa-pastoris?\_S12\_Abisko 69pp77\_Arabis\_alpina?\_S12\_Abisko 68pp 75\_Capsella\_bursa-pastoris?\_S12\_Abisko

64pp60\_Barbaea\_vulgaris?\_S11\_Abisko Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 68pp61 Capsella bursa-pastoris? S12 Abisko REVonly 68PP71\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 64\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 68\_Capsella\_bursa-pastoris?\_S12\_Abisko Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 66pp 66\_Cardaminopsis\_arenosa\_S11\_Abisko 66pp 69 Cardaminopsis arenosa S11 Abisko 66pp 67\_Cardaminopsis\_arenosa\_S11\_Abisko Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF3\_AT4G25480.1 66pp 77\_Cardaminopsis\_arenosa\_S11\_Abisko 68PP76\_Capsella\_bursa-pastoris?\_S12\_Abisko 69pp 77\_Arabis\_alpina?\_S12\_Abisko 68pp75\_Capsella\_bursa-pastoris?\_S12\_Abisko



Figure 3.1.1.1.1 b. Clustal alignment of *CBF* nucleotide sequences isolated from members of the *Brassicaceae* compared to *Arabidopsis thaliana CBF*1-4 genbank sequences. Sequences are ordered by Clustal sorting. Names are in the form "Species code pp primer pair code\_Genus\_Species\_Site code\_Location". Genbank gene names are in the form "Genus\_Species\_Gene\_Genbank code".

#### 3.1.1.2 Ericaceae

All Ericaceae sequences were more similar to one another than to the *Arabidopsis thaliana* sequences (Fig. 3.1.1.2.1 a-b). Only one primer pair was used for all *Ericaceae CBF* amplification (primer pair 22 – For primer pair list see section 2.5.1.1 and Table 2.5.1a-g)

All *Andromeda polifolia* sequences from sample 43 (bands A-D) (Abisko, Sweden) and sample 18 (Abisko, Sweden) were very similar to one another (Fig. 3.1.1.2.1 a-b). The sequence isolated from band A of sample 43 was more similar to the sequence from sample 18pp22 than to bands B, C and D isolated from sample 43 (Fig. 3.1.1.2.1 a-b). However, similarity was still high across all sequences, more-so than seen between the different *CBF* genes (1-4) within *Arabidopsis thaliana* (Fig. 3.1.1.2.1 a-b).

*Vaccinium vitis-idaea* samples 10 (Abisko, Sweden), 40 (Abisko, Sweden) and 118 (Weardale, UK) had identical *CBF* sequences except for two degenerate bases seen in sample 10 (Fig. 3.1.1.2.1 a-b).

The sequence from *Vaccinium myrtillis* sample 114 was more similar to *Vaccinium vitis-idaea* sequences than sequences from other species but was definitely a distinct sequence to the *Vaccinium vitis-idaea* sequence (Fig. 3.1.1.2.1 a-b).

The three *Empetrum nigrum* sequences from sample 5 (sequences A-C isolated by TOPO cloning) were almost identical to one another except for a small number of substitutions between sequences (Fig. 3.1.1.2.1 a-b).

Sample 3 *Arctostaphylos alpina* and sample 2 *Diapensia lapponica* had identical sequences to sequence A from sample 5 (Fig. 3.1.1.2.1 a-b).

Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 Arabidopsis\_thaliana\_CBF3\_AT4G25480.1 43PP22A\_Andromeda\_polifolia\_S6\_Abisko 18pp 22\_Andromeda\_polifolia\_S3\_Abisko 43PP22B\_Andromeda\_polifolia\_S6\_Abisko 43pp22D\_Andromeda\_polifolia\_S6\_Abisko 43pp22c Andromeda polifolia S6 Abisko 114pp 22\_Vaccinium\_myrtillus\_S25\_Weardale&Teesdale 10pp22\_Vaccinium\_vitis-idaea\_S2\_Abisko 40pp22\_Vaccinium\_vitis-idaea\_S6\_Abisko 118pp22\_Vaccinium\_vitis\_idea\_S25\_Weardale&Teesdale 5pp 22B\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 5pp22C Empetrum nigrum S2 Abisko TOPO 5pp 22A\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 3pp 22\_Arctostaphylos\_alpina\_S2\_Abisko 2pp 22\_Diapensia\_lapponica\_S2\_Abisko

Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 Arabidopsis thaliana CBF3 AT4G25480.1 43PP22A\_Andromeda\_polifolia\_S6\_Abisko 18pp 22\_Andromeda\_polifolia\_S3\_Abisko 43PP22B\_Andromeda\_polifolia\_S6\_Abisko 43pp22D Andromeda polifolia S6 Abisko 43pp22c\_Andromeda\_polifolia\_S6\_Abisko 114pp 22\_Vaccinium\_myrtillus\_S25\_Weardale&Teesdale 10pp22\_Vaccinium\_vitis-idaea\_S2\_Abisko 40pp 22\_Vaccinium\_vitis-idaea\_S6\_Abisko 118pp 22\_Vaccinium\_vitis\_idea\_S25\_Weardale&Teesdale 5pp 22B\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 5pp 22C\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 5pp22A\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 3pp 22\_Arctostaphylos\_alpina\_S2\_Abisko 2pp 22\_Diapensia\_lapponica\_S2\_Abisko

Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 Arabidopsis thaliana CBF3 AT4G25480.1 43PP22A\_Andromeda\_polifolia\_S6\_Abisko 18pp22\_Andromeda\_polifolia\_S3\_Abisko 43PP22B\_Andromeda\_polifolia\_S6\_Abisko 43pp 22D\_Andromeda\_polifolia\_S6\_Abisko 43pp22c\_Andromeda\_polifolia\_S6\_Abisko 114pp 22\_Vaccinium\_myrtillus\_S25\_Weardale&Teesdale 10pp22\_Vaccinium\_vitis-idaea\_S2\_Abisko 40pp 22\_Vaccinium\_vitis-idaea\_S6\_Abisko 118pp22\_Vaccinium\_vitis\_idea\_S25\_Weardale&Teesdale 5pp 22B\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 5pp 22C\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 5pp 22A\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 3pp 22\_Arctostaphylos\_alpina\_S2\_Abisko 2pp 22\_Diapensia\_lapponica\_S2\_Abisko



Figure 3.1.1.2.1.a. Clustal alignment of *CBF* nucleotide sequences isolated from members of the *Ericaceae* compared to *Arabidopsis thaliana CBF*1-4 genbank sequences. Sequences are ordered by Clustal sorting. Names are in the form "Species code pp primer pair code\_Genus\_Species\_Site code\_Location". Genbank gene names are in the form "Genus Species Gene Genbank code". Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 Arabidopsis\_thaliana\_CBF3\_AT4G25480.1 43PP22A\_Andromeda\_polifolia\_S6\_Abisko 18pp22\_Andromeda\_polifolia\_S3\_Abisko 43PP22B\_Andromeda\_polifolia\_S6\_Abisko 43pp 22D\_Andromeda\_polifolia\_S6\_Abisko 43pp22c\_Andromeda\_polifolia\_S6\_Abisko 114pp 22\_Vaccinium\_myrtillus\_S25\_Weardale&Teesdale 10pp22\_Vaccinium\_vitis-idaea\_S2\_Abisko 40pp 22\_Vaccinium\_vitis-idaea\_S6\_Abisko 118pp22\_Vaccinium\_vitis\_idea\_S25\_Weardale&Teesdale 5pp22B\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 5pp 22C\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 5pp 22A\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 3pp 22\_Arctostaphylos\_alpina\_S2\_Abisko 2pp 22\_Diapensia\_lapponica\_S2\_Abisko

Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 Arabidopsis\_thaliana\_CBF3\_AT4G25480.1 43PP22A\_Andromeda\_polifolia\_S6\_Abisko 18pp22\_Andromeda\_polifolia\_S3\_Abisko 43PP22B\_Andromeda\_polifolia\_S6\_Abisko 43pp 22D\_Andromeda\_polifolia\_S6\_Abisko 43pp22c\_Andromeda\_polifolia\_S6\_Abisko 114pp 22\_Vaccinium\_myrtillus\_S25\_Weardale&Teesdale 10pp22\_Vaccinium\_vitis-idaea\_S2\_Abisko 40pp 22\_Vaccinium\_vitis-idaea\_S6\_Abisko 118pp22\_Vaccinium\_vitis\_idea\_S25\_Weardale&Teesdale 5pp 22B\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 5pp 22C\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 5pp 22A\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 3pp 22\_Arctostaphylos\_alpina\_S2\_Abisko 2pp 22\_Diapensia\_lapponica\_S2\_Abisko

Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 Arabidopsis\_thaliana\_CBF3\_AT4G25480.1 43PP22A\_Andromeda\_polifolia\_S6\_Abisko 18pp22\_Andromeda\_polifolia\_S3\_Abisko 43PP22B\_Andromeda\_polifolia\_S6\_Abisko 43pp 22D\_Andromeda\_polifolia\_S6\_Abisko 43pp22c\_Andromeda\_polifolia\_S6\_Abisko 114pp 22\_Vaccinium\_myrtillus\_S25\_Weardale&Teesdale 10pp22\_Vaccinium\_vitis-idaea\_S2\_Abisko 40pp 22\_Vaccinium\_vitis-idaea\_S6\_Abisko 118pp22\_Vaccinium\_vitis\_idea\_S25\_Weardale&Teesdale 5pp22B\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 5pp 22C\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 5pp 22A\_Empetrum\_nigrum\_S2\_Abisko\_TOPO 3pp 22\_Arctostaphylos\_alpina\_S2\_Abisko 2pp 22\_Diapensia\_lapponica\_S2\_Abisko

322	2 A	AT	т	тс	G	С	r g	A	ТΤ	С	G	т	r G I	GC	G G	ст	тс	G T	ΓA Τ	ТΤ	c c	ΤG	AG	AC	ΤA	C	ΤТ	G T	сс	т	ΑA	G G	A	ЭA	ΤТ	C A	GA	٩A	A G	ст	G C	G T	r <mark>c</mark> 1	r <mark>g</mark> A	AA	СТ	GC	ΑA	GG	CG	ΤТ	тс	A <mark>G</mark> A	AT	GΑ	G <mark>A </mark> C	ТА	C G	AC	G <mark>A</mark>	GG	GA	т с т	441
313	3 A	ΑT	T.	тс	G	c.	r g	A	СТ	С	G	т :	r G I	GC	GG	СТ	AC	G /	٩A	гс	c c c	GG	<u> </u>	тс	ΑA	C	ст	GΤ	GC	c <mark>/</mark>	ΑA	G G	A/	A A	тс	CA	AA	٩A	G G	сG	GC	G G	C 1	r <mark>g</mark> A	۸A	СС	GC	G T '	r g /	A T	ΤТ	тси	A A <mark>G</mark>	ΑT	GΑ	G A 1	r					- G	т <mark>б</mark> т	420
304	1 A	AC	T.	тс	G	C.	r g	A	СТ	C	G	T I	r G I	GC	G G	СТ	AC	G 🖌	۹A	гс	00	GG	AG	тс	ΑA	C.	ΑT	GC	GC	c o	ΑA	GG	A	ΤA	тс	CA	AA	٩A	A G	сG	GC	ΤG	C 1	r <mark>g</mark> A	۸A	CG	GC	G T '	r G G	СТ	ТΤ	тси	A A G	ΑT	GΑ	GAC	<b>.</b>					- G	т <mark>с</mark> т	411
313	3 <mark>A</mark>	AT	т.	тс	G	С	r G	A	СТ	C	G	<u>T</u>	r G I	G A	G A	СТ	C C	G /	A A I	ГС	СС	GG	ΑA	тс	ΑA	(C)	ТΤ	GC	GC	т,	ΑA	G G	A (	CA	тс	CA	AA	٩A	GG	СG	GC	G G	C	r <mark>g</mark> A	A	СТ	GC	G T '	GG	G C	ТΤ	тс	🖌 G G	ΑT	GA	G A 1	r					- G	T <mark>G</mark> T	420
342	2 A	AT	т.	ТС	G	C /	٩G	A	СТ	CC	G	G	r G I	GC	GG	ТΤ	GC	0.0	GG	ΤG	с с ,	AG	ТΤ	тс	ΑA	۱A	GG	AG	GO	A	GA.	AC	A	ΤA	ΤA	AG	G <mark>A</mark>	A G (	GG	СG	GC	AG	00	G	GG	CG	GC	ΤG	٩AA	TG	ТΤ	cc	G -	• •		CAA	A C A					- G (	<mark>c</mark>	444
155	5 A	AT	т.	ΤY	G	c /	٩G	A	СТ	CC	G	G	T G I	GC	GG	ΤТ	GC	0.0	GG	ΤG	c c <mark>,</mark>	AG	ТΤ	тс	ΑA	۱A	GG	AG	GO	A	ЭA.	AC	A	ΤA	ΤA	AG	G <mark>/</mark>	A G (	GG	сG	GC	AG	00	G G 🖌	GG	CG	GC	ΤG	٩A/	TG	ТΤ	сс	G -			CAA	A C A					- G (	<mark>c</mark>	257
342	2 A	AT	т.	ТΤ	G	C /	٩G	A	СТ	CC	G	C	r G I	GC	GG	ΤТ	GC	0.0	GG	ΤG	c c <mark>,</mark>	AG	ТΤ	тс	ΑA	۱A	GG	AG	GC	A	GA.	AC	A	ΤA	ΤA	AG	G <mark>A</mark>	A G (	GG	СG	GC	AG	TO	G G A	GG	CG	GC	ΤG	٩AA	TG	ТΤ	сс	G -	• •		CAA	A C A				• •	- G (	с	444
342	2 A	AT	т.	ТΤ	G	C /	٩G	A	СТ	CC	G	C	r G I	GC	GG	ΤТ	GC	0.0	GG	ΤG	c c <mark>,</mark>	AG	ТΤ	тс	ΑA	۱A	GG	AG	GO	A	GA.	AC	A	ΤA	ΤA	AG	G <mark>/</mark>	A G (	GG	сG	GC	AG	00	G G A	GG	CG	GC	T G /	۱AA	TG	ТΤ	сс	۶G -			CAA	A C A					- G (	<mark>c</mark>	444
342	2 A	AT	т.	тт	G	C /	٩G	A	СТ	CC	G	C	r G I	GC	GG	ΤТ	GC	0.0	GG	ΤG	с с ,	A G	ТΤ	тс	A A	۱A	GG	AG	GC	A	ЭA.	AC	A	ΤA	ΤA	AG	G <mark>A</mark>	A G (	GG	СG	GC	AG	00	G G A	GG	CG	GC	Τ <mark>G</mark>	A A A	TG	ТΤ	сс	G -			CAA	A C A				· ·	- G (	<mark>c</mark>	444
343	3 A	AT	т.	ТС	G	C /	٩G	A	СТ	C	G	G	r G I	GC	G G	ΤТ	GC	0.0	GG	ΤG	00	GG	ΤA	тс	G A	۱A	GG	AG	GO	A	GA.	AC	A	ΤA	ΤA	AG	G <mark>A</mark>	A G (	GG	CG	GC	GG	00	GG	GG	CG	GC	ΤG,	۱AA	TG	ТΤ	сс	۶G -			CAA	A C A					- G (	<mark>c</mark>	445
343	3 A	AT	т.	ТΤ	G	c,	٩G	A	СТ	C/	G	G	r G I	GC	AG	ТΤ	GC	0.0	GG	ΤG	00	GG	ΤA	тс	ΑA	۱A	GG	AG	GO	A	ЭA.	AC	A	ΤA	ΤA	AG	G <mark>A</mark>	A G (	GG	сG	GC	GG	) C (	GG	GG	CR	GC	T G /	٩AA	TG	ТΤ	cc	G -	• •	• •	CAA	A C A				• •	- G (	с	445
343	3 A	A	Т.	ТΤ	G	C /	٩G	A	СТ	C/	G	G	r G I	GC	AG	ТΤ	GC	0.0	GG	ΤG	000	GG	ΤA	тс	ΑA	۱A	GG	AG	GO	A	GA.	AC	A	ΤA	ΤA	AG	G <mark>A</mark>	A G (	GG	CG	GC	GG	00	G G A	GG	CA	GC	T G /	A A A	TG	ТΤ	cc	G -	• •		CAA	A C A				· ·	- G (	с	445
343	3 A	AT	Т.	TT	G	С	A G	A	СТ	C,	G	G	r G I	GC	AG	ТΤ	GC	C C	GG	ΤG	000	GG	ΤA	тс	AA	۱A	GG	AG	GC	A	G A	AC	A	ΤA	ΤA	AG	GA	A G (	GG	сG	GC	GG	00	GGA	GG	CA	GC	TG,	<b>۱</b> Α 4	TG	ТΤ	cc	G -				A C A					- G (	С	445
361	i A	AT	Т.	тс	G	C (	G	A	СТ	CC	G	G	r G I	GC	GG	ТΤ	GC	CC	G G	ΤG	000	GG	ТΤ	тс	GA	۱A	GG	AG	GO	GG	G A	GC	A	ΤA	ΤA	AG	AA	٩A	GG	CG	GC	GG	00	GGA	GG	CA	GC	C G ,	٩A	CG	ТΤ	cc	i G T	СС	тс	cge	C A					- G	TAT	470
361	i A	AT	Т.	тс	G	C (	G	A	СТ	CC	G	G	r G I	GC	GG	ΤТ	GC	CC	GG	ΤG	000	GG	ТΤ	тс	GA	۱A	GG	AG	GC	GG	G A	GC	A 1	ΤA	ΤA	AG	AA	٩A	GG	сG	GC	GG	00	GGA	GG	CA	GC	C G .	1 A C	CG	ТΤ	cc	i G T	СС	тс	cge	C A				• •	- G	TAT	470
361	I A	AT	Т.	тс	G	C (	G	A	СТ	CC	G	G	r G I	GC	GG	ТΤ	GC	CC	G G	ΤG	000	GG	ТΤ	тс	GA	۱A	G G	AG	GO	GG	3 A	GC	A	ΤA	ΤA	AG	AA	٩A	GG	cG	GC	GG	• C C	GGA	GG	CA	GC	C G ,	٩A	CG	ТΤ	ccc	i G T	СС	тс	cge	C A					- G	TAT	470
331	i A	AT	Т.	тс	G	C (	G	A	СТ	CC	G	G	r G I	GC	GG	ΤТ	GC	CC	GG	ΓG	000	GG	ТΤ	тс	GA	۱A	GG	AG	GO	GG	G A	GC	Α.	ΤA	ΤA	AG	AA	٩A	GG	CG	GC	GG	00	GGA	GG	CA	GC	C G ,	A A C	CG	ТΤ	cc	i G T	СС	тс	cge	C A					- G	TAT	440
331	I A	AT	Т.	ТС	G	C (	G	A	CT	CC	G	G	T G I	GC	GG	ΤT	GC	CO	G G	TG	00	GG	ТΤ	TC	GA	۱A	GG	AG	GC	GG	3 A	GC	A	ΤA	ΤA	AG	AA	ΑA	GG	CG	GC	GG	00	GG	GG	CA	GC	CG	۱A	CG	ТΤ	CC	G T	CC	TC	CGG	CA					- G	ТАТ	440
						-																																																										
442	2 A	A 4	A		G	CC	e G	C	GG		G	<u> </u>	• A	GG	AG	GC	GG	0/	AGO	3 G (	1	GG	GG	GI	GA	G	GG	AG	GG	GG	A	GA	GC		GG	GC	Ge	A	GG	AG	CA	94		GG		GI	GI	GI		A .	A I	GG	AIG	AT	GA	GGC	360			GGG	GA	TGO		561
421		A 1	<b>.</b>	G	Â	CC	12	C	GG	A	G		A	TG	GI	C I	I G	Ê,	A.	GG	<b>^</b>	GG	AG	AC	CI	-	GG	TG	GA	G	90	10			A 1	AC	G		9 G 9 G	<u>аа</u>	CA	G,	GU		A		GC	G I		<u>.</u>	A	GG	A I G		GA	GGC		TG		GG	GA	IG		540
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421	10	AI	9		A	0						1					00					0 0 ~ T		A C	TO	+		TO					÷.,		AC	AU				ŶŶ	CA							9 I T T -			<u>^</u>							T T .		GA	GA		666	5.40
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440			1		0	0				<u>^</u> .	5	1			<b>.</b>	- 0	00					3 <u> </u>		10	10	1	۶Â	10		Ľ,	A 1	66	÷ .		AG		00		- A	<u>^</u>	00		\					 			<u>^</u>	G · ·		2.2				÷÷.	+ + +		• •			542
440			-			~			00	Ω.		14			<u>.</u>		00					3 <u>+</u>		10	10	+	20	10	2		<u>,</u>	00	÷ .				00	1	<b>`</b> `	21	00	2	)		-						<u>^</u>	<u> </u>		22	22			+ + +	+ +		• •			542
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448					0	~		í,	66			14	20		č .							3 T	C T	00	T o	+	22	TG	2	Ľ.	<u>,</u>	66	÷			<u> </u>			22	2÷	66						a T	тт.		î,	2÷	a		<u>A</u>	22			÷÷.	+ +					543
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441	iĞ	0.0	÷	c G	Δ	c (		c.	C A	c (	GO		G	AC	ĞΑ	. c	GG	C.		GG	гт)	ΑT	GG	τŤ	τc	0	сс	тG	GT	Ge	зŤ	тс	G I	A G	G A	τŤ	GZ	Δ.	тĞ	ĀŤ	GG	GA	T T		T	AG	ĞТ	τŤ	GO	AT	AT	Ğ.,	ď		GG	GGT	GT	τŤ.	τ τ d					546
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562	2 A	AC	Т	тт	т	т	r g	A	G A	A	A	G	эс	GG	AG	GG	G A	Т	3 C	тт	гτ	sс	сg	сс	GC	c	GG	АA	GT	т	GG	ст	GO	зA	АT	CA	TA	٩A	c -		G A	СТ	гтт	G A	cc	GA	GТ	GG	Т	AC	G-		ГБТ	CA	ст	стс	GA	GT	ттт	GA	CG	AG	ТАА	675
541	I A	GT	т	тG	т	т	G	A	ΤА	A	A	GG	эс	сG	AA	GG	G A	Т	3 C	тт	тт	AC	сg	тс	GC	c	GΤ	сG	GT	т	C A	АТ	GO	зA	AC	ΤА	LT Α	AΑ	ΤТ	тт	GΑ	TG	т	GA	GG	GA	GΑ	ΤG	A T C	AC	<b>G</b> -	•	Г <mark>Б</mark> Т	сс	ΤТ	ATC	G A	GC	ТΑТ	TA	A ·			651
532	2 A	ст	т	ΤG	т	т	G G	A	τА	A	A	GO	эс	ΤG	AА	GG	C A	Т	зс	тт	τт	АС	сg	сс	GС	: c	GТ	ст	GТ	т.	CA.	А Т	GO	зA	АT	C A	Υ.Τ.A	AΑ	тт	АТ	GΑ	ce	GO	GA	A	GA	GΑ	TGO	Т	AC	<b>G</b> -	•	ГБТ	CG	сτ	ттс	GA	GT	τА	ТА	А-			642
538	3 A	GТ	т.	ΤG	т	т	e G	С	тΑ	A	A	GG	ЭС	AG	ΑA	GG	G A	т	ЭС	тт	τт	GС	сG	ст	тс	c	GТ	сс	GТ	A	CA	GТ	GO	βA	АT	C A	ι Τ A	AΑ	тс	АТ	GΑ	AG	т	GG	C C	GC	GΑ	тg	A T C	AC	GA	CG	ГАТ	CG	ΤТ	ATC	G 🗚	G T T	ТΑТ	ТА	A -			651
543	3.								. A	A	A	GG	ЭС	GG	ΑA	G G	G C	CC	ЭТ.	ΤA	сτ	ст	ст	сс	CA	C	GC	СТ	тс	т.	ΤТ	ст	T	A G	GΤ	CG	т	GG	GТ	тс	AG	CI	GG	GA	Т	ΑT	GТ	TGO	AA	GT														620
357	7 -		-						- A	A	A	r G (	ЭС	GG	ΑA	G G	G C	C C	Э Т 1	ΤA	сΤ	ст	ст	сс	CA	C	GC	СТ	тс	т	ΑT																																	397
543	3 -		-		÷			·	- A	A	A	GG	ЭС	GG	ΑA	GG	GC	CC	ЭТ.	T A	сΤ	ст	СТ	сс	CA	C	GC	СТ	тс	т.	ΤТ	СТ	ти	AG	GΤ	CG	тс	GG	GТ	тс	AG	СТ	GG	G G <mark>A</mark>	Т	ΑT	GΤ	ΤGO	AA	GT														620
543	3.		-		-			•	- A	A	A	GG	ЭС	GG	ΑA	G G	G C	C C	Э Т 1	ΤA	сΤ	ст	СТ	сс	CA	C	GC	СТ	тс	т.	ΤТ	ст	тZ	AG	GΤ	CG	т	GG	GТ	тс	AG	СТ	r <mark>G G</mark>	G G <mark>A</mark>	Т	ΑT	GΤ	TGO	AA	GT														620
543	3 -		-	• •	·		• •	·	- A	A	A	GG	ЭС	GG	ΑA	GG	GC	CC	ЭТ.	T A	сΤ	ст	СТ	сс	CA	C	GC	СТ	тс	т.	ΤТ	СТ	C/	A G	GΤ	СG	тс	GG	ЭΤ	тс	AG	СТ	r G G	G G <mark>A</mark>	Т	ΑT	GΤ	ΤGO	AA	GT														620
544	4 -		-		-		• •	•	- A	A	A	GG	ЭC	GG	ΑA	G G	GC	CC	Э Т <sup>-</sup>	T A	сΤ	ст	СТ	сс	СТ	C	GC	СТ	тс	т.	ΤA	СТ	CC	GG	GΤ	CG	т	GG	GΤ	тс	AG	СТ	r G G	G G <mark>A</mark>	T	ΑT	GΤ	TGO	AA	GT				• •										621
544	4 -	• •	-		-		• •	•	- A	A	A	GO	ЭС	GG	ΑA	GG	GC	CC	Э Т 1	ΤA	СТ	СТ	СТ	СС	СТ	C	GC	СТ	тс	T	ΤA	СТ	CC	ЭG	G T	CG	Т	GG	GТ	тс	AG	CT	GG	G G 🖌	T	AT	GΤ	TGO	AA	GT					• •					• •	· ·			621
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544	+ -	• •	-		-		• •	•	- A	A	A	GO	G C	GG	ΑA	GG	GC	CG	3 T 1	ΤA	Т	СТ	СТ	сс	СТ	C	GC	СТ	TC	Т.	ТΑ	СТ	CC	GG	G T	CG	T	GG	GT	тс	AG	СТ	GG	G G <mark>/</mark>	T	ΑT	G T	TGO	AA	GT														621
577	/ -	• •	-	• •	-		• •	•	• A	C/	T	GO	9 C	GG	AA	GG	GC	CC	9 T 1	TG	СТС	GT	СТ	СС	СТ	C	GC	СС	CC	T	ТС	GT	Т	GG	GT	CG	T A	AG	ТΤ	ΤT	TG	ТТ	GG	G A	T	AT	GT	GGO	G 🦌	GT	GA	T G T	TG	AG	GΤ	СТС	G	TG	TGO	AG	TT.	AG.		681
577	′ -	• •	-	• •	-		• •	•	- A	C/	T	GO	9 C	GG	ΑA	GG	GC	CC	3 T 1	TG	СТС	GT	СТ	СС	СТ	C	GC	СС	сc	T I	ТС	G T	T	GG	GT	CG	T A	AG	ТΤ	ТΤ	ΤG	ТТ	GG	G G A	T	ΑT	GT	GGO	G G 🖌	GT	GA	T G '	I T G	AG	GΤ	СТС	G	TG	TGO	AG	TT	AG.		681
577		• •	-	• •	-		• •	•	• A	C/	TI	GO	ЭC	GG	AA	GG	GC	CC	9 T 1	ΤG	СТС	GT	СТ	сc	СТ	C	GC	СС	CC	T	тс	GT	Т	G G	GT	CG	T A	AG	ТТ	ΤT	TG	TT	GG	G G A	T	AT	GT	GGO	G	GT	GA	TG	ΓTG	AG	GT	CTC	GT	TG	TGO	AG	ΤŤ,	AG		681
547		• •	-	• •	-		• •	•	· A	C/	TI	GO	) C	GG	A A	GG	GC	CC	• T ·	TG	T	GT	CT	C C	CT	C	GC	CC	CC	T	TC	GT	TC	GG	GT	CG	T A	A G	ΤŤ	TT	TG	TI	GG	G A	TO	AT	GT	GGO	G A	GT	• •				• •	• • •				• •	• •			624
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Figure 3.1.1.2.1 b. Clustal alignment of *CBF* nucleotide sequences isolated from members of the *Ericaceae* compared to *Arabidopsis thaliana CBF*1-4 genbank sequences. Sequences are ordered by Clustal sorting. Names are in the form "Species code pp primer pair code\_Genus\_Species\_Site code\_Location". Genbank gene names are in the form "Genus\_Species\_Gene\_Genbank code".

#### **3.1.1.3 All Other Species**

Two distinct sequences were isolated from *Trifolium repens*. One sequence isolated from samples 58 (Abisko, Sweden), 77 (Weardale, UK) and 94 (Weardale, UK) using primer pair 13 (Table 2.5.1a-g) contained an 18 base insertion (Fig. 3.1.1.3.1 b) not seen in other *CBF* sequences (which would result in an additional 6 amino acids). The sequences were mostly identical to one another except for some degenerate bases. The second sequence was isolated from the same samples using different primer pairs; sample 58 using primers 19 and 23, sample 77 using primer pairs 19, 23 and 24 and sample 94 using primer pairs 19 and 23(Table 2.5.1a-g), these sequences were very similar to one another with some substitutions and degenerate bases (Fig. 3.1.1.3.1 a-c).

All sequences isolated from *Betula nana* (sample 4 and sample "nula" (both from Abisko, Sweden)) using primer pairs CBF1, CBF2 and 59 (Table 2.5.1a-g) were very similar to one another (Fig. 3.1.1.3.1 a-c).

*Fragaria vesca* samples 74 (Abisko, Sweden) and 78 (Weardale, UK) had identical *CBF* sequences (Fig. 3.1.1.3.1 a-c) amplified using primer pair 50 (Table 2.5.1a-g).

Two distinct sequences were amplified from *Salix herbacea* sample 51 using primer pairs 51 and 53 (Table 2.5.1a-g). Not only were these sequences very different from one another they were also very different from any of the other sequenced *CBFs* (Fig. 3.1.1.3.1 a-c). However, megablast (NCBI, 2018) indicated high similarity to *CBF* sequences from a variety of *Populus* species.

*CBF* sequences isolated from sample 63, *Matricaria recutita* using primer pairs 5, 6 and 17 (Table 2.5.1a-g) were very similar to one another (Fig. 3.1.1.3.1 a-c) except for some degenerate bases and variation at the start of the sequence (Fig. 3.1.1.3.1 a).

Sample 90, *Leucanthemum vulgare*, had a *CBF* sequence (amplified by primer pair 12 – See section 2.5.1.1) distinct from but very similar to that of sample 63 *Matricaria recutita* (Fig. 3.1.1.3.1 a-c).

Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 Arabidopsis\_thaliana\_CBF3\_AT4G25480.1 Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 90pp12\_Leucanthemum\_vulgare?\_S19\_Weardale&Teesdale 4bpeCBF2\_Betula\_nana\_S2\_Abisko nulabpeCBF1\_Betula\_nana\_nula\_Abisko 4bpeCBF1 Betula nana S2 Abisko 4PP59 Betula nana S2 Abisko Nulapp59\_Betula\_nana\_nula\_Abisko\_REVonly 74pp50\_Fragaria\_vesca\_S14\_Abisko 78pp50\_Fragaria\_vesca\_S16\_Weardale&Teesdale 51PP51\_Salix\_herbacea\_S6\_Abisko 51pp53\_Salix\_herbacea\_S6\_Abisko 77pp 19\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp19\_Trifolium\_repens\_S22\_Weardale&Teesdale 77PP23&24B\_Trifolium\_repens\_S16\_Weardale&Teesdale 77PP23&24A\_Trifolium\_repens\_S16\_Weardale&Teesdale 58pp23B\_Trifolium\_repens\_S9\_Abisko 58pp 19\_Trifolium\_repens\_S9\_Abisko\_FWDonly 94pp23\_Trifolium\_repens\_S22\_Weardale&TeesdaleFWDonly 58pp23C\_Trifolium\_repens\_S9\_Abisko 63pp17\_Matricaria\_recutita?\_S11\_Abisko 63pp6\_Matricaria\_recutita?\_S11\_Abisko 63pp5\_Matricaria\_recutita?\_S11\_Abisko\_REVonly 58pp13\_Trifolium\_repens\_S9\_Abisko 77pp13\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp 13\_Trifolium\_repens\_S22\_Weardale&Teesdale\_FWDonly

Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 Arabidopsis\_thaliana\_CBF3\_AT4G25480.1 Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 90pp 12\_Leucanthemum\_vulgare?\_S19\_Weardale&Teesdale 4bpeCBF2 Betula nana S2 Abisko nulabpeCBF1\_Betula\_nana\_nula\_Abisko 4bpeCBF1\_Betula\_nana\_S2\_Abisko 4PP59\_Betula\_nana\_S2\_Abisko Nulapp59\_Betula\_nana\_nula\_Abisko\_REVonly 74pp50\_Fragaria\_vesca\_S14\_Abisko 78pp50\_Fragaria\_vesca\_S16\_Weardale&Teesdale 51PP51\_Salix\_herbacea\_S6\_Abisko 51pp53\_Salix\_herbacea\_S6\_Abisko 77pp19\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp19\_Trifolium\_repens\_S22\_Weardale&Teesdale 77PP23&24B\_Trifolium\_repens\_S16\_Weardale&Teesdale 77PP23&24A\_Trifolium\_repens\_S16\_Weardale&Teesdale 58pp23B\_Trifolium\_repens\_S9\_Abisko 58pp19\_Trifolium\_repens\_S9\_Abisko\_FWDonly 94pp 23\_Trifolium\_repens\_S22\_Weardale&TeesdaleFWDonly 58pp 23C\_Trifolium\_repens\_S9\_Abisko 63pp17\_Matricaria\_recutita?\_S11\_Abisko 63pp6\_Matricaria\_recutita?\_S11\_Abisko 63pp5\_Matricaria\_recutita?\_S11\_Abisko\_REVonly 58pp13\_Trifolium\_repens\_S9\_Abisko 77pp13\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp 13\_Trifolium\_repens\_S22\_Weardale&Teesdale\_FWDonly

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1			- ATGAACTCAT	TTT···CTGC	CTTTTCTGAAAT	G T T T	GGCTCCGAT	TACGAGTCTCC	G G T T T C C	T C A G G C G G T G A	AT 72
1			- AT <mark>G</mark> AACTCAT	TTT · · · CAGC	ΤΤΤΤΤ <mark></mark> ΟΤ <mark>Θ</mark> ΑΑΑΤ	Г <mark> </mark>	G G C T C C <mark>G A</mark> T '	TACGA <mark>GCCTC</mark> A	<u> <u></u></u>	<mark>A</mark> GGCGG <mark>A</mark> GA	<b>T</b> 63
1			- AT <mark>G</mark> AACTCAT	TTTCTGC	ΤΤΤΤΤ <mark></mark> ΟΤ <mark>Θ</mark> ΑΑΑΤ	Г <mark>G</mark> Т Т Т	<mark>g g c t c c g</mark> a t 1	TACGAGTCTTC	<mark>6</mark> <mark>6</mark> Т Т Т <mark>С С</mark>	T C A G G C G G T G A	T 72
1			- AT <mark>G</mark> AATCCAT	TTTACTCTAC	ATTCCCA <mark>G</mark> ACTC	G T T T C T C T C A	ATCTCC <mark>G</mark> AT(	CATAGATCTCC	<u> 6</u> <mark>G Т Т Т С</mark> А	S A C A <mark>G T A G T G</mark> A	G 81
1								A A C A <mark>G</mark> A C T C T T	G · · · · <u>· · C T C A</u> .	A C A T C T <mark>G</mark> A C <mark>G</mark> A	G 28
1						<mark>T C G T C G G</mark>	AGTCGGAGAG	TGGGGCG-AT	<u> 6</u> <mark>С А С</mark> Т Т <mark>Б</mark>	TCGG <mark>ACG</mark> AGG -	G 44
1						· · · · <mark>T C G T C G G</mark>	AGTCGGAGAG	TGGGGCG-AT	<u> Э</u> <mark>С А С</mark> Т Т <mark>Б</mark>	TCGG <mark>A</mark> CG <mark>A</mark> GG -	G 44
1						<mark>ТССТССС</mark>	AGTCGGAGAG	T G G G G C G - A T	<u> 5</u> <mark>С А С Т Т </mark>	TCGG <mark>ACG</mark> AGG -	G 44
1					<u></u>		A	T G G G G C G - A T	<u> 6</u> <mark>С А С</mark> Т Т <mark>Б</mark>	TCGG <mark>ACG</mark> AGG -	G 29
1					<mark>с</mark> дд	A T A T T C G T A G T	A G T C G G A N A G	TGGGGCG-AT	<u> 6</u> <mark>С</mark> А С Т Т <mark>С</mark>	T C <mark>G G A C G A G G A</mark>	G 51
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1 <u></u>	AGTACTATGATO	CACAACCCA <mark>G</mark> T	T <mark>gg</mark> tt <mark>ggg</mark> att:	TGCTGGAATC	TTCGTCTCATTC	C G A T A A C A A C	AGCGGCGGGG	TTGGACGGAT	<u> 6</u> <mark>6</mark> С С <mark>Т Т </mark> 6	T C A <mark>G A C G A </mark> G G -	A 106
1 <mark>CAATT<mark>GGG</mark>TCCAT<mark>C</mark>AATTTI</mark>	TT <mark>GCCA</mark> GTTATI	T <mark>g a c g</mark> a <mark>g c a a</mark> g	AATGTTCTTAT	TCTTCTGCTC	TTTCAGATAGTA	A G C A T C A C T A G	TAGTGTTAC	T <mark>aaa<mark>gg</mark>a<mark>g</mark>ttc.</mark>	A A C A A <mark>G C C G</mark> C A.	A T T T T T T C T <mark>G</mark> A	T 128
1				TCTCAGCTGA	CAGACAAGAATC	A T C T T T <mark>G C T</mark> A	TCC <mark>G</mark> ACA <mark>G</mark> T/	A G C G G T T C T C A	G - CAA <mark>G</mark> ATTCG	C C C A <mark>G</mark> T T C <mark>G</mark> A A	C 74
1	]	TCACACCCCAT	TACCTCAATAC	сттсстсттс	CTCCCACAAAAT	T <mark>atct</mark> cttcca	.AATTTA- <mark>G</mark> A'	I <mark>G T C T C T C A T G</mark> .	а <mark>Т а Т </mark>	Τ <mark>ς α α ς</mark> ττςς α α	C 90
1		<u></u> .				<mark>СТТССА</mark>	AATTTA <mark>TG</mark> A'	I <mark>G T C T C T C A T G</mark> .	<u> </u>	Τ <mark>ς αα</mark> ς ττςς αα	C 44
1		· · · · · <mark>CCCC</mark> AA	TTCCTCAATAC	сттсстсттс	CTCCCACAAAA	CATCTCTTCCG	AATTTA - <mark>G</mark> A'	I G T C T C T C A T G I	<u> 3</u> <mark>Т А Т </mark>	T C A A C T T C C A A	C 85
1		· · · · · <mark>CCCC</mark> AA	TTCCTCAATAC	сттсстсттс	CTCCCACAAAA	CATCTCTTCC <mark>G</mark>	ΑΑΤΤΤΑ - <mark>Θ</mark> Α΄	I <mark>G T C T C T C A T</mark> G I	3 <mark>T A T G</mark>	T C A A C T T C C A A	C 85
1			····CCATCTC	ATACTTCTCT	TCTCCACAAAA	ATCTCTTCCR	A - TTTA - <mark>G</mark> A '	I G T C T C T C A T G	R · · · · · · TATG	T C A A C T T C C A A	C 74
1						<u></u> .	ΑΑΤΤΤΑ - <mark>Θ</mark> Α΄	I <mark>G T C T C T C A T G</mark> .	A · · · · · · <b>T A T G</b>	Τ <mark>ς αας</mark> ττςς αα	C 36
1						ATCTCTTCCR	A - TTTA - <mark>G</mark> A '	I <mark>G T C T C T C A T G</mark>	R · · · · · · <mark>T A T G</mark>	T C A A C T T C C A A	AC 45
1					· · · · · · · · · · · · · · ·	ATCTCTTCCG	A · TTTA · GA	I G T C T C T C A T G	R · · · · · · TATG	TCAACTTCCAA	C 45
1		<u></u> .	- TCTTTTCTGT	C T T C G T C G T C	CAT GAAAC -	- <mark>G C C C T T T A</mark> -	- CGTGTACCO	AC-GGAA <mark>GT</mark> G	G TA <mark>G C C C</mark> T T C A	T C A C A A C T T <mark>G</mark> -	- 75
1		· · ATCTTACAA	CTCTTTTCTGT	CTTCGTCGTC	CATAT - GAAAC -	- GCCCTTTA-	- YGTGTACCO	AC-GGAA <mark>GT</mark> GI	G T A G C Y Y T T C A	T C A C A A C T T <mark>G</mark> -	- 87
1				<mark>6 T T C </mark> 6 T C	CATATCGAAACC	GACCCTTTAA	C T G T G T A C C C	A CAGGAAGTG	G TAGCCYTTCA	TCASAACTT <mark>G</mark> -	- 70
1						· · · · · <mark>CACTTC</mark>	TTCTTCTGA/	A A C A A <mark>G</mark> T T C T T	с <u>тт</u> сааа	C C <mark>G</mark> T A T A A <mark>G</mark> T T	C 45
1						···· <mark>CACTTC</mark>	TTCTTCTGA/	A A C A A <mark>G</mark> T T C T T	С <mark>Т Т С</mark> ААА	C C <mark>G</mark> T A T A A <mark>G</mark> T T	C 45
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73 TACA - GTCCGAAGCTTGCCA	- C <mark>GAGCTG</mark> CCCC	CAA <mark>G</mark> AAACCA <mark>G</mark>	CGGG <mark>AA</mark> GG <mark>AA</mark> G	- AA <mark>G</mark> TTTC <mark>G</mark> T	GAGACTCGTCAC	CCAATTTACA	GAGGAGTTC			З	T 107
											107
64 A G CCGACG GGCCA	- CGAGTTGTCCG	3 A A G A A A C C G G	CGGGCCGTAAG	- AAGTTTCGT	GAGACTCGTCAC	CCAATTTACA	GAGGAGTTC	TCAAAGAAAC	TCCGGTAAGTG	GTTTCTGAAG	T 188
73 TATA - TTCCGACGTTGGCCA	- CGAGTTGTCCG	SAAGAAACCGG CAAGAAACCGG	CGGGCCGTAAG	- <mark>AAGTTTCG</mark> T - AAGTTTCGT	GAGACTCGTCAC GAGACTCGTCAC	CCAATTTACA	GAGGAGTTCC GAGGAGTTCC	T CAAAGAAAC	TCCGGTAAGTG	S G T T T <mark>C</mark> T G A A G S G T T T G T G A G G	T 188
73 TATA - TTCCGACGTTGCCA 82 TGTT - CACCAAAGTTAGCT	A - COAGTTOTCCO A - OCAGCTOCCCO - CAAGTTOTCCA	3 A A G A A A C C G G C A A G A A A C C G G A A A G A A A C G A G	CGGGCCGTAAG CGGGTCGTAAG CTGGGAGGAAG	- AA <mark>G TTTCG T</mark> - AAG TTTCG T - AAG TTTCG T	G A G A C T C G T C A C G A G A C T C G T C A C G A G A C A C G T C A T	CCAATTTACA CCAATATACA CCC <mark>G</mark> ATTTACA	GAGGAGTTC GAGGAGTTC GAGGAGTTC	T CAAAGAAAC T CGGAGAAAC	T C C G G T A A G T G T C C G G T A A G T G T C T G G T A A A T G	G G T T T <mark>C T G A A G</mark> G G T T T G T G A G G G G T T T G T G A A G	T 188 T 197
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54 TATT- GTCCGACGTTGGCCA 73 TATA- TTCCGACGCTTGCCA 82 TGTT-CACCAAGGTTAGCT 29 GATG-TCC ACCTGGCG 45 GATG-TCC CCTGGCG	CGAGTTGTCCG CAGCTGCCCC CAAGTTGTCCA CCCAAAACCCCC CCCAAAACCCCCCCCCC	3 A A G A A A C C G G C A A G A A A C C G G A A A G A A A C G A G 3 A A A A A A G C G A G 3 A A G A A A C G G G	CGGGCCGTAAG CGGGTCGTAAG CTGGGAGGAAG CAGGCAGGAAG CGGGGAGGAAG	- AAGTTTCGT - AAGTTTCGT - AAGTTTCGT - AAGTTTAGA - AAGTTCAAG	GAGACTCGTCAC GAGACTCGTCAC GAGACACGTCAT GAGACACGTCAC GAGACGAGGCAC	CCAATTTACA CCCAATATACA CCCGATTTACA CCCGGTGTATC CCCGGTGTACY	GAGGAGTTCC GAGGAGTTCC GAGGAGTTCC GTGGGGTTCC GGGGAGTGAC	TCAAAGAAAC TCGGAGAAAC TCAGAGGAAAC AAARAGGGAT GAGGARGAAW	TCCGGTAAGTG TCCGGTAAGTG TCTGGTAAATG TCGGGTAAATG TCCGGGAAGTG	G G T T T C T G A A G G G T T T G T G A G G G G T T T G T G A G G G T T T G T G A A G G G T C T G C G A R G G G T C T G C G A R G	T 188 T 197 T 206 T 150
64   A   I - CEC A CG   TG C C A 73   A T A - T T C CG A CG C T TG C C A 82   TG T T - C A C C A A A G T T A G C T 29   G A T G - T C C A C C T G C C G 45   S A T T - C T G C T G C C G 45   S A T T - C T G C T G C C G 45   S A T T - C T	A - CG AG T T G T C C A - G C AG C T G C C C - C A AG T T G T C C - C C A AAAC C C C - C G AG G AAC C C C - C G AG G AAC C C C	5 A A G A A A C C G G C A A G A A A C C G G A A A G A A A C G A G 5 A A A A A A C G A G 5 A A G A A A C G G G 5 A A G A A A C G G G	C G G G C C G T A A G C G G G T C G T A A G C T G G G A G G A A G C A G G C A G G A A G C G G G G A G G A A G C G G G G A G G A A G	- AAG T T T C G T - AAG T T T C G T - AAG T T T C G T - AAG T T T AG A - AAG T T CAAG - AAG T T CAAG	GAGACTCGTCAC GAGACTCGTCAC GAGACACGTCAT GAGACACGTCAC GAGACGAGGCAC GAGACGAGGCAC	CCAATTTACA CCCAATATACA CCCGATTTACA CCCGGTGTATC CCCGGTGTACY	GAGGAGTTCC GAGGAGTTCC GAGGAGTTCC GTGGGGTTCC GGGGAGTGAC GGGGAGTGAC	TCAAAGAAAC TCGGAGAAAC TCAGAGGAAT AAARAGGGAT GAGGARGAAW	T C C G G T A A G T G T C C G G T A A G T G T C T G G T A A A T G T C G G G T A A A T G T C G G G A A G T G T C C G G G A A G T G	G G T T T C T G A A G G G T T T G T G A G G G G T T T G T G A A G G G T R T G T G A A G G G T C T G C G A R G G G T C T G C G A R G	T 188 T 197 T 206 T 150 T 164
64   A T - G   C C G A C G   T G C C / 73 T A T A - T T C C G A C G C T G C G / 82 T G T - C A C C A A G T T A G C T 29 G A T G - T C C A C C T G C C G 45 G A T - C T G C T G C C G 45 G A T - C T G C T G C C G	A - CGAGTTGTCC - GCAGCTGCCC - CAAGTTGTCCA - CCAAGACCCC - CGAGGAACCCC - CGAGGAACCCC - CGAGGAACCCC	3 A A G A A A C C G G C A A G A A A C C G G A A A G A A A C G A G 3 A A A A A G G G G 3 A A G A A A C G G G 3 A A G A A A C G G G 3 A A G A A A C G G G	CGGGCCGTAAG CGGGTCGTAAG CGGGCAGGAAG CAGGCAGGAAG CGGGGAGGAAG CGGGGAGGAAG	- AAGTTT CGT - AAGTTT CGT - AAGTTT CGT - AAGTTT AGA - AAGTT CAAG - AAGTT CAAG - AAGTT CAAG	GAGACTCGTCAC GAGACTCGTCAC GAGACACGTCAT GAGACACGTCAC GAGACGAGGCAC GAGACGAGGCAC GAGACGAGGCAC	CCCAATTTACA CCCAATATACA CCCGATTTACA CCCGGTGTATC CCCGGTGTACY CCCGGTGTACY CCCGGTGTACY	GAGGAGTTCC GAGGAGTTCC GAGGAGTTCC GTGGGGTTCC GCGGAGTGAC GGGGAGTGAC GGGGAGTGAC	T CAAAGAAAAC T CGGAGAGAAAC T CAGAGGAAAC AAARAGGGAT G AGGARGAAW G AGGARGAAW	T C C G G T A A G T G T C C G G T A A G T G T C T G G T A A A T G T C G G G T A A A T G T C C G G G A A G T G T C C G G G A A G T G T C C G G G A A G T G	SGTTTCTGAAG SGTTTGTGAAG SGTTTGTGAAG SGTTTGTGAAG SGTCTGCGARG SGTCTGCGARG SGTCTGCGARG	T 188 T 197 T 206 T 150 T 164 T 164
1         1         1         C	- CAASTTGTCCC - GCAGCTGCCCC - CAASTTGTCCCC - CCAAAACCCC - CGAGGAACCCC - CGAGGAACCCC - CGAGGAACCCC - CGAGGAACCCC	3 A A G A A A C C G G C A A G A A A A C G A G 3 A A A A A A C G A G 3 A A G A A A C G A G 3 A A G A A A C G G G 3 A A G A A A C G G G 3 A A G A A A C G G G	C G G G C C G T A A G C G G G T C G T A A G C G G G T C G T A A G C G G G C A G G A A G C G G G G A G G A A G C G G G G A G G A A G C G G G G A G G A A G	- AAGTTTCGT - AAGTTTCGT - AAGTTTCGT - AAATTTAGA - AAGTTCAAG - AAGTTCAAG - AAGTTCAAG - AAGTTCAAG	GAGACTCGTCAC GAGACTCGTCAC GAGACACGTCAT GAGACACGTCAC GAGACGAGGCAC GAGACGAGGCAC GAGACGAGGCAC GAGACGAGGCAC GAGACGAGGCAC	CCCAATTTACA CCCAATATACA CCCGATTTACA CCCGGTGTATC CCCGGTGTACY CCCGGTGTACY CCCGGTGTACY	GAGGAGTTCC GAGGAGTTCC GAGGAGTTCC GTGGGGTTCC GGGGAGTGAC GGGGAGTGAC GGGGAGTGAC	T CAAAGAAAC T CGGAGAGAAAC T CGGAGGAGAAAC AAARAGGAA G AGGARGAAW G AGGAGGAGAAW G AGGAGGACGAAW	T C C G G T A A G T G T C C G G T A A G T G T C T G G T A A A T G T C C G G G A A G T G T C C G G G A A G T G T C C G G G A A G T G T C C G G G A A G T G	G T T T C T G A A G G G T T T G T G A G G G T T T G T G A A G G G T C T G C G A A G G G T C T G C G A R G G G T C T G C G A R G G G T C T G C G A R G G G T C T G C G A R G	T 188 T 197 T 206 T 206 T 150 T 164 T 164 T 164
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141       1       1       1       1       0       1       0	- CAASTTGTCCC - CAASTTGTCCC - CAASTTGTCCC - CCAAAACCCC - CCAASGAACCCC - CCAASGAACCCC - CCAASGAACCCC - CCAASGAACCCC - CCAASGAACCCC - CCAASGAACCCC - CCAASGAACCCC - CCAASGAACCCC - CCAASGAACCCC - CCAASGAACCCC	3 A A G A A A C C G G C A A G A A A C C G G 3 A A G A A A C G A G 3 A A G A A A C G A G 3 A A G A A A C G G G 3 A A G A A A C G G G 3 A A G A A A C G G G 3 A A G A A A C G G G 3 A A G A A A C G G G 3 A A G A A A C G G G 3 A A G A A A C G G G 3 A A G A A A C G G G 3 A A G A A A C G G G 3 A A G A A A C G G G 3 A A G A A A C G G G	C G G C C G T A A G           C G G C T C G T A A G           C G G G T C G T A A G           C G G G A G A A G           C G G G G A G A A G           C G G G A G A A G           C G G G A G A G A A G           C G G G A G A G A A G           C G G G A G A G A A G           C G G G A G A G A A G           C G G G A G A G A A G           C G G G A G A G A A G           C G G G G A G A G A A G           C G G G G A G A G A A G           C G G G G A G A G A A G           C G G G G A G A G A A G	- AAG TTTCGT - AAG TTTCGT - AAG TTTCGT - AAG TTCAAG - AAG TTCAAG - AAG TTCAAG - AAG TTCAAG - AAG TTCAAG - AAG TTCAAG	G AG A C T CG T CA C G AG A C T CG T CA C G AG A C A CG T CA T G AG A C A CG T CA T G AG A CG AG G C A C G AG A CG AG G C A C	CCGG TG TACC	GAGGAGTTCC GAGGAGTTCC GAGGAGTTCC GGGGGGTTCC GGGGAGTGAC GGGGAGTGAC GGGGAGTGAC GGGGAGTGAC GGCGCGCGAGTGAC	T C AAAG AAAC T C G G AG AAAC T C AG AG G AAAC G AG G AG G A G AG G AG	TCCGGTAAGTG TCCGGTAAATG TCGGGTAAATG TCCGGGAAGTG TCCGGGAAGTG TCCGGGAAGTG TCCGGGAAGTG TCCGGGAAGTG TCCGGGAAGTG	G G TTT C T G A A G G G TTT G T G A A G G G T T T G T G A A G G G T C T G C G A R G G T C T G C G A R G G T C T G C G A R G G T C T G C G A R G G T C T G C G A R G G T C T G C G A R G G T T T G C G A R	T 188 T 197 T 206 T 150 T 164 T 164 T 164 T 164 T 164 T 149 T 149 T 171
14       14       1	- CAAGTTGTCCC - CAAGTTGTCCC - CAAGTTGTCCC - CAAGTTGTCCC - CAAGAACCCC - CAAGAACCCCC - CAAGAACCCCCC - CAAGAACCCCC - CAAGAACCCCC - CAAGAACCCCC - CAAGAACCCCC - CAAGAACCCCCC - CAAGAACCCCCC - CAAGAACCCCCC - CAAGAACCCCCC - CAAGAACCCCCC	A A 9 A A A C C C 6 C C A 4 9 A A A C C 6 C 6 A A 9 A A A C 6 A 6 A 4 9 A A A 6 C 6 A 6 A 4 9 A A A 6 C 6 A 6 A 4 9 A A A C 6 C 6 A 4 9 A A A C 6 C 6 A 4 9 A A A C 6 C 6 A 4 9 A A A C 6 C 6 A 4 9 A A A C 6 C 6 A 4 9 A A A C 6 C 6 A 4 9 A A 6 A 6 C 6 A 4 9 A A 6 A 6 C 6 C 6 A 4 9 A 4 6 A 6 C 6 C 6	C         G         C         C         T         AAG         C         C         T         AAG         C         C         T         C         C         T         AAG         C </td <td>- AAGTTTCGT - AAGTTTCGT - AAGTTTCAT - AAGTTTCAAG - AAGTTCAAG - AAGTTCAAG - AAGTTCAAG - AAGTTCAAG - AAGTTCAAG - AAATTCAAG</td> <td></td> <td>CCGG TG TACC</td> <td>GAGGAGTTCC GAGGAGTTCC GTGGGAGTTCC GGGGAGTGAC GGGGAGTGAC GGGGAGTGAC GGGGAGTGAC GGGGAGTGAC GGCGCGTGAC</td> <td>T C AAAG AAAC           T C G G G AAAAC           T C A G G AAAAC           T C A G AAAAC           T C A G AAAAC           A A A A A G G AA           G A G G A G AAW           G A G G A G A AAAC           G A G G A G A AAAC           G A G G A G A AAAC</td> <td>TCCGCTAAGTG TCCGCTAAGTG TCGGTAAATG TCGGGAAATG TCCGGGAAGTG TCCGGGAAGTG TCCGGGAAGTG TCCGGGAAGTG TCCGGCAAGTG TCCGGCAAGTG</td> <td>G TTT CTCAAG G TTT CTCAAG G TTT CTCAAG G CTCGCAAAG G CTCGCCAAC G CTCGCCAAC G CTCGCCAAC G CTCGCCAAC G CTCGCCAAC G CTTGCCAAC G CTTGCCAAC G CTTGCCAAC</td> <td>T 188 T 197 T 206 T 150 T 150 T 164 T 164 T 164 T 164 T 164 T 149 T 149 T 171 T 226</td>	- AAGTTTCGT - AAGTTTCGT - AAGTTTCAT - AAGTTTCAAG - AAGTTCAAG - AAGTTCAAG - AAGTTCAAG - AAGTTCAAG - AAGTTCAAG - AAATTCAAG		CCGG TG TACC	GAGGAGTTCC GAGGAGTTCC GTGGGAGTTCC GGGGAGTGAC GGGGAGTGAC GGGGAGTGAC GGGGAGTGAC GGGGAGTGAC GGCGCGTGAC	T C AAAG AAAC           T C G G G AAAAC           T C A G G AAAAC           T C A G AAAAC           T C A G AAAAC           A A A A A G G AA           G A G G A G AAW           G A G G A G A AAAC           G A G G A G A AAAC           G A G G A G A AAAC	TCCGCTAAGTG TCCGCTAAGTG TCGGTAAATG TCGGGAAATG TCCGGGAAGTG TCCGGGAAGTG TCCGGGAAGTG TCCGGGAAGTG TCCGGCAAGTG TCCGGCAAGTG	G TTT CTCAAG G TTT CTCAAG G TTT CTCAAG G CTCGCAAAG G CTCGCCAAC G CTCGCCAAC G CTCGCCAAC G CTCGCCAAC G CTCGCCAAC G CTTGCCAAC G CTTGCCAAC G CTTGCCAAC	T 188 T 197 T 206 T 150 T 150 T 164 T 164 T 164 T 164 T 164 T 149 T 149 T 171 T 226
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1       A       A       A       A       C       C       C       C       C       C       C       C       C       C       C       C       C       C       C       C       C       C       T       C       C       C       T       C       C       C       T       C       T       C       T       C       C       C       T       C	<ul> <li>CAAGITTOTCOCC</li> <li>CAAGITTOTCOCC</li> <li>CAAGITTOTCOCC</li> <li>CAAGITTOTCOCC</li> <li>CAAGAACCOC</li> <li>CAACACCOC</li> <li>CAAGAACCOC</li> </ul>	CAAGAAACCCCC AAGAAACCCCC AAGAAACCCAC SAAAAACCCCC SAAGAAACCCCC SAAGAAACCCCC SAAGAAACCCCC SAAGAAACCCCC SAAGAACCCCC SAAGAACCCCC SAAGAACCCCC SAAGAACCCCCC SAAGAACCCCCC	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	- AAG TT TCG T - AAG TT TCG T - AAG TT TCG T - AAG TT TCAAG - AAG TT CAAG - AAG TT TCAAG - AAG TT TCAAG - ATT TCAAG			GAGGAGTTCC GAGGAGTTCC GAGGAGTTCC GGGGAGTGA GGGGAGTGA GGGGAGTGA GGGGAGTGA GGGGAGTGA GCGGCGTGA GAGGGGTGTA	T         C         AAAA         AAAA         C           T         C         G         AAAA         AAAA         C           T         C         G         AAAAA         G         AAAT         C           G         AAGAA         G         G         AAAAA         G         G         G         G         AAAAA         G         G         AAAAA         G         G         G         G         AAAAA         G         G         G         AAAAA         G         G         G         G         AAAAA         G         G         G         AAAAAA         G         G         G         G         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	TCCGCTAAGTG TCCGCTAAATG TCGGCTAAATG TCCGCGAAGTG TCCGGCAAGTG TCCGGCAAGTG TCCGGCAAGTG TCCGGCAAGTG TCCGGCAAGTG TCCGCCAAGTG TCCGCCAAGTG TCCGCCAAGTG	SG TTT CTG AAG SG TTTG TG AAG SG TRTG TG AAG SG TCTG CG ARG SG TCTG CG ARG SG TCTG CG ARG SG TCTG CG AAG SG TCTG CG AAG SG TTTG CG AAG SG TTTG CG AAG SG TTTG CG AAG	T       188         T       197         T       206         T       150         T       164         T       126         T       226         T       226         T       253         T       199
141       1       1       1       1       1       0       1       0       0       0       1       0       0       1       1       0       1       1       1       0       1	. CAAGTTOTCCC CAAGTTOTCCC . CAAGTTOTCCC . CCAAGACCCC . CCAAGACCCC . CCAAGACCCC . CCAGGAACCCC . CCAGGAACCCC . CCAGGAACCCC . CCAGGAACCCC . CCAGGAACCCC . CCAGGAATCCC . CCAGGAATCCC . CCAGCCCCCC	A A G A A A C C A G           A A G A A A C A A C           A A G A A A C A A           A A G A A A C A A           A A G A A A C A A           A A G A A A C A A           A A G A A A C A A           A A G A A A C A A           A A G A A A C A C A           A A G A A A C A C A           A A G A A A C A C A           A A G A A A C A C A C A           A A G A A A C A C A C A           A A G A A A C A C A C A           A A G A A A C A C A C A           A A G A A A C A C A C A           A A G A A A C A C A C A           A A G A A A C A C A C A C A           A A G A A A C A C A C A C A C A C A C A	C C C C C C C C T A A C C C C C C C C T A A C C C C C C C C C A A C C A C C A C A	- AAG TT TCG T - AAG TT TCG T - AAG TT TCG T - AAG TT TCG A - AAG TT CAAG - AAG TT TAGG - ATT CAAG - AAG TT CAAG - AAG TT CAAG	A         A         C         T         C         T         C         T         C         A         A         C         T         C         A         A         C         T         C         A         A         C         T         C         A         A         C         T         C         A         A         C         A         C         A         C         A         C         A         A         A         C         A         C         A		G A G G A G T T C G A G G A G T T C G A G G A G T T C G G G G A G T G A G G G G G G T G A G A G G G G T G A	T         C         AAAA         C           T         C         G         AAAA         C           T         C         G         AAAA         AAAA         C           AAAAA         AAAAA         G         AAAAA         G         AAAAA         G         AAAAA         AAAAA         G         AAAAAA         G         AAAAAA         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	TC C C C TAAG TG TC C C TAAG TG TC TG C TAAA TG TC C C C TAAA TG TC C C C C AAA TG	C C C C C C C C C C C C C C C C C C C	T       188         T       197         T       206         T       150         T       164         T       126         T       226         T       226         T       253         T       199         T       215
14         14<	. C G A G T T C T C C C . C A A G T T C T C C C . C A A G T T C T C C C . C A A G T T C T C C A . C C A A C C C . C A A G A A C C C . C A A G A A C C C . C A A G A A C C C . C A A G A A C C C . C A A G A C T A C C C . C A A G A C T A C C C . C A A G T A C C C C . C A A G T A C C C C . C A A G T A C C C C C C . C A A G T A C C C C C . C A A G T A C C C C C C . C A A G T A C C C C C C . C A A G T A C C C C C C C . C A A G T A C C C C C C . C A A G T A C C C C C C C C C C C C C C C C C C	C A G A A A C C C G C C A Q A A A C C G A C A A Q A A A C G A C S A A A A A A C C G C G S A A G A A A C C G C G S A A G A A A C C G C G S A A G A A A C C G C G S A A G A A A C C G C G S A A G A A A C C G C G S A A G A A A C C G C G S A A G A A G C C C T G S A A G A A G C C T G S A A G A A G C C T G	• • • • • • • • • • • • • • • • • • •	- AAG TIT C GT - AAG TIT C GT - AAG TIT C GT - AAG TIT C GT - AAG TI C AAG - AAG TI C AAG - AAG TI C AAG - AAG TI C C AAG - AAG TI C C AAG - AAG TI C AAG - AAG TI C AAG - AAG TI C AAG - AAG TI C AAG		A       A       T       T       A	A & G & A & G & A & G & T & T & C & G & G & A & G & T & T & C & G & G & A & G & A & G & G & G & A & G & G	T         C         AAAA         AAAA         C           T         C         G         AAAA         AAAA         C           T         C         G         AAAA         AAAA         AAAAA         C         AAAA         AAAA         AAAAA         AAAAAA         AAAAA         AAAAAA         AAAAAA         AAAAAAAA         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	TC C G C TAAG TG TC C G C TAAG TG TC TG C TAAATG TC C G C TAAATG TC C G C G AAG TG TC C G C CAAG TG TC C G C CAAG TG TC C G C CAAG TG TC C G C C AAG TG TTAG C TAAATG	CONTROLOGY	T       187         T       197         T       206         T       150         T       164         T       126         T       226         T       226         T       226         T       226         T       226         T       226         T       253         T       199         T       215         T       169
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141       141       141       141       140       141       140       1	. C G A G T T C T C C C C C A G T T C T C C C . C A G T T C T C C C . C A G A A C C C . C G A G A A C C C . C G A G A A C C C . C G A G A A C C C . C G A G A A C C C . C G A G A C T A C C C . C G A G A C T A C C C . C A G G A C T C C C . C A G G A C T C C C C . C A G G A T A C C C C C . C G A G T A C C C C C . C G A C T A C C C C C . C G A T A C C C C C . C G A T A C C C C C . C G A T A C C C C C . C G A T A C C C C C . C G A T A C C C C C . C G A T A C C C C C . C G A T A C C C C C . C G A T A C C C C C . C G A T A C C C C C C . C G A T A C C C C C C . C G A T A C C C C C C . C G A T A C C C C C C . C G A T A C C C C C C . C G A T A C C C C C C C C C C C C C C C C C		C G G G C C G T A A G           C G G G T C G T A G           C G G G T C G T A G           C G G G T C G T A G           C G G G G A G A A G           C G G G G A G G A A G           C G G G G A G G A A G           C G G G G A G G A A G           C G G G G A G G A A G           C G G G G A G G A A G           C G G G G A G G A A G           C G G G A G G A A G           C G G G A G G A A G           C G G G A G G A A G           C G G G A G G A A G           C G G G A G G A A G           C G G G A G G A A G           C G G G A G G A A G           C A G G A A G A A A G           G A G G G A G A A A G           G A G G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G	- AAG TIT CG T - AAG TIT CG T - AAG TIT CG T - AAG TIT CA - AAG TI CAAG - AAG TI CAAG	A         A         C         T         C         T         C         T         C         A         A         C         T         C         A         A         C         T         C         A         A         C         T         C         A         A         C         T         C         A         A         A         C         T         C         A         A         C         A         C         A         C         A		A & G & A & G & A & G & T & T C            C & A & G & A & C & T & T C            G & G & A & G & T & T & C            G & G & A & G & G & T & C            G & G & A & G & A & T & A            G & G & A & G & A & T & A            G & G & A & G & A & T & A            G & G & A & G & A & T & A            G & G & A & C & G & A & T & A            G & G & A & C & G & A & T & A            G & G & G & G & G & T & A            G & G & G & T & G & T & T & A            G & G & G & T & T & T & A            G & G & G & T & T & T & A            G & G & G & T & T & T & A            G & G & G & T & T & T & A            G & G & G & T & T & T & A            G & G & G & T & T & T & A            G & G & G & T & T & T & A            G & G & G & T & T & T & A            G & G & G & T & T & T & A            G & G & G & T & T & T & A            G & G & T & T & T & T & A            G & G & T & T & T & T & A            G & G & T & T & T & T & A            G & G & T & T & T & T & A            G & G & T & T & T & T & A            G & G & T & T & T & A            G & G & T & T & T & A	T         C         AAAA         AAAA         C           T         C         G         AAAA         AAAA         AAAAA         C         C         T         C         G         AAAAA         AAAAA         C         C         AAAAA         AAAAAA         G         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	C C C C TAAG TG C C C TAAG TG C C C C TAAG TG C C C C TAAA TG T C C C C AAA TG T C C C C C AAG TG T C C C C C AAG TC T C C C C C C C C C C C C C C C C C C C	C C C C C C C C C C C C C C C C C C C	T       187         T       197         T       206         T       150         T       164         T       149         T       226         T       199         A       216         A       210         A       210
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14       14 <td< td=""><td>. C G A G T T C T C C C C C A G T T C T C C C C C A G T T C T C C C C C A G G A C C C C G A G G A C C C C C A G G A C C C C C C A G G A C C C C C C A G T A C C C C C C A G T A C C C C C C A G T A C C C C C C A G T A C C C C C C A G T A C C C C C C C A G T A C C C C C C C A G T A C C C C C C C A G T A C C C C C C C A G T A C C C C C C C A G T A C C C C C C C A G T A C C C C C C C C A G T A C C C C C C C C A G T A C C C C C C C C C C C C C C C C C C</td><td></td><td>C G G G C C G TAAG           C G G G T C G TAAG           C T G G G AG G AAG           C G G G T G G G AG           C G G G T G G G AG           C G G G G G G AG           C G G G G G G AG           C G G G G G G G AG           C G G G G G G G AG           C G G G G G G AAG           C G G G A G G AAG           C G G G G G G AAG           C AG G G C G G AAAG           C AG G G G G AAAG           G AG G G A G AAAG</td><td>- AAG TTT CG T - AAG TTT CG T - AAG TTT CG T - AAG TTT CAT - AAG TT CAAG - AAG TT CAAG</td><td>A         A         C         T         C         T         C         A         A         C         T         C         A         A         A         C         T         C         A         A         A         C         T         C         A         A         C         A         A         A         C         A</td><td></td><td>G A G G A G T T C           G A G G A G T T C           G A G G A G T T C           G G G G A G T G A           G G G G A G G A           G G G G A G G A           G G G G A G G A           G G G G A G G A           G G G G A G G A           G G G G A G G A           G G G G A G G A           G G G G G G G G G A           G G G G G G G G G G G G G G G G G G G</td><td>T         C         AAAA         AAAA           T         C         G         AAAA           T         C         G         AAAA           T         C         AAAA         AAAA           C         AAAAA         AAAA         AAAA           C         AAAAA         AAAAA         AAAAA           C         AAAAA         AAAAA         AAAAA           C         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA</td><td>TC C C C TAAG TG         TC C C C TAAG TG         TC C C C TAAG TG         TC C C C C AAA TAAA TG         TTAA ATAAA TG         TTAA ATAAATG</td><td>0       TTT       TT       T&lt;</td>       TT       TT       <td< td=""><td>T 188 T 197 T 206 T 150 T 164 T 164 T 164 T 164 T 164 T 164 T 164 T 164 T 226 T 226 T 226 T 226 T 253 T 199 A T 216 A T 216 A T 210 A T 200 A T 200 A</td></td<></td<>	. C G A G T T C T C C C C C A G T T C T C C C C C A G T T C T C C C C C A G G A C C C C G A G G A C C C C C A G G A C C C C C C A G G A C C C C C C A G T A C C C C C C A G T A C C C C C C A G T A C C C C C C A G T A C C C C C C A G T A C C C C C C C A G T A C C C C C C C A G T A C C C C C C C A G T A C C C C C C C A G T A C C C C C C C A G T A C C C C C C C A G T A C C C C C C C C A G T A C C C C C C C C A G T A C C C C C C C C C C C C C C C C C C		C G G G C C G TAAG           C G G G T C G TAAG           C T G G G AG G AAG           C G G G T G G G AG           C G G G T G G G AG           C G G G G G G AG           C G G G G G G AG           C G G G G G G G AG           C G G G G G G G AG           C G G G G G G AAG           C G G G A G G AAG           C G G G G G G AAG           C AG G G C G G AAAG           C AG G G G G AAAG           G AG G G A G AAAG	- AAG TTT CG T - AAG TTT CG T - AAG TTT CG T - AAG TTT CAT - AAG TT CAAG - AAG TT CAAG	A         A         C         T         C         T         C         A         A         C         T         C         A         A         A         C         T         C         A         A         A         C         T         C         A         A         C         A         A         A         C         A		G A G G A G T T C           G A G G A G T T C           G A G G A G T T C           G G G G A G T G A           G G G G A G G A           G G G G A G G A           G G G G A G G A           G G G G A G G A           G G G G A G G A           G G G G A G G A           G G G G A G G A           G G G G G G G G G A           G G G G G G G G G G G G G G G G G G G	T         C         AAAA         AAAA           T         C         G         AAAA           T         C         G         AAAA           T         C         AAAA         AAAA           C         AAAAA         AAAA         AAAA           C         AAAAA         AAAAA         AAAAA           C         AAAAA         AAAAA         AAAAA           C         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	TC C C C TAAG TG         TC C C C TAAG TG         TC C C C TAAG TG         TC C C C C AAA TAAA TG         TTAA ATAAA TG         TTAA ATAAATG	0       TTT       TT       T<	T 188 T 197 T 206 T 150 T 164 T 164 T 164 T 164 T 164 T 164 T 164 T 164 T 226 T 226 T 226 T 226 T 253 T 199 A T 216 A T 216 A T 210 A T 200 A
14       14 <td< td=""><td>. C G AG T T C T C C C C C A G T T C T C C C . C A G T T C T C C C . C C A G A A C C C . C G A G A A C C C . C G A G A A C C C . C G A G A A C C C . C G A G A A C C C . C G A G A C T A C C C . C G A G A C T A C C C . C A G G A C T A C C C . C A G G A C T A C C C C . C A G T A C C C C C . C G A G T A C C C C C C . C G A C T A C C C C C C . C G A C T A C C C C C C C C C C C C C C C C</td><td></td><td>C G G G C C G TAAG           C G G G T C G TAAG           C G G G T C G TAAG           C G G G G C G AG           C G G G G G G G AG           C G G G G AG           C AG G G AG           G AG G AG           G AG G AG      <t< td=""><td>- AAG TIT CG T - AAG TIT CG T - AAG TIT CG T - AAG TIT CA - AAG TI CAAG - AAG TI CAAG</td><td>A         A         C</td><td></td><td>G A G G A G T T C           G A G G A G T T C           G G G G G T T C           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G G G G G G G G G G A           G G G G G T G T A A           G G G G T G T A A           G A G G G T G T A A           G A G G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T G T A A           G G G T G T G T A A           G G G T G T G T A A           G G G T G T G T A</td></t<></td></td<> <td>T         C         AAAA         AAAA           T         C         G         AAAA         AAAA           T         C         G         AAAA         AAAA           AAAAA         AAAA         G         AAA           G         AG         G         AAAAA         AAAAA           G         AG         G         AAAAA         AAAAA           G         AG         G         AAAAA         AAAAA           G         AG         G         AAAAAA         AAAAAAAA           G         AG         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA</td> <td>C C C C TAAG TG C C C TAAG TG C C C C TAAA TG T C C C C TAAA TG T C C C C C AAA TG T C C C C C AAG TG T AAA TAAATG T T AG ATAAATG T T AG ATAAATG T T AG ATAAATG T T AG ATAAATG</td> <td>C C T T C T C AAC C T T T C T C AAC C T T T C T C AAC C T T C T C C AAC C T T C C C AAC C T C C C AAC C C T C C C AAC C C C C C C AAA C C C C C C C C AAA C C C C C C C C AAA C C C C C C C C C C C C C C C C C C C</td> <td>T       188         T       197         T       206         T       150         T       164         T       210         A       210         A       210         A       2100         A       2100         A       2100         A       169</td>	. C G AG T T C T C C C C C A G T T C T C C C . C A G T T C T C C C . C C A G A A C C C . C G A G A A C C C . C G A G A A C C C . C G A G A A C C C . C G A G A A C C C . C G A G A C T A C C C . C G A G A C T A C C C . C A G G A C T A C C C . C A G G A C T A C C C C . C A G T A C C C C C . C G A G T A C C C C C C . C G A C T A C C C C C C . C G A C T A C C C C C C C C C C C C C C C C		C G G G C C G TAAG           C G G G T C G TAAG           C G G G T C G TAAG           C G G G G C G AG           C G G G G G G G AG           C G G G G AG           C AG G G AG           G AG G AG           G AG G AG <t< td=""><td>- AAG TIT CG T - AAG TIT CG T - AAG TIT CG T - AAG TIT CA - AAG TI CAAG - AAG TI CAAG</td><td>A         A         C</td><td></td><td>G A G G A G T T C           G A G G A G T T C           G G G G G T T C           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G G G G G G G G G G A           G G G G G T G T A A           G G G G T G T A A           G A G G G T G T A A           G A G G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T G T A A           G G G T G T G T A A           G G G T G T G T A A           G G G T G T G T A</td></t<>	- AAG TIT CG T - AAG TIT CG T - AAG TIT CG T - AAG TIT CA - AAG TI CAAG - AAG TI CAAG	A         A         C		G A G G A G T T C           G A G G A G T T C           G G G G G T T C           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G G G G G G G G G G A           G G G G G T G T A A           G G G G T G T A A           G A G G G T G T A A           G A G G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T A A           G G G T G T G T A A           G G G T G T G T A A           G G G T G T G T A A           G G G T G T G T A	T         C         AAAA         AAAA           T         C         G         AAAA         AAAA           T         C         G         AAAA         AAAA           AAAAA         AAAA         G         AAA           G         AG         G         AAAAA         AAAAA           G         AG         G         AAAAA         AAAAA           G         AG         G         AAAAA         AAAAA           G         AG         G         AAAAAA         AAAAAAAA           G         AG         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	C C C C TAAG TG C C C TAAG TG C C C C TAAA TG T C C C C TAAA TG T C C C C C AAA TG T C C C C C AAG TG T AAA TAAATG T T AG ATAAATG T T AG ATAAATG T T AG ATAAATG T T AG ATAAATG	C C T T C T C AAC C T T T C T C AAC C T T T C T C AAC C T T C T C C AAC C T T C C C AAC C T C C C AAC C C T C C C AAC C C C C C C AAA C C C C C C C C AAA C C C C C C C C AAA C C C C C C C C C C C C C C C C C C C	T       188         T       197         T       206         T       150         T       164         T       210         A       210         A       210         A       2100         A       2100         A       2100         A       169
14       14 <td< td=""><td>C C A A C T T C T C C C C C A A C T T C T C</td><td></td><td>C G G G C C G T A A G           C G G G T C G T A A G           C T G G G A G C A G A A G           C T G G G A G A G A A G           C G G G T G A G A A G           C G G G G A G A G A A G           C G G G G A G A G A A G           C G G G A G A G A A G           C G G G A G A G A A G           C G G G A G A G A A G           C G G G A G A G A A G           C G G G A G A A G A A G           C G G G A G A A G A A G           C G G G A G A A G A A A G           C G G G A G A A G A A A G           C A G G A A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G G A G A A A G           G A G G G G A A A G           G A G G</td><td>- AAG TTT CG T - AAG TTT CG T - AAG TTT CG T - AAG TTT CG T - AAG TT CAAG - AAG TT CAAG</td><td>A A A C T C A C           A A A C T C A C T C A T C A C           A A A A C T C A C T C A T           A A A A C A C A C T C A T           A A A A C A C A C T C A T           A A A C A C A A C T C A T           A A A C A C A A C C A C T C A T           A A A C A C A A C C A C C A C C           A A A C A A C A A C C A C C A C C           A A A C A A C A A C C A C C A C C A C C           A A A C A A C A A C C A C C C A C C           A A A C A A C A A C C A C C C A C C           A A A C A A C C A C C C A C C C A C C A C C A C C A C C A C C A C C C A C C A C C A C C C A C A C A C A C C A C C A C C A C C A C A C A C A C A C C A C</td><td></td><td>G A G G A G T T C           G A G G A G T T C           G A G G A G T T C           G T G G G T T C           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G C G G G A T G A           G C G G G A T G A           G C G G G A T G A           G C G G G A T G A           G C G G T G T A A           G A G G T G T G A           G A G G T G T G A           G A G G T G T G A           G A G G T G T G A           G A G G T G T G A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A           G A G G T G T A           G A G G T G T A           G A G G T G T A           G A G G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A</td><td>T         C         AAAA         AAAA         C           T         C         G         AG         AAAA         C         AAAA         AAAA         C         AAAA         AAAA         G         C         AAAAA         G         G         AAG         G         AAAAA         G         G         AAG         G         AAG         G         AAAAA         G         G         AAG         G         AAG         AAAAA         G         AAG         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA</td><td>1       C       C       T       AAA       T         1       C       C       G       AAA       T         1       C       G       C       AAA       TG         1       C       G       G       AAA       TG         1       C       G       C       AAA       TG         1       C       G       C       AAA       TG         1       T       G       C       AAA       TG         1       T       G       C       AAA       TG         1       T       G       T       AAA       TG         1       T       G       T       AAAA       TG         1       T       G<td>C TT C T C AAG C TT C T C AAG C TT C T C AAG C T T C T C AAG C T C C C C AAG C T C C C C AAG C C T C C C AAG C</td><td>T 1989 T 1975 T 206 T 150 T 164 T 164 T 164 T 164 T 164 T 164 T 226 T 216 T 21</td></td></td<>	C C A A C T T C T C C C C C A A C T T C T C		C G G G C C G T A A G           C G G G T C G T A A G           C T G G G A G C A G A A G           C T G G G A G A G A A G           C G G G T G A G A A G           C G G G G A G A G A A G           C G G G G A G A G A A G           C G G G A G A G A A G           C G G G A G A G A A G           C G G G A G A G A A G           C G G G A G A G A A G           C G G G A G A A G A A G           C G G G A G A A G A A G           C G G G A G A A G A A A G           C G G G A G A A G A A A G           C A G G A A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G A G A A A G           G A G G G A G A A A G           G A G G G G A A A G           G A G G	- AAG TTT CG T - AAG TTT CG T - AAG TTT CG T - AAG TTT CG T - AAG TT CAAG - AAG TT CAAG	A A A C T C A C           A A A C T C A C T C A T C A C           A A A A C T C A C T C A T           A A A A C A C A C T C A T           A A A A C A C A C T C A T           A A A C A C A A C T C A T           A A A C A C A A C C A C T C A T           A A A C A C A A C C A C C A C C           A A A C A A C A A C C A C C A C C           A A A C A A C A A C C A C C A C C A C C           A A A C A A C A A C C A C C C A C C           A A A C A A C A A C C A C C C A C C           A A A C A A C C A C C C A C C C A C C A C C A C C A C C A C C A C C C A C C A C C A C C C A C A C A C A C C A C C A C C A C C A C A C A C A C A C C A C		G A G G A G T T C           G A G G A G T T C           G A G G A G T T C           G T G G G T T C           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G C G G G A T G A           G C G G G A T G A           G C G G G A T G A           G C G G G A T G A           G C G G T G T A A           G A G G T G T G A           G A G G T G T G A           G A G G T G T G A           G A G G T G T G A           G A G G T G T G A           G A G G T G T A A           G A G G T G T A A           G A G G T G T A           G A G G T G T A           G A G G T G T A           G A G G T G T A           G A G G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A           G R G T G T G T A	T         C         AAAA         AAAA         C           T         C         G         AG         AAAA         C         AAAA         AAAA         C         AAAA         AAAA         G         C         AAAAA         G         G         AAG         G         AAAAA         G         G         AAG         G         AAG         G         AAAAA         G         G         AAG         G         AAG         AAAAA         G         AAG         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	1       C       C       T       AAA       T         1       C       C       G       AAA       T         1       C       G       C       AAA       TG         1       C       G       G       AAA       TG         1       C       G       C       AAA       TG         1       C       G       C       AAA       TG         1       T       G       C       AAA       TG         1       T       G       C       AAA       TG         1       T       G       T       AAA       TG         1       T       G       T       AAAA       TG         1       T       G <td>C TT C T C AAG C TT C T C AAG C TT C T C AAG C T T C T C AAG C T C C C C AAG C T C C C C AAG C C T C C C AAG C</td> <td>T 1989 T 1975 T 206 T 150 T 164 T 164 T 164 T 164 T 164 T 164 T 226 T 216 T 21</td>	C TT C T C AAG C TT C T C AAG C TT C T C AAG C T T C T C AAG C T C C C C AAG C T C C C C AAG C C T C C C AAG C	T 1989 T 1975 T 206 T 150 T 164 T 164 T 164 T 164 T 164 T 164 T 226 T 216 T 21
141       - 11       - 11       - 12       - 14	CAAGITACCCCCC CAAGITACCCCCCCAAAACCCCC CCAAGAACCCCCCCCAAAACCCCCCCCCAAAACCCCCCCC		C G G G C C G TAAG           C G G G T C G TAAG           C T G G G AG G AAG           C G G G T G G G AG G AAG           C G G G G G G AG G AAG           C G G G G G G AG G AAG           C G G G G G G AG G AAG           C G G G G G G AG G AAG           C G G G G G G G AG G AAG           C G G G G G G AG G AAG           C G G G G G G AG G AAG           C G G G G G G G AAG           C G G G G G G G AAG           C G G G G G G G AAG           C G G G G G G G AAG           C AG G G G G AAAG           C AG G G A G AAAG           C G AG G A G AAAG	- AAG TIT CGT - AAG TIT CGT - AAG TIT CGT - AAG TIT CAG - AAG TI CAAG - AAG TI CAAG	A         A         C		A         A	T         C         AAAA         AAAA         C           T         C         G         AAAA         AAAA         C           T         C         AAAA         AAAA         AAAA         AAAA         AAAA         AAAAA         AAAAAA         AAAAAA         AAAAAAAAA         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	TC C C C TAAG TG         TC C C C TAAG TG         TC C C C TAAG TG         TC C C C C AAA TG         TTAA ATAAA TG         TTAA ATAAA TG         TTAA ATAAA TG         TTAAA TAAA TG         TTAA ATAAA TG         TTAA ATAAA TG         TTAAA TAAA TG         TAAA TAAA TG AAA TG	0       TTT 0 T 0 AAG         6       TTT 0 T 0 AAG         6       TTT 0 T 0 AAG         6       TT 0 T 0 AAG         6       TT 0 T 0 C AAG         6       T 0 T 0 C AAG	1         1
14       14 <td< td=""><td><ul> <li>C G A G T T G T C G C C</li> <li>C C A G T T G T C G C C C</li> <li>C C A G T T G T C C C</li> <li>C C A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A A C C C</li> <li>C G A G A C T A C C C</li> <li>C G A G T A C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C 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   G AG G AAAG           G AG G AAAG           G AG G AG AAAG           G AG G AAAAG           G AG G AAAAG&lt;</td><td>- AAG TIT CG T - AAG TIT CG T - AAG TIT CG T - AAG TIT CA - AAG TI CAAG - AAG TI CAAG</td><td>A A A A C T C A C T C A C           A A A A C T C A C T C A C           A A A A C T C A C T C A T           A A A A C A C A C T C A T           A A A A C A C A C T C A T           A A A A C A C A C T C A T           A A A A C A A C A C A C A C A C           A A A A C A A C A C A C C A C C A C           A A A C A A C A A C C A C C A C C A C C A C C A C C A C C A C C A C C A C C A C C A C C A C C A C C A C C A C C A C C C A C C A C C C A C C A C C C A C C C A C C C A C C C A C C C A C C C A C C C A C C C A C C C A C C C A C C C A C C C A C C C A C C C A C C A C C C C C C C C C C A C C A C C C C C C C C C C C C C C C C C C A C C A C</td><td></td><td>G A G G A G T T C           G A G G A G T T C           G T G G G G T T C           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G A G T G A           G G G G G G G G G G G G A           G G G G G T G T A           G G G G T G T G A           G G G G T G T A           G G G G T G T G A           G A G G G T G T A           G G G G T G T G A           G G G G T G T A           G G G G T G T A           G G G G T G T A           G G G G T G T A           G G G G T G T G A           G G G T G T G A           G G G T G T G T A           G G G T G T G T A           G G G G T G T T A           G G G T G T G T A           G G G T G T G T A           G G G T G T G T A           G G G T G T G T A           G G G T G T T A           G G G T G T T A           G G G T G T T A           G G G T G T T A           G G G T G T T A           G G G T G T T A           G G G G T G T T A           G G G G T G T T A</td><td>T         C         AAAAA         C           T         C         G         AAAAA         C           T         C         G         AAAAA         C         G         AAAAA         C         G         AAAAAA         C         G         AAAAAAA         C         G         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA</td><td>TC C C C TAAG TG         TC C C C TAAG TG         TC C C C TAAA TG         TC C C C C AAA TG         TC C C C AAA TAAA TG         TT AC AT AAAA TG         TT AC AT AAAATG         TT AC AT AAATG         TT AC AT AAATG      <tr< td=""><td>C TT C T C AAG C TT C T C AAG C TT T C T C AAG C T T C T C AAG C T T C T C C AAG C T T C C C ARG C T T C C AAG C T T C C AAG C T T C C AAG C T C C C AAG C C C C C C C C C C C C C C C C C C C</td><td>T         186         17         186           T         187         186         187         186           T         186         1         167         186           T         186         1         184         186           T         186         1         184         186           T         186         1         149         187        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T A C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G A G A C C C C</li> <li>C G A G A G A C C C C</li> <li>C G A G A G A C C C C</li> <li>C G A G A G A C C C C</li> <li>C G A G A G A C C 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T A           G G G T G T G T A           G G G T G T G T A           G G G T G T G T A           G G G T G T T A           G G G T G T T A           G G G T G T T A           G G G T G T T A           G G G T G T T A           G G G T G T T A           G G G G T G T T A           G G G G T G T T A	T         C         AAAAA         C           T         C         G         AAAAA         C           T         C         G         AAAAA         C         G         AAAAA         C         G         AAAAAA         C         G         AAAAAAA         C         G         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	TC C C C TAAG TG         TC C C C TAAG TG         TC C C C TAAA TG         TC C C C C AAA TG         TC C C C AAA TAAA TG         TT AC AT AAAA TG         TT AC AT AAAATG         TT AC AT AAATG         TT AC AT AAATG <tr< td=""><td>C TT C T C AAG C TT C T C AAG C TT T C T C AAG C T T C T C AAG C T T C T C C AAG C T T C C C ARG C T T C C AAG C T T C C AAG C T T C C AAG C T C C C AAG C C C C C C C C C C C C C C C C C C C</td><td>T         186         17         186           T         187         186         187         186           T         186         1         167         186           T         186         1         184         186           T         186         1         184         186           T         186         1         149         187           T         126         7         228         123           T         129         228         123         187           T         189         T         110         147         180           T         189         T         110         187         100         187         100           T         161         189         T         110         189         110         199         111         199         111         199         111         110         111</td></tr<>	C TT C T C AAG C TT C T C AAG C TT T C T C AAG C T T C T C AAG C T T C T C C AAG C T T C C C ARG C T T C C AAG C T T C C AAG C T T C C AAG C T C C C AAG C C C C C C C C C C C C C C C C C C C	T         186         17         186           T         187         186         187         186           T         186         1         167         186           T         186         1         184         186           T         186         1         184         186           T         186         1         149         187           T         126         7         228         123           T         129         228         123         187           T         189         T         110         147         180           T         189         T         110         187         100         187         100           T         161         189         T         110         189         110         199         111         199         111         199         111         110         111
141       - 11       C C C A C C C T C C C C C T C C C C T C C C C	<ul> <li>C.G.A.G.T.T.G.T.C.G.Z.G.C.G.A.G.T.T.G.T.C.G.Z.G.G.A.G.A.G.C.C.G.C.G.G.G.G.G.G.G.G.G.G</li></ul>		C G G G C C G TAAG           C G G G T C G TAAG           C T G G G A G G AAG           C T G G G A G G AAG           C G G G T G G G A G G AAG           C G G G G A G G AAG           C G G G G A G G AAG           C G G G G A G G AAG           C G G G G A G G AAG           C G G G G A G G AAG           C G G G G A G G AAG           C G G G G A G G AAG           C G G G G A G G AAG           C A G G A G AAAG           G A G G A A G G AAAG           G A G G A A G G AAAG           G A G G A A G G AAAG           G A G G A A G G AAAG           G A G G A A G G AAAG	- AAG TTT CG T - AAG TTT CG T - AAG TTT CG T - AAG TTT CG T - AAG TT CAAG - AAG TT CAAG	A         A         C		G A G G A G T T C         G A G G A G T T C           G A G G A G T T C         G G G A G T A C           G T G G G G T C         G G G A G T G A           G G G G A G T G A         G G G A G T G A           G G G G A G G A G T G A         G G G G A G T G A           G G G G A G T G A G         G G G G G G G G G G G G G G G G G G G	T         C         AAAA         AAAA           T         C         AAAA         AAAA           T         C         AAAA         AAAA           T         C         AAAA         AAAA           C         AAAA         AAAA         AAAA           G         AG         G         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	1       C       C       T       AAA       TG         1       C       C       T       AAA       TG       TAAA       TG         1       C       C       G       TAAA       TG       TG       C       GG       AAA       TG       TC       CG       GAAG       TG       TG       CG       GAAG       TG       TC       CG       GAAG       TG       GAAG       TG       TG       TG       TG       TG       GAAG       TG	C TT C T C AAG C T C T C C AAG C T C T C C AAG C T C T C C AAG C T C C C AAG C C C C C C C C AAG C C C C C C C C C C C C C C C C C C C	1         16         17         186           1         186         1         197         186           1         197         166         1         197           1         197         166         1         197           1         164         1         164         1           1         164         1         164         1           1         164         1         164         1           1         164         1         164         1           1         164         1         200         1         200           1         168         1         210         1         168           1         168         1         169         1         11           1         168         1         168         1         168           1         168         1         168         1         169           1         168         1         170         1         169           1         170         1         11         1         1         1
141       1.41	<ul> <li>C G A G T T G T C G C C</li> <li>C C A G T T G T C G C C C</li> <li>C C A G T T G T C C C</li> <li>C C A G A A C C C</li> <li>C G A G G A A C C C</li> <li>C G A G G A A C C C</li> <li>C G A G G A C C C C</li> <li>C G A G G A C C C C</li> <li>C G A G G A C C C C</li> <li>C G A G G A C C C C</li> <li>C G A G G A C C C C</li> <li>C G A G G A C C C C</li> <li>C G A G G A C C C C</li> <li>C G A G G A C C C C</li> <li>C G A G G A C C C C</li> <li>C G A G G A C C C C</li> <li>C G A G T A C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G T A C C C C C</li> <li>C G A G A C C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <li>C A G A G A C C C C</li> <lic a="" c="" c<="" g="" li=""> <li>C A G A G A C C C C</li></lic></ul>		C G G G C C G TAAG           C G G G T C G A G AAG           C T G G G A G A G AAG           C G G G T C G A G AAG           C G G G A G A G AAG           C G G G A G G A G G AAG           C G G G A G G A G G AAG           C G G G A G G A G G AAG           C G G G A G G A G G AAG           C G G G A G G A AG           C G G G A G G A AG           C G G G A G G A AG           C G G G A G G A AG           C G G G A G G A AG           C G G G A G G A AG           C G G G A G G A AG           C G G G A G G A AG           C A G G A G A AAG           C A G G A G A AAG           C A G G A G A AAG           G A G G A G A AAG	- AAG TIT CG T - AAG TIT CG T - AAG TIT CG T - AAG TIT CAG - AAG TI CAAG - AAG TI CAAG	A         A         C		G         A         G         A         T         C           G         A         G         A         T         C           G         A         G         A         T         C           G         G         G         G         T         C           G         G         G         G         T         C           G         G         G         G         G         T         C           G         G         G         A         T         A         G	T         C         AAAA         AAAA         C           T         C         G         AGAAA         AAAA           AAAA         AAAA         AAAA         AAAA         AAAA           AAAA         AGGAA         AAAA         AAAA         AAAA           AAAA         AGGAA         AAAA         AAAA         AAAA           AAAA         AGGAA         AAAA         AAAAA         AAAAA           G         AGGAA         AAAAA         AAAAA         AAAAA           G         AGGAAA         G         AAGAA         AAAA           G         AGGAA         G         AAGAAA         G         AAGAAAAA           G         AGGAA         G         AAGAAAAA         G         AAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	1       C       C       T       AAA       T         1       C       C       C       T       AAA       T         1       C       C       C       T       AAA       T         1       C       C       C       AAA       T       T         1       T       C       C       AAA       T       T       T       T       T       T       T       T       T       T       T       T       T       T       T       T       T	C TT C T C AAG C TT C T C AAG C TT C T C AAG C T T C T C C AAG C T C C C C A C C T C C C A C C T C C C A C C C T C C C A C	1         16         17           1         188         1         187           1         187         188         197         180           1         197         160         197         160           1         164         1         164         1         164           1         164         1         164         1         164           1         164         1         164         1         164           1         126         1         226         1         226         1         226         1         226         1         164         1         164         1         164         1         164         1         164         1         164         1         166         1         164         1         166         1         164         1         166         1         165         1         126         1         126         1         126         1         161         1         166         1         165         1         165         1         165         1         126         1         126         1         126         1         126         1         126
141       1.11       0.11       CORACE       1.10       CORACE       1.10       CORACE       1.10       CORACE       1.10       CORACE       1.10       CORACE       CORACE       1.10       CORACE       CORACE <td><ul> <li>C.G.A.G.T.T.G.T.C.G.C.C.</li> <li>C.G.A.G.T.T.G.T.C.G.C.C.</li> <li>C.G.A.G.A.A.C.C.G.</li> <li>C.G.A.G.G.A.C.C.G.</li> <li>C.G.A.G.T.A.C.C.G.</li> <li>C.G.A.G.T.A.C.C.C.G.</li> <li>C.G.A.G.T.A.C.C.G.G.</li> <li>C.G.A.G.T.A.C.C.G.G.</li> <li>C.G.A.G.T.A.C.C.G.G.</li> <li>C.G.A.G.A.G.C.G.G.</li> <li>C.G.A.G.A.G.C.G.G.</li> <li>C.T.T.C.T.A.A.C.G.G.</li> </ul></td> <td></td> <td>C G G G C C G T AAG           C G G G T C G T AAG           C T G G A G A G A AG           C T G G G A G G A AG           C G G G T C G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G A AG           C G G G G A G A AG           C G G G G A G A AG           C G G G A G A AAG           C G G G A G A AAG           C A G G A G A AAG           G A G G A A AG           G A G G A A AG</td> <td>- AAG TIT CG T - AAG TIT CG T - AAG TIT CG T - AAG TIT CA - AAG TI CAAG - AAG - AAG TI CAAG - AAG TI</td> <td>A         A         C         C         C         C         A         A         C         C         C         A         A         C         C         C         A         A         C         C         C         C         C         A         A         C         C         C         C         C         A         A         C         C         C         C         A         A         C         C         C         A         C         C         C         C         A         C         C         C         C         C         A         C         C         C         C         C         C         C         C         C         C         A         C         C         C         C         C</td> <td></td> <td></td> <td>T         C         AAAAA         C           T         C         G         AAAAA         C           T         C         G         AAAAA         C         G         AAAAAA         C           G         AAAAAAA         G         G         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA</td> <td>T C C C T AAA T G T C C C T AAA T G T C C C C T AAA T G T C C C C AAA T G T C T AAA T AAAA T G T T AAAA T AAAA T G T T AAAAA T AAAA T G AAAA G C AAAA T G AAT AAAC AAAA T G</td> <td>C TT C T C AAC C TT C T C AAC C TT T C T C AAC C T T C T C AAC C T T C T C C AAC C T T C T C C AAC C C T C C C AAC C T C C C C C AAC C T C C C C C C C C C C C C C C C C C C</td> <td>1         18         1         18         1         18         1         18         1         19         1         19         1         19         1         19         1         19         1         19         1         19         1         19         1         19         1         19         1         10         1         10         1         10         1         10         1         10</td>	<ul> <li>C.G.A.G.T.T.G.T.C.G.C.C.</li> <li>C.G.A.G.T.T.G.T.C.G.C.C.</li> <li>C.G.A.G.A.A.C.C.G.</li> <li>C.G.A.G.G.A.C.C.G.</li> <li>C.G.A.G.T.A.C.C.G.</li> <li>C.G.A.G.T.A.C.C.C.G.</li> <li>C.G.A.G.T.A.C.C.G.G.</li> <li>C.G.A.G.T.A.C.C.G.G.</li> <li>C.G.A.G.T.A.C.C.G.G.</li> <li>C.G.A.G.A.G.C.G.G.</li> <li>C.G.A.G.A.G.C.G.G.</li> <li>C.T.T.C.T.A.A.C.G.G.</li> </ul>		C G G G C C G T AAG           C G G G T C G T AAG           C T G G A G A G A AG           C T G G G A G G A AG           C G G G T C G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G G A AG           C G G G G A G A AG           C G G G G A G A AG           C G G G G A G A AG           C G G G A G A AAG           C G G G A G A AAG           C A G G A G A AAG           G A G G A A AG	- AAG TIT CG T - AAG TIT CG T - AAG TIT CG T - AAG TIT CA - AAG TI CAAG - AAG - AAG TI CAAG - AAG TI	A         A         C         C         C         C         A         A         C         C         C         A         A         C         C         C         A         A         C         C         C         C         C         A         A         C         C         C         C         C         A         A         C         C         C         C         A         A         C         C         C         A         C         C         C         C         A         C         C         C         C         C         A         C         C         C         C         C         C         C         C         C         C         A         C         C         C         C         C			T         C         AAAAA         C           T         C         G         AAAAA         C           T         C         G         AAAAA         C         G         AAAAAA         C           G         AAAAAAA         G         G         AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	T C C C T AAA T G T C C C T AAA T G T C C C C T AAA T G T C C C C AAA T G T C T AAA T AAAA T G T T AAAA T AAAA T G T T AAAAA T AAAA T G AAAA G C AAAA T G AAT AAAC AAAA T G	C TT C T C AAC C TT C T C AAC C TT T C T C AAC C T T C T C AAC C T T C T C C AAC C T T C T C C AAC C C T C C C AAC C T C C C C C AAC C T C C C C C C C C C C C C C C C C C C	1         18         1         18         1         18         1         18         1         19         1         19         1         19         1         19         1         19         1         19         1         19         1         19         1         19         1         19         1         10         1         10         1         10         1         10         1         10

Figure 3.1.1.3.1 a. Clustal alignment of *CBF* nucleotide sequences isolated from all other species that were not members of the *Ericaceae* or *Brassicaceae* compared to *Arabidopsis thaliana CBF*1-4 genbank sequences. Sequences are ordered by Clustal sorting. Names are in the form "Species code pp primer pair code\_Genus\_Species\_Site code\_Location". Genbank gene names are in the form "Genus\_Species\_Gene\_Genbank code".

Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 Arabidopsis thaliana CBF3 AT4G25480.1 Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 90pp 12\_Leucanthemum\_vulgare?\_S19\_Weardale&Teesdale 4bpeCBF2\_Betula\_nana\_S2\_Abisko nulabpeCBF1\_Betula\_nana\_nula\_Abisko 4bpeCBF1\_Betula\_nana\_S2\_Abisko 4PP59 Betula nana S2 Abisko Nulapp59\_Betula\_nana\_nula\_Abisko\_REVonly 74pp50\_Fragaria\_vesca\_S14\_Abisko 78pp 50\_Fragaria\_vesca\_S16\_Weardale&Teesdale 51PP51 Salix herbacea S6 Abisko 51pp53\_Salix\_herbacea\_S6\_Abisko 77pp 19\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp 19\_Trifolium\_repens\_S22\_Weardale&Teesdale 77PP23&24B\_Trifolium\_repens\_S16\_Weardale&Teesdale 77PP23&24A\_Trifolium\_repens\_S16\_Weardale&Teesdale 58pp 23B\_Trifolium\_repens\_S9\_Abisko 58pp 19\_Trifolium\_repens\_S9\_Abisko\_FWDonly 94pp 23\_Trifolium\_repens\_S22\_Weardale&TeesdaleFWDonly 58pp23C\_Trifolium\_repens\_S9\_Abisko 63pp 17\_Matricaria\_recutita?\_S11\_Abisko 63pp6\_Matricaria\_recutita?\_S11\_Abisko 63pp5\_Matricaria\_recutita?\_S11\_Abisko\_REVonly 58pp13\_Trifolium\_repens\_S9\_Abisko 77pp13\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp 13\_Trifolium\_repens\_S22\_Weardale&Teesdale\_FWDonly

Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 Arabidopsis\_thaliana\_CBF3\_AT4G25480.1 Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 90pp 12\_Leucanthemum\_vulgare?\_S19\_Weardale&Teesdale 4bpeCBF2\_Betula\_nana\_S2\_Abisko nulabpeCBF1\_Betula\_nana\_nula\_Abisko 4bpeCBF1\_Betula\_nana\_S2\_Abisko 4PP59 Betula nana S2 Abisko Nulapp59\_Betula\_nana\_nula\_Abisko\_REVonly 74pp50\_Fragaria\_vesca\_S14\_Abisko 78pp 50\_Fragaria\_vesca\_S16\_Weardale&Teesdale 51PP51\_Salix\_herbacea\_S6\_Abisko 51pp53\_Salix\_herbacea\_S6\_Abisko 77pp 19\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp19 Trifolium repens S22 Weardale&Teesdale 77PP23&24B\_Trifolium\_repens\_S16\_Weardale&Teesdale 77PP23&24A\_Trifolium\_repens\_S16\_Weardale&Teesdale 58pp 23B\_Trifolium\_repens\_S9\_Abisko 58pp 19\_Trifolium\_repens\_S9\_Abisko\_FWDonly 94pp 23\_Trifolium\_repens\_S22\_Weardale&TeesdaleFWDonly 58pp23C Trifolium repens S9 Abisko 63pp 17\_Matricaria\_recutita?\_S11\_Abisko 63pp6\_Matricaria\_recutita?\_S11\_Abisko 63pp5\_Matricaria\_recutita?\_S11\_Abisko\_REVonly 58pp13\_Trifolium\_repens\_S9\_Abisko 77pp13\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp 13\_Trifolium\_repens\_S22\_Weardale&Teesdale\_FWDonly

	· · A A G A A A A A C C A G G A T T T G G C	CGGGACTTTC - CAAACCGCT	GAGA GGCAGC CG GC CAU	GACGICGCIGCA IAGCCC	CCGTGGCCGATCAGCA 297
	AAGAAAACAAGAATTTGGC	COGOACATTT CAAACCOCT	GAGATGGCAGCTCGAGCTCA	CACOTTOCCOCTTTACCCCT	TCATAGCCAATCAGCC 208
		ACCTACTTT CORACCCTT			
207 140 40 40 40 40 40 40 40 40 40 40 40 40 4	· · AAGAAATCTAGGATTTGGT	AGGTACTTTT. CCGACGGTT	GAAATGGCTGCTCGTGCTCA	I A I O I I O C I O C I I I A O C I C I	
151 AAGAGAGCCTAAG	· · AAGAAAAACAAGAGTGTGGC	YGGTACGTAT - CCTACTGCT	GACATGGCRGCTAGAGCTCAT	IGACG TGG CGG C TT TGG C TA 1	GAGGGGTCGGTCYGCG 259
165 CCGGGAGCCCAAC	AAGCAGTCCAGGATATGGC	GGGGACGTTT-CCGACGGCG	GAGATGGCGGCTCGGGCCCAT	T <mark>G A C G T G G C G G C G A T A</mark> G C <u>K</u> C T	IGCGGGGGAAACTCTGCC 273
165 CCGGGAGCCCAAC	AAGCAGTCCAGGATATGGC	GGGG <mark>ACGTTT-CCGACGG</mark> CG	GAGATGGCGGCTCGGGCCCAT	T G A C G T G G C G G C G A T A G C G C T	GCGGGGGAAACTCTGCC 273
165 CCGGGGGGGCCCAAC	- AAGCAGTCCAGGATATGGC	GGGGACGTTT-CCGACGGCG	GAGATGGCGGCTCGGGCCCAT	TO A COTO CO O CO O CO O CO O CO O CO O	
150 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	AAGCAGTCCAGGATATGGC	ACCOLOUTE CCOLOGCO	GAGATGGCGGCTCGGGCCCCAT	TO A COTO O COO CO A TAO CO CI	GCGGGGAAACTCTCCC 259
	- · AAGCAGTCCAGGATATGGC	GGGGACGTTT-CCGACGGCG	GAGATGGCGGCTCGGGCCCA	IGACGIGGCGGCGATAGCGCI	GCGGGGGAAACTCYGCC 280
227 TCGTGAGCCGAAC · · · · · · · · · · · · · · · · · ·	- · AAGAAGACGAGGATTTGGC	TGGGACTTTC - CCCACTGCC	GAAATGGCTGCGCGTGCGCA	CGACGTGGCGGCGATTGCCTI	IGAGGGGCCGGTCTGCG 335
227 TCGTGAGCCGAAC	AAGAAGACGAGGATTTGGC	T <mark>GGGACTTTC-CCCACTG</mark> CC	GAAATGGCTGCGCGTGCGCA	C G A C G T G G C G G C G A T T G C C T T	GAGGGGCCGGTCTGCG 335
254 TAGAGAGCCTAAT	AAGAAATCAAGGATTTGGT	A <mark>GGGACTTTT-CCTACCG</mark> CT	G A T A T G G C T G C T A G G G C A C A 1	T <mark>GATGTTGCTGCTTTGGCG</mark> TT	Г <mark>САСССССАССТСТССТ</mark> 362
	AAGAAGTCACGTATTTGGC	AGGCACKTAT, CCTACAGCC	GAGATGGCTGCCCGGGCACA(	GACGTAGCTGCTTTAGCATT	
	· · AAGAAAACTAAAATTIGGC	AGGGACATTT. CAAACGGCC	GAAATGGCAGCCCGAGCCCCA	IGATGICGCGGCATTGGCATT	GAGAGGICGCAATGCI 324
170 GAGAGAGCCCTAAC	· · AAGAAAACTAAAATTTGGC	AGGGACATTT CAAACGGCC	GAAATGGCAGCCCGAGCCCAT	T <mark>GATGTCGCGGCATT</mark> GGCATT	IGAGAGGICGCAAIGCI 278
211 GAGAGAACCTAAT · · · · · · · · · · · · · · · · · ·	AAAAA <mark>G</mark> ACTAA <mark>G</mark> ATTTGGC	A <mark>gggactttt</mark> -ccaacggcc	GAAATGGCAGCCCGAGCACAT	T <mark>G A T G T C G C G G C A T T</mark> G G C A T T	T <mark>G A G A G G T C G C A A T G C T</mark> 319
211 GAGAGAACCTAAT	AAAAAGACTAAGATTTGGC	AGGGACTTTT-CCAACGGCC	GAAATGGCAGCCCGAGCACAT	T <mark>G A T G T C G C G G C A T T G G C A T T</mark>	IGAGN <mark>GGTCGCAATGCT</mark> 319
201 GAGAGARCCTAAY	· · AARAARACTAARATTTGGC	AGGGACATTTTCCAACGGCC	GAAATGGCAGCCCGAGC		
		ACCONTTL CAAACOCC	CAAATEC CACCCCCACCCCA	-	225
1/0 GAGAGARCCTAAY	AARAARACTAARATTIGGC	AGGGACTITI - CCAACGGCC	GAAATGGCAGCCCGAGCCCA	IGAIGICGCGGCAIIGGCAII	IGAGAGGICGCAATGCI 278
171 GAGAGAACCTAAT	AAAAAGACTAANATTTGGC	AGGGACTTTT - CCAACGGCC	GAAATGGCAGCCCGAGCCCAT	T <mark>G A T G T C N C G G C A T T G G C A T T</mark>	IGAGNGGTCGCAATGCT 279
200 GAGACACCCGATC	ACTCAGGCGAGGGTTTGGC	A <mark>gggacacat</mark> - Ccaaat <mark>g</mark> cc	G A A A T G G C A G C C C G G G C T C A 1	T <mark>G A C G T G G C C G</mark> T T T T A <mark>G</mark> C C A T	GAGAGGACGAAACGCG 308
212 GAGACACCCGATC	· · ACTCAGGCGAGGGTTTGGC	WGGGACWCAT - CCAAATGCY	G A A A T G G C A G C C C G G G C T C A 1	T <mark>G A Y G T G G C S G T T T T A G C C A T</mark>	GAGAGGACGAAACGCG 320
	ACTCAGGCGAGGGTTTGGC	WGGGACWCAT, CCAAATGCY	GAAATGGCAGCCCGGGCTCAT	IGAXGIGGCSGITTTAGCCAT	GAGAGGRCGAAACGCG 307
172 GAGAGITTCCTAATTGTAATAACAAAAACA	TTATTAAGTCAAGGATTTGGC	TGGATCATAT. CCAACACCT	GAAATGGCTGCACGTGCACA	IGAIGTIGCIGCACTTACTCI	TAAGGGAAAATCAGCT 298
1/2 GAGAGIICCIAAIIGIAAIAACAAAAACA	TTATTAAGTCAAGGATTIGGC	TGGATCATAT-CCAACACCT	GAAAIGGCIGCACGIGCACA	IGATGI IGCI GCACI IACI CI	TAAGGGAAAATCAGCT 298
106 <mark>ο α</mark> ο ο ο ο ο ο ο ο ο ο ο ο ο ο ο ο ο ο	TTATTAA <mark>G</mark> TCAA <mark>GG</mark> ATTT <mark>GG</mark> C	T <mark>gg</mark> atcatat - ccaacacct	G A A A T G G C T G C A C G T G C A C A T	T <mark>G</mark> AT <mark>G</mark> TTGCTGCACTTACTCT	TTAA <mark>ggg</mark> aaaatca <mark>gct</mark> 232
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307 G C CAA I CGC GAC CGGC GGCG	GC ACGAA CCCCGGAA CAACU	CAAGGAAA CCAAAA	GGUGGUGGU GAAGUUGUG		· · · · · · · · · · · · · · · · · · ·
	GCTACGAATCCCGGAATCAAC		AGCGGCTGCTGAAGCGGCGT	IGAATTTTCAAGATGAGAGAC.	
298 TGTCTCAACTTCGCTGACTCGGCTTGGCG	G C TACGAATCCCGGAATCAACC G C TACGAATCCCGGAGTCAAC	TGCGCCAAGGAAATCCAAAA TGCGCCAAGGATATCCAAAA	AGCGGCTGCTGAAGCCGCGT	IGG CTTTTCAAGATGAGAC	
307 TOTOTOTATTTCCCTGACTCGCCTTGGCC 298 TOTOTCACTTCGCTGACTCGGCTTGGCG 307 TOTCTCAATTTCGCTGACTCGGCTTGGAG	G CTACGAATCCCCGGAATCAACC G CTACGAATCCCCGGA <mark>G</mark> TCAAC A CTCCGAATCCCCGGAATCAAC	TE CECCAAEGAAATCCAAAA TE CECCAAEGATATCCAAAA TE CECTAAEGACATCCAAAA	AGCGGCTGCTGAAGCCGCCGTT GGCGGCCGCCTGAAGCCGCCGTT GGCGGCCGCCTGAAGCTGCGTT	r <mark>gg c t t t t caagat gagac</mark> r <mark>gg c g</mark> t t t <mark>caagat gagat</mark>	GTGTGA 422 GTGTGA 413 GTGTGA 413
307 TOTCTCAATTTCCCTGACTCGGCTTGGCG 298 TOTCTCAACTTCGCTGACTCGGCTTGGCG 307 TOTCTCAATTTCGCTGACTCGGCTTGGGG 316 TOTCTCAATTTCGCTGATTCTGCTTGGGG	G CTACGAATCCCGGAATCAAC G CTACGAATCCCGGGAGTCAAC A CTCCGAATCCCGGAATCAAC G CTTCGTATTCCTGAGACTAC	TG CG C CAAGGAAAT C CAAAA TG CG C CAAGGATAT C CAAAA TG CG C TAAGGACAT C CAAAA TG T C C TAAGGAG <mark>A C</mark> AT T CA <mark>G</mark> AA	A G C G G C C G C C T G A A G C G G C G T G G C G G C G G C T G A A G C T G C G C T A G C T G C G T C T G A A G C T G C A A T	IGG C T T T T C A G A T G A G A T A T I G G C G T T T C A G G A T G A G A C I G G C G T T T C A G G A T G A G A G A C T A I G G C G T T T C A G A A T G A G A C T A	CA 422 CA 413 CA 413 CA 413 CA 423 CA 423 CA 423 CA 423 CA 423 CA 423 CA 423 CA 423 CA 423 CA 422 CA 422 CA 422 CA 422 CA 422 CA 422 CA 422 CA 422 CA 422 CA 423 CA 413 CA 413 CA 413 CA 413 CA 413 CA 413 CA 413 CA 413 CA 413 CA 422 CA 413 CA 413 CA 423 CA 413 CA 423 CA 413 CA 423 CA 413 CA 423 CA
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307 TO TC TC AATTTC & CTG ACTC & GC TG & C 288 TG TC TC AATTTC & CTG ACTC & GC TG & C 307 TG TC TC AATTTC & CTG ACTC & GC TTG & AG 318 TG TC TC AATTTC & CTG ACTC & CTTG C TG & C 260 TG TTT & AATTTC & CTG ACTC & TTATG & C 274 TG CC TC AACTTC & CCG ACTC & GC TTG & C	GCTACCAATCCCCGAATCAAC ACTCCGAATCCCCGAATCAAC GCTTCGTATCCCGGAATCAAC GCTTCGTATTCCTGAGACTAC ATTGCCGGTTCCTGAGACTAC GCTGCCAATGCCGGCGTCGGG	TO TO C CAAGO AAAT C CAAAA TO C C C CAAGO ATAT C CAAAA TO C C TAAGO ACAT C CAAAA TO T C C TAAGO ACAT C CAAAA AATO T C AAGO ATAT T CAAAA AATO C C GAAGO ATAT T CAAAA	A C C G C T G C T G A A G C C G C G T G C G G C G G C T G A A G C G C G T A G C T G C G G C T G A A G C T G C A A G G C T G C G G T T A A G C C G C A G G C T G C G G T T A A G C C G G C A G C T G G A C R G C G G C G G G A G C C G G C A	IGGCITITICAAGATGAGAT. IGGCCTITICAAGATGAGAT. IGGCGTITICAGGATGAGAT. IGGCGTITICAGGATGAGACTA AGGCGTITICCGGCCTG	GTGTCA 422 GTGTCA 413 GTGTCA 413 GTGTCA 422 CGACGGAGGGATCTAA 443 362 376
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30' T G T C T C A A C T T C G C T G A C T C G C C T T G G C G 30' T G T C T C C A A T T T C G C T G A C T C G C C T T G G C G 30' T G T C T C C A A T T T C G C T G A C T C G C C T T G G C G 30' T G T C T C A A T T T T C G C T G A C T C G C T T G G C G 30' T G T C T C A A C T T C G C C G A C T C G G C T T G G C G 274 T G C C T C A A C T T C G C C G A C T C G G C T G G C G 274 T G C C T C A A C T T C G C C G A C T C G G C T G G C G 30' G C T C A A C T T C G C C G A C T C G G C T G G C G 30' G C T C A A C T T C G C C G A C T C G G C T G G C G 33' G C T T G A A C T T C G C C G A C T C G G C T G G C G 33' G C T T G A A C T T T G C C G A C T C G C C T G G C G 33' G C T T G A A C T T T G C C G A C T C G G C T G G C G 33' G C T T G A A C T T T G C C G A C T C G G C T G G C G 33' G C T T G A A C T T T G C C G A C T C G G C T G G C G 33' G C T T G A A C T T T G C C G A C T C G G C T G G C G 33' G C T T G A A C T T T G C G G A C T C G G C T G G C G 32' G T C T C A A C T T T G C A G A C T C A G C T T G G C G 32' G T C T C C A A C T T T G C A G A C T C A G C T T G C G C 32' G T C T C C A A C T T T G C A G A C T C A G C T T G C G C 32' G T C T C C A A C T T T G C A G A C T C A G C T T G C G C 30' G T C T C C A A C T T T G C A G A C T C A G C T T G C G C 30' G T C T C C A A C T T T G C A G A C T C A G C T T G C G C 30' G T C T C C A A C T T T G C A G A C T C A G C T T G C G C 30' G T C T C C A A C T T T G C A G A C T C A G C T T G C G C 30' G T C T C A A C T T T G C A G A C T C A G C T T G C G C 30' G T T T A A A C T T T G C T G A T T C A G C T T G C G C 30' G T T T A A A C T T T G C T G A T T C A G T A T G C G C 30' G T T T A A A C T T T G C T G A T T C A G T A T G C G C 30' G T T T A A A C T T T G C T G A T T C A G T A T G C G C 30' G T T T A A A C T T T G C T G A T T C A G C T T G C G C G T G C C C T C A C T T A G C G C G T G C C C T C A T T C G C G C G T G C C C C C G C G C G	G C T A C G A A T C C C G A A T C A A C G C T A C G A A T C C C G A A T C A A C G C T T C G A A T C C C G A A T C A A C G C T C C G A T T C C T G A G T C T G G G C T G C C A G T G C C G C G T C G G G G C T G C C A G T G C C G C C T C G G G G C T G C C A G T G C C G C C T C G G G G C T G C C A G T G C C G C C T C G G G G C T G C C A G T G C C G C C T C T A A G C T G C C A G T G C C G C C T C T A A G C T G C C A G T G C C G C C T C C A A G C T G C C A G T G C C G C C T C C A A G C T C C C A G T G C C G C C T C C A A G C T C C C A G T G C C G C C T C C A A G C T C C C A G T G C C C A C T A C G C T C C T A T G C C T A C C A C T A C G C T C C T A T G C C T A C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C C A C T A C G C T T C G T A T G C C T G C A A T C T A A C T G C C T G T C C C T G A A T C T A A C T G C C T G T C C C T G C A G T C G A A T C T A A C T G C C T G T C C C T A C C G A G T C T A A C T G C C T G T C C C T A C C G A G T C T A A C T G C C T G T C C C T A C C G A G T C A A C T T G A G T T A C C G G A T C C G A G T C A A C T T G A G T T A C C G G A T C C G A G T C A A C T T G A G T T A C C G G A T C C G A G T C A A C T G C C T G T A C C G G A C T C G A G T C A A C T G C C T G T C C C T A C C G G A T C G A G T C A A C T G A G T T A C C G G A T C C G A G T C A A C T G C C T G C T A C C G G A T C G A G T C A A C T G C C T G T C C C T A C C G G A T C G A G T C A A C T G C C T G C T C C C T C C C T G C C T C C C C	TO GO COA AC GAAA TO COA AAAA TO CO COA AC GA CATT COA AAA ATO CO CAA GO ACATT COA AAA AC GO COA AC GA CATT COA AAA AC GO COA AC GA CATT TO AAAA AC GO COA AC GA CATATT CAA AC AC GO COA AC GA CATACAC AC GO COA AC GA CATATT CAA AC CACACACAAC AC ATATT CAA AC CACACAAAA GA CATTT CAA AA CACACAAAA GA CATTT CAA AA CACACAAAAC AC ATTT CAAAA CACACAAAAC AC ATTT CAAAA CAAACACAAAAC AC ATTT CAAAA CAAACACAAAACACACAATTT CAAAA CAAACACAAAACACACACAATT CAAAAA CAAAACACAAAACACACAATTT CAAAAA CAAAACACAAAACACACAATTT CAAAAA CAAAACACAAAACACACAATTT CAAAAA CAAAACACAAAACACACAACACAATTT CAAAAA CAAAACACAAAACACACAATTT CAAAAA CAAAACACAAAACAACACACAATTT CAAAAA CAAAACACAAAACAACACAACAACAACAAAAAAAA	A C C C C C C C C C A A C C C C C C C T           A C C C C C C C C C A A C C T C C A A C C T C C A A C C T C C A A C C T C C A A C C T C C A A C C T C A A C C T C C A A C C T C A A C C T C C A A C C T C C A A C C T C C A A C C C C	CARAGA TO AGA TO AGA TO TO CAGA TO AGA AGA AGA TO AGA AGA AGA AGA AGA AGA AGA AGA AGA AG	Image: Constraint of the state of

Figure 3.1.1.3.1 b. Clustal alignment of *CBF* nucleotide sequences isolated from all other species that were not members of the *Ericaceae* or *Brassicaceae* compared to *Arabidopsis thaliana CBF*1-4 genbank sequences. Sequences are ordered by Clustal sorting. Names are in the form "Species code pp primer pair code\_Genus\_Species\_Site code\_Location". Genbank gene names are in the form "Genus\_Species\_Gene\_Genbank code".

Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 Arabidopsis\_thaliana\_CBF3\_AT4G25480.1 Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 90pp12\_Leucanthemum\_vulgare?\_S19\_Weardale&Teesdale 4bpeCBF2\_Betula\_nana\_S2\_Abisko nulabpeCBF1\_Betula\_nana\_nula\_Abisko 4bpeCBF1\_Betula\_nana\_S2\_Abisko 4PP59 Betula nana S2 Abisko Nulapp59\_Betula\_nana\_nula\_Abisko\_REVonly 74pp50\_Fragaria\_vesca\_S14\_Abisko 78pp50\_Fragaria\_vesca\_S16\_Weardale&Teesdale 51PP51\_Salix\_herbacea\_S6\_Abisko 51pp53\_Salix\_herbacea\_S6\_Abisko 77pp 19\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp 19\_Trifolium\_repens\_S22\_Weardale&Teesdale 77PP23&24B\_Trifolium\_repens\_S16\_Weardale&Teesdale 77PP23&24A\_Trifolium\_repens\_S16\_Weardale&Teesdale 58pp 23B\_Trifolium\_repens\_S9\_Abisko 58pp 19\_Trifolium\_repens\_S9\_Abisko\_FWDonly 94pp 23\_Trifolium\_repens\_S22\_Weardale&TeesdaleFWDonly 58pp 23C\_Trifolium\_repens\_S9\_Abisko 63pp 17\_Matricaria\_recutita?\_S11\_Abisko 63pp6\_Matricaria\_recutita?\_S11\_Abisko 63pp5\_Matricaria\_recutita?\_S11\_Abisko\_REVonly 58pp13\_Trifolium\_repens\_S9\_Abisko 77pp13\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp13\_Trifolium\_repens\_S22\_Weardale&Teesdale\_FWDonly

Arabidopsis\_thaliana\_CBF2\_AT4G25470.1 Arabidopsis\_thaliana\_CBF1\_AT4G25490.1 Arabidopsis\_thaliana\_CBF3\_AT4G25480.1 Arabidopsis\_thaliana\_CBF4\_AT5G51990.1 90pp 12\_Leucanthemum\_vulgare?\_S19\_Weardale&Teesdale 4bpeCBF2\_Betula\_nana\_S2\_Abisko nulabpeCBF1\_Betula\_nana\_nula\_Abisko 4bpeCBF1\_Betula\_nana\_S2\_Abisko 4PP59 Betula nana S2 Abisko Nulapp59\_Betula\_nana\_nula\_Abisko\_REVonly 74pp50\_Fragaria\_vesca\_S14\_Abisko 78pp50\_Fragaria\_vesca\_S16\_Weardale&Teesdale 51PP51\_Salix\_herbacea\_S6\_Abisko 51pp53\_Salix\_herbacea\_S6\_Abisko 77pp 19\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp 19\_Trifolium\_repens\_S22\_Weardale&Teesdale 77PP23&24B\_Trifolium\_repens\_S16\_Weardale&Teesdale 77PP23&24A\_Trifolium\_repens\_S16\_Weardale&Teesdale 58pp 23B\_Trifolium\_repens\_S9\_Abisko 58pp 19\_Trifolium\_repens\_S9\_Abisko\_FWDonly 94pp 23\_Trifolium\_repens\_S22\_Weardale&TeesdaleFWDonly 58pp 23C\_Trifolium\_repens\_S9\_Abisko 63pp17\_Matricaria\_recutita?\_S11\_Abisko 63pp6\_Matricaria\_recutita?\_S11\_Abisko 63op 5\_Matricaria\_recutita?\_S11\_Abisko\_REVonly 58pp13\_Trifolium\_repens\_S9\_Abisko 77pp13\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp13\_Trifolium\_repens\_S22\_Weardale&Teesdale\_FWDonly

42	23 TATGACGACGGATGCTCATGGTCTTGACATGGAGGAGGACCTTGGTGGAGGCTATTTATACGCCGGGAACAG.AGCCGATTTATATGGATGAAGAGGCGATGTTGGGGATGTC	TAGT 543
41		GACT 534
42		C A C T 540
		040
44		CAAC 564
36	63 ····································	GGGG 480
38	80 · · · · · · · · · · · · · · · · · · ·	GGGG 482
38	80 · · · · · · · · · · · · · · · · · · ·	GGGG 482
38	80 · · · · · · · · · · · · · · · · · · ·	GGGG 482
36	65 · · · · · · · · · · · · · · · · · · ·	GGGG 447
38	87 · · · · · · · · · · · · · · · · · · ·	GGGG 489
44	46 · · · · · · · · · · · · · · · · · · ·	GGGG 551
44		6666 551
47		ACCT 594
42		000 519
42		
43		AGAG 546
39	90 · · · · · · · · · · · · · · · · · · ·	
42	25 · · · · · · · · · · · · · · · · · · ·	AGAG 529
42	25 · · · · · · · · · · · · · · · · · · ·	AGAA 532
41	15 · · · · · · · · · · · · · · · · · · ·	AGGG 500
42		AGGG 512
41		4666 499
41		TGAT 505
		TGAT 505
		TOAT 100
34		438
54	44 TTGTTGGATAACATGGCCGAAGGGATGCTTTTACCGTCGCCGTCGCGTCGAATGGAACTATAATTTTGATGTCGAGGGAGATGATGACGTGTCCTTATGGAGCCTATTAA	651
53	35 TTO TTO CATACTATA TA TO COTO A COCOTO TO TTA COCOCO COTO TO CAATO CATACTATATTA TO ACOOCO A AGOA O ATO COTO A COTO COTO TA CTAA ·····	642
54	41 TTGTTGGCTAATATGGCAGAAGGGATGCTTTTGCCGCTTCCGTCCG	651
56	65 TTTTTTGAGAATATGGCGGAGGGGATGCTTTTGCCGCCGGAAGTTGGCTGGAATCATAAC···GACTTTGACGGAGTGGGTGAGGC···TGTCACTCTGGAGTTTTGACGAGTAA	675
46	61 TTTTTTGCTGACATGGCACAAGGGTTGATGTTACCGCCACC······CCAGGAGYGACAACT···GCTTCGATGACGTG······	529
46	63 TT <mark>G</mark> CTGGTAAATATGGCGGGGGGGGGGGGGGCCCTCCTCCCCCCCC	568
46	63 TT C CT C C T C C T C C C C C C C C C	568
46	63 TTGCTGGTAAATATGGCGGAGGGGATGCTCTTGCCTCCACCCTACTGTGGGAGATGATGATGGCGGGGTATGGCGGGGATGACATGGAAGCCCCACGCCGACGTTTCAT	568
44	48 TTGCTGGTGGTGGTGGGGGGGGGGGGGGGGGGGGGGGG	542
47	70 TTGCTGGTGGTGGTGGGGGGGGGGGGGGGGGGGGGGGG	547
55	52 TTGCTGGCCGAACATGGCGGAAGGGATGCTACTGCCGCCTCCTCAAT.	634
55	52 TTACTOR CAACATOR CAACATOR CAACATOR CACTOR COCCTOC CAAT	634
59		679
50		078
51		005
54		620
50	UT I CATTGAGAAATATGGCACTAATGTCCCCAACACATATGGGCATGAGTATGAGCATATGATCATCATGT	574
53	30 TCATTGAGAAATATGGCACTAATGTCCCCAACACATAGGCCTGGGCATGAGTATGAGCATATTGATCATGTAGACTTTCAAGATGTG	619
53	33 TCATTGAGGAATATGGCACTAATGTCCCCCAACACATAGCTTAGGGCATGAGTATGAACATATTGATC···ATGTAGACTTT	610
50	01 TTTCTTGATAGCATGGCTGAAGCACTCATGCTACCACCCTCAAAGTGTTGGATATGGCAACTTTTTTTGAAGATGTGGAATCA	584
50 51	01 TTTCTTGATAGCATGGCTGAAGCACTCATCCTACCACCACCTCAAAGTGTTGGATATGGCAACTTTTTTTGAAGATGTGGAATCA	584 596
50 51 50	01 TTTC TTG ATAS CATGS CTG AAS CACT CATS CTACCACCAC CT CAAAS TS TTG ATATS CAAC TTTTTTTG AAS ATS TS AAATCA. 13 TTTC TTG ATAS CATGS CTG AAS CACT CATS CTACCACCACCACCT CAAAST CTTG ATATS CAACTTTTTTTG AAS ATS TS GAATCA. 0 TTTC TTG ATAS CATGS CTG AAS CACT CATS CTACCACCACCT CAAAST CTTG ATATS CAACTTTTTTTG AAS ATS TS GAATCA.	584 596 535
50 51 50	01 TTTCTTGATAGCATGGCTGAAGCACTCATGCTACCACCACCTCAAAGTGTTGGATATGGCAACTTTTTTTGAAGATGTGGAATCA 13 TTTCTTGATAGCATGGCTGAAGCACTCATGCTACCACCACCTCAAAGTGTTGGATATGGCAACTTTTTTGAAGATGTGGAATCA 00 TTTCTTGATAGCATGGCTGAAGCACTCATGCTGCCA 01 TTGCTTTTGATATGCTAATGACCTTTACGTTCACTTCCCACCTTCCCCACCAAATTATTATGGTGGAACTTTTTAACTGGGGTGACTTGGAATGT.	584 596 535 603
50 51 50 50	01 TTTCTTGATASCATGCTGAASCACTCATCCTACCACCTCAAASTGTTGGATATGGCAACTTTTTTGAAGATGTGGAATCA 13 TTTCTTGATASCATGCCTGAASCACTCATCCTACCACCACCTCGAAGTGTTGGATATGGCAACTTTTTTGAAGATGTGGAATCA 00 TTTCTTGATASCATGCCTGAASCACTCATSCTGCCA 00 TTGCTTTTTGAGTATTGCTAACGACCTTTACGTTCCTACTTCCCCACCAACTATTATTATGGTGGTACTTGTAACTGGGGTGACATGGACATGCT 00 TTGCTTTTTGAGTATTGCTAATGAGCCTTTACGTTCCCACTTCCCCACCAACTATTATTGGTGGTACCTGGACGTGACATGGACATGGACATGCT 00 TTGCTTTTTGAGTATTGCTAATGAGCCTTTACGTTCCCCACTGCCCACCAACTATTATTGTGGTGGTACCTGGACATGGACATGGACATGCT 00 TTGCTTTTTGAGTATTGCTAATGAGCCTTTACGTTCCCCACTGCCAACTATTATTGCGGTGCTACCTGGACATGGACATGGACATGCT 00 TTGCTTTTGAGTATTGCTAATGAGCCTTTACGTTCCCCCCCC	584 596 535 603
50 51 50 50	01 TTT C TT & A TAO CATGO CT & A AO CAC T CATGO TAC CAC CAC CT CAAAO T & TT & G ATATGO CAAC TTTTT TT & AAG AT & TG G AAT CA 13 TTT C TT & A TAO CATGO CT & AA CAC T CATGO TAC CAC CC CCACAC TT TT G TAGO CAAC TTTTTT TO AAG AT & TG G AAT CA 01 TTT C TT & A TAO CATGO CT & AA CAC T CATGO CT AC CAC CC CCAACAC TTTT TT G AGA T & TG G AAT CA 05 TT & G TTTT & AAT A G CATGO CT AA CATGO CT CCAC T C CTAC T C CCAACAAAT TATTAT & G T & G T & G T AA CT & G G & CATGO AC AT & CT 06 TT & CTTTT & AA TATTO CTAAT & AA CC TTTC AC TT C CTAC T T C CCAACAAAT TATTAT & G T & G T & G T AA CT & G G & CATGO AC AT & CT 06 TT & CTTTT & AA TATTO CTAAT & AA CC TTTC AC TT C CTAC T T C CCAACAAAT TATTAT & G T & G T & G T AA CT & G G & CATGO AC AT & CT 07 TT & CTTTT & AA TATTO CTAAT & AA CC TTTACO TT C C T AC TT C CCAACAAAT TATTAT & G T & G T & G T & AA CT & G G & CATGO AC AT & CT 07 TT & CTTTT & AA TTATTO CTAAT & AA CC TTT C & CTT & CTACTT C CCAACAAAT TATTAT & G T & G T & G T & AA CT & G G & CATGO AC AT & CT 07 TT & CTTTT & AA TTATTO CTAAT & CTT &	584 596 535 603 603

Figure 3.1.1.3.1 c. Clustal alignment of *CBF* nucleotide sequences isolated from all other species that were not members of the *Ericaceae* or *Brassicaceae* compared to *Arabidopsis thaliana CBF*1-4 genbank sequences. Sequences are ordered by Clustal sorting. Names are in the form "Species code pp primer pair code\_Genus\_Species\_Site code\_Location". Genbank gene names are in the form "Genus\_Species\_Gene\_Genbank code".

# **3.1.2 Translated CBF sequences**

\*Note: The terms substitution, insertion or deletion are used simply relative to *Arabidopsis thaliana* CBF1; they do not have any evolutionary significance.\*

There was greater amino acid sequence conservation across all species than nucleotide conservation (Fig. 3.1.2.1a-b vs Fig. 3.1.1.1.1 a-b, Fig. 3.1.1.2.1 a-b, Fig. 3.1.1.3.1 a-c).

All sequences, where sequence reads covered those regions, had CBF signature sequences with the possible exception of 63pp5 *Matricaria recutita* which had numerous differences in this region relative to other sequenes (Fig 3.1.2.2).

The majority of variation between species was in the COOH-terminus (Fig. 3.1.2.1 b). Members of the same family tend to be similar to one another.

*Arabidopsis thaliana* CBF1-3, *Cardaminopsis arenosa* 66pp66, 66pp69, 66pp67 and *Capsella bursa-pastoris* 68pp61, 68pp71 and 68pp64 were all very similar to one another (Fig. 3.1.2.1 b). *Arabidopsis thaliana* CBF4 and *Barbaea vulgaris* (64pp60) were also somewhat similar to this group of *Brassicaceae* sequences however they display greater similarity to one another than to the other members of this group (Fig. 3.1.2.1 b).

A secondary distinct pairing of *Brassicaceae* sequences was seen consisting of sequences *Cardaminopsis arenosa* 66pp77 and *Arabis alpina* 69pp77 (Fig. 3.1.2.1 b).

*Capsella bursa-pastoris* 68pp75 had a distinct COOH-terminus which was not similar to any other sequence (Fig. 3.1.2.1 b).

All *Ericaceae* samples were very similar to one another; *Vaccinium vitis-idaea* 10pp22, 118pp22, 40pp22, *Vaccinium myrtillus* 114pp22, *Andromeda polifolia* 43pp22C, 43pp22D, 43pp22A, 43pp22B and 18pp22 with the exception of *Empetrum nigrum* 5a, b and c which displayed a different COOH-terminus sequence (Fig. 3.1.2.1 b). This COOH-

terminus sequence did, however, show greater similarity to the other members of the *Ericaceae* than to any other group (Fig. 3.1.2.1 b). 2pp22 *Diapensisa lapponica* and 3pp22 *Arctostaphylos alpina* had identical sequences to *Empetrum nigrum* 5a, b and c (Fig. 3.1.2.1 b).

Two distinct groups of COOH-terminus sequences were seen for *Trifolium repens*, one group consisted of sequences 58pp13, 7pp13, and 94pp3 with almost identical COOH-termini (Fig. 3.1.2.1 b). The second group consisted of sequences 77pp19, 94pp19, 77pp23&24B, 77pp23&24A, which had very similar, but not identical, COOH-termini to one another (Fig. 3.1.2.1 b).

All Betula nana sequences were identical to one another (Fig. 3.1.2.1 a-b).

*Fragaria vesca* 74 (Abisko, Sweden) and 78 (Weardale) had identical sequences (Fig. 3.1.2.1 a-b).

*Salix hebacea* had two distinct sequences 51pp51 and 51pp53 which had little similarity to one another (relative to all other sequences). 51pp53 had a distinct COOH-terminus which was very different from other species (Fig. 3.1.2.1 b).

66pp66\_Cardaminopsis\_arenosa\_S11\_Abisko 66pp 69\_Cardaminopsis\_arenosa\_S11\_Abisko 66pp67 Cardaminopsis arenosa S11 Abisko Arabidopsis\_thaliana\_AT4G25470.1\_CBF2 Arabidopsis\_thaliana\_AT4G25480.1\_CBF3 Arabidopsis\_thaliana\_AT4G25490.1\_CBF1 68pp 61\_Capsella\_bursa-pastoris?\_S12\_Abisko\_REVonly 68PP71\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 64 Capsella bursa-pastoris? S12 Abisko 68pp 68\_Capsella\_bursa-pastoris?\_S12\_Abisko Arabidopsis\_thaliana\_AT5G51990.1\_CBF4 64pp60\_Barbaea\_vulgaris?\_S11\_Abisko 10pp 22\_Vaccinium\_vitis-idaea\_S2\_Abisko 118pp 22\_Vaccinium\_vitis\_idea\_S25\_Weardale&Teesdale 40pp22\_Vaccinium\_vitis-idaea\_S6\_Abisko 114pp22\_Vaccinium\_myrtillus\_S25\_Weardale&Teesdale Andromeda\_polifolia\_Abisko\_43pp22C\_Topo Andromeda\_polifolia\_Abisko\_43pp22D\_Topo Andromeda\_polifolia\_Abisko\_43pp 22A\_Topo Andromeda\_polifolia\_Abisko\_43pp 22B\_Topo 18pp22 Andromeda polifolia S3 Abisko 2pp 22\_Diapensia\_lapponica\_S2\_Abisko 5bTOPO\_Empetrum\_nigrum\_S2(north)\_Abisko 3pp22\_Arctostaphylos\_alpina\_S2\_Abisko 5aTOPO\_Empetrum\_nigrum\_S2(north)\_Abisko 5cTOPO\_Empetrum\_nigrum\_S2(north)\_Abisko 58pp13\_Trifolium\_repens\_S9\_Abisko 77pp13\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp 13\_Trifolium\_repens\_S22\_Weardale&Teesdale\_FWDonly 66pp77\_Cardaminopsis\_arenosa\_S11\_Abisko 68PP76\_Capsella\_bursa-pastoris?\_S12\_Abisko 69pp77\_Arabis\_alpina?\_S12\_Abisko 68pp 75\_Capsella\_bursa-pastoris?\_S12\_Abisko 51pp53\_Salix\_herbacea\_S6\_Abisko 77pp 19\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp19\_Trifolium\_repens\_S22\_Weardale&Teesdale 77PP23&24B\_Trifolium\_repens\_S16\_Weardale&Teesdale 77PP23&24A\_Trifolium\_repens\_S16\_Weardale&Teesdale 58pp 19\_Trifolium\_repens\_S9\_Abisko\_FWDonly 94pp 23\_Trifolium\_repens\_S22\_Weardale&Teesdale\_FWDonly 58pp 23C\_Trifolium\_repens\_S9\_Abisko\_FWDonly 4bpeCBF2\_Betula\_nana\_S2\_Abisko nnulabpeCBF1\_Betula\_nana\_nula\_Abisko 4bpeCBF1\_Betula\_nana\_S2\_Abisko 4PP59\_Betula\_nana\_S2\_Abisko Nulapp59\_Betula\_nana\_nula\_Abisko\_REVonly 74pp50 Fragaria vesca S14 Abisko 78pp50\_Fragaria\_vesca\_S16\_Weardale&Teesdale 51PP51\_Salix\_herbacea\_S6\_Abisko 90pp12\_Leucanthemum\_vulgare?\_S19\_Weardale&Teesdale 63pp5\_Matricaria\_recutita?\_S11\_Abisko\_REVonly 63pp6\_Matricaria\_recutita?\_S11\_Abisko 63pp 17\_Matricaria\_recutita?\_S11\_Abisko

1	1 · · · · · · · · · · · · · · · · · · ·	G S D Y E S · · · · S G G D Y S	PKLATSCPKKPAG -	RKKFRETRHPIN	YRGVRQRNSGH	KWVSELREPN	· · KKTR	IWLGTFQ	TAEMAAR	AHDVAAIAL	RGRS · · · ACL	NEA 8	94
4	1	GSDYES	PKLATSCPKKPAG	RKKERETRUPI	ROVRORNSON		KKTP			AHDVAALAL	C.P.S.	NEA	۵d
	· · · · · · · · · · · · · · · · · · ·	0000 T3	REALD CENTRO							ANDUARIAL			
1	1			· · KERETRHPIN	ROVRORNSG	KWVSELKEPN · · ·	· · KKTR		TAEMAAR	AHDVAATAL	KORS ACL	INFA C	26
1	1 · · · · · · · · · · · · · · · · · MNSFSAFSEMF	GSDYESP - VS <mark>SGGD</mark> YS	PKLATSCPKKPAG	RKKFRETRHPIN	YRG V RQ R N SG P	KWVCEL <mark>R</mark> EP <mark>N</mark> ····	· · KKTR	IWL <mark>GT</mark> FQ	TAEMAAR	AHDVAAIAL	RGRS · · · ACL	_ <mark>N</mark> F A 10	7נ
1	1 · · · · · · · · · · · · · · · · MNSFSAFSEMF	GSDYESS-VS <mark>S</mark> GGD <mark></mark> YI	PTLA <mark>SS</mark> CP <mark>KK</mark> PAG -	RKKFRETRHPIN	Y R G V R R R N S G P	KWVCEV <mark>REPN</mark> ····	· · KKTR	IWL <mark>GT</mark> FQ	TAEMAAR	AHDVAALAL	RGRS · · · ACL	_ <mark>N</mark> F A 10	37
1	1	G S D Y E <mark>P</mark> · · · · Q <mark>G G D</mark> Y C	PTLA <mark>TS</mark> C <mark>PKKP</mark> AG -	RKKFRETRHPIN	Y R G V R Q R N S G P	KWVSEVREPN	KKTR	IWLGTFQ	TAEMAAR	AHDVAALAL	RGRSACL	NFA 10	04
1	1 • • • • • • • • • • • • • • • • • • •				G	· · VSELREPN · · · ·			TXEMAAR	AHDVAAITL	GRS · · · ACL	NEA 4	46
1		G S D Y E S PG S S G G G D Y C	PTLATSCPKKPAG.	RKKERETRHPIN	ROVRORSSOL		KKTR		TAEMAAR	AHDVAALTL	GRS ACI	NEAS	97
4				RKKERETRHPI	ROVERENSO	WVCEVREPN	KK CP		TAEMAAP	AHDVAALAL	C.R.S.	NEA	70
		COVERD VERGERVE		PVVCPCTPVC	PAVPPPMCA				TAEMAA			NEA /	07
1	· · · · · · · · · · · · · · · · · · ·	SUTESP VSBOODYC	NILAAS OF KKPAG	BEEF REIRHPIN	ROVERRUSO		· · · · · · · · · · · · · · · · · · ·		TUEMAAR	ANDVAATAL	AUL		21
1	1 · · · · · · · · · · · · · · · · MN <mark>P</mark> FYSIF <mark>P</mark> DSFLSI	ISDHRS <mark>P</mark> · VSDSSECS	PREASSCPRERAG-	RECEPTION	YRGVR <mark>QRN</mark> SGP	KWVCEVREPN	· · KKSR	IWLGIFP		AHDVAALAL	<b>(GRS</b> ACL	- NFA 11	10
1	1 · · · · · · · · · · · · · · · · · · ·	TSDHRSP-VSEGSECS	PKLASSCPKKRAG -	RKKFRETRHPIN	YRGVR <mark>QRN</mark> SGP	KWVCEVREPN	· · KKSR	IWLGTFP	TVEMAAR	AHDVAALAL	GRS · · · ACL	NFA 9	99
1	1 <mark>G</mark> Y <mark>P</mark> N <mark>G</mark> SNS <mark>G</mark> SKSISLEYVE	E <mark>G</mark> STSR <mark>G</mark> S <mark>P</mark> K <mark>S</mark> DE <mark>E</mark> LM	ITLA <mark>SSQPKKR</mark> AG -	RKKFKETRHPIN	Y R G V R R R <mark>N</mark> N D F	RWVCEL <mark>REPN</mark> · · · ·	· · KKSR	LWL <mark>GTY</mark> P	TAEMAAR	AHDVAALAL	GESST-ACL	_ <mark>N</mark> FA 11	17
1	1 <mark>G</mark> Y <mark>P</mark> N <mark>G</mark> S N S <mark>G</mark> S K S I S L E Y V E	E <mark>G</mark> STSR <mark>G</mark> S <mark>P</mark> K <mark>S</mark> DE <mark>E</mark> LM	ITLA <mark>SS</mark> QPKKRAG -	RKKFKETRHPIN	Y R G V R R R <mark>N</mark> N D F	RWVCEL <mark>REPN</mark> ····	· · KKSR	LWL <mark>GTY</mark> P	TAEMAAR	AHDVAALAL	KGESST · ACL	_ <mark>N</mark> F A 11	17
1	1 · · · · · · · · · · · · · · · · · · ·	E <mark>G</mark> STSR <mark>G</mark> S <mark>P</mark> K <mark>S</mark> DE <mark>E</mark> LM	ITLA <mark>SSQPKKR</mark> AG-	RKKFKETRHPIN	Y R G V R R R N N D F	RWVCELREPN	KKSR	LWL <mark>GTY</mark> P	TAEMAAR	AHDVAALAL	GESST-ACL	NFA 11	17
1	1 · · · · · · · · · · AF <mark>P</mark> IMSNS <mark>G</mark> SKS <mark>P</mark> SLEYVE	E <mark>g</mark> stsr <mark>g</mark> spk <mark>s</mark> de <mark>e</mark> lm	ITLASSOPKKRAG -	RKKFKETRHPIN	Y R G V R R R N N D F	RWVCELREPN	· · KKSR		TAEMAAR	AHDVAALVL	GESST - ACL	NFA 11	17
1	1 · · · · · · · · · ISNRVNLRIKISFIRIRG	ORFNOPRLPOSDEELM	ITLASSOPKKRAG	RKKEKETRHPIN	RGVRRRNNN		· · KKSR		TAEMAAR	AHDVAALAL	GESSTACI	NEA 11	16
4	1ISNRVNLRIKISFIRIRG		ITLASSOPKKRAG.	RKKEKETRHPIN			KKSR		TAEMAAR	AHDVAALAL	GESST. ACI	NEA 11	16
			LTLASSORKKRAG.	PREFET PUPIN					TACMAAR		A FRAT ACL	NEA 4	10
1			TTLASSOPKKRAG.	DENERGERRET			KASK		TACMAAR	AHDVAALAL	CEDST-AUL		10
1	1 ISNKVNLKIKISFIRIRG	urrnu <mark>m</mark> rl <b>mus</b> de <b>e</b> lm	TILASSUPKKRAG -	KKK KEKPIN	ROVERENNE	WV CELKEPN	· · KKSR	LWLGTYP	TAEMAAR	ANDVAALAL	COESSI · ACL	- NFA 11	10
1	1 • • • • • • • • • • • • • • • • • • •	· • · · · · • • • • • • • • • • • • • •			<b>RR</b> XNN	KWVCELREPN	· · KKSR	LWLGTYP		AHDVAALAL	CGESST-ACL	NFA 6	54
1	1 · · · · · · · · · · · · · · · <mark>G</mark> N <mark>P</mark> ISKS <mark>P</mark> ISEYIC	D <mark>G</mark> STSE <mark>P</mark> SAQ <mark>S</mark> DE <mark>E</mark> TM	ITLA <mark>SS</mark> RPKKPAG -	RKKFKETRHPVY	Y R G V R K R <mark>N</mark> N D P	KWVCELREPN · · · ·	· · KKSR	LWL <mark>GTY</mark> P	TAEMAAR	AHDVAALAL	CG D S SL P P C L	- <mark>N</mark> FA 11	13
1	1 · · · · · · · · · · · · · · · <mark>G</mark> N <mark>P</mark> ISKS <mark>P</mark> ISEYID	D <mark>G</mark> STSE <mark>P</mark> SAQ <mark>S</mark> DE <mark>E</mark> TM	ITLA <mark>SS</mark> R <mark>PKK</mark> PAG -	RKKFKETRHPV	Y R G V R K R <mark>N</mark> N D P	KWVCEL <mark>REPN</mark>	KK <mark>S</mark> R	LWL <mark>GTY</mark> P	TAEMAAR	AHDVAALAL	KG D S S L P P R L	_ <mark>N</mark> F A 11	13
1	1 · · · · · · · · · · · · · · · <mark>G</mark> N <mark>P</mark> I S K S <mark>P</mark> I S E Y I [	D <mark>G</mark> STSE <mark>P</mark> SAQ <mark>S</mark> DE <mark>E</mark> TM	ITLA <mark>SS</mark> R <mark>PKK</mark> PAG -	RKKFKETRHPVY	Y R G V R K R <mark>N</mark> N D H	KWVCEL <mark>REPN</mark> ····	· · KKSR	LWL <mark>GTY</mark> P	TAEMAAR	AHDVAALAL	<pre>GDSSLPPCL</pre>	NFA 11	13
1	1 · · · · · · · · · · · · · · · · G <mark>n P</mark> isks <mark>P</mark> iseyi	D <mark>G</mark> STSE <mark>P</mark> SAQ <mark>S</mark> DE <mark>E</mark> TM	ITLASSRPKKPAG -	RKKFKETRHPV	Y R G V R K R N N D P	KWVCELREPN · · · ·	· · KKSR		TAEMAAR	AHDVAALAL	GDSSLPPCL	NFA 11	13
1	1 · · · · · · · · · · · · · · · · · · ·	DGSTSEPSAQSDFFTM	ITLASSRPKKPAG	RKKEKETRHPVN	RGVRKRNND		· · KKSR		TAEMAAR	AHDVAALAL	GDSSLPPCI	NEA 11	13
4	1	SSSETSSSSNRISSSO	VILASSKPKKKAG	RRVEKETRHPV					TPEMAAP	AHDVAALTL	GKS	NEA 10	n4
	,	CONTRACTOR	VILASSKPKKKAG	PRVEKETRURVA					TREMAAR			NEA 10	
	4 4	333E13333NR13339	PUPULAD.	DRVENE TRUCK					TREMAAR				24 00
1	1		·····	REVERENCE	ROVERRNNN			TWLGSYP	TEMAAR	AHUVAALILI	CORS ACL	N A 8	32
1	1		· · · · EMKPKKRAG ·	RRIFKETRHPIN	RGVRRRDGD	KWVCEVREP · · · ·	- THORR		TAEMAAR	AHDVAVLAL	KGRS · · · ACL	INFS 7	77
1	1		• • • • EMK <mark>PKKR</mark> AG •	RRIFKETRHPIN	<b>YRGVRRRDGD</b>	KWVCEVREP · · · · ·	- I H <mark>Q</mark> R <mark>R</mark>	VWL GTYP	TAEMAAR	AHDVAVLAL	GRS · · · ACL	NFS 7	77
1	1		••••EMK <mark>PKKR</mark> AG•	RKIFKETRHPIN	Y R G V R R R D G D H	KWVCEV <mark>REP</mark> ····	- I H <mark>Q</mark> R <mark>R</mark>	VWLGTYP	TADMAAR	AHDVAVLAL	RGRS ACL	NFS 7	77
1	1	· · · · <u>·</u> · · · · · <u>·</u> · · · · ·	• • • • EMRPKKRAG •	RTVFKETRHPVY	Y R G I R R R <mark>N</mark> G D P	KWVCEV <mark>R</mark> EP · · · · ·	- THQRR	IWL <mark>GTY</mark> P	TADMAAR	AHDVAVLSL	RGRS · · · ACL	NFA 7	77
1	1 · · · · · · · · · · · · · · · · · · SADRQESSLLS	S D S S <mark>G</mark> S Q Q D S <mark>P</mark> S S N E E	VLLA <mark>TS</mark> HPKRRAG -	RRIFKETRHPIN	Y R G V R K R <mark>N</mark> G D H	KWVCEL <mark>R</mark> EPN · · · ·	· · KKSR	IWLGTYP	TAEMAAR	AHDVAALAF	RGKS · · · ACL	NFA 10	92
1	1 SH <mark>P</mark> I T S I <mark>P</mark> S S S S H K I S L	L <mark>P</mark> NLDVSHDM <mark>S</mark> TSNXE	VRLAASTPKKRGG	RKKFKETRHPV	Y R G V R S R N L D P	KWVCEM <mark>REPN</mark> ····	· · KKTK	IWLGTFQ	TAEMAAR	AHDVAALAL	GRN ACL	NFA 11	13
1	1 · · · · · · · · · · · · · · · · · · ·	LMN·····MSTSNXF	VRLAASTPKKRGG	RKKEKETRHPVN	REVESENIDE		· · KKTK		TAEMAAR	AHDVAALAL	GRN · · · ACI	NEAS	97
1	1PNSSIPSSSSHKTSI		VELAASTPKKRGG	RKKEKETRHPV	REVETENIDE		KKTK		TAEMAAR	AHDVAALAL	GRN ACI	NEA 1	11
4	1PNSSIPSSSSHKTSI		VELAASTEKKEGG	RKKEKETRHPV	ROVETENLOP		KKTK		TAEMAAR		GRN ACI	NEA 11	11
	1		VPLAASTPKKPAA	PVVYVETPUPVY	POVPSPNID				TAEMAAB				70
1			VOLAASTEKKKOG.	DEVENETBUS					TAFMAAR				20 00
1		LDVSHAMSISNAE	VRLAASTPKKRGG.	REALERAPVY		WV CEMKEPN	· · · · · · · · · · · · · · · · · · ·	I WLOIFP	AEMAAR	ANDVAALAL	ORN ACL	INFA S	98 98
1	1 · · · · · I S S · · · ·	· · DLDVSHXMSTSNEE	VRLAASTPKKRGG -	REFEETRIPVY	Y A G V R T R N L D P	KWVCEMREPN	••• <b>KKT</b> X	IWLGTF P		AHDVXALAL	G R N A C L	NFA S	98
1	1 · · · · · · · · · · · · · · · · · · ·	E · · · · · S <mark>G</mark> AMHLS <mark>D</mark> E <mark>G</mark>	ILLASRNPKKRAG -	RKKFKETRHPVY	Y XG V R R X X S G P	KWVCEV <mark>REPN</mark>	KQSR	IWLGTFP	TAEMAAR	AHDVAAIAL	<b>(</b> G N <mark>S</mark> A C L	NFA 9	96
1	1 · · · · · · · · · · · · · · · · · · ·	E · · · · · S <mark>G</mark> AMHLS <mark>D</mark> E <mark>G</mark>	ILLA <mark>S</mark> RNPKKRAG -	RKKFKETRHPVY	Y X G V R R X X S G P	KWVCEV <mark>R</mark> EP <mark>N</mark> ····	• • KQ S R	IWL <mark>GT</mark> FP	TAEMAAR	AHDVAAIAL	RGN <mark>S</mark> ···ACL	NFA 8	96
1	1 · · · · · · · · · · · · · · · · · · ·	E S <mark>G</mark> AMHL S <mark>D</mark> E <mark>G</mark>	ILLA <mark>S</mark> RN <mark>PKKR</mark> AG -	RKKFKETRHPV	Y X G V R R R X S G P	KWVCEV <mark>R</mark> EPN · · · ·	KQ S R	IWLGTFP	TAEMAAR	AHDVAAIAL	G N S A C L	NFA 8	96
1	1	SGAMHLSDEG	ILLA <mark>S</mark> RNPKKRAG -	RKKFKETRHPVY	Y X G V R R R X S G H	KWVCEV <mark>REPN</mark>	KQ S R	IWLGTFP	TAEMAAR	AHDVAAIAL	3 G N S A C L	NFA 8	91
1	1 NIRSSR)	X · · · · · VGRCTCRTRR	IXLASRNPKKRAG -	RKKFKETRHPV	YRG VRRRKSG P	KWVCEVREPN · · ·	KQ S R		TAEMAAR	AHDVAAIAL	GNS · · · ACL	NFA 8	98
1	1 · · · · · · QYYDHN · · PVGSDLLESSSHSD	DNNS <mark>ggvg</mark> rmals <mark>d</mark> ee	VMLAGTYPKKRAG	RKKEKETRHPV	REVERENSE	KWVCEVREPN · · · ·	· · KKTR		TAEMAAR	AHDVAALAL	GRS · · · ACL	NEA 11	16
1	1 OYYDHN PVGSDI I ESSSHS	DNNSGGVGRMAL SDEE	VMLAGTYPKKRAG	RKKEKETRHPV	RGVRRRNSG		KKTR		TAEMAAR	AHDVAALAL	GRS ACI	NEA 11	16
		SVTKGVOOAALESDEE	VMLASENEKKPAG	RKKERETRHPV	ROVERENSOL		KK CP		TADMAAB	AHDVAALAL	GRS	NEA 1	25
	<u></u>		VULASON PKKPAG	PVVCPCTPUPVX	PAVPKPDSA				TADMAAD			NEA C	01
1	· · · · · · · · · · · · · · · · · · ·			A KEY KET DUDY	ROVKKRUSGP		TOAR		LA DIMAAR	ANDVAALAM	V N N N N N N N N N N N N N N N N N N N		21
1	I · · · · · · · · · · · · · · · · · · ·	VYRUEVVAX · HXNLKS		GKKAKETRHPVN	RAVAARAKGP	WVCEVRHP1		V W A G A H P	NAEMAAR	AHAVAVLAM	ARN	NFA 10	37
1	1 · · · · · · · · · · · · LTTLFCLRR <mark>P</mark> YET <mark>P</mark> F	FXCTDGSGSXSSQLEV	PALAXEXPKKRAGR	KKX-KEXRHPV	YRXVXXRXKG	KWVCEVRHPI	TQAR	VWXGXHP	NXEMAAR	AHXVXVLAM	GRN ACL	NFA 11	11
		F T C T D C C C C C C C C C C C C C C C C		DIZIZE DE TOUDY/N	POVDEDDVAL			VM/LGTHD	N A ELIA A B		C D N A C I	MEA 40	07

Figure 3.1.2.1.a Clustal alignment of translated CBF nucleotide sequences isolated from collected samples compared to *Arabidopsis thaliana CBF*1-4 genbank sequences. Sequences are ordered by Clustal sorting. Names are in the form "Species code pp primer pair code\_Genus\_Species\_Site code\_Location". Genbank gene names are in the form "Genus\_Species\_Gene\_Genbank code". 66pp66 Cardaminopsis arenosa S11 Abisko 66pp 69\_Cardaminopsis\_arenosa\_S11\_Abisko 66pp 67\_Cardaminopsis\_arenosa\_S11\_Abisko Arabidopsis thaliana AT4G25470.1 CBF2 Arabidopsis\_thaliana\_AT4G25480.1\_CBF3 Arabidopsis\_thaliana\_AT4G25490.1\_CBF1 68pp 61\_Capsella\_bursa-pastoris?\_S12\_Abisko\_REVonly 68PP71\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 64\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 68 Capsella bursa-pastoris? S12 Abisko Arabidopsis\_thaliana\_AT5G51990.1\_CBF4 64pp60\_Barbaea\_vulgaris?\_S11\_Abisko 100 DSAWRLRIPD 10pp 22\_Vaccinium\_vitis-idaea\_S2\_Abisko V<mark>S</mark>KEAEHIR<mark>R</mark>AAAEAAEMF 118pp 22\_Vaccinium\_vitis\_idea\_S25\_Weardale&Teesdale 118 DSVWQLPVP 118 DSVWQLPVF 40pp22 Vaccinium vitis-idaea S6 Abisko 114pp22 Vaccinium myttillus S25 Weardale&Teesdale 118 DSVWRLPVP Andromeda\_polifolia\_Abisko\_43pp22C\_Topo 117 DSVWRLPVF Andromeda\_polifolia\_Abisko\_43pp22D\_Topo 117 DSVWRLPVP Andromeda\_polifolia\_Abisko\_43pp22A\_Topo 117 D Andromeda\_polifolia\_Abisko\_43pp 22B\_Topo 117 DSVWRLPVF 55 DSVWRLPVP 18pp22\_Andromeda\_polifolia\_S3\_Abisko 2pp22\_Diapensia\_lapponica\_S2\_Abisko 114 DSVWRLPVF 114 DSVWRLPVP 5bTOPO Empetrum nigrum S2(north) Abisko 3pp 22\_Arctostaphylos\_alpina\_S2\_Abisko 114 DSVWRLPVF 5aTOPO Empetrum nicrum S2(north) Abisko 114 DSVWRLPVE 5cTOPO\_Empetrum\_nigrum\_S2(north)\_Abisko 114 DSVWRLPVF 58pp13\_Trifolium\_repens\_S9\_Abisko 77pp13\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp13 Trifolium repens S22 Weardale&Teesdale FWDonly 78 DSAWRLPTPE 66pp77 Cardaminopsis arenosa S11 Abisko 78 DSAWBLPTPE 68PP76 Capsella bursa-pastoris? S12 Abisko 69pp77\_Arabis\_alpina?\_S12\_Abisko 78 D 68pp 75\_Capsella\_bursa-pastoris?\_S12\_Abisko 51pp53\_Salix\_herbacea\_S6\_Abisko 77pp19\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp19\_Trifolium\_repens\_S22\_Weardale&Teesdale 77PP23&24B\_Trifolium\_repens\_S16\_Weardale&Teesdale 112 DSAWRLRMPA 77PP23&24A\_Trifolium\_repens\_S16\_Weardale&Teesdale 58pp19 Trifolium repens S9 Abisko FWDonly 94pp 23\_Trifolium\_repens\_S22\_Weardale&Teesdale\_FWDonly 58pp23C\_Trifolium\_repens\_S9\_Abisko\_FWDonly 4bpeCBF2\_Betula\_nana\_S2\_Abisko nnulabpeCBF1\_Betula\_nana\_nula\_Abisko 4bpeCBF1 Betula nana S2 Abisko 4PP59 Betula\_nana\_S2\_Abisko Nulapp59\_Betula\_nana\_nula\_Abisko\_REVonly 74pp50\_Fragaria\_vesca\_S14\_Abisko 78pp 50\_Fragaria\_vesca\_S16\_Weardale&Teesdale 51PP51 Salix herbacea S6 Abisko 90pp12 Leucanthemum vulgare? S19 Weardale&Teesdale 63pp5\_Matricaria\_recutita?\_S11\_Abisko\_REVonly 63pp6\_Matricaria\_recutita?\_S11\_Abisko 112 DXVWRLPVP 63pp 17\_Matricaria\_recutita?\_S11\_Abisko



Figure 3.1.2.1 b. Clustal alignment of translated CBF nucleotide sequences isolated from collected samples compared to Arabidopsis thaliana CBF1-4 genbank sequences. Sequences are ordered by Clustal sorting. Names are in the form "Species code pp primer pair code Genus Species Site code Location". Genbank gene names are in the form "Genus Species Gene Genbank code".

66pp 77\_Cardaminopsis\_arenosa\_S11\_Abisko 68PP76\_Capsella\_bursa-pastoris?\_S12\_Abisko 69pp77\_Arabis\_alpina?\_S12\_Abisko 68pp 75\_Capsella\_bursa-pastoris?\_S12\_Abisko 66pp 66\_Cardaminopsis\_arenosa\_S11\_Abisko 66pp 67\_Cardaminopsis\_arenosa\_S11\_Abisko 66pp 69\_Cardaminopsis\_arenosa\_S11\_Abisko 68pp 61\_Capsella\_bursa-pastoris?\_S12\_Abisko\_REVonly 68PP71\_Capsella\_bursa-pastoris?\_S12\_Abisko Arabidopsis\_thaliana\_AT4G25470.1\_CBF2 Arabidopsis\_thaliana\_AT4G25490.1\_CBF1 Arabidopsis\_thaliana\_AT4G25480.1\_CBF3 68pp 64\_Capsella\_bursa-pastoris?\_S12\_Abisko 68pp 68\_Capsella\_bursa-pastoris?\_S12\_Abisko Arabidopsis\_thaliana\_AT5G51990.1\_CBF4 64pp60\_Barbaea\_vulgaris?\_S11\_Abisko 51PP51\_Salix\_herbacea\_S6\_Abisko 4bpeCBF2\_Betula\_nana\_S2\_Abisko nnulabpeCBF1\_Betula\_nana\_nula\_Abisko 4bpeCBF1\_Betula\_nana\_S2\_Abisko 4PP59\_Betula\_nana\_S2\_Abisko Nulapp59\_Betula\_nana\_nula\_Abisko\_REVonly 74pp50\_Fragaria\_vesca\_S14\_Abisko 78pp50\_Fragaria\_vesca\_S16\_Weardale&Teesdale 77pp19\_Trifolium\_repens\_S16\_Weardale&Teesdale 58pp19\_Trifolium\_repens\_S9\_Abisko\_FWDonly 94pp19\_Trifolium\_repens\_S22\_Weardale&Teesdale 77PP23&24B\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp 23\_Trifolium\_repens\_S22\_Weardale&Teesdale\_FWDonly 77PP23&24A\_Trifolium\_repens\_S16\_Weardale&Teesdale 58pp 23C\_Trifolium\_repens\_S9\_Abisko\_FWDonly Andromeda\_polifolia\_Abisko\_43pp22B\_Topo 18pp 22\_Andromeda\_polifolia\_S3\_Abisko Andromeda\_polifolia\_Abisko\_43pp22A\_Topo Andromeda\_polifolia\_Abisko\_43pp22D\_Topo Andromeda\_polifolia\_Abisko\_43pp22C\_Topo 10pp22\_Vaccinium\_vitis-idaea\_S2\_Abisko 118pp 22\_Vaccinium\_vitis\_idea\_S25\_Weardale&Teesdale 40pp 22\_Vaccinium\_vitis-idaea\_S6\_Abisko 114pp 22\_Vaccinium\_myrtillus\_S25\_Weardale&Teesdale 2pp 22\_Diapensia\_lapponica\_S2\_Abisko 5bTOPO\_Empetrum\_nigrum\_S2(north)\_Abisko 3pp 22\_Arctostaphylos\_alpina\_S2\_Abisko 5aTOPO\_Empetrum\_nigrum\_S2(north)\_Abisko 5cTOPO\_Empetrum\_nigrum\_S2(north)\_Abisko 51pp53\_Salix\_herbacea\_S6\_Abisko 58pp13\_Trifolium\_repens\_S9\_Abisko 77pp13\_Trifolium\_repens\_S16\_Weardale&Teesdale 94pp 13\_Trifolium\_repens\_S22\_Weardale&Teesdale\_FWDonly

90pp12\_Leucanthemum\_vulgare?\_S19\_Weardale&Teesdale 63pp6\_Matricaria\_recutita?\_S11\_Abisko\_REVonly 63pp6\_Matricaria\_recutita?\_S11\_Abisko 63pp17\_Matricaria\_recutita?\_S11\_Abisko

10 20 30	40	50 6	0 70	80	
PKKRAGR - RIFKETRHPIYRGVRRRDGDKWVCEVRE	P   H (	Q R R VWL G T Y P T A E MA	ARAHDVAVLALRGRS	· · · ACLNFS	DSAWR
PKKRAGR - RIFKETRHPIYRGVRRRDGDKWVCEVRE	P   H (	Q R R VWL G T Y P T A E MA	ARAHDVAVLALRGRS	· · · ACLNES	DSAWR
PKKRAGR KIEKETRHPINGVRRRDGDKWVCEVR	P I H	OR RVWL GTYPTADMA	ARAHDVAVLALEGES	· · · ACL NES	DSAWR
PREPAGE TVERETERPYYEGIERENGORWUCEVER	р ти	OPPLWLGTYPTADMA	A DAH DVAVLEL DODS		DEAWP
PREASE - KERELAHFITAS VAGANSSKWUSELA		TRIWLOTFOTAEMA	ARAD VAAIAL NORS	AULNEA	
·····KFHETHHPTYRGVRURNSGKWVSELRE	P · · · NK · · ·	KTRIWLGIFQIAEMA	ARAHDVAATALRGRS	· · · AULNEA	DSAWR
PKKPAGR · KKFRETRHPIYRGVRORNSGKWVSELRE	P···NK···	KTRIWLXTFQTAEMA	ARAHDVAAIALRGRS	· · · ACLNFA	
· · · · · · · · · · · · · · · · · · ·	P · · · N K · · · I	KTRIWLGTFQTXEMA	ARAHDVAAITLRGRS	A C L N F A	
PKKPAGR - KKFRETRHPIYRGVRORSSGKWVSELRE	P N K I	KTRIWLGTFQTAEMA	A RAHDVAAITLRGRS	A <mark>C</mark> L <mark>N</mark> F A	DSAWR
PKKPAGR - KKFRETRHPIYRGVRQRNSGKWVCELRE	P • • • N K • • • I	KTRIWLGTFQTAEMA	A RAHDVAAIAL RG RS	· · · ACLNFA	DSAWR
PKKPAGR - KKFRETRHPIYRGVRQRNSGKWVSEVRE	P N K	KTRIWLGTFQTAEMA	A RAHDVAALAL RG R S	ACLNFA	DSAWR
PKKPAGR - KKFRETRHPIYRGVRRRNSGKWVCEVRE	P · · · N K · · · I	KTRIWLGTFQTAEMA	ARAHDVAALALRGRS	· · · ACLNFA	
· · · · AGR · KKFRETRHPIYRGVRRRNSGKWVCEVR	P N K I	KSRIWLGTFOTAFMA	ARAHDVAAIALRGRS		DSAWR
PKKPAGE, KKERETEHPLYRGVERENSGKWVCEVER	P NK I	KSR LWLGTFOTAEMA	ARAHDVAALALRGRS		DSAWR
					DEAWP
					DEAW
PKKRAGR•KKFREINHFITRGVRUKNSGKWVCEVRE	P NK	K SKIWLGIFPIVEMA	ARAHDVAALALKGRS	· · · AULNEA	DSAWR
PKKRAGR·KKFRETRHPVYRGVRRRNSGKWVCEVRE	P · · · NK · · ·	KSRIWLGTFPTADMA	ARAHDVAALALRGRS	· · · ACLNFA	DSAWR
PKKRAGR-KKFKETRHPVYXGVRRXXSGKWVCEVRE	P · · · NK · · · (	Q S R I WLGTFPTAEMA	A RAHDVAAIAL RGNS	A C L N F A	
PKKRAGR · KKFKETRHPVYXGVRRXXSGKWVCEVRE	P · · · N K · · · (	Q S R I WLGTFPTAEMA	A RAHDVAAIAL RG NS	• • • A C L N F A	
PKKRAGR - KKFKETRHPVYXGVRRRXSGKWVCEVRE	P NK (	Q S <mark>R</mark> I W L G T F P T A E M A	A RAHDVAAIAL RG N S	· · · ACLNFA	DSAWR
PKKRAGR - KKFKETRHPVYXGVRRRXSGKWVCEVRE	P N K (	Q S <mark>R</mark> IWL <mark>G T F P T A E</mark> MA	A RAHDVAAIAL RG N S	· · · ACLNFA	DSAWR
PKKRAGR - KKFKETRHPVYRGVRRRKSGKWVCEVRE	P · · · N K · · · (	Q S R I W L G T F P T A E M A	A RAHDVAAIAL RG N S	ACLNFA	DSAWR
PKKRAGR - KKFKETRHPVYRGVRRRNSGKWVCEVRE	P N K	KTRIWLGTFPTAEMA	ARAHDVAAIALRGRS	· · · ACLNFA	
PKKRAGR - KKFKETRHPVYRGVRRRNSGKWVCEVRE	P · · · N K · · · I	KTRIWLGTFPTAEMA	ARAHDVAAIALRGRS	· · · ACLNFA	
PKKRGGR-KKEKETRHPVYRGVRSRNLDKWVCEMRE	P N K I	KTKIWLGTFOTAEMA	ARAHDVAALALRGRN		DSAWR
PKKRGGR, KKXKETRHPVYRGVRSBNIDKWVCEMRE	P NK	KTK IWLGTFOTAEMA	ARAH		
PKKROOP, KKEKETPHPYYROVRSPNI DKWVCEMP	P NK	KTK IWLGTEOTAEMA			DSAWR
PKKROOR KKEKETPHPYYPCVPTPNI DKWVCEMP					
					DEAWD
PKKROCK-KKFKETRHFVTROVRTRNLDKWVCEMRE	P NK	KTKTWLGTFPTAEMA	ARAHDVAALALKGRN	AULNEA	DSAWR
PKKRGGR·KKFKETRHPVYRGVRIRNLDKWVCEMRE	P NK	KTK IWLGTFPTAEMA	ARAHDVAALALXGRN	· · · ACLNFA	DSAWR
PKKROGR·KKFKETRHPVYXGVRTRNLDKWVCEMRE	P · · · NK · · ·	KIXIWLGIFPIAEMA	ARAHDVXALALXGRN	· · · ACLNEA	DSAWR
PKKRAGR - KKFKETRHPIYRGVRRRNNNKWVCELRE	P · · · N K · · · I	KSRLWLGTYPTAEMA	A RAHDVAALAL KGES	ST - ACLNFA	
· · · · · · · · · · · · · · · · · · ·	P · · · N K · · · I	KSRLWLGTYPTAEMA	A RAHDVAALAL KGES	ST · ACLNFA	
PKKRAGR - KKFKETRHPIYRGVRRRNNNKWVCELRE	P • • • N K • • • I	K S R L W L G T Y P T A E M A	A RAHDVAALAL KGES	ST · ACLNFA	
PKKRAGR - KKFKETRHPIYRGVRRRNNNKWVCELRE	P N K I	K S R L W L G T Y P T A E M A	A RAHDVAALAL <mark>KGES</mark>	ST - ACLNFA	
PKKRAGR - KKFKETRHPIYRGVRRRNNNKWVCELRE	P • • • <mark>N K</mark> • • •	K S R L W L G T Y P T A E M A	A <mark>R</mark> AHDVAALAL <mark>K</mark> GES	ST - ACLNFA	
PKKRAGR - KKFKETRHPIYRGVRRRNNDRWVCELRE	P N K	K S R L W L G T Y P T A E M A	A <mark>r</mark> ahdvaalal <mark>k</mark> ges	ST - ACLNFA	
PKKRAGR - KKFKETRHPIYRGVRRRNNDRWVCELRE	P N K I	K S R L W L G T Y P T A E M A	A <mark>r</mark> ahdvaalal <mark>k</mark> ges	ST - ACLNFA	
PKKRAGR - KKFKETRHPIYRGVRRRNNDRWVCELRE	P · · · N K · · · I	KSRLWLGTYPTAEMA	ARAHDVAALAL <mark>KGES</mark>	ST · ACLNFA	
PKKRAGR - KKFKETRHPIYRGVRRRNNDRWVCELRE	P N K	KSRLWLGTYPTAEMA	ARAHDVAALVLKGES	ST · ACLNFA	
PKKPAGR - KKFKETRHPVYRGVRKRNNDKWVCEL RE	P N K I	KSRIWIGTYPTAEMA	ARAHDVAALAL KGDS	SI PPCINEA	
PKKPAGE, KKEKETEHPYYEGVEKENNDKWVCELEE	P NK I	KSRLWLGTYPTAEMA	ARAHDVAALALKGDS	SUPPRINEA	DSVWR
PKKPAGE, KKEKETRHPVYRGVEKENNDKWVCELRE	P NK	KSPLWLGTYPTAEMA	ARAHDVAALALKGDS	SUPPOLNEA	DSVWR
PKKPAGP, KKEKETPHPYYPGYPKPNND KWYCEL PE		KSPLWLGTYPTAEMA		SUPPOLNEA	
			A BALLEVAALAL KGDS	SL PROLINEA	DEVICE
		CORLINE OT YPTAEMA	ARAND VAALAL KGDS	SC FFGLNFA	D D O V V R
KKKKAGK · KIFKETKHPIYKGVKKKNGDKWVCELRE		A SHIWLGIYPIAEMA	ARAHD VAALAF RGKS	· · · AULNEA	SAWR
PKKKAGK·RVFKETRHPVYRGVKRKNNNKWVCEMR	PNCNNKNII	KSKIWLGSYPTPEMA	ARAHDVAALTLKGKS	· · · ACLNFA	DSAWR
PKKKAGR - RVFKETRHPVYRGVRRRNNNKWVCEMR	PNCNNKNII	KSR IWLGSYPTPEMA	ARANDVAALTLKGKS	A C L N F A	DSAWR
PKKKAGR · RVFKETRHPVYRGVRRRNNNKWVCEMR	PNCN <mark>NK</mark> NII	KSR IWLGSYPTPEMA	ARAHDVAALTLKGKS	• • • A C L N F A	DSAWR
PKKRAGR · KKFRETRHPVYRGVRKRDSGKWVCEVR	P • • • K K • • • •	KTRVWLGTYPTADMA	ARAHDVAALAM <mark>RGRS</mark>	••• A <mark>C</mark> L <mark>N</mark> F A	DSLWR
TRRREPEGKK <mark>×KETRHPVYR</mark> ×V××R×KGKWVCEVR	1 <mark>P</mark>   T	Q A <mark>R</mark> VWX G X H P N X E M A	A RAHXVXVLAMRXR N	· · · ACLNFA	XXVWR
PKKRAGR · KKXKEXRHPVYRXVXXRXKGKWVCEVR	<mark>P</mark>   T	QARVWXGXHPNXEMA	A RAHXVXVLAMRGRN	· · · ACLNFA	
PKKRAGR - KKFKETRHPVYRGVRSRDKGKWVCEVR	1 <mark>P</mark>   T (	Q A R VWL G T H P N A E M A	ARAHDVAVLAMRGRN	· · · ACLNFA	

CBF Signature Sequences 🔍

Figure 3.1.2.2. Clustal alignment of the CBF signature sequences and AP2 domain only of translated CBF nucleotide sequences isolated from collected samples compared to *Arabidopsis thaliana* CBF1-4 genbank sequences. CBF signature sequences highlighted. Sequences are re-ordered by Clustal sorting. Names are in the form "Species code pp primer pair code\_Genus\_Species\_Site code\_Location". Genbank gene names are in the form "Genus\_Species\_Gene\_Genbank code".

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## **3.1.2.1 CBF Signature Sequences and the AP2 Domain.**

The CBF signature sequences and AP2 domain (the sequence between the CBF signature sequences) was highly conserved across all species (Fig 3.1.2.2).

# **3.1.2.1.1 Differences Between** *Arabidopsis thaliana* CBF1-4 AP2 and CBF Signature Sequences, and Comparison to All Other Sequences.

In *Arabidopsis thaliana* the only differences in the CBF signature sequences and AP2 domain between the 4 CBFs was that CBF4 had an R instead of P (4 amino acids into the CBF signature sequence) and P instead of Q (55 amino acids in) relative to *Arabidopsis thaliana* CBF1-3 (Fig 3.1.2.2). Both of these differences were unconserved.

These two substitutions (R<sub>4</sub> to P and P<sub>55</sub> to Q) were seen in multiple species however they were not constant in their pairing (i.e. some sequences had both substitutions and some only had one). The majority of sequences had R rather than P (Fig 3.1.2.2). The exceptions being *Arabidopsis thaliana* CBF1-3, *Cardaminopsis arenosa* 66pp66, pp69, (pp77 has R), *Capsella bursa-pastoris* 68pp 71 and 68 (pp75 and 76 have R), *Diapensia lapponica* 2pp22 *Empetrum nigrum* 5 TOPO(a-c) and *Arctostaphylos alpina* 3pp22 (Fig 3.1.2.2). The three *Trifolium repens* sequences with an insert in the AP2 domain (58pp13, 77pp13 and 94pp13) displayed a substitution not seen in any other at this locus, a conserved (relative to R) substitution to K (Fig 3.1.2.2).

The majority of sequences also had P rather than Q, the exceptions being *Arabidopsis thaliana* CBF1-3, *Cardaminopsis arenosa* 66pp66, pp67 and pp69, *Capsella bursa- pastoris* 68pp61, pp71, pp64 and pp68 and *Trifolium repens* 77pp19, 58pp19, 94pp19 (Fig 3.1.2.2).

## 3.1.2.1.2 The AP2 Domain

Numerous differences not seen between the four *Arabidopsis thaliana* CBF sequences were seen in the AP2 region when they were compared to all other sequences.

The most notable differences in the AP2 domain were a 6 amino acid insertion (sometimes aligned as two 3 amino acids insertions) seen in *Trifolium repens* 77pp19, 58pp19, 94pp19 (Fig 3.1.2.2) and the different two or three amino acid insertion seen in all *Ericaceae* and *Diapensia lapponica* (Fig 3.1.2.2).

#### **3.1.2.1.3** The N terminus CBF signature sequence

Aside from the previously discussed R to P substitution 4 amino acids into the sequence (Section 3.2.1.1), two other differences in the N-terminus CBF signature sequences were seen. There was an unconserved substitution at amino acid 10, the majority of sequences had K at this point (including all *Arabidopsis thaliana* sequences) however some sequences had either I or V (Fig 3.1.2.2). Those sequences with I were *Cardaminopsis arenosa* 66pp77, *Capsella bursa-pastoris* 68pp76, *Arabis alpina* 69pp77 and *Salix herbacea* 51pp53) (Fig 3.1.2.2). Those sequences with V were *Trifolium repens* 58pp13 and 77pp13 and *Capsella bursa pastoris* 66pp66 (Fig 3.1.2.2).

A conserved R to K substitution at amino acid 12 occurs in all non-*Brassicaceae* species except *Salix herbacea* 51pp51 (note *Salix herbacea* 51pp53 has this substitution) and *Leucanthemum vulgare* 90pp12 (Fig 3.1.2.2). Several *Brassicaceae* also show this substitution namely *Cardaminopsis arenosa* 66p77, *Capsella bursa-pastoris* 68pp76, 68pp75 and *Arabis alpina* 69pp77 (Fig 3.1.2.2).

#### **3.1.2.1.4 The C-Terminus CBF Flanking Sequence**

The C-terminus CBF flanking sequence consists of five amino acids (DSAWR in *Arabidopsis thaliana*). D was conserved across all species (Fig 3.1.2.2). S was either conserved or unknown (X) across all species (Fig 3.1.2.2). A was mostly conserved across all species with the conserved amino acid substitution V (All Ericaceae, *Diapensia lapponica* and *Matricaria recrutita*) or L (*Leucanthemum vulgare*) also seen (Fig 3.1.2.2). W was conserved across all species (Fig 3.1.2.2). R was conserved across all species

except for the unconserved substitution of Q in all *Vaccinium vitis-idaea* sequences (Fig 3.1.2.2).

# 3.1.3 MATK sequences

*MATK* sequences were isolated from several samples to check for the possibility of contamination, following the sequencing of identical *CBF* sequences (matching that of an *Empetrum nigrum CBF* sequence) from all *Calluna vulgaris* DNA extracted prior to a certain date. It was not possible to isolate *CBF* from DNA extracted or re-extracted on subsequent dates (data not shown). Sequence 173\_C.vulgaris\_NagsHead\_MATK (amplified from the older DNA extraction) displayed 100 % identity to *Empetrum nigrum* MATK sequences rather than the expected *Calluna vulgaris* sequence (Fig 3.1.3.1 a-c). All other *Calluna vulgaris* sequences were obtained from subsequent DNA extractions and did not match the *Empetrum nigrum* MATK sequences and had a unique sequence. This sequence had high similarity to the complete MATK coding sequence for *Calluna vulgaris* (U61326.1) from the NCBI database with a few differences (Fig 3.1.3.1 a-c) (see section 3.1.3.1 for further details).

The isolated *Phyllodoce caerulea* MATK sequence predominantly matched the NCBI (U61318.1) sequence, with two key differences. In the NCBI sequence there was an insertion of a G that was not seen in the isolated *Phyllodoce caerulea* sequence or any other aligned MATK sequence (Fig 3.1.3.1b). Eight bases downstream from this the NCBI sequence also had a deletion of a T that is present in all other MATK sequences (Fig 3.1.3.1b). Several substitutions also occur towards the end of the sequence (Fig 3.1.3.1b) whilst they differ from one another, these substitutions are seen in other MATK sequences (Fig 3.1.3.1b).

*Cassiope tetragona* showed 100 % identity between the isolated sequence, from Abisko, Sweden, and the NCBI sequence (KC474423.1), from Canada.

All *Empetrum sp.* sequences had 100 % identity to one another (Fig 3.1.3.1 a-c). This includes sequences sampled from across the UK, Abisko; Sweden, Canada and Boston; North America (N.B. the North American sample was from the Harvard arboretum so could have originally been collected from elsewhere). This includes MATK sequences from two samples recorded as *Empetrum hermaphroditum* (also referred to as *Empetrum nigrum subsp. hermaphroditum*) all others being *Empetrum nigrum* (also referred to as *Empetrum nigrum subsp. nigrum*) (Please see discussion section 5.2.6 on nonclamature and identification of *Empetrum nigrum & hermaphroditum*).



Figure 3.1.3.1 a. Clustal alignment of MATK nucleotide sequences from collected Ericaceae samples compared to one another and NCBI sequences. Only the section of NCBI sequences that are cover the sequenced section from collected samples is shown. Names are in the form genbank Code (NCBI sequences) or species code (study samples)\_Species\_Location (where known)

362\_C.vulgaris\_Derby\_MATK 221\_C.vulgaris\_BenMacdui\_MATK 299 269\_C.vulgaris\_Portpatrick\_MATK 295 178\_C.vulgaris\_Abisko\_MATK 276 374\_C.vulgaris\_DerbyshireBleaklow\_MATK 275 342 C.vuluaris Breconbeacons MATK 274 329\_C.vulgaris\_Westwales\_MATK 294 289\_C.vulgaris\_Fylingdales\_MATK 276 293 284\_C.vulgaris\_Glen Trool\_MATK 238\_C.vulgaris\_Exmoor\_MATK 281 208\_C.vulgaris\_Yorkshire\_MATK 288 294 182\_C.vulgaris\_Hedleyhopefell\_MATK 180 C.vulgaris Hedleyhopefell MATK 294 179\_C.vulgaris\_Teesdale\_MATK 266 266 309 C.vulgaris Yorkshire MATK 274 257\_C.vulgaris\_NorwayGrødalen\_MATK 342a\_C.vulgaris\_Breconbeacons\_MATK 259 173\_C.vulgaris\_NagsHead\_MATK U61318.1 Phyllodoce caerulea ? 259 Phyllodoce caerulea NorwayKongsvoll MATK KC474423.1\_Cassiope\_tetragona\_Canada 324\_Cassiopetetragona\_Abisko\_MATK AF519561.1\_empetrumhermaphroditum\_Arboretum,Harvard,l 321 E.hermaphroditum Abisko MATK HQ115642.1\_empetrumnigrum\_Newfoundland\_canada 224\_E.nirum\_BenMacdui\_\_MATK 445 251\_E.nigrum\_Exmoor\_MATK 277\_E.nigrum\_Portpatrick\_MATK 363\_E.nigrum\_Derby\_MATK 343\_E.nigrum\_Breconbeacons\_MATK 310\_E.nigrum\_Yorkshire\_MATK U61326.1\_callunavulgaris\_? 837 250\_C.vulgaris\_Exmoor\_MATK 436 362 C.vulgaris Derby MATK 221 C.vulgaris BenMacdui MATK 447 269\_C.vulgaris\_Portpatrick\_MATK 178\_C.vulgaris\_Abisko\_MATK 374\_C.vulgaris\_DerbyshireBleaklow\_MATK 342\_C.vulgaris\_Breconbeacons\_MATK 329 C.vulgaris Westwales MATK 289\_C.vulgaris\_Fylingdales\_MATK 284 C.vulgaris GlenTrool MATK 238\_C.vulgaris\_Exmoor\_MATK 208\_C.vulgaris\_Yorkshire\_MATK 182\_C.vulgaris\_Hedleyhopefell\_MATK 180\_C.vulgaris\_Hedleyhopefell\_MATK 442 179 C.vulgaris Teesdale MATK 414 309\_C.vulgaris\_Yorkshire\_MATK 257 C.vuluaris NorwavGrødalen MATK 342a\_C.vulgaris\_Breconbeacons\_MATK 173\_C.vulgaris\_NagsHead\_MATK

U61318.1 Phyllodoce caerulea ?

324\_Cassiopetetragona\_Abisko\_MATK

321\_E.hermaphroditum\_Abisko\_MATK

343\_E.nigrum\_Breconbeacons\_MATK

224 E.nirum BenMacdui MATK

251 E.niurum Exmoor MATK

363\_E.nigrum\_Derby\_MATK

277\_E.nigrum\_Portpatrick\_MATK

310\_E.nigrum\_Yorkshire\_MATK

U61326.1\_callunavulgaris\_?

250\_C.vulgaris\_Exmoor\_MATK

689 836 CAACCAATOTTOTCAATATOTTATGACTATATOTTOTAAAGAACCATTOTTTTATAGAAAAATAGAACTAGAACTAGAACTAGAACTAGAATTTTAAGGCCATTOTTATGACTAGGCCATTOTTCTGAAGAACTOTTTCTGCAAGAATTOTTACTAAGAACTAGAATTOTTACTAAGAACTAGAATTOTAAGAACCATTOTTACTAAGAATTOTTACTAAGAACTAGAATTOTTACTAGAATTOTTACTAAGAATTOTTACTAGAATTOTTACTAAGAATTOTTACTAGAATTOTTACTAAGAATTOTTACTAGAATTOTTACTAGAATTOTTACTAGAATTOTTACTAGAATTOTTACTAGAACTAGAATTOTTACTAGAATTOTTACTAGAATTOTTACTAGAATTOTTACTAGAATTOTTACTAGAATTAGAACTAGAATTAGAACTAGAACTAGAATTAGAACTAGAATTAGAA 260 407 259 Phyllodoce caerulea NorwavKongsvoll MATK 167 KC474423.1\_Cassiope\_tetragona\_Canada 314 258 405 AF519561.1 empetrumhermaphroditum Arboretum.Harvard.U 689 836 272 419 HQ115642.1\_empetrumnigrum\_Newfoundland\_canada 689 836 297 CAACCAATCTTATCATTTAC 444 CATTATGI GOT CAATAT CITY CTGAAAC CITTY CTAGAAC GAAT CTCTTTY CTATAAAAAAATAGAAC TAGAAC TCTTTACTAAAGATTTTAAGG GOT CAATAT CITY CTGAAAC CITTY CTAGAAC GAAT CTCTTTY CTATAAAAAAATAGAAC TAGAAC TCTTTACTAAAGATTTTAAGG CATTCTATGGGTGTTTAAAGAG CAACCAATCTTATCATTTAC CTGCATTATG 299 446 CATTCTATGGGTGTTTAAAGAG 295 CAACCAATCTTATCATTTAC TTTCCTGCATTATG 442 296 CAACCAATCTTATCATTTACCCCTCAATATCTTCTGAAACCTTTCTAGAACCAATCTCTTTCTATAAAAAAATAGAACTAGAACTCTTTTACTAAAGAATTTTAAGG CAACCAATCTTATCATTTACCCCTCAATATCTTCTGAAACCTTTCTAGAACGAATCTCTTTCTATAAAAAAATAGAACTAGAACTCTTTACTAAAGATTTTAAGG CATTCTATGGGTGTTTAAAGA 443 CATTAT 273 CATTCTATGGGTGTTTAAAGAG TTTCCT<mark>G</mark>CATTAT<mark>G</mark> 420 264 CATTCTATGGGTGTTTAAA<mark>G</mark>A CATTAT 411 689 TACCCAATCTTCTCATTTACCCTTAATATCTTCTGAAAACTTTTCTAGAACGAATCTCTTTCTATCAAAAAATCGAACTAGAACTCTTTACTAATGAATTTAAGGCCATTCTATGGGACTTTAAAAGA TTTCATGCATTATG 836 288 CAATCTTCTCATTTACSCTTAATATCTTCTSGAAACTTTTCTAGAACGAATCTCTTTCTATCAAAAAATCGAACTAGAAGTCTTTAACTAAGAATTTTAAGG CATTCTATGGGTATTTAAAGA TTTCATGCATTATGT 435 C TTAATATCTTT CTGAAACTTTTCTAGAACGAATCTCTTTCTATCAAAAAATCGAACTAGAACTCTTTACTAGAATTTTAAGG C TTAATATCTTCTGGAAACTTTTCTAGAACGAATCTCTTTCTATCAAAAAATCGAACTAGAAGTCTTTACTAAGAATTTTAAGG C TTAATATCTTCTGAAACTTTTCTAGAACGAATCTCTTTCTATCAAAAAATCGAACTAGAAGTCTTTACTAAGAATTTTAAGG C TTAATATCTTCTGAAACTTTTCTAGAACGAATCTCTTTCTATCAAAAAATCGAACTAGAAGTCTTTACTAAGAATTTTAAGG C TTAATATCTTCTGAAACTTTTCTAGAACGAATCTCTTTCTATCAAAAAATCGAACTAGAAGTCTTTACTAAGAATTTTAAGG CAATCTTCTCATTTACC CATTCTAT<mark>GGG</mark>TATTTAAA<mark>G</mark>A 298 TTTCATGCATTATG T 445 CAATCTTCTCATTTAC CATTCTAT<mark>GGG</mark>TATTTAAA<mark>G</mark>AG TTTCATGCATTATGT 446 CAATCTTCTCATTTAC CATTCTATGGG TATTTAAAGA TTTCATCCATTATC1 442 CAATCTTCTCATTTAC CATTCTAT<mark>GGG</mark>TATTTAAA<mark>G</mark>A TTTCATGCATTATG 423 CAATCTTCTCATTTAC AAACTTTTCTA<mark>G</mark>AACGAATCTCTTTCTATCAAAAAATCGAACTAGAAGTCTTTACTAAGAATTTTAAGG CATTCTATGGGTATTTAAAGA TTTCATG CATTAT 422 CTTAATATCTTCTGAAACTTTTCTAGAACGAATCTCTTTCTATCAAAAAATCGAACTAGAAGTCTTTACTAAGAATTTTAAGG CAATCTTCTCATTTAC CATTCTAT<mark>GGG</mark>TATTTAAA<mark>G</mark>A TTTCATGCATTATG 421 CTTAATAT CTTCTGAAACTTTTCTAGAACGAATCTCTTTCTATCAAAAAATCGAACTAGAAGTCTTTACTAAGAATTTTAAGG CAATCTTCTCATTTAC CATTCTAT<mark>GGG</mark>TATTTAAA<mark>G</mark>A TTTCAT GCATTATGT 441 CTTAATATCTTCTGAAACTTTTCTAGAACGAATCTCTTTCTATCAAAAAATCGAACTAGAAGTCTTTACTAAGAATTTTAAGG CAATCTTCTCATTTACG CATTCTATGGGTATTTAAAGA TTTCATGCATTATGT 423 CAAT CIT CT CATTING OCT TAATATCIT CT GAAAACTITT CTAGAAACAACTIT CTATCAAAAAAAT CG AACTAGAAG TCTTTAAAGAATTITAAAGACCATTCTATGOGTATTAAAGAC TTTCATGCATTATGT 440 CAATCTTCTCATTTAC CATT<mark>CTATGGG</mark>TATTTAAA<mark>G</mark>A TTTCATGCATTATG 428 AATCTTCTCATTAC CATTCTATGGGTATTTAAAGA TTTCATGCATTATG 435 CTTAATATCTTCTGAAACTTTTCTAGAACGAATCTCTTTCTATCAAAAAATCGAACTAGAAGTCTTTACTAAGAATTTTAAGG C C A T T <mark>C T A T G G G T A T T T A A A G</mark> A G AATCTTCTCATTTAC TTTCATGCATTATGT 441 CAAT CTT CTC ATTTACE CTTAATAT CTT CTE GAAACTTTT CTAGAACGAAT CTC TTT CTATCAAAAAAT CGAACTAGAAGT CTTTACTAAGAATTTTAAGG CATTCTAT<mark>GGG</mark>TATTTAAA<mark>G</mark>A TTTCATGCATTATGT 441 CAATCTTCTCATTTACCCTTAATATCTTCTGAAACTTTTCTAGAACGAATCTCTTTCTATCAAAAAATCGAACTAGAAGTCTTTACTAAGAATTTTAAGGC CATTCTATGGGTATTTAAAGA TTTCATGCATTATGT1 413 CAATCITC TCATITAC C TTAATATCITI CTE AAAACTITIC TAGAACCAATCITCITIC TATCAAAAAATCGAACTAGAAGTCITITAAGGAATTITAAGGCCATTCIATGGGTATTIAAAGA TTTCATECATTATETTA 413 CAATCTTCTCATTTAC<mark>S</mark>CTTAATATCTTCT<mark>S</mark>AAACTTTTCTA<mark>S</mark>AAC<mark>S</mark>AATCTCTTTCTATCAAAAAATCSAACTASAASTCTTTACTAAS<mark>A</mark>AATTTTAAS<mark>S</mark>CCATTCTAT<mark>SSST</mark>TAATAAS<mark>AAC</mark> CTTTCATGCATTATGTTA 421 CAATO TTO TO ATTTACE OTTAATATOTTO TE AAACOTTTTO TAG AACGAATOTO TTTO TATCAAAAAATO SAACTAGAASTO TTTACTAAGAATTTTAAGGOOCATTOTATGOO TATTAAAGA TTTCATGCATTATGT 406 837 GTAT 985 408 GTATO 556 315 GTATCGAGGAAAATCCCTTTTGGCTTCAAAAGGGACGTCTCTTTGATGAATAAATGGAAATATTA 463 TTOTCAATTTCTGGCAATGTTATTTTTACATGTGGTCTCAACCAAGAAGGATCCATATAAACCAATTATCCAATCATTC CTTTTGGCTTCAAAAGGGACGTCTCTTTGATGAATAAATGGAAAATATTAC 406 GTAT GAGGAAAATC 554 GAAAAG CTTTTAGTTTCAAAAGGGGACGTCTCTTTTGATGAATAAATGGAAAATATTAC T C A A C C A A G A A G G A T C C A T A T A A A T C A A T T A G C C A A T C A T T A 837 GTAT 985 TTGTCAATTTATGGCAATGTTATTTTACATGTGGT 420 GTAT GAAAAG CTTTTAGTTTCAAAAGGGACGTCTCTTTTGATGAATAAATGGAAAATATTAC CTT<mark>6</mark> T CAATTTAT<mark>66</mark> CAAT<mark>6</mark> T TATTTTTACAT<mark>6 T 66</mark> T C T CAACCAA<mark>6</mark> AA<mark>66</mark> AT C CATATAAAT CAATTA<mark>6</mark> C CAAT CATTCCC 568 TT<mark>G</mark>TCAATTTAT<mark>GG</mark>CAAT<mark>G</mark>TTATTTTTACATGTGGTCTCAACCAA<mark>G</mark>AAGGATCCATATAAATCAATTAG 837 GTAT GAAAAG CAATCATT 985 ЗТАТ GAAAAG TTTCAAAAGGGACGTCTCTTTTGATGAATAAATGGAAATATTA TTGTCAATTTATGGCAATGTTATTTTTACATGTGGTCTCAACCAAGAAGGATCCATATAAATCAATTAG CAATCATT 593 447 GTATO GAAAAG CTT<mark>6</mark> T CAATTTAT<mark>66</mark> CAAT<mark>6</mark> TTATTTTTACAT<mark>66</mark> T CTCAACCAA<mark>6</mark> AA<mark>66</mark> ATCCATATAAATCAATTA6 CAATCATTCCC 595 GAAAAG TTGTCAATTTATGGCAATGTTATTTTTACATGTGGTCTCAACCAAGAAGGATCCATATAAATCAATTAG 443 G T A T CAATCATT 591 CTTTTAGTTTCAAAAGGGACGTCTCTTTTGATGAATAAATGGAAATATTAC GAAAAG TT<mark>GT</mark>CAATTTAT<mark>GG</mark>CAAT<mark>G</mark>TTATTTTTACAT<mark>G</mark>TGGTCTCAACCAAGAA<mark>GG</mark>ATCCATATAAATCAATTAG 444 G T A T ( CAATCATTCC 592 GAAAAG TT<mark>O</mark>TCAATTTAT<mark>GO</mark>CAAT<mark>O</mark>TTATTTTTACAT<mark>O</mark>T<mark>GO</mark>TCTCAACCAA<mark>O</mark>AA<mark>GO</mark>AT<u>CCATATAAAT</u>CAATTAG 421 GTAT CAATCATTC 569 C C T T T T AG T T T C AAAAGGG A C G T C T C T T T T G A T G AA T AAAT G G AAAT AT T A C TTGTCAATTTATGGCAATGTTATTTTTACATGTGGTCTCAACCAAGAAGGATCCATATAAATCAATTAG 412 GTAT GAAAAG CAATCATT 560 CTATTTTGGCTTCAAAA<mark>gggacatctcttttgatg</mark>aataaat<mark>gg</mark>aaatatta AATTTTT<mark>GG</mark>CAAT<mark>G</mark>TTATTTTTACAT<mark>GTGGTC</mark>TCAACCCAAAA<mark>GG</mark>ATCT<mark>G</mark>TATAAACCAATTATCCAACCAT 3 T A T C GAAAAG 985 GAAAA<mark>G CTATTTT G G CTT CAAAA G G G A CATCT CTTTT G</mark> AT<mark>G</mark> AATAAAT G G AAATATTA C TTO TAAATTTTTO O CAATO TTATTTTTACATO TO O TCTCAACCCAAAAO O ATCTO TATAAACCAATTATCCAACCATT этатс 584 GAAAAG CTATTTTGG CTT CAAAAGGGGACATCT CTTTTGATGAATAAATGGAAATATTAC TT<mark>G</mark>TAAATTTTTT<mark>GG</mark>CAAT<mark>G</mark>TTATTTTTTACAT<mark>GTGG</mark>TCTCAACCCAAAA<mark>GG</mark>ATCT<mark>G</mark>TATAAACCAATTATCCAACCATTCC 446 GTATCGA 594 TATCGAGGAAAAGCTATTTTGGCTTCAAAAGGGGACATCTCTTTTGATGAATAAATGGAAAATATTA TTO TAAATTTTT SO CAATO TTATTTTTACATO TO O TCTCAACCCAAAAO CATCTO TATAAACCAATTATCCAACCATTC 595 443 GTATCGAGGAAAAGCTATTTTGGCTTCAAAAGGGACATCTCTTTTGATGAATAAATGGAAAAATATTAC CTTO TAAATTTTTTOCCAATO TTATTTTTACATO TOC CAAACCCAAAAGO AT CTO TATAAACCAATTAT CCAACCATTCC 591 424 GTAT EGA GAAAAG CTATTTT GGCTT CAAAAG GGACATCT CTCTTT GATGAATAAAT GGAAATATTAC 423 GTATCG A GGAAAAG CTATTTT GGCTT CAAAAG GGACATCT CTCTTT GATGAATAAAT GGAAATATTAC CTCAACCCAAAA<mark>gg</mark>at<mark>ctg</mark>tataaaccaattatccaaccat TTGTAAATTTTTGGCAATGTTATTTTTACATGTGGT 572 TT<mark>O</mark>TAAATTTTT<mark>GO</mark>CAAT<mark>O</mark>TTATTTTTACAT<mark>OTGOTCT</mark>CAACCCAAAA<mark>GO</mark>AT<mark>CTO</mark>TATAAACCAATTATCCAACCAT 571 422 GTATCGAGGAAAAGCTATTTTGGCTTCAAAAGGGACATCTCTTTTGATGAATAAATGGAAAATATTA CTT<mark>O</mark>TAAATTTTTT<mark>GO</mark>CAAT<mark>O</mark>TTATTTTTACAT<mark>O</mark>T<mark>GO</mark>TCTCAACCCAAAA<mark>OO</mark>ATCT<mark>O</mark>TAT<mark>AAACCAATTATCCAACCAT</mark> 570 442 GTATCGAGGAAAAGCTATTTTGGCTTCAAAAGGGACATCTCTTTTGATGAATAAATGGAAATATTAC TT<mark>O</mark>TAAATTTTTT<mark>OO</mark>CAAT<mark>O</mark>TTATTTTTTACAT<mark>O TOO</mark>TCTCAACCCAAAA<mark>GG</mark>ATCT<mark>O</mark>TATAAACCAATTATCCAACCATT 590 424 GTATCGAGGAAAAGCTATTTTGGCTTCAAAAGGGACATCTCTTTTGATGAATAAATGGAAATATTAC CTT<mark>G</mark>TAAATTTTT<mark>GG</mark>CAAT<mark>G</mark>TTATTTTTACAT<mark>G</mark>T<mark>GG</mark>TCTCAACCCAAAA<mark>GG</mark>ATCT<mark>G</mark>TATAAACCAATTATCCAACCATTC 572 441 GTAT CGAGGAAAAG CTATTTTGG CTT CAAAAG GGACAT CT CTTTTGATGAATAAAT GGAAATATTAC TCAACCCAAAA<mark>GG</mark>ATCT<mark>G</mark>TATAAACCAATTATCCAACCATT TTGTAAATTTTTGGCAATGTTATTTTTACATGTGGT 589 429 GTATCGAGGAAAAGCTATTTTGGCTTCAAAAGGGACATCTCTTTTGATGAATAAATGGAAAATATTAC CTT<mark>G</mark>TAAATTTTT<mark>GG</mark>CAAT<mark>G</mark>TTATTTTTACAT<mark>GTGG</mark>TCTCAACCCAAAA<mark>GG</mark>ATCT<mark>G</mark>TATAAACCAATTATCCAACCATTC 577 TCAACCCAAAA<mark>gg</mark>atctgtataaaccaattatccaaccat 584 CTTGTAAATTTTTTGGCAATGTTATTTTTTACATGTGGTCTCAACCCAAAAGGATCTGTATAAACCCAATTATCCAACCATT 590 TATCGAGGAAAAGCTATTTTGGCTTCAAAAGGGGACATCTCTTTTGATGAATAAATGGAAATATTAC C T T <mark>G T A A A T T T T T G G C A A T G</mark> T T A T T T T T A C A T <mark>G T G G</mark> T C TCAACCCAAAA<mark>gg</mark>atct<mark>g</mark>tataaaccaattatccaaccatt 590 T CAACCCAAAA<mark>GG</mark>ATCT<mark>G TATAAACCAATTATCCAACCATTC</mark> TT<mark>G</mark> TAAA TTTTT<mark>G G CAATG</mark> TTA TTTTTA CAT<mark>G T</mark>G 562 414 GTATCGAG GAAAAG CTATTTTGG CTT CAAAAG GGACATCT CTTTTGATGAATAAATGGAAATATTAC T CAACCCAAAA<mark>GG</mark>ATCTGTATAAACCAATTATCCAACCATTCCC TT<mark>G</mark>TAAATTTTT<mark>GGCAATG</mark>TTATTTTTACATGTGGT 562 422 GTATCGAGGAAAAGCTATTTTGGCTTCAAAAGGGACATCTCTTTTGATGAATAAATGGAAAATATTA TATTTTTACAT<mark>G TGG</mark>TCTCAACCCAAAA<mark>GG</mark>ATCTGTATAAACCAATTATCCAACCATTCCC 570 407 GTATCGAGGAAAAGCTATTTTGGCTTCAAAAGGGACATCTCTTTTGATGAATAAATGGAAAATATTAC T CAACCCAAAA<mark>GGATCTGTAT</mark>AAACCAATTATCCAACCATTCCC TTGTAAATTTTTGGCAATGTTATTTTTACATGTGGT 555 418 GTATCGCGGAAAAGCCCCTTTTAGTTTCAAAAGGGACGTCTCTTTTGATGAATAAATGGAAAATATTAC TT<mark>G</mark>T CAATTTAT<mark>GG</mark> CAAT<mark>G</mark>TTATTTTTACAT<mark>G</mark>T GG TCT CAACCAA<mark>G</mark>AAG<mark>G</mark>ATCCATATAAATCAATTAGCCAATCATTCCC

Figure 3.1.3.1 b. Clustal alignment of MATK nucleotide sequences from collected Ericaceae samples compared to one another and NCBI sequences. Only the section of NCBI sequences that are cover the sequenced section from collected samples is shown. Names are in the form genbank Code (NCBI sequences) or species code (study samples)\_Species\_Location (where known)

U61318 1 Phyllodoce caenulea ?	986 TCGACTTTCTG	GOCTATCTT	ТСАА <mark>СТСТС</mark> СС	SATTAAA <mark>G</mark> CCTTT	AATGTTACG	GAGTCAAATG		TCATTTCTAATAG	AGAATGC	TAGTAAGAA	TTCGATACTCTAAT	CCAATTACTCCAAT	ATTTTATCATTOTATAAA	1134
259 Phyllodoce caerulea NonyayKongovoli MATK	557 TOGACTTTCTC	GOCTATOTT	TCAAGTGTGC	ATTAAAGCOTTT	AATGTTACG	GAGTCAAATC		TCATTCTAATAG	AGAATOC	TAGTAAGAA	TTCGATACTCTAAT	CCAATTACTCCAAT	ATTTTATCATTOTATAAA	705
VC474492.4 Cassiana tatavana Casada								TOATTTOTAATAO						810
NC474423.1_Cassiope_tetragona_Canada				OATTAAAO COTTO	AATOOTACO	O A O T C A A A T O			AGAATOC	TAUTAADAA	STICOATACIC TAOTA	ACCAATTACTCCAAT		012
324_Cassiopetetragona_Abisko_MATK	555 TCGACTTTATG	GGCTATCTT		GATTAAAGCCTTC	AATGGTACG	GAGICAAAIG		TCATTICIAATAG	AGAATGC		STICGATACICIA <mark>G</mark> I	ACCAATTACTCCAAT	JATTGGATCGTTGTCTAAA	703
AF519561.1_empetrumhermaphroditum_Arboretum,Harvard,U	986 TT <mark>GACTTTCTG</mark>	G G C T A T C T T I	T C A A G T G T G C C	<mark>gattaaaaccttt</mark>	AATGGTACG	GAGTCAAATG	A TA <mark>G</mark> AAAA T	TCATTTCTAATAG	AGAATGC	TATTAAGAAG	STTCGATACTCTAAT	CCAATTACTCCAAT	SATTGAATCATTGGTTAAA	1134
321_E.hermaphroditum_Abisko_MATK	569 TTGACTTTCTG	<mark>g g c t a t c t t 1</mark>	T C A A <mark>G T G T G C C</mark>	<mark>g</mark> attaaaa <mark>cc</mark> ttt	AATGGTACG	GAGTCAAATG	A TA <mark>G</mark> AAAA T	TCATTTCTAATAG	AGAATGC	TATTAA <mark>G</mark> AA	STTCGATACTCTAAT	CCAATTACTCCAAT	SATT <mark>G</mark> AATCATT <mark>GG</mark> TTAAA	717
HQ115642.1_empetrumnigrum_Newfoundland_canada	986 TT <mark>GACTTTCT</mark> G	<mark>g g c t a t c</mark> t t 1	Τ C A A <mark>G T G T G C C</mark>	<mark>g</mark> attaaaa <mark>cc</mark> ttt	AATGGTACG	GAGTCAAATG	Α <mark>ΤΑ</mark> ΘΑΑΑΑΤ	TCATTTCTAATAG	AGAATGC	TATTAAGAA	3 T T C <mark>G</mark> A T A C T C T A A T C	CCAATTACTCCAAT	<mark>Β</mark> ΑΤΤ <mark>Θ</mark> ΑΑΤ <mark>ΟΑΤΤΘΘ</mark> ΤΤΑΑΑ	1134
224E.nirum_BenMacduiMATK	594 TTGACTTTCTG	<mark>g g c t a t c</mark> t t 1	ТСАА <mark>СТСТС</mark> СС	<mark>g</mark> attaaaa <mark>cc</mark> ttt	AATGGTACG	GAGTCAAATG	ΑΤΑ <mark>Θ</mark> ΑΑΑΑΤ	TCATTTCTAATAG	AGAATGC	ΤΑΤΤΑΑ <mark>Θ</mark> ΑΑ	STTCGATACTCTAAT	CCAATTACTCCAAT	<mark>ΒΑΤΤ</mark> ΘΑΑΤ <mark>CΑΤΤΘΘ</mark> ΤΤΑΑΑ	742
251 E.nigrum Exmoor MATK	596 TTGACTTTCTG	<mark>g g c t a t c</mark> t t i	Т С А А <mark>С Т С Т С</mark> С С	<mark>G</mark> Α Τ Τ Α Α Α Α <mark>C C</mark> T T T	AATGGTACG	GAGTCAAATG	ΑΤΑ <mark>Θ</mark> ΑΑΑΑΤ	TCATTTCTAATAG	AGAATGC	TATTAAGAA	STTCGATACTCTAAT	CCAATTACTCCAAT	<mark>ΒΑΤΤ</mark> ΘΑΑΤ <mark>ΟΑΤΤΘΟ</mark> ΤΤΑΑΑ	744
277 E.nigrum Portpatrick MATK	592 TTGACTTTCTG	<mark>с с татс</mark> тт 1	т с а а <mark>с т с т с</mark> с с	G A T T A A A A C C T T T	AATGGTACG	GAGTCAAATG	A T A G A A A A T	TCATTTCTAATAG	AGAATGC	TATTAAGAA	зттс <mark>с</mark> атастстаат	CCAATTACTCCAAT	ΒΑΤΤ <mark>Θ</mark> ΑΑΤ <mark>ΟΑ</mark> ΤΤ <mark>ΘΘ</mark> ΤΤΑΑΑ	740
363 E nicum Derby MATK	593 TTGACTTTCTG	GOTATOTT	TCAAGTGTGC	SATTAAAACCTTT	AATGGTACG	GAGTCAAATG		TCATTTCTAATAG	AGAATGO	TATTAAGAA	STICGATACTCTAAT	CCAATTACTCCAAT	SATTGAATCATTGGTTAA4	741
242 E piceum Broopho soono MATK	570 TTGACTTTCTC	GOCTATOTT	TCAAGTGTGC	ATTAAAACCTTT	AATGGTACG	GAGTCAAATC	TACAAAT	TCATTCTAATAG	AGAATOC	TATTAACAA	TTCGATACTCTAAT	CCAATTACTCCAAT	ATTO AATCATTOGTTAA/	710
240 5 since Vedebie MATK														700
STO_E.mgram_ronksmie_mATK					AATOOTACO				AGAATOC		TTOOLTACTOTACT			1104
U61326.1_callunavulgans_?	986 TCGATTTTATG.	AGCTATCTT	TCAAGIGIGIGC	GATTACAGUTTTT	AATGGTACG	GAGTAAAATG	ATAGAAAAT	TCATTTTAATAG	AGAATGU	TAGTAAAAA	STICGATACICIAAT	JCCAATTACTCCAATC	JATTGGATACTTGTCTAAA	1134
250_C.vulgaris_Exmoor_MATK	585 TCGATTTTATG	AGCTATCTTI	T C A A G T G T G C C	GATTACAGCTTTT	AATGGTACG	GAGTAAAATG	A TAGAAAAT	TCATTTTTAATAG	AGAATGC	TAGTAAAAA	STTCGATACTCTAAT	CCAATTACTCCAAT	JATTGGATCATTGTCTAAA	733
362_C.vulgaris_Derby_MATK	595 TCGATTTTATG	A <mark>GCTATCTTI</mark>	T C A A <mark>G T G T G C C</mark>	G A T T A C A <mark>G C T T T T</mark>	AATGGTACG	GAGTAAAATG	A TA <mark>G</mark> AAAA T	T CATTTTTAATAG	AGAATGC	TAGTAAAAA	STTCGATACTCTAAT	BCCAATTACTCCAAT	3 A T T <mark>G G</mark> A T C A T T <mark>G T C T</mark> A A A	743
221_C.vulgaris_BenMacdui_MATK	596 TCGATTTTATG	A <mark>G C T A T C T T 1</mark>	Τ C A A <mark>G T G T G</mark> C C	G A T T A C A <mark>G C</mark> T T T T	AATGGTACG	GAGTAAAATG	Α <mark>ΤΑ</mark> ΘΑΑΑΑΤ	T CATTTTTAA TAG	AGAATGC	ΤΑ <mark>σ</mark> ΤΑΑΑΑΑ	STTCGATACTCTAAT	CCAATTACTCCAAT	<mark>BATTGG</mark> AT <mark>CATTG</mark> TCTAAA	744
269_C.vulgaris_Portpatrick_MATK	592 TCGATTTTATG	Α <mark>Θ C</mark> T A T C T T 1	T C A A <mark>G T G T G C C</mark>	G A T T A C A <mark>G C</mark> T T T T	AATGGTACG	GAGTAAAATG	ATA <mark>G</mark> AAAAT	T CATTTTTAATAG	AGAATGC	ΤΑ <mark>σ</mark> ΤΑΑΑΑΑ	STTC <mark>G</mark> ATACTCTAAT	CCAATTACTCCAAT	<mark>ΒΑΤΤ<mark>ΘΘ</mark>ΑΤ<mark>CΑΤΤ</mark>ΘΤCΤΑΑΑ</mark>	740
178_C.vulgaris_Abisko_MATK	573 TCGATTTTATG	Α <mark>ς с</mark> та тс тт 1	ТСАА <mark>СТСТС</mark> СС	G A T T A C A <mark>G C</mark> T T T T	AATGGTACG	GAGTAAAATG	ΑΤΑ <mark>Θ</mark> ΑΑΑΑΤ	TCATTTTTAATAG	AGAATGC	ΤΑ <mark>σ</mark> ΤΑΑΑΑΑ	STTCGATACTCTAAT	CCAATTACTCCAAT	BATT <mark>GG</mark> ATCATT <mark>G</mark> TCTAAA	721
374 C.vulgaris DerbyshireBleaklow_MATK	572 TCGATTTTATG	Α Θ C Τ Α Τ C Τ Τ Ι	Т С А А <mark>С Т С Т С</mark> С С	<mark>G ΑΤΤΑ CΑ</mark> G C ΤΤΤΤ	AATGGTACG	GAGTAAAATG	ATAGAAAAT	TCATTTTTAATAG	AGAATGC	ΤΑ <mark>σ</mark> ΤΑΑΑΑΑ	S T T C G A T A C T C T A A T C	CCAATTACTCCAAT	<mark>BATTGGATCATTGTC</mark> TAAA	720
342 C.vulgaris Breconbeacons MATK	571 TCGATTTTATG	А <mark>ССТАТС</mark> ТТІ	т с а а <mark>с т с т с</mark> с с	GATTACAGCTTTT	AATGGTACG	GAGTAAAATG	A T A G A A A A T	TCATTTTTAATAG	AGAATGC	ΤΑΘΤΑΑΑΑΑ	зттс <mark>с</mark> атастстаат	CCAATTACTCCAAT	BATT <mark>GG</mark> ATCATT <mark>G</mark> TCTAAA	719
329 C vulgaris Westwales MATK	591 TCGATTTTATG	ACTATOTT	TCAAGTGTGC	SATTACASC TTTT	AATGGTACG	GAGTAAAATG		TCATTTTAATAG	AGAATGO	TAGTAAAAA	STICGATACTCTAAT	CCAATTACTCCAAT	SATTGGATCATTGTCTAA4	739
299 C wulgaria Evlingdaloa MATK	572 TCGATTTATA		TCAAGTGTGC	ATTACASCTTT	AATGGTACG	GAGTAAAATG	TACAAAT	TCATTTTAATAG	AGAATOC	TACTAAAAA	TTCGATACTCTAAT	CCAATTACTCCAAT	ATTOGATCATTOTOTAA/	721
205_0.vulgans_ryimguales_mArk	500 100 11111													720
284_C.Vulgans_Glen Indol_MATK	SOUTCOATTITATO.	AGUTATUTT	TUAAGIGIGIGU	GATTACAGUTTTT	AATGGTACG	GAGTAAAATG		TCATTTTAATA0	AGAATOU		JITCGATACICIAAT	JCCAATTACTCCAAT	JAT I GOAT CATTOT CTAAA	738
238_C.Vulgans_Exmoor_MATK	578 TCGATTTTATG.	AGCTATCTT	TCAAGIGIGIGC	GATTACAGUTTTT	AATGGTACG	GAGTAAAATG	ATAGAAAAT	TCATTTTAATAG	AGAATGU	TAGTAAAAA	STICGATACICIAAT	JCCAATTACTCCAATC	JATTGGATCATTGTCTAAA	720
208_C.vulgaris_Yorkshire_MATK	585 TCGATTTTATG	AGCTATCTTI	T C A A G T G T G C C	GATTACAGCTTTT	AATGGTACG	GAGTAAAATG	A TAGAAAAT	TCATTTTTAATAG	AGAATGC	TAGTAAAAA	STTCGATACTCTAAT	CCAATTACTCCAAT	JATTGGATCATTGTCTAAA	733
182_C.vulgaris_Hedleyhopefell_MATK	591 TCGATTTTATG	A <mark>GCTATCTTI</mark>	T C A A <mark>G T G T G C C</mark>	G A T T A C A <mark>G C T T T T</mark>	AATGGTACG	GAGTAAAATG	A TA <mark>G</mark> AAAA T	T CATTTTTAATAG	AGAATGC	TAGTAAAAA	STTCGATACTCTAAT	BCCAATTACTCCAAT	3 A T T <mark>G G</mark> A T C A T T <mark>G T C T</mark> A A A	739
180_C.vulgaris_Hedleyhopefell_MATK	591 TCGATTTTATG	A <mark>G C T A T C T T 1</mark>	Τ C A A <mark>G T G T G</mark> C C	G A T T A C A <mark>G C</mark> T T T T	AATGGTACG	GAGTAAAATG	Α <mark>ΤΑ</mark> ΘΑΑΑΑΤ	T CATTTTTAA TAG	AGAATGC	ΤΑ <mark>σ</mark> ΤΑΑΑΑΑ	STTCGATACTCTAAT	CCAATTACTCCAAT	<mark>BATTGG</mark> AT <mark>CATTG</mark> TCTAAA	739
179_C.vulgaris_Teesdale_MATK	563 TCGATTTTATG	Α <mark>Θ C</mark> T A T C T T 1	T C A A <mark>G T G T G C C</mark>	G A T T A C A <mark>G C</mark> T T T T	AATGGTACG	GAGTAAAATG	ATA <mark>G</mark> AAAAT	T CATTTTTAATAG	AGAATGC	ΤΑ <mark>σ</mark> ΤΑΑΑΑΑ	STTC <mark>G</mark> ATACTCTAAT	CCAATTACTCCAAT	<mark>ΒΑΤΤ<mark>ΘΘ</mark>ΑΤ<mark>CΑΤΤ</mark>ΘΤCΤΑΑΑ</mark>	711
309_C.vulgaris_Yorkshire_MATK	563 TCGATTTTATG	Α <mark>ς с</mark> та тс тт 1	ТСАА <mark>СТСТС</mark> СС	<mark>G</mark> Α Τ Τ Α <mark>C</mark> Α <mark>G C</mark> Τ Τ Τ Τ	AATGGTACG	GAGTAAAATG	Α <mark>ΤΑ</mark> ΘΑΑΑΑΤ	TCATTTTTAATAG	AGAATGC	ΤΑ <mark>σ</mark> ΤΑΑΑΑΑ	STTCGATACTCTAAT	CCAATTACTCCAAT	BATT <mark>GG</mark> ATCATT <mark>G</mark> TCTAAA	711
257 C.vulgaris NorwayGrødalen MATK	571 TCGATTTTATG	Α Θ C Τ Α Τ C Τ Τ Ι	Т С А А <mark>С Т С Т С</mark> С С	<mark>G ΑΤΤΑ CΑ</mark> G C ΤΤΤΤ	AATGGTACG	GAGTAAAATG	ATAGAAAAT	TCATTTTTAATAG	AGAATGC	ΤΑ <mark>σ</mark> ΤΑΑΑΑΑ	S T T C G A T A C T C T A A T C	CCAATTACTCCAAT	<mark>BATTGGATCATTGTC</mark> TAAA	719
342a C.vuluaris Breconbeacons MATK	556 TCGATTTTATG	А <mark>ССТАТС</mark> ТТІ	т с а а <mark>с т с т с</mark> с с	GATTACAGCTTTT	AATGGTACG	GAGTAAAATG	A T A G A A A A T	TCATTTTTAATAG	AGAATGC	ΤΑΘΤΑΑΑΑΑ	зттс <mark>с</mark> атастстаат	CCAATTACTCCAAT	BATT <mark>GG</mark> ATCATT <mark>G</mark> TCTAAA	704
173 Couldarie NaceHead MATK														
		GGUIAIUII	TCAAGTGTGCC	GATTAAAACCTTT	AATGGTACG	GAGTCAAATG	ATAGAAAAT	TCATTTCTAATAG	AGAATGC	TATTAAGAA	STTCGATACTCTAAT(	CCAALACICCAAL	JAIIGAAICAIIGGIIAAA	715
Ing_o.wigana_waganead_wikink	007 TIGACIIICIG	GGCTATCTT	TCAA <mark>GTGTG</mark> CC	GATTAAAACCTTT	AATGGTACG	GAGTCAAATG	ATA <mark>G</mark> AAAAT	TCATTTCTAATAG	AGAATGC	TATTAAGAA	STTCGATACTCTAAT	CCAATTACTCCAAT	ATTGAATCATTGGTTAAA	715
1/61318 1 Phyllodore caenules 2		TAACGTGTTA		GATTAAAACCTTT		GAGTCAAATG	ATAGAAAAT	TATTGAACGATTC	AGAATGC	TATTAAGAA	ATTCGATACTCTAAT	AT AR	ATTGAATCATTGGTTAAA	1253
U61318.1_Phyllodoce_caerulea_? 259_Bhyllodoce_caerulea_NorvayKoncevoll_MATK	1135 GCGAAATTTG		AGGACATCCC	GATTAAAACCTTT ATGAGTAAGCCGG	CCTGGAGCG	GAGTCAAATG	ATAGAAAAT CTCTGATAT	TATTGAACGATTC	GGGCGTA	TATTAAGAA	ATCTTTCGATACTCTAAT	ATAG	SATIGAATCATIGGITAAA	715 1253 799
U61318.1_Phyllodoce_caerulea_? 259_Phyllodoce_caerulea_NonvayKongsvoll_MATK	1135 G CGAAATTTTG 708 G CGAAATTTTG	TAACG TGTTA TAATG TGGTA	AGGACATCCC AGGACATCCCA	GATTAAAACCTTT ATGAGTAAGCCGG ATGAGTAAGCCGG	AATGGTACG CCTGG <mark>A</mark> GCG CCTGGGCCG	GAGTCAAATG	ATAGAAAAT CTCTGATAT TTCTGATAT	TATTGAACGATTC TATTGAACGATTC	GGGCGTA	TATTAAGAA TATATAGAA TAT	ATCTTTCTCATTATT		SATIGATUATIGG I FAAA	715 1253 799 721
U6 1318 1_Phyllodoce_caenue_? 259_Phyllodoce_caenuea_NonwayKongovoll_MATK KC474423.1_Cassiope_tetragona_Canada	1135 GCGAAATTTG 706 GCGAAATTTG 813 GCGAAATTTG	TAACG TG TTA TAATG TGG TA TAATG TGG TA	T C A A G T G T G C C A G G A C A T C C C A A G G A C A T C C C A A G G A C A T C C T A	GATTAAAACCTTT ATGAGTAAGCCGG ATGAGTAAGCCGG ATGAGTAAGCCAG	AATGGTACG CCTGGAGCG CCTGGGCCG TATGGGCTG	GAGTCAAATG GTTTATCAGA CTTTATCAGA ATTTATCAGA	ATAGAAAAT CTCTGATAT TTCTGATAT TTCTGATAT	TATTGAACGATTC TATTGAACGATTC TATTGACCGATTC TATTGACCGATTC	AGAATGC GGGCGTA GGGCGTA GGGCGTA	TATTAAGAA TATATAGAA TAT TATATAGAA	ATCTTTCTCATTATE	ATAG ATAG	SATIGAATUATIGGITAAA	715 1253 799 731
U61318.1_Phyllodoce_caenulea_? 259_Phyllodoce_caenulea_NonvayKongevoll_MATK KC474423.1_Caesiope_tetragona_Canada 324_Caesiopetetragona_Abisko_MATK	1135 G CG AAATTTTG 706 G CG AAATTTTG 613 G CG AAATTTTG 704 G CG AAATTTTG	TAACG TG TTA TAATG TGG TA TAACG TCTTA TAACG TCTTA	T CAAGTGTGTGC AGGACATCCCA AGGACATCCCA AGGACATCCTA AGGACATCCTA	GATTAAAACCTTT ATGAGTAAGCCGG ATGAGTAAGCCGG ATGAGTAAGCCAG ATGAGTAAGCCAG	AATGGTACG CCTGGACCG CCTGGGCCG TATGGGCTG TATGGGCTG	GAGTCAAATG GTTTATCAGA CTTTATCAGA ATTTATCAGA ATTTATCAGA	ATAGAAAAT CTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT	TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC TATTGACCGATTC TATTGACCGATTC	AGAATGC GGGCGTA GGGCGTA GGGCGTA	TATTAAGAA TATATAGAA TAT TATATAGAA	ATTCGATACTCTAAT ATCTTTCTCATTATT; ATCTTTCTCATTATC;	ATAG	9 A I I 9 A A I VA I I 9 9 I I A A A	715 1253 799 731 791
17.5	1135 6 C GAAATTTT6 708 6 C GAAATTTT6 813 6 C GAAATTTT6 704 6 C GAAATTTT6 1135 6 C GAAATTTT6	TAACGTGTTA TAATGTGGTA TAACGTCTTA TAACGTCTTA TAACGTCTTA	T C A A G T G T G C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C T A G G A C A T C C C	GATTAAAACCTTT ATGAGTAAGCCGG ATGAGTAAGCCGG ATGAGTAAGCCAG ATGAGTAAGCCAG ATGAGTAAGCCAG	AATGGTACG CCTGGGCCG CCTGGGCCG TATGGGCTG TATGGGCTG TCTGGGCCG	GAGT CAAATG GTTTAT CAGA CTTTAT CAGA ATTTAT CAGA ATTTAT CAGA CTTTAT CAGA	ATAGAAAAT CTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT	TCATTTCTAATAG TATTGAACGATTC TATTGAACGATTC TATTGACCGATTC TATTGACCGATTC TATTGAACGATTC	AGAATGC GGGCGTA GGGCGTA GGGCGTA GGGCGTA	TATTAAGAA TATATAGAA TAT TATATAGAA TATATAGATCC	ATTCOATACTCTAAT ATCTTTCTCATTATT ATCTTTCTCATTATC ATCTTTCTCATTATC	ATAG	5A   0AA   0A   0G   AAA	715 1253 799 731 791 1253
U61318.1_Phyllodose_caenulea_? 259_Phyllodose_caenulea_NonvayKongsvoll_MATK KC474423.1_Cassiope_tetragona_Canada 324_Cassiopetetragona_Abisko_MATK AF519561.1_empetrumkemaphroitum_Arboretum,Harvard,{ 321_E.tremaphroitum_Abisko_MATK	1135         6 C G AAATTTTG           708         6 C G AAATTTTG           813         6 C G AAATTTTG           704         6 C G AAATTTTG           714         6 C G AAATTTTG           1135         6 C G AAATTTTG           704         8 C G AAATTTTG           704         8 C G AAATTTTG           704         8 C G AAATTTTG	TAACGTGTTA TAATGTGGTA TAACGTCTTA TAACGTCTTA TAACGTCTTA TAATGTCTTA	T C A A G T G T G C G A G G A C A T C C C / A G G A C A T C C C / A G G A C A T C C T / A G G A C A T C C C / A G G A C A T C C C /	GATTAAAACCTTT ATGAGTAAGCCGG ATGAGTAAGCCGG ATGAGTAAGCCAG ATGAGTAAGCCAG ATGAGTAAGCCAG	AATGGTACG CCTGGGCCG CCTGGGCCG TATGGGCTG TATGGGCCG TCTGGGCCG TCTGGGCCG	GAGTCAAATG CTTTATCAGA ATTTATCAGA ATTTATCAGA CTTTATCAGA CTTTATCAGA	ATAGAAAAT TTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT	TCATTTCTAATAG TATTGAACGATTC TATTGAACGATTC TATTGACCGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC	AGAATGC GGGCGTA GGGCGTA GGGCGTA GGGCATA GGCATA	TATTAAGAA TATATAGAA TAT TATATAGAA TATATAGAA TATCGATCC	NTTCOATACTCTAAT ATCTTTCTCATTATC ATCTTTCTCATTATC STOCTTTTTTCAAAA	ATAG	<b>7</b> A   1 <b>0</b> AA   <b>0</b> A   1 <b>00</b>   1AAA	715 1253 799 731 791 1253 805
U61318.1_Phyllodoce_caenulea_? 259_Phyllodoce_caenulea_NorwayKongevoll_MATK KC477423.1_Cassiope_tetragona_Canada 324_Cassiopetetragona_Abisko_MATK AF519561.1_enpetrumhermaphroditum_Arboretum,Harvard,L 321_E.hermaphroditum_Abisko_MATK HQ115642.1_empetrumigrum_Newfoundland_canada	1135 GCGAAATTTTG 706 GCGAAATTTTG 704 GCGAAATTTTG 704 GCGAAATTTTG 1135 GCGAAATTTTG 718 GCGAAATTTTG 1135 GCGAAATTTTG	TAACOTOTTA TAATGTOGTA TAACGTCTTA TAACGTCTTA TAACGTCTTA TAATGTCTTA TAATGTCTTA	T C A A G T G T G C G A G G A C A T C C C / A G G A C A T C C C / A G G A C A T C C T / A G G A C A T C C C / A G G A C A T C C C / A G G A C A T C C C /	GATTAAAACCTTT ATGAGTAAGCCGG ATGAGTAAGCCGG ATGAGTAAGCCAG ATGAGTAAGCCAG ATGAGTAAGCCAG ATGAGTAAGCCAG	AATGGTACG CCTGGGCCG CATGGGCCG TATGGGCTG TATGGGCTG TCTGGGCCG TCTGGGCCG TCTGGGCCG	GAGTCAAATG CTTTATCAGA ATTTATCAGA ATTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA	ATAGAAAAT CTCTGATAT ITCTGATAT ITCTGATAT ITCTGATAT ITCTGATAT ITCTGATAT	TCATTTCTAATAG TATTGAACGATTC TATTGAACGATTC TATTGACCGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC	AGAATGC GGGCGTA GGGCGTA GGGCGTA GGGCATA GGGCATA	TATTAGAA TATATAGAA TAT TATATAGAA TATCGATCC TATATAGAA	AT CONTROLATION AT CONTROLOGICATION AT CONTROLOGICATION CONTROLOGICATION AT CONTROLOGICATION AT CONTROLOGICATION	ATAG ATAG SATT ATAG	<b>241 1944 104 109 1</b> 244	715 1253 799 731 791 1253 805 1253
U6 1318 1_Phyllodose_caenulea_? 259_Phyllodose_caenulea_? 259_Phyllodose_caenulea_NonvayKongsvoll_MATK KC474423.1_Cassiope_tetragona_Canada 324_Cassiopetetragona_Abisko_MATK AF51956.1_empetrumhemaphroditum_Arboretum_Harvard,U 321_E.hermaphroditum_Abisko_MATK HQ115642.1_empetrumnigrum_Newfoundland_canada 224_E.nirum_BenMacdui_MATK	1135 GCGAAATTTTG 706 GCGAAATTTTG 613 GCGAAATTTTG 704 GCGAAATTTTG 1135 GCGAAATTTTG 718 GCGAAATTTTG 1135 GCGAAATTTTG 1135 GCGAAATTTTG	TAACG TO TTA TAATG TOG TA TAACG TO TTA TAACG TO TTA TAACG TO TTA TAATG TO TTA TAATG TO TTA TAATG TO TTA	T C A A G T G T G C C A G G A C A T C C C / A G G A C A T C C C / A G G A C A T C C T / A G G A C A T C C T / A G G A C A T C C C / A G G A C A T C C C / A G G A C A T C C C /	GATTAAAACCTTT A TG AG TAAAG C C G G A TG AG TAAG C C G G A TG AG TAAG C C AG A TG AG TAAG C C AG	AATGGTACG CCTGGGCCC CATGGGCCG TATGGGCTG TATGGGCCG TCTGGGCCG TCTGGGCCG TCTGGGCCG	GAGTCAAATG CTTTATCAGA CTTTATCAGA ATTTATCAGA ATTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA	ATAGAAAAT TTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT	TATTGAACGATTC TATTGACCGATTC TATTGACCGATTC TATTGACCGATTC TATTGACCGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC	AGAATGC GGGCGTA GGGCGTA GGGCGTA GGGCATA GGGCATA GGGCATA	TATTAAGAA TATATAGAA TAT TATAGAA TATCGATCCC TATATAGAA TATATAGAA	AT CONTROLOGIAL ACTORNAL AT CITIT CICATTATC AT CITIT CICATTATC TOCITITIT CAAAAA AT CITIT CICATTATC ATMITIC CATTATC	ATA ATA ATA ATA ATA ATA	24   19/44   UA   1999   1444	715 1253 799 731 791 1253 805 1253 860
11.5_v.migannegor.eau_mkr.X U61318.1_Phyllodoce_caenulea_? 259_Phyllodoce_caenulea_NonvayKongavoll_MATK KC474423.1_Cassiope_tetragona_Canada 324_Cassiopetetragona_Abisko_MATK AF519561.1_empetrumnemaphroditum_Arboretum,Harvard,I 321_E.hemaphroditum_Abisko_MATK HQ115642.1_empetrumnigrum_Newroundland_canada 224_E.ninum_BenMacdui_MATK 251_E.nigum_Exmoor_MATK	1135 G C GAAATTTT 1135 G C GAAATTTTG 1135 G C GAAATTTTG 113 G C G GAAATTTTG 1135 G C GAAATTTTG 1135 G C GAAATTTTG 1135 G C GAAATTTTG 1135 G C GAAATTTTG 1245 G C GAAATTTTG	TAACG TO TTA TAACG TO GTA TAACG TO TTA TAACG TO TTA TAACG TO TTA TAATG TO TTA TAATG TO TTA TAATG TO TTA	T C A A G T G T G C C A A G G A C A T C C C A A G G A C A T C C T C A G G A C A T C C T T A G G A C A T C C C T A G G A C A T C C C A A G G A C A T C C C C A G G A C A T C C C C	A T A A O T A A O C C O O A T G A O T A A O C C O O A T G A O T A A O C C C O A T G A O T A A O C C A O A T G A O T A A O C C A O A T G A O T A A O C C A O A T G A O T A A O C C A O A T G A O T A A O C C A O	AATGGTACG CCTGGGCCG TATGGGCCG TATGGGCTG TATGGGCTG TCTGGGCCG TCTGGGCCG TCTGGGCCG TCTGGGCCG TCTGGGCCG	GAGITCAAAIG CITITATCAGA ATTTATCAGA ATTTATCAGA CITITATCAGA CITITATCAGA CITITATCAGA CITITATCAGA CITITATCAGA	ATAGAAAAT TTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT TTCTGATAT	TATTO AACGATTC TATTG AACGATTC TATTG AACGATTC TATTG ACCGATTC TATTG AACGATTC TATTG AACGATTC TATTG AACGATTC TATTG AACGATTC	AGAATGC GGGCGTA GGGCGTA GGGCGTA GGGCATA GGGCATA GGGCATA	TATTAAGAA TAT TAT TATATAGAA TATCGATCC TATATAGAA TATATAGAA TATATAGAA	AT CONTROLOGIA TA CICATA TA AT CITICICATTATT AT CITICICATTATC AT CITICICATATC AT CITICICATTATC ATMITTCICATTATC	ATAG ATAG ATT ATAG ATT	<b>24   1944   UA   199</b>   1844	715 1253 799 731 1253 805 1253 860 845
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11.5_Unigamregoriesto_meritk U6 13 18.1_Phyllodoce_caenulea_? 258_Phyllodoce_caenulea_NorwayKongsvoll_MATK KC474423.1_Cassiope_letragona_Canada 324_Cassiopetetragona_Abisko_MATK AF51956.1_empetrumihemaphroditum_Arboretum_Harvard,I 321_E.hemaphroditum_Abisko_MATK 40115642.1_empetrumingum_NewYoundland_canada 224_E ninum_BenhAsodui_MATK 251_E.ningrum_Dotpatiok_MATK 363_E.ningrum_Detrop_MATK 310_E.ningum_Beconbeacons_MATK 310_E.ningum_Statistic_MATK <td>1135 G C GAAATTITG 1135 G C GAAATTITG 1136 G C GAAATTITG 1136 G C GAAATTITG 1136 G C GAAATTITG 1135 G C GAAATTITG 1135 G C GAAATTITG 1141 G C GAAATTITG 1141 G C GAAATTITG 1141 G C GAAATTITG 119 G C GAAATTITG 119 G C GAAATTITG 119 G C GAAATTITG</td> <td>TAACO TO TT TAACO TO TT TAACO TO TT TAACO TO TT TAATO TO TT</td> <td>A G A C A T C C C / A G A C A T C C C / A G A C A T C C T / A G A C A T C T T / A G A C A T C T / A G A C A T C C /</td> <td>ATTAAAACCCT ATGAGTAACCCG ATGAGTAACCCG ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA</td> <td>CTGGACC</td> <td>CARTCAAATG CTTTATCAGA ATTTATCAGA ATTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA</td> <td>TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT</td> <td>TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO ACCGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO</td> <td>A G A A T G C           C G G C G T A           C G G C G T A           C G G C G T A           G G C G T A           G G C G T A           G G C G T A           G G C G T A           G G C G T A           G G C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G G C A T A           G G G C A T A           G G G C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G C C A T A</td> <td>TATTAGGAA TATATAGGAA TATATAGGAA TATATAGGAA TATATAGGAA TATATAGAA TATATAGAA TATATAGAA</td> <td>AT CITT CICATTATI AT CITT CICATTATI AT CITT CICATTATI AT CITT CICATTATI AT CITT CICATTATI AT CITT CICATTATI AT MITT CICATTATI AT MITT CICATTATI AT MITT CICATTATI AT MITT CICATTATI</td> <td>ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA</td> <td><b>241 1944 104 109</b>   1444</td> <td>1253 799 731 791 1253 805 1253 860 845 867 860 806 797 1253</td>	1135 G C GAAATTITG 1135 G C GAAATTITG 1136 G C GAAATTITG 1136 G C GAAATTITG 1136 G C GAAATTITG 1135 G C GAAATTITG 1135 G C GAAATTITG 1141 G C GAAATTITG 1141 G C GAAATTITG 1141 G C GAAATTITG 119 G C GAAATTITG 119 G C GAAATTITG 119 G C GAAATTITG	TAACO TO TT TAACO TO TT TAACO TO TT TAACO TO TT TAATO TO TT	A G A C A T C C C / A G A C A T C C C / A G A C A T C C T / A G A C A T C T T / A G A C A T C T / A G A C A T C C /	ATTAAAACCCT ATGAGTAACCCG ATGAGTAACCCG ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA ATGAGTAACCCA	CTGGACC	CARTCAAATG CTTTATCAGA ATTTATCAGA ATTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA CTTTATCAGA	TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT TTCTOATAT	TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO ACCGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO TATTO AACGATTO	A G A A T G C           C G G C G T A           C G G C G T A           C G G C G T A           G G C G T A           G G C G T A           G G C G T A           G G C G T A           G G C G T A           G G C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G G C A T A           G G G C A T A           G G G C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G C C A T A           G G C C A T A	TATTAGGAA TATATAGGAA TATATAGGAA TATATAGGAA TATATAGGAA TATATAGAA TATATAGAA TATATAGAA	AT CITT CICATTATI AT CITT CICATTATI AT CITT CICATTATI AT CITT CICATTATI AT CITT CICATTATI AT CITT CICATTATI AT MITT CICATTATI AT MITT CICATTATI AT MITT CICATTATI AT MITT CICATTATI	ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA	<b>241 1944 104 109</b>   1444	1253 799 731 791 1253 805 1253 860 845 867 860 806 797 1253
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11.5	1135 G C GAAATTITG 706 G C GAAATTITG 718 G C GAAATTITG 743 G C GAAATTITG 744 G C GAAATTITG 744 G C GAAATTITG 749 G C GAAATTITG 710 G C GAAATTITG 710 G C GAAATTITG 734 G C GAAATTITG 734 G C GAAATTITG 744 G C GAAATTITG	TAACG TG TT A TAACG TG TT T TAACG TC TT T TAACG TC TT T TAATG TC TT T	A C A A B T C T C C I A C A A C T C C C I A C A A C A T C C T I A C A A C A T C C T I A C A A C A T C C T I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A C A T C C C I A C A C A T C C C I A C A C A T C C C I A C A C A T C C C I A C A C A T C C C I A C A C A T C C C I	AT T A A A A C C T C A C A T A A C C A C C A A T A A C C A C C A C A			TTC TO ATAT TTC TO ATAT	TATTIGAACGATTC TATTGAACGATTC TATTGACCGATTC TATTGACCGATTC TATTGACCGATTC TATTGACCGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC TATTGAACGATTC	A G A A T G C         C G G C G T A         C G G C G T A         C G G C G T A         C G C C T A         C G C C T A         C G C C T A         C G C C T A         C G C C T A         C G C C T A         C G C C T A         C G C C T A         C G C C A T A         C G C C A T A         C G C C A T A         C G C C A T A         C G C C A T A         C G C C A T A         C G C C A T A         C G C C A T A         C G C C A T A         C G C C A T A         C G C C A T A         C G C C A T A         C G C C C A T A         C G C C C A T A         C G C C C A T A         C G C C C A T A         C G C C C A T A         C G C C C T A         C G C C T A         C G C C T A         C G C G T A	TATTAGAA TATATAGAA TATCGATCCI TATATAGAA TATCGATCCI TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA	AT CITT CI CATATI AT CITT CI CATATIC AT CITT CI CATATIC ATMITICI CATATIC ATMITICI CATATIC ATMITICI CATATIC	ATAG ATAG ATAG ATAG ATA ATAG ATAG ATAG	24   19/44   UA   1999   1444	715 799 731 791 1253 805 845 860 845 860 806 806 807 797 1253 828 831
<ul> <li>U6 1318.1_Phyllodoxe_caenulea_?</li> <li>U6 1318.1_Phyllodoxe_caenulea_?</li> <li>250_Phyllodoxe_caenulea_NonvayKongsvoll_MATK</li> <li>KC474423.1_Cassiope_tetragona_Canada</li> <li>224_Cassiopetetragona_Abisko_MATK</li> <li>AF51956.1.gempetnuhemaphroitium_Arboretum,Harvard,I</li> <li>321_E.hermaphroditum_Abisko_MATK</li> <li>HQ 115642.1_empetrunmigrum_Newfoundland_canada</li> <li>224_E.ning.BenMadout_MATK</li> <li>251_E.nigrum_Dentopatrick_MATK</li> <li>263_E.nigrum_Dentopatrick_MATK</li> <li>363_E.nigrum_Dentopatrick_MATK</li> <li>363_E.nigrum_Dentopatrick_MATK</li> <li>310_E.nigrum_Dentopatrick_MATK</li> <li>310_E.nigrum_Vorkahire_MATK</li> <li>361_C.cullopata_Exmoor_MATK</li> <li>362_C.vulgata_Emory_MATK</li> <li>221_C.vulgata_Dentoy_MATK</li> </ul>	1135 G C G AAATTTTG 706 G C G AAATTTTG 706 G C G AAATTTTG 718 G C G AAATTTTG 718 G C G AAATTTTG 718 G C G AAATTTTG 718 G C G AAATTTTG 743 G C G AAATTTTG 744 G C G AAATTTTG 710 G C G AAATTTTG 710 G C G AAATTTTG 711 35 G C G AAATTTTG 712 G C G AAATTTTG 744 G C G AAATTTTG 744 G C G AAATTTTG	TAACO TO TT TAACO TO TT TAACO TO TT TAACO TO TT TAACO TO TT TAATO TO TT	A G G A G A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C T ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G C C A T C C C ( A G G T C A T C C C ( A G T C C C C C C C C C C C C C C C C C C	AT TAAAAC C C TA AT G AG TAAG C C G G AT G AG TAAG C C AG AT TAG TAAG C C AG AG G AG TAAG C C AG AG G AG TAAG C C G G AG G AG TAAG C C G G	CTGGACCC CTGGACCC CTGGCCG CTGGCCG TATGGCTG TCTGGCCG CTGGGCCG TCTGGCCG TCTGGCCG TCTGGCCG TCTGGCCG TCTGGCCG TCTGGCCG TCTGGCCG TCTGGCCG TCTGGCCG TCTGGCCG	GARTCAAACG CTTTATCACA ATTTATCACA ATTTATCACA CTTTATCACA CTTTATCACA CTTTATCACA CTTTATCACA CTTTATCACA CTTTATCACA CTTTATCACA CTTTATCACA CTTTATCACA CTTTATCACA CTTTATCACA CTTTATCACA CTTTATCACA		TATTO AACGATTO TATTO AACGATTO TATTO AACCATTO TATTO AACCATTO TATTO AACCATTO TATTO AACGATTO TATTO AACGATTO	C G A A T G C           C G G C G T A           C G G C G T A           C G G C G T A           C G G C G T A           C G G C G T A           C G G C A T A           C G G C A T A           C G G C A T A           C G G C A T A           C G G C A T A           C G G C A T A           C G G C A T A           C G G C A T A           C G G C A T A           C G G C A T A           C G G C A T A           C G C C A T A           C G C C A T A           C G C C A T A           C G C C A T A           C G C C A T A           C G C C A T A           C G C C A T A           C G C C A T A           C G C C A T A           C G C C C T A           C G C C G T A           C G C C G T A           C G C C G T A           C G C C G T A           C G C C G T A           C G C C G T A           C G C C G T A           C G C C G T A           C G C C G T A           C G C C G T A           C G C C G T A           C G C C G T A	TAT TAGGAA           TAT AT AGGAA	AT CITT CICATTATI AT CITT CICATTATI AT CITT CICATTATC I CITT CICATTATC I CITT CICATTATC AT CITT CICATTATC AT CITT CICATTATC AT MITT CICATTATC AT MITT CICATTATC AT ATTICICATTATC	ATTA ATTA ATTA ATTA ATTA ATTA ATTA ATT	24   19/44   UA   1999   1 444	715 799 731 791 1253 805 1253 860 845 867 860 806 797 1253 828 831 828 831 846
U6 3181 [Phyllodoce_caenulea.? 259_Phyllodoce_caenulea.? 259_Phyllodoce_caenulea. 259_Phyllodoce_caenulea. 241_Cassiopetetragona_Abisko_IMATK AF51956.1.2 empetruminemaphroditum_Aboretum,Harvard,U 321_E.hem.aphroditum_Abisko_IMATK 470115622.1 empetrumingrum_NewYoundland_canada 224_E.nirgum_Econoc_IMATK 251_E.nirgum_Dotpatisk_MATK 251_E.nirgum_Dotpatisk_MATK 363_E.nirgum_Dotpatisk_MATK 363_E.nirgum_Detby_MATK 363_E.nirgum_Detby_MATK 363_E.oulguma_Exmoor_MATK 362_C.vulgata_Exmoor_MATK 250_C.vulgata_Exmoor_MATK 221_C.vulgata_BetMacdui_MATK	1135 G C GAAATTITG 706 G C GAAATTITG 718 G C GAAATTITG 743 G C GAAATTITG 744 G C GAAATTITG 745 G C GAAATTITG 746 G C GAAATTITG 747 G C GAAATTITG 746 G C GAAATTITG 744 G C GAAATTITG	TAACG TG TT A TAACG TG TT T TAACG TG TT T TAACG TG TT T TAACG TG TT T TAATG TC TT TAATG TG TT TAATG TG TT TAATG TG TG T	A C A A B T C T C C I A C A A C A T C C C I A C A A C A T C C T I A C A A C A T C C T I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A A C A T C C I A C A C A T C C A C A C A C C A C A C C C A C A	T T A A A A C C T T A A A A C C C C C C			TT CT C ATAT TT CT C ATAT	TATTIGAACGATTC           TATTGAACGATTC           TATTGACCGATTC           TATTGACCGATTC           TATTGACCGATTC           TATTGACCGATTC           TATTGACCGATTC           TATTGACCGATTC           TATTGACCGATTC           TATTGACCGATTC           TATTGACCGATTC           TATTGAACGATTC           TATTGACGATTC           TATTGAACGATTC	A G A A T G C           A G G A C G T A           A G G A C G T A           A G G A C G T A           A G G A C G T A           A G G A C G T A           A G G A C A T A           A G G C G T A           A G G C G T A           A G G C G T A           A G G C G T A           A G G C G T A           A G G C G T A           A G G C G C A T A           A G G G C G A T A           A G G G C G A T A           A G G G C G A T A           A G G G C G C A           A G G G G G C A           A G G G G G T A           A G G G G G T A           A G G G G G T A	TAT TAGAA TAT ATAGAA TAT CGATCCI TAT ATGAA TAT CGATCCI TAT ATAGAA TAT ATAGAA TAT ATAGAA TAT ATAGAA TAT ATAGAA TAT ATAGAA TAT ATAGAA	AT C TTT CT CATTATT AT C TTT CT CATTATT AT C TTT CT CATTATC AT C TTT CT CATTATC AT C TTT CT CATTATC AT C TTT CT CATTATC AT MTTT CT CATTATC AT MTTT CT CATTATC AT MTTT CT CATTATC	ATAG ATAG ATAG ATA ATAG ATA ATAG ATAG A	24   19/44   UA   1999   1444	715 799 731 791 1253 860 845 850 845 860 846 860 806 797 1253 828 831 846 831
<ul> <li>U6 1318.1_Phyllodoce_caenulea_?</li> <li>U6 1318.1_Phyllodoce_caenulea_?</li> <li>259_Phyllodoce_caenulea_?</li> <li>259_Phyllodoce_caenulea_Canada</li> <li>324_Cassiopetetragona_Abisko_MATK</li> <li>KC474423.1_Cassiope_tetragona_Canada</li> <li>324_Enium_Benklaodui_MATK</li> <li>HQ115642.1_empetrunhempumoitum_Arboretum_Harvard, U</li> <li>321_E.hermaphroditum_Abisko_MATK</li> <li>HQ115642.1_empetrunhemu_Mewfoundland_canada</li> <li>224_E.nium_Benklaodui_MATK</li> <li>251_E.nigrum_Derby_MATK</li> <li>332_E.nigrum_Breconbeacons_MATK</li> <li>330_E.nigrum_Yontohire_MATK</li> <li>331_E.nigrum_Protopatrick_MATK</li> <li>332_E.nigrum_Breconbeacons_MATK</li> <li>331_E.nigrum_Yontohire_MATK</li> <li>343_E.nigria_cberby_MATK</li> <li>254_C.vulgaria_berby_MATK</li> <li>254_C.vulgaria_Derby_MATK</li> <li>264_C.vulgaria_Derby_MATK</li> <li>264_C.vulgaria_Derby_MATK</li> <li>265_C.vulgaria_Derby_MATK</li> <li>269_C.vulgaria_Derby_MATK</li> <li>269_C.vulgaria_Derby_MATK</li> <li>269_C.vulgaria_Derby_MATK</li> <li>260_C.vulgaria_Derby_MATK</li> <li>260_C.vulgaria_Derby_MATK</li> <li>260_C.vulgaria_Derby_MATK</li> <li>260_C.vulgaria_Derby_MATK</li> <li>260_C.vulgaria_Derby_MATK</li> <li>260_C.vulgaria_Derby_MATK</li> </ul>	1135 G C AAATTTTG 706 G C AAATTTTG 706 G C AAATTTTG 718 G C AAATTTTG 718 G C AAATTTTG 718 G C AAATTTTG 718 G C AAATTTTG 743 G C AAATTTTG 743 G C AAATTTTG 745 G C AAATTTTG 746 G C AAATTTTG 747 G C AAATTTTG 748 G C AAATTTTG 738 G C AAATTTTG 734 G C AAATTTTG 744 G C AAATTTTG 744 G C AAATTTTG 745 G C G AAATTTTG 745 G C G AAATTTTG 746 G C G AAATTTTG 747 G C G AAATTTTG 746 G C G AAATTTTG	TAACO TO TT TAATO TO CT TAATO TO TO TAATO TO TT TAATO TO TO TA	A C A A B C T C C C A A C A A C A T C C C A A C A A C A T C C C A A C A A C A T C C T A A C A A C A T C C T A A C A A C A T C C C A A C A T C C A T C C C A A C A T C C C A T C C C A A C A T C C C A T C C C A A C A T C C C A T C C C A A C A T C C C A T C C C A T C C C A T C C C A T C C C A T C C C A T C C C A T C C C A T C C C A T C C C A T	ATTAAAACCCTT ATTAACTAACCCAC ATTAACTAACCCAC ATTAACTAAC			TT CT G ATAT TT CT G ATAT	TATTIG AACGATTC         TATTG AACGATTC         TATTG ACCGATTC         TATTG AACGATTC	1464A190C           1600000           1600000           1600000           1600000           1600000           1600000           1600000           1600000           1600000           1600000           1600000           16000000           16000000000           16000000000000000000000000000000000000	ТАТТААĞАА ТАТ ТАĞАА ТАТ СĞАТССІ ТАТАТАĞАА ТАТ СĞАТССІ ТАТАТАĞАА ТАТАТАĞАА ТАТАТАĞАА ТАТАТАĞАА ТАТАТАĞАА ТАТАТАĞАА	AT CTTT CT CATTATT AT CTTT CT CATTATC T CTTT CT CATTATC T CTTTT CT CATTATC T CTTTT CT CATTATC AT CTTTT CT CATTATC AT MTTTCT CATTATC AT MTTTCT CATTATC AT MTTTCT CATTATC AT ATTTCT CATTATC	TAC TAC TAC TAC TAC TAC TAC	24   19/44   UA   1999   1 444	715 799 731 791 1253 805 1253 860 845 857 860 806 797 1253 828 831 828 831 845 831 845 831 845 831
VG 101981_Program Exec_merror     VG 13181_Phyllodooce_caenulea_?     S59_Phyllodooce_caenulea_NonvayKongsvoll_MATK     KC474423.1_Cassiope_tetragona_Canada     324_Cassiopetetragona_Abisko_MATK     AF51956.1_eempetruminemphroditum_Arboretum_Harvard,I     321_E.hem.aphroditum_Abisko_MATK     Y0115642.1_eempetruminemphroditum_Aboretum_Harvard,     V115642.1_eempetruminemphroditum_Aboretum_Harvard,     21_E.hem.aphroditum_Abisko_MATK     Y21_E.nignum_Dorbpatrick_MATK     332_E.nignum_Benohaeaona_MATK     313_E.nignum_Benohaeaona_MATK     313_E.nignum_Benohaeaona_MATK     313_E.nignum_Benohaeaona_MATK     314_E.nignum_Streampart     YA3E.nignum_Streampart     YA3E.nignum_Streampart     YA3E.nignum_Benohaeaona_MATK     321_C.vulgata_E.maNord(I_MATK     250_C.vulgata_BenMadout_MATK     251_C.vulgata_BenMadout_MATK     178_C.vulgata_Derby_INATK     274_C.vulgata_DerbyATK	1135 6 C 6 AAATTIT6 706 6 C 6 AAATTIT6 613 6 C 6 AAATTIT6 718 6 C 6 AAATTIT6 718 6 C 6 AAATTIT6 718 6 C 6 AAATTIT6 718 6 C 6 AAATTIT6 743 6 C 6 AAATTIT6 743 6 C 6 AAATTIT6 742 6 C 6 AAATTIT6 710 6 C 6 AAATTIT6 711 6 C 6 AAATTIT6 712 6 C 6 AAATTIT6 712 6 C 6 AAATTIT6 712 6 C 6 AAATTIT6 713 6 C 6 AAATTIT6 714 6 C 6 AAATTIT6 714 6 C 6 AAATTIT6 714 6 C 6 AAATTIT6 714 72 6 C 6 AAATTIT6 714 72 6 C 6 AAATTIT6 715 72 6 C 6 AAATTIT6 716 72 6 C 7 AAATTIT6 717 72 6 C 7 AAATTIT6 718 72 72 72 72 72 72 72 72 72 72 72 72 72	TAACC TC TT A TAACC TC TT A TAATC TC TT T TAATC TC TC TT TAATC TC TC TT TAATC TC TC TT	A G G A A T C C C A G G A C A T C C C T A G G A C A T C C T A G G A C A T C C T A G G A C A T C C T A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G T C A T C C C A G T C A T C C C A G T C A T C C C C A G T C A T C C C C A G T C A T C C C C A G T C A T C C C C C C C C C C C C C C C C	A T T A A A A C C T T A T G A C T A A C C C A C A T G A C T A A C C C A C A T G A C T A A C C A C A T G A C T A A C C A C A T G A C T A C C A C A T G A C T A C C A C A T G A C T A C C A C A T G A C T A C C A C A T G A C T A C C A C A T G A C T A C C C A C A T G A C T A C C C A C A T G A C T A C C C A C A T G A C T A C C C A C A T G A C T A C C C A C A C A C T A C C C C C A C A C T A C C C C C A C A C T A C C C C C A C A C T A C C C C C A C A C T A C C C C C A C A C T A C C C C C A C A C T A C C C C C A C C A C T A C C C C C A C A C T A C C C C C C A C A C T A C C C C C C A C A C T A C C C C C C A C A C T A C C C C C C A C A C T A C C C C C C A C A C T A C C C C C C C C C C C C C C			11 C C O ATAT 11 C C O ATAT	TATTIGAACGATIC         TATTGAACGATIC         TATTGACCATTC         TATTGACCATTC         TATTGACCATTC         TATTGACCATTC         TATTGACCATTC         TATTGACCATTC         TATTGACCATTC         TATTGACCATTC         TATTGACCATTC         TATTGAACGATTC	1000 A A T G C           1000 A C G C G T A           1000 C G C G T A           1000 C G C G T A           1000 C G C G A A           1000 C G C A A           1000 C G C A T A           1000 C G C A A           1000 C G C A A           1000 C G T A           <	TAT TAGAA TAT AT AGAA TAT AT AGAA	AT CTITCTCATTATC AT CTITCTCATTATC BTGCTTTTTCTCATTATC BTGCTTTTTTTGAAAAA AT CTITCTCATTATC ATMTTTCTCATTATC ATMTTTCTCATTATC ATMTTTCTCATTATC	ATAG ATAG ATAG ATAG ATA ATAG ATA ATAG ATA ATA	24   19/44   UA   1999   1444	715 799 731 791 1253 805 1253 860 846 806 797 1253 828 831 846 831 846 831 846 831 846 831
<ul> <li>U6 1318 1_Phyllodoce_cae.ulea_?</li> <li>U6 1318 1_Phyllodoce_cae.ulea_abixo.uMATK</li> <li>U6 13642.1_empetrumenaphroitum_Abixo.MATK</li> <li>U6 13642.1_empetrumenigrum_Newfoundland_canada</li> <li>U24_E_nirgrum_Denhatoick_MATK</li> <li>U6 1326.1_callunawulgaria_?</li> <li>U6 1326.1_callunawulgaria_?</li> <li>U6 1326.1_callunawulgaria_?</li> <li>U6 1326.1_callunawulgaria_?</li> <li>U6 1326.1_callunawulgaria_?</li> <li>U6 120.cwlgaria_Denhy.MATK</li> <li>U7 _ Loringrum_Statick_MATK</li> <li>U6 1326.1_callunawulgaria_?</li> <li>U6 _cwlgaria_Denhy.MATK</li> <li>U7 _ Loringria_Phylathick_MATK</li> <li>U7 _ Loringria_Phylathick_MATK</li> <li>U7 _ Loringria_Phylathick_MATK</li> <li>U7 _ Loringria_BechyshireBleaMow_MATK</li> <li>U7 _ Loringria_Breconbeacon_MATK</li> <li>U6 1326.1_callunawulgaria_Phylathick_MATK</li> <li>U8 _ U14garia_Breconbeacon_MATK</li> <li>U8 _ U14garia_Breconbeacon_MATK</li> </ul>	1135 G C GAAATTITG 706 G C GAAATTITG 706 G C GAAATTITG 718 G C GAAATTITG 718 G C GAAATTITG 718 G C GAAATTITG 718 G C GAAATTITG 743 G C GAAATTITG 744 G C GAAATTITG 745 G C GAAATTITG 745 G C GAAATTITG 746 G C GAAATTITG 747 G C GAAATTITG 746 G C GAAATTITG 726 G C GAAATTITG 727 G C G GAAATTITG 720 G C G GAAATTITG	TAACG TG TT A TAACG TG TT T TAACG TC TT A TAACG TC TT A TAACG TC TT A TAATG TC TT T TAATG TG TG T TAATG TG TG T TAATG TG TG T	A C A A C T C C C A A C A A C T C C C A A C A A C A T C C T A A C A A C A T C C T A A C A A C A T C C T A A C A A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C T C C A T C C C A A C T C C A T C C C A A C T C C A T C C C A A C T C C A T C C C A A C T C C C A T C C C A T C C C A A C T C C C A T C C C A T C C C A T C C C A T C C C A T C C C A T C C C A T C C C A T C C C A T C C	AT T A A A A C C T T A A A A C C C C G G AT G A G T A A C C C A G AT G A G T A A C C A C A A C C A C A A C A A C C A C A A C A A C C A C A A C A A C C A C A A C A A C C A C A A C A A C C A C A A C A A C C C C C A C A A C A A C C A C A A C A A C C A C A A C A A C C A C A A C A A C C A C A A C A A C C C C C A C A A C A A C C A C A A A C C C C C A C A A C A A C C A C A A C A A C C C C C A C A C A A C A A C C A C A C A A C C A C C A C A A C A A C C A C A C A A C C A C C A C A C A A C C A C C A C A A C C A C C A C A A C C A C C A C A A C C A C C A C A A C C A C C A C A A C C A C C A C A A C C A C C A C A A C C A C C A C A C A A C C C C C A C A C A A C C A C C A C A A C C A C			TT CT G ATAT TT CT G ATAT	TATTIGAACGATTC         TATTGAACGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGAACGATTC	146644760           1666460           1666660           166660           166670           166670           166670           166670           166670           166670           166700           166700           166700           166700           166700           166700           166700           166700           166700           166700           166700           166700           166700           166700           166700           166700           166700           166700           167000           167000           167000           167000           167000           167000           167000           167000           167000           167000           167000           167000           167000           167000           167000           167000           167000           167000           167000           <	TATTAAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA	AT CTTT CT CATTATT AT CTTT CT CATTATT AT CTTT CT CATTATC AT CTTT CT CATTATC AT CTTTT CT CATTATC AT CTTTT CT CATTATC AT MTTTCT CATTATC AT MTTTCT CATTATC AT MTTTCT CATTATC AT ATTTCT CATTATC	TAG TAG TAG TAG TAG TAG	24   19/44   UA   1999   1 AA4	715 799 731 791 1253 805 1253 805 1253 845 845 860 806 806 806 806 807 797 1253 828 831 848 837 815 813
<ul> <li>H.G., Kingam, Zingay Kato, Jinki K.</li> <li>U6 13 18.1, Phyllodoce_caerulea_?</li> <li>259_Phyllodoce_caerulea Annva yKongsvoll_MATK</li> <li>KC474423.1, Cassiope_letragona_Canada</li> <li>224_Cassiopetetragona_Abisko_MATK</li> <li>AF51956.1.1_empetruminemaphroditum_Arboretum_Harvard,I</li> <li>321_E.herm.aphroditum_Abisko_MATK</li> <li>HQ115642.1_empetrumineum_Newboundland_canada</li> <li>224_E.nium_Benhkaodui_MATK</li> <li>251_E.niigrum_Dotopathok_MATK</li> <li>251_E.niigrum_Dotopathok_MATK</li> <li>363_E.niigrum_Benobaeoong_MATK</li> <li>310_E.niigrum_Yonkohire_MATK</li> <li>326_L.cailunawulgaria_?</li> <li>250_O.vulgaria_Benkaodui_MATK</li> <li>251_C.vulgaria_Benkaodui_MATK</li> <li>252_C.vulgaria_Benkaodui_MATK</li> <li>250_O.vulgaria_Benkaodui_MATK</li> <li>251_C.vulgaria_Benkaodui_MATK</li> <li>252_C.vulgaria_Derby_MATK</li> <li>274_O.vulgaria_Derby_MATK</li> <li>374_O.vulgaria_DerbyahireBlaaklow_MATK</li> <li>374_O.vulgaria_Benkaodui_MATK</li> <li>322_C.vulgaria_Westwalea_MATK</li> <li>322_C.vulgaria_Westwalea_MATK</li> <li>322_O.vulgaria_Benkaodui_MATK</li> <li>323_O.vulgaria_Benkaodui_MATK</li> <li>324_O.vulgaria_Benkaodui_MATK</li> <li>325_O.vulgaria_Benkaodui_MATK</li> <li>326_O.vulgaria_Benkaodui_MATK</li> <li>322_O.vulgaria_Westwalea_MATK</li> </ul>	00/         00 AAATTTTG           1135         00 C AAAATTTTG           013         00 AAATTTTG           013         00 AAATTTTG           1135         00 C AAAATTTTG           1135         00 C AAATTTTG           1136         00 C AAATTTTG           1137         00 C AAATTTTG           1138         00 C AAATTTTG           1139         00 C AAATTTTG           1140         00 C AAATTTTG           1150         00 C AAATTTTG           1160         00 C AAATTTTG           1170	TAACC TC TT TAACC TC TT TAACC TC TT TAACC TC TT TAACC TC TT TAACC TC TT TAATC TC TC TT	A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C T ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G A C A T C C C ( A G G C A T C C C ( A G T C C C C C C C C C C	AT TAAAAC C C TA AT TAAG TAAG C C G G AT G AG TAAG C C AG AT G AG TAAG C C AG AG G AG TAAG C C G AG G AG TAAG C C G G G AG G AG TAAG C C G G G G G G G G G G G G G G G G				TATTO AACGATTC           TATTO AACGATTC           TATTO AACGATTC           TATTO ACCGATTC           TATTO AACGATTC	146.44.19         100         1	TAT TAGGAA TAT AT AGGAA TAT AT AGGAA TAT AGGAA TAT AGGAA TAT AT AGGAA	AT CTITCTCATTATT AT CTITCTCATTATC AT CTITCTCATTATC TCCTTTCTCATTATC AT CTITTCTCATTATC AT CTITTCTCATTATC AT MITTCTCATTATC AT MITTCTCATTATC AT MITTCTCATTATC AT ATTTCTCATTATC	ATTA ATTA ATT ATTA ATT ATTA ATTA ATTA	24   1944   UA   1999   1444	715 729 731 729 1253 805 845 853 860 846 857 860 806 797 1253 828 831 848 831 848 837 815 816 813 815 817
<ul> <li>U6 1318 1_Phyllodoce_caenulea_?</li> <li>U6 1318 1_Phyllodoce_caenulea_caenada</li> <li>U2 1_caenotunhemaphroditum_Abtoretum_Harvard, U</li> <li>U3 1_c.E.nemetumhemaphroditum_Abtoretum_Harvard, U</li> <li>U1 1562 1_caenotunhemaphroditum_Abtoretum_Harvard, U</li> <li>U6 1318 1_caenotunhemaphroditum_Abtoretum_Harvard, U</li> <li>U1 1562 1_caenotunhemaphroditum_Abtoretum_Harvard, U</li> <li>U6 1312 1_caenotunhemaphroditum_Abtoretum_Harvard, U</li> <li>U6 1328 1_calluna vulgaria_?</li> <li>U6 0_culgaria_Exmoor_MATK</li> <li>U6 0_culgaria_Exmoor_MATK</li> <li>U6 0_culgaria_Exmoor_MATK</li> <li>U6 0_culgaria_Exmoor_MATK</li> <li>U6 0_culgaria_Exmoor_MATK</li> <li>U6 0_culgaria_BotophateBotow_MATK</li> <li>U6 0_culgaria_Abtoremation ATK</li> <li>U6 0_culgaria_FyllingAttoremation ATK</li> <li>U6 0_culgaria_FyllingAttoremation ATK</li> <li>U6 0_culgaria_FyllingAttoremation ATK</li> <li>U6 0_culgaria_FyllingAt</li></ul>	1135 G C GAAATTITG 706 G C GAAATTITG 706 G C GAAATTITG 718 G C GAAATTITG 718 G C GAAATTITG 718 G C GAAATTITG 718 G C GAAATTITG 743 G C GAAATTITG 744 G C GAAATTITG 745 G C GAAATTITG 746 G C GAAATTITG 747 G C GAAATTITG 747 G C GAAATTITG 748 G C GAAATTITG 749 G C GAAATTITG 740 G C GAAATTITG 720 G C GAAATTITG	TAACG TG TT A TAACG TG TT T TAACG TC TT T TAACG TC TT T TAATG TG TT T TAATG TG TG T	A G G A C A T C C C J A G G A C A T C C T J A G G A C A T C C T J A G G A C A T C C T J A G G A C A T C C T J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G T C A T C C C J A G T C A T C C J	T T A A A A C C T T A A A A C C C C G C A T G A G T A A G C C A G A T G A G T A A G C C A G A T G A G T A A G C A A G A T G A G T A A G C A A G A T G A G T A A G C A A G A T G A G T A A G C A A G A T G A G T A A G C A A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A T G A G T A A G C A G A G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G A C G A G T A A G C C G G G A C G A G T A A G C C G A G C A				TATTIG AACGATTC         TATTG AACGATTC         TATTG ACCGATTC         TATTG ACCGATTC         TATTG ACCGATTC         TATTG ACCGATTC         TATTG ACCGATTC         TATTG ACCGATTC         TATTG AACGATTC	146 A T 9 C           166 C 0 T A           167 C 0 T A           168 C 0	TATTAGAA TATATAGAA TATCGATCCI TATATAGAA TATCGATCCI TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA	AT CONTROLOGIATION CONTRACT AT CITIT CIT CATATATO AT CITIT CIT CATATATO AT CITIT CIT CATATATO AT CITIT CIT CATATATO AT MITIT CIT CATATATO AT MITIT CIT CATATATO AT ATTITICIT CATATATO AT ATTITICIT CATATATO	AT AG AT AG AT AG AT AG AT AG AT AG AT AG AT AG AT AG AT AG	24   19/44   UA   1999   1444	715 799 791 1263 805 8460 8467 860 806 867 860 806 806 806 806 807 815 831 816 813 815 816 813 811
<ul> <li>The Chingking Ingoin East Control of the Control of Contr</li></ul>	1135         C         AAATTTTG           706         G         C         AAATTTTG           706         G         C         AAATTTTG           706         G         C         AAATTTTG           706         G         C         AAATTTTG           713         G         C         AAATTTTG           713         G         C         AAATTTG           713         G         C         AAATTTG           743         G         C         AAATTTG           743         G         C         AAATTTG           744         G         C         AAATTTG           745         G         G         AAATTTG           746         G         C         AAATTTG           747         G         C         AAATTTG           748         G         C         AAATTTG           744         G         C         AAATTTG           744         G         C         AAATTTG           722         G         C         AAATTTG           721         G         C         AAATTTG           722         G         C         AAATTTTG     <	TAACO TO THE TAACO TO THE TAACO TO THE TAACO TO THE TAACO TO THE TAATO TO THE	A G G A G T C C C A G G A C A T C C C C A G G A C A T C C T C C C A G G A C A T C C T C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G A C A T C C C A G G T C A T C C C A	AT T AA AAA C C T T AA AAA C C C T A AT G AO T AAO C C AO C AT G AO T AAO C C AO C AC G AO T AAO C C O C AC G AO T AAO C C C O C AC G AO T AAO C C O C C AC G AO T AAO C C O C C AC G AO T AAO C C C O C AC G AO T AAO C C C O C AC G AO T AAO C C C O C C AC G AO T AAO C C C O C C AC G AO T AAO C C C O C C AO C AO C C C O C C C C				TATTG AACGATTC         TATTG AACGATTC         TATTG AACGATTC         TATTG ACCGATTC         TATTG AACGATTC         TATG AACGATTC         TATG AACGATTC         TATG AACGATTC         TATG AACGATTC         TATG AACGATTC         TATG AACGAC	146:44.19         100         1	TATTAGAA TAT ATAGAA TAT ATAGAA TAT CGATCCI TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATA TATATA TATATA TATATA TATATA	AT CTTT CT CATTATT AT CTTT CT CATTATC AT CTTT CT CATTATC T CTTTT CT CATTATC AT CTTTT CT CATTATC AT MTTTC CATTATC AT MTTTC CATTATC AT MTTTC CATTATC AT MTTTC CATTATC	TAG TAG TAG TAG TAG TAG	<b>24   19</b> 44   <b>1</b> 4   <b>199</b>   1444	715 799 731 791 1263 805 845 845 860 806 797 1263 828 831 845 828 831 845 815 815 813 817 801 813 817 801 824
<ul> <li>U6 1318 1_ Phyllodoce_caenulea_?</li> <li>U6 1318 1_ Phyllodoce_caenulea_?</li> <li>U6 1318 1_ Phyllodoce_caenulea_?</li> <li>U6 1318 1_ Phyllodoce_caenulea_Canada</li> <li>224_Cassiopetetragona_Abisko_MATK</li> <li>KC474423 1_ Cassiope_tetragona_Canada</li> <li>232 1_E.hem.aphroditum_Abisko_MATK</li> <li>M215E.hem.aphroditum_Abisko_MATK</li> <li>M215E.hem.aphroditum_Abisko_MATK</li> <li>M215E.hem.aphroditum_Abisko_MATK</li> <li>M215E.hem.aphroditum_Abisko_MATK</li> <li>M315_E.nigrum_Dotpatisko_MATK</li> <li>M335_E.nigrum_Dotpatisko_MATK</li> <li>M345_E.nigrum_Dotpatisko_MATK</li> <li>M356_1_callunavulgaria_?</li> <li>250_c.vulgaria_Exmoor_MATK</li> <li>250_c.vulgaria_Exmoor_MATK</li> <li>250_c.vulgaria_Exmoor_MATK</li> <li>250_c.vulgaria_Exmoor_MATK</li> <li>221_C.vulgaria_Dotpatisko_MATK</li> <li>221_C.vulgaria_Dotpatisko_MATK</li> <li>322_c.vulgaria_Dotpatisko_MATK</li> <li>324_C.vulgaria_Dotpatisko_MATK</li> <li>325_c.vulgaria_Dotpatisko_MATK</li> <li>326_c.vulgaria_Dotpatisko_MATK</li> <li>3274_C.vulgaria_Dotpatisko_MATK</li> <li>328_C.vulgaria_Ereorobeacone_MATK</li> <li>329_c.vulgaria_Fylingdates_MATK</li> <li>328_C.vulgaria_Fylingdates_MATK</li> <li>328_C.vulgaria_Filingdates_MATK</li> <li>328_C.vulgaria_Filingdates_MATK</li> <li>328_C.vulgaria_Filingdates_MATK</li> </ul>	00/         IDAC           1135         CC         GAAATTITG           016         CC         GAAATTITG           1070         GC         GAAATTITG           108         CC         GAAATTITG           108         CC         GAAATTITG           108         CC         GAAATTITG           1185         CC         GAAATTITG           1185         CC         GAAATTITG           1185         CC         GAAATTITG           1185         CC         GAAATTITG           745         CC         GAAATTITG           746         CC         GAAATTITG           747         GC         GAAATTITG           748         GC         GAAATTTG           749         GC         GAAATTTG           740         GC         GAAATTTG           741         GC         GAAATTTG           742         GC         GAAATTTG           743         GC         GAAATTTG           744         GC         GAAATTTG           721         GC         GAAATTTG           720         GC         GAATTTG           720         GC         GAATTTG <td>TAACG TG TT A TAACG TG TT T TAACG TC TT T TAACG TC TT T TAATG TG TT T TAATG TG TG T</td> <td>A G A A A G T C C C J           A G A A A T C C T J           A G A A C A T C C T J           A G A A C A T C C T J           A G A A C A T C C T J           A G A A C A T C C T J           A G A A C A T C C T J           A G A A C A T C C C J           A G A A C A T C C C J           A G A A C A T C C C J           A G A A C A T C C C J           A G A A C A T C C C J           A G A A C A T C C C J           A G A A C A T C C C J           A G A A C A T C C C J           A G A C A T C C C J           A G A C A T C C C J           A G A C A T C C C J           A G A C A T C C C J           A G A C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C C J           A G T C A T C C J</td> <td>T T A A A A C C T T A A A A C C C C G G A T G A G T A A G C C A G A T G A G T A A G C C A G A T G A G T A A G C C A G A T G A G T A A G C C A G A T G A G T A A G C C A G A T G A G T A A G C C A G A T G A G T A A G C C A G A T G A G T A A G C C A G A T G A G T A A G C C A G A T G A G T A A G C C A G A T G A G T A A G C C A G A T G A G T A A G C C A G A T G A G T A A G C C A G A C A G T A A G C C A G A C A G T A A G C C G G A C A G T A A G C C G G A C G A G T A A G C C G G A C C G G A C C G G A C C G G A C C G G A C C G G A C C G G A C C G G A C C G G C G G C G G G C G G G C G G C G G G C G G G G G G G G G G G G G G G G C G</td> <td></td> <td></td> <td></td> <td>TATTIGAACGATTC         TATTGAACGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGAACGATTC         <t< td=""><td>1464A19C           166676           166676           166767           16777           16777           16777           16777           16777           16777           16777           16777           16777           16777           16777           16777           16777           16777           16777           167777           167777           167777           167777           167777           167777           167777           167777           167777           167777           167777           167777           167777           167777           167777           167777           167777           1677777           1677777           16777777           1677777777777           16777777777777777777777777777777777777</td><td>TAT TAGAA. 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<ul> <li>U6 1318 1_Phyllodoce_caenulea_?</li> <li>U6 1318 1_Phyllodoce_caenulea_phroditum_Abtoretum_Harvard, U</li> <li>U2 1_E. hermaphroditum_Abtoko_MATK</li> <li>U4 15642.1_empetrumenaphroditum_Marvard, U</li> <li>U4 15642.1_empetrumenaphroditum_Abtoko_MATK</li> <li>U4 15642.1_empetrumenaphroditum_Newfoundland_canada</li> <li>U2 4_E. ninum_Benhaodui_MATK</li> <li>U4 15642.1_empetrumenaphroditum_Abtoko_MATK</li> <li>U6 1326.1_calluna vulgaria_?</li> <li>U50_C. vulgaria_Exmoor_MATK</li> <li>U6 1326.1_calluna vulgaria_?</li> <li>U60_C. vulgaria_Benhaodui_MATK</li> <li>U61326.1_calluna vulgaria_?</li> <li>U60_C. vulgaria_Benhaodui_MATK</li> <li>U61326.1_calluna vulgaria_?</li> <li>U60_C. vulgaria_Benhaodui_MATK</li> <li>U61326.1_calluna vulgaria_?</li> <li>U10_C. nigraria_Becohaecon_MATK</li> <li>U61326.1_calluna vulgaria_?</li> <li>U61326.1_calluna vulgaria_?</li> <li>U61326.1_calluna vulgaria_?</li> <li>U61326.1_calluna vulgaria_?</li> <li>U61326.1_culgaria_Becohaecon_MATK</li> <li>U61326.1_culgaria_Becohaecon_MATK</li> <li>U61326.1_culgaria_Becohaecon_MATK</li> <li>U61326.1_culgaria_Becohaecon_MATK</li> <li>U61326.1_culgaria_Becohaecon_MATK</li> <li>U61326.1_culgaria_DethyphineBleaklow_MATK</li> <li>U61326.1_culgaria_Becohaecon_MATK</li> <li>U61326.1_culgaria_Becohaecon_MATK</li> <li>U61326.1_culgaria_Becohaecon_MATK</li> <li>U61326.1_culgaria_PhyphineBleaklow_MATK</li> <li>U61326.1_culgaria_Becohaecon_MATK</li> <li>U61326.1_culgaria_Becohaecon_MATK</li> <li>U61326.1_culgaria_Glen_Tool_MATK</li> <li>U20.1_culgaria_Exponor_MATK</li> <li>U20.1_culgaria_Yondokie_MATK</li> <li>U20.1_Culgaria_Solemoor_MATK</li> <li>U20.1_Culgaria_Tool_MATK</li> <li>U20.1_Culgaria_Tool_MATK</li> <li>U20.1_Culgaria_Solemoor_MATK</li> <li>U20.1_Culgaria_Solemoor_MATK</li> <li>U20.1_Culgaria_Yo</li></ul>	00/         00/         00/         00/           1135         00         00         00           00         00         00         00         00           1135         00         00         00         00         00           1135         00         00         00         00         00         00           1135         00	TAACO TO TT A TAACO TO TT A TAACO TO TT A TAACO TO TT A TAACO TO TT A TAATO TO TT A	A G G A C A T C C C J           A G G A C A T C C C J           A G G A C A T C C T J           A G A C A T C C T J           A G A C A T C C T J           A G A C A T C C J           A G A C A T C C T J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G A C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C A T C C J           A G T C	AT T AA AAA C C T T A T G AG T AAG C C AG AT G AG T AAG C C G AG G AG T AAG C C G G G AG C AG G AG G A			TT CT G ATAT TT CT G ATAT	TATTIGAACGATTC         TATTGAACGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGAACGATTC         TATGAACGATTC         TATGAACGATTC         TATGAACGATTC         TATGAACGATTC         TATGAACGATTC         TATGAACGATTC         TATGAACGATTC         TATGAACGATTC         TATGAA	A G A A T G C           C G G C G T A           C G G C G G T A           C G G C G T A           C G C G C T A           C G C G C T A           C G C G C A T A           C G C G C A T A           C G C G C A T A           C G C G C A T A           C G C C A T A           C G C C A T A           C G C C A T A           C G C C A T A           C G C C A T A           C G C C A T A           C G C C T A           C G C C T A           C G C C T A           C G C C T A           C G C C T A           C G C C T A           C G C C T A           C G C C T A           C G C C T A           C G C C T A           C G G C C T A           C G G C C T A           C G G C C T A           C G G C C T A           C G G C C T A           C G G C C T A           C G G C C T A           C G G C C T A           C G G C C T A           C G G C C T A           C G G C C T A           C G G C C T A           C G G C C T A	TATTAGAA           TATATAGAA           TATATAGAA           TATATAGAA           TATCGATCCI           TATATAGAA           TATCGATCCI           TATATAGAA           TATCGATCCI           TATATAGAA	AT CTTT CT CATTATT AT CTTT CT CATTATC T CTTT CT CATTATC T CTTTT CT CATTATC T CTTTT CT CATTATC T MTTTCT CATTATC ATMTTTCT CATTATC ATMTTTCT CATTATC ATMTTTCT CATTATC	TAG TAG TAG TAG TAG TAG TAG	24   19/44   U 4   1999     444	715 799 731 791 1263 805 1263 867 860 845 867 860 797 1253 828 831 845 831 845 831 845 831 845 813 817 815 813 817 801 848 837 815
VG 10. Virgan 2, may a keb _ mkr/k      VG 13.18.1_Phyllodooc_caerulea_?     S59_Phyllodooc_caerulea_NonvayKongsvoll_MATK     KC474423.1_Cassiope_tetragona_Canada     324_Cassiopetetragona_Abisko_MATK     AF51956.1_eenpetruminemaphroditum_Aboretum_Harvard,I     321_E.herm.aphroditum_Abisko_MATK     Y0115642.1_eenpetruminemaphroditum_Aboretum_Harvard,2     Y1_E.nignum_Sonoo_MATK     Y251_E.nignum_Dontpatisko_MATK     Y33E_figum_Dontpatisko_MATK     Y33E_figum_Betooheaons_MATK     Y33E_figum_Betooheaons_MATK     Y33E_figum_Betooheaons_MATK     Y33E_figum_Secondeaons_MATK     Y32E_figum_Secondeaons_MATK     Y32E_figum_Secondeaons_MATK     Y32E_figum_Secondeaons_MATK     Y32E_orulgata_Emotor_MATK     Y32C_vulgata_Derby_MATK     Y32C_vulgata_Derby_MATK     Y32C_vulgata_Derby_MATK     Y32C_vulgata_Derby_MATK     Y32C_vulgata_Derby_MATK     Y32C_vulgata_Derby_MATK     Y32C_vulgata_Derby_MATK     Y32C_vulgata_DerbyMATK     Y32C_	00/         00         00         00           1135         00         00         00           013         00         00         00         00           014         00         00         00         00         00           1135         00         00         00         00         00         00           1135         00 <td< td=""><td></td><td>A G A A G T C C C J           A G A A A T C C T           A G A A C A T C C T           A G A A C A T C C T           A G A A C A T C C T           A G A A C A T C C T           A G A A C A T C C T           A G A A C A T C C T           A G A A C A T C C T           A G A A C T C C T           A G A A C T C C T           A G A A C T C C T           A G A A C T C C T           A G A A C T C C T           A G A A C T C C T           A G A C A T C C T           A G A C A T C C T           A G A C A T C C T           A G A C A T C C T           A G A C A T C C T           A G A C A T C C T           A G A C A T C C T           A G A C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A T C C T           A G T C A</td><td>AT T A A A A C C T T AT G A G T A A C C C A G AT G A G T A A C C C A G AT G A G T A A C C C A G AT G A G T A A C C A A AT G A G T A A C C A A AT G A G T A A C C A A AT G A G T A A C C A A AT G A G T A A C C A A AT G A G T A A C C A A AT G A G T A A C C A A AT G A G T A A C C A A AT G A G T A A C C A A AT G A G T A A C C A A AT G A G T A A C C C A A C A G T A A C C C A A C A G T A A C C C A A C A G T A A C C C A A C A G T A A C C C A A C A G T A A C C C A A C A G T A A C C C A A C A G T A A C C C A A C A C T A A C C C A A C A G T A A C C C A A C A G T A A C C C C A A C A G T A A C C C C A A C A G T A A C C C C A A C A G T A A C C C C A A C A G T A A C C C C A A C A G T A A C C C C A A C A G T A A C C C C A A C A G T A A C 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ATA ATA ATA ATA ATA ATA ATA</td><td>24   19/44   U 4   1999     444</td><td>715 1253 799 791 1253 805 1253 800 845 867 886 867 880 806 797 1253 800 806 797 1253 845 845 845 845 831 848 837 813 845 813 847 813 847 813 847 813 847 847 847 847 847 847 847 847 847 847</td></t<></td></td<>		A G A A G T C C C J           A G A A A T C C T           A G A A C A T C C T           A G A A C A T C C T           A G A A C A T C C T           A G A A C A T C C T           A G A A C A T C C T           A G A A C A T C C T           A G A A C A T C C T           A G A A C T C C T           A G A A C T C C T           A G A A C T C C T           A G A A C T C C T           A G A A C T C C T           A G A A C T C C T           A G A C A T C C T           A G A C A T C C T           A G A C A T C C T           A G A C A T C C T           A G A C A T C C T           A G A C A T C C T           A G A C A T C C T           A G A C A T C C T           A G T C A T C C T           A G T C A T C C T         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<ul> <li>U6 13 18 1, Phyllodoce_caenulea;</li> <li>U6 13 18 1, Phyllodoce_caenulea;</li> <li>259_Phyllodoce_caenulea; NonvayKongsvoll_MATK</li> <li>KC474423.1, Cassiope_tetragona_Canada</li> <li>324_Cassiopetetragona_Abisko_MATK</li> <li>AF51956.1.2, Leepetrumingenaphroditum_Abtoretum, Harvard, I</li> <li>321_E.hermaphroditum_Abisko_MATK</li> <li>471_E.nigrum_Denbarduic_MATK</li> <li>471_E.nigrum_Denbarduic_MATK</li> <li>472_E.nigrum_Denbarduic_MATK</li> <li>473_E.nigrum_Denbarduic_MATK</li> <li>473_E.nigrum_Denbarduic_MATK</li> <li>473_E.nigrum_Denbarduic_MATK</li> <li>473_E.nigrum_Denbarduic_MATK</li> <li>473_E.nigrum_Denbarduic_MATK</li> <li>474_E.nigrum_Denbarduic_MATK</li> <li>475_C.vulgaria_Exmoor_MATK</li> <li>474_C.vulgaria_Exmoor_MATK</li> <li>474_C.vulgaria_Denbarduic_MATK</li> <li>474_C.vulgaria_Enconbeacona_MATK</li> <li>474_C.vulgaria_Enconbeacona_MATK</li> <li>474_C.vulgaria_Fylingdata_MATK</li> <li>474_C.vulgaria_Fylingdata_MATK</li> <li>474_C.vulgaria_Fylingdata_MATK</li> <li>474_C.vulgaria_Exmoor_MATK</li> <li>474_C.vulgaria_Fylingdata_MATK</li> <li>474_C.vulgaria_Fylingdata_MATK</li> <li>474_C.vulgaria_Fylingdata_MATK</li> <li>474_C.vulgaria_Helleyhopefel_MATK</li> <li>475_C.vulgaria_Helleyhopefel_MATK</li> <li>476_C.vulgaria_Helleyhopefel_MATK</li> <li>476_C.vulgaria_Helleyhopefel_MATK</li> <li>476_C.vulgaria_Helleyhopefel_MATK</li> <li>476_C.vulgaria_Helleyhopefel_MATK</li> <li>476_C.vulgaria_Helleyhopefel_MATK</li> <li>476_C.vulgaria_Helleyhopefel_MATK</li> <li>476_C.vulgaria_Helleyhopefel_MATK</li> <li>476_C.vulgaria_Helleyhopefel_MATK</li> <li>476_C.vulgaria_Helleyhopefel_MATK</li> <li>476_C.vulg</li></ul>	00/         00 A A A TITIO           1135 G C G A A A TITIO         00 G C G A A A TITIO           013 G C G A A A TITIO         01 G C G A A A TITIO           1135 G C G A A A TITIO         01 G C G A A A TITIO           1135 G C G A A A TITIO         01 G C G A A A TITIO           1135 G C G A A A TITIO         01 G C G A A A TITIO           1135 G C G A A A TITIO         01 G C G A A A TITIO           1135 G C G A A A TITIO         01 G C G A A A TITIO           743 G C G A A A TITIO         01 G C G A A A TITIO           744 G C G A A A TITIO         01 G C G A A TITIO           745 G C G A A A TITIO         01 G C G A A TITIO           746 G C G A A A TITIO         01 G C G A A TITIO           747 G C G A A A TITIO         02 G C G A A TITIO           748 G C G A A A TITIO         02 G C G A A TITIO           749 G C G A A A TITIO         02 G C G A A TITIO           740 G C G A A A TITIO         02 G C G A A TITIO           740 G C G A A A TITIO         02 G C G A A TITIO           740 G C G A A A TITIO         02 G C G A A TITIO           740 G C G A A A TITIO         02 G C G A A TITIO           740 G C G A A A TITIO         02 G C G A A TITIO           740 G C G A A A TITIO         02 G C G A A TITIO           740 G C G A A A TITIO         02 G C G A		A G G A C A T C C C J A G G A C A T C C C J A G G A C A T C C T J A G G A C A T C C T J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G T C A T C C C J A G T C A T	AT T A A A A C C T T A A A A C C C C G G AT G A G T A A C C C C G G AT G A G T A A C C C A G AT G A G T A A C C A C A C AT G A G T A A C C A C A C AT G A G T A A C C A C A C AT G A G T A A C C A C A C AT G A G T A A C C A C A C AT G A G T A A C C A C A C AT G A G T A A C C A C A C AT G A G T A A C C A C A C AT G A G T A A C C A C A C A C A C A C A C A C A				TATTIG AACGATTC         TATTG AACGATTC         TATTG ACCGATTC         TATTG ACCGATTC         TATTG ACCGATTC         TATTG AACGATTC         TATTG	146 A T 19 C           166 6 C 0 T A           166 6 C 0 T A           166 7 C 0 -           167 7 C 0 -           168 7 C 0 -           16	TAT TAGAA. TAT CAA TAGAA. TAT CAA TAGAA. TAT CAA TAGAA. TAT CAA TAGAA. TAT CAA TAGAA. TAT AT AGAA. TAT AT AGA. TAT AT AGA. TAT AT AGA. TAT AT AGA. TAT AT AGA.	AT C TIT CT CATTAAT AT C TIT CT CATTAAT AT C TIT CT CATTAATC AT C TIT CT CATTAATC	AT A G AT	24   19/44   U 4   1999     444	715 1263 709 731 791 1253 800 845 860 806 806 806 806 807 800 808 807 800 808 807 808 813 813 816 813 813 816 813 824 825 832 835 801
<ul> <li>H.G., Longan, C. Hagar Lea, C. M.K.Y.</li> <li>U6 1318. 1, Phyllodoce_caerulea_?</li> <li>259_Phyllodoce_caerulea Canada</li> <li>324_Cassiopetetragona_Canada</li> <li>324_Cassiopetetragona_Canada</li> <li>324_Cassiopetetragona_Abisko_MATK</li> <li>AF51356.1. 1_empetruminemaphroditum_Arboretum_Harvard, I</li> <li>321_E. herm.aphroditum_Abisko_MATK</li> <li>421_E. herm.aphroditum_Abisko_MATK</li> <li>421_E. nigrum_BendhascduiMATK</li> <li>251_E. nigrum_Derbpathick_MATK</li> <li>332_E. nigrum_Benobaeaons_MATK</li> <li>333_E. nigrum_Benobaeaons_MATK</li> <li>334_E. nigrum_Benobaeaons_MATK</li> <li>335_E. nigrum_Benobaeaons_MATK</li> <li>336_L. nigrum_Seconbaeaons_MATK</li> <li>337_C. vulgaris_BenMacdui_MATK</li> <li>250_C. vulgaris_BenMacdui_MATK</li> <li>250_C. vulgaris_BenMacdui_MATK</li> <li>250_C. vulgaris_Derby_MATK</li> <li>250_C. vulgaris_Derby_MATK</li> <li>250_C. vulgaris_BenMacdui_MATK</li> <li>250_C. vulgaris_Derby_MATK</li> <li>260_C. vulgaris_Derby_MATK</li> <li>271_C. vulgaris_Derby_MATK</li> <li>282_C. vulgaris_Derby_MATK</li> <li>292_C. vulgaris_Derby_MATK</li> <li>293_C. vulgaris_Abidow_MATK</li> <li>294_C. vulgaris_Abidow_MATK</li> <li>294_C. vulgaris_Abidow_MATK</li> <li>294_C. vulgaris_Glen Tool_MATK</li> <li>294_C. vulgaris_Glen Tool_MATK</li> <li>294_C. vulgaris_Hedleyhopetel_MATK</li> <li>295_C. vulgaris_T. Feedale_MEMATK</li> <li>296_C. vulgaris_T. Feedale_MEMATK</li> <li>296_C. vulgaris_T. Feedale_MEMATK</li> <li>296_C. vulgaris_T. Feedale_MEMATK&lt;</li></ul>	00/         0 & C & AAATTTTG           1135 & C & C & AAATTTTG           013 & C & C & AAATTTTG           014 & C & C & AAATTTTG           1135 & C & C & AAATTTG           743 & C & C & AAATTTG           744 & C & AAATTTG           1135 & C & C & AAATTTG           1140	TAACO TO TH TAACO TO TH TAACO TO TH TAACO TO TH TAACO TO TH TAATO TO TH	A G G A C A T C C C A G G A C A T C C C C A G G A C A T C C T C C C A G G A C A T C C T C A G G A C A T C C C C A G G A C A T C C C C A G G A C A T C C C C A G G A C A T C C C C A G G A C A T C C C C A G G A C A T C C C C A G G A C A T C C C C A G G A C A T C C C C A G G A C A T C C C C A G G A C A T C C C C A G G A C A T C C C C A G G A C A T C C C C A G G T C A T C C C C A C C C C C C C C C C C	AT T AA AAA C C T T AA AAA C C C T A AT G AG T AAA C C C AG AT G AG T AAA C C C AG AT G AG T AAG C C AG AC AG T AAG C C G AG C AG T AAG C C G G AG T AAG C C G G AG C AG T AAG C C G G AG T AAG C C G G AG C AG T AAG C C G G G AG T AAG C C G G G G AG T AAG C C G G G G G G G G G G G G G G G G				TATTG AACGATTC         TATTG AACGATTC         TATTG AACGATTC         TATTG ACCGATTC         TATTG ACCGATTC         TATTG ACCGATTC         TATTG ACCGATTC         TATTG ACCGATTC         TATTG ACCGATTC         TATTG AACGATTC         TATTG	A G A A T G C           C G G C G T A           C G G C G C T A           C G G C G C T A           C G G C G C T A           C G G C G C A           C G G C G C A T A           C G C C C A T A           C G C C C A T A           C G C C C A T A           C G C C C A T A           C G C C C A T A           C G C C C A T A           C G C C C A T A           C G C C C A T A           C G C C C A T A           C G C C C A A           C G C C C A A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G G C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A           C G C C C T A	TATTAGAA TAT CGATCCI TATATAGAA TATCGATCCI TATATAGAA TATCGATCCI TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATA TATATAGAA TATATA TATATAGAA TATATAGAA TATATAGAA TATATAGAA TATATAGAA	AT CTTT CTCATTATC AT CTTT CTCATTATC AT CTTT CTCATTATC TOCTTT CTCATTATC TOCTTTT CTCATTATC AT CTTTT CTCATTATC AT MTTTCTCATTATC AT MTTTCTCATTATC AT MTTTCTCATTATC AT AT TTCTCATTATC AT AT TTCTCATTATC	TAG TAG TAG TAG TAG TAG TAG TAG	24   1944   UA   1999   1444	715 1253 799 731 761 1253 805 1253 845 860 845 860 846 837 707 707 707 815 813 845 813 815 813 817 801 817 803 824 824 824 824 824 824 824 825 807 707 707
<ul> <li>U6 13 18 1, Phyllodoce_caenulea_?</li> <li>U6 13 18 1, Phyllodoce_caenulea_Canada</li> <li>24 L Cassiopetetragona_Abisko_MATK</li> <li>AF5 1956 1, 1_empetrumhemaphroditum_Aboretum_Harvard, I</li> <li>32 1, E. hermaphroditum_Abisko_MATK</li> <li>14 19 1562 2, Lempetrumhemaphroditum_Aboretum_Harvard, I</li> <li>24 _ E. nigrum_Exmoor_MATK</li> <li>25 1, E. nigrum_Dorby MATK</li> <li>26 1, Cangentum-Botto, MATK</li> <li>27 1, E. nigrum_Dorby MATK</li> <li>28 2, E. nigrum_Dorby MATK</li> <li>29 2, E. nigrum_Dorby MATK</li> <li>20 2, Culgania_Exmoor_MATK</li> <li>20 2, Culgania_Exmoor_MATK</li> <li>20 2, Culgania_Dorby_MATK</li> <li>21 C, Culgania_Emotohaeong_MATK</li> <li>22 1, C. vulgania_Derby_MATK</li> <li>22 1, C. vulgania_Dorby_MATK</li> <li>23 2, Culgania_Dorby_MATK</li> <li>24 2, Culgania_Dorby_MATK</li> <li>25 0, Culgania_Dorby_MATK</li> <li>26 0, Culgania_Dorby_MATK</li> <li>27 4, C. vulgania_Dorby_MATK</li> <li>28 0, Culgania_Dorby_MATK</li> <li>29 C, vulgania_Dorby_MATK</li> <li>20 C, vulgania_Exmoor_MATK</li> <li>20 C, vulgania_Exmoor_MATK</li> <li>20 C, vulgania_Hedleyhopefell_MATK</li> <li>20 C, vulgania_Teedoleyhopefell_MATK</li> <li>20 C, vulgania_Toedolae_MATK</li> <li>20 C, vulgania_Toedolaephope</li></ul>	00/         00 AAATTITO           1135         00 AAATTITO           000         00 AAATTITO           1135         00 AAATTITO           114         00 AAATTITO           115         00 AAATTITO           1135         00 AAATTITO           1136         00 AAATTITO           1137         00 AAATTITO           1136         00 AAATTITO           1137         00 AAATTITO           1136         00 AAATTITO           1137         00 AAATTITO           1138         00 AAATTITO           11390         00 AAATTITO		A G G A C A T C C C J A G G A C A T C C T J A G G A C A T C C T J A G G A C A T C C T J A G G A C A T C C T J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G G A C A T C C J A G T C A T C C J A G	AT T A A A A C C T T A A A A C C C C G G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G AT G A G T A A G C C A G A C A G T A A G C C A G A C A G T A A G C C G G A C G A G T A A G C C G G A C A G T A A G C C G G A C A G T A A G C C G G A C A G T A A G C C G G A C A G T A A G C C G G A C A G T A A G C C G G A C A G T A A G C C G G G C G G G T A G C C G G A C A G T A A G C C G G A C A G T A A G C C G G A C A G T A A G C C G G A G T A A G C C G G G G G A G T A A G C C G G G G G G G G G G G G G G G				TATTIGAACGATTC         TATTGAACGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGAACGATTC         TATTGAACGATTC <t< td=""><td>140:44.410           160:64.60           160:65.60           160:65.60           160:65.60           160:65.60           160:65.60           160:65.60           160:65.60           <t< td=""><td></td><td>AT C TTT CT CATA TA TA A A A A A A A A A</td><td>AT A G AT A G AT</td><td>24   19/44   U 4   1999     444</td><td>715 1263 799 731 791 1253 800 845 860 867 860 808 867 860 808 847 860 833 817 816 813 813 813 813 813 814 824 822 833 815 814 824 825 825 825 807 877 828 825 837 840 843 845 845 845 845 845 845 845 845 845 845</td></t<></td></t<>	140:44.410           160:64.60           160:65.60           160:65.60           160:65.60           160:65.60           160:65.60           160:65.60           160:65.60 <t< td=""><td></td><td>AT C TTT CT CATA TA TA A A A A A A A A A</td><td>AT A G AT A G AT</td><td>24   19/44   U 4   1999     444</td><td>715 1263 799 731 791 1253 800 845 860 867 860 808 867 860 808 847 860 833 817 816 813 813 813 813 813 814 824 822 833 815 814 824 825 825 825 807 877 828 825 837 840 843 845 845 845 845 845 845 845 845 845 845</td></t<>		AT C TTT CT CATA TA TA A A A A A A A A A	AT A G AT	24   19/44   U 4   1999     444	715 1263 799 731 791 1253 800 845 860 867 860 808 867 860 808 847 860 833 817 816 813 813 813 813 813 814 824 822 833 815 814 824 825 825 825 807 877 828 825 837 840 843 845 845 845 845 845 845 845 845 845 845
<ul> <li>U6 1318 1, Phyllodoce_cae.ulea_?</li> <li>U6 1316 1, Caestope_tragona_Ganada</li> <li>U2 1, Caestope_tragona_GANATK</li> <li>U6 1364 2, Leenpetruhmenaphrolitum_Abioretum_Horvard, I0 1324Enirum_BenAtaodui_MATK</li> <li>U6 1326 1, Caelunavulgaria_?</li> <li>U6 1326 1, Caelunavulgaria_MATK</li> <li>U6 1326 1, Caelunavulgaria_MATK</li> <li>U6 1326 1, Caelunavulgaria_MATK</li> <li>U6 1326 1, Caelunavulgaria_MATK</li> <li>U6 1326 1, Caelunavulgaria_Wathine_MATK</li> <li>U6 1326 1, Caulgaria_Nonxing (CaelunaMATK</li> <li>U6 1326 1, Caulgaria_Nonxing (CaelunaMATK</li> <li>U6 1326 1, Caelunavulgaria_MATK</li> <li>U6 1326 1, Caelunavulgaria_MATK</li> <li>U6 1326 1, Caelunavulgaria_MATK</li> <li>U6 13276 1, Ualgaria_Yotchine_MATK</li> <li>U6 13276 1</li></ul>	1135         C         GAAATTITG           706         C         GAAATTITG           707         G         C         GAAATTITG           708         C         GAAATTITG           708         G         C         GAAATTITG           718         G         GAAATTITG           718         G         C         GAAATTITG           718         G         G         GAAATTITG           748         G         G         GAAATTTG           749         G         G         GAAATTTG           740         G         G         G         GAATTTG           749         G         G         G         G           740         G         G         G         G         G           741         G         G         G         G         TTG           745         G         G         G         G         G         TTG           745         G         G         G         G         TTG         TTG           746         G         G         G         G         G         TTG           720         G         G         G		A C A A C T C C C A A C A A C T C C C A A C A A C T C C C A A C A A C T C C C A A C A A C T C C C A A C A A C T C C C A A C A A C T C C C A A C A A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C A C A T C C C A A C T C A T C C C C A A C T C A T C C C A A	AT GAG TAAG C C TA TG AG TAAG C C AG AT GAG TAAG C C G AG AG TAAG C C G AG C AG TAAG C C G G G TAAG C C G G AG C AG TAAG C C G G G TAAG C C G G G G TAAG C C G G G G TAAG C C G G G G G G G G G G G G G G G G				TATTIGAACGATTC         TATTGAACGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCGATTC         TATTGACCATTC         TATGACCATTC	A G A A T G C           A G A A T G C           A G A A T G C           A G A A T G C           A G A G C G T A           A G G C G C A T           A G G C G C A T           A G G C G T A <td>TATTAGGA           TATATAGGA           TATATAGAA           TATATAGAA           TATCGATCCI           TATATAGAA           TATCGATCCI           TATATAGAA           TATATAGAA</td> <td>AT CTTT CT CATTATT AT CTTT CT CATTATT AT CTTT CT CATTATC AT CTTT CT CATTATC AT CTTTT CT CATTATC AT MTTTCT CATTATC AT MTTTCT CATTATC AT MTTTCT CATTATC AT MTTTCT CATTATC AT MTTTCT CATTATC</td> <td>TAG TAG TAG TAG TAG TAG TAG</td> <td>24   1944   U 4   1999     444</td> <td>715 1263 799 731 761 1253 805 845 860 866 866 866 867 886 867 886 867 887 813 815 816 813 815 815 816 813 815 816 813 815 813 815 816 813 817 815 816 817 817 817 817 817 817 817 817 817 817</td>	TATTAGGA           TATATAGGA           TATATAGAA           TATATAGAA           TATCGATCCI           TATATAGAA           TATCGATCCI           TATATAGAA	AT CTTT CT CATTATT AT CTTT CT CATTATT AT CTTT CT CATTATC AT CTTT CT CATTATC AT CTTTT CT CATTATC AT MTTTCT CATTATC AT MTTTCT CATTATC AT MTTTCT CATTATC AT MTTTCT CATTATC AT MTTTCT CATTATC	TAG TAG TAG TAG TAG TAG TAG	24   1944   U 4   1999     444	715 1263 799 731 761 1253 805 845 860 866 866 866 867 886 867 886 867 887 813 815 816 813 815 815 816 813 815 816 813 815 813 815 816 813 817 815 816 817 817 817 817 817 817 817 817 817 817

Figure 3.1.3.1 c. Clustal alignment of MATK nucleotide sequences from collected *Ericaceae* samples compared to one another and NCBI sequences. Only the section of NCBI sequences that are cover the sequenced section from collected samples is shown. Names are in the form genbank Code (NCBI sequences) or species code (study samples)

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#### Species\_Location (where known)

### **3.1.3.1** Collected vs NCBI Database *Calluna vulgaris* Sequences

The Calluna vulgaris MATK sequences from the more recent DNA extractions did not perfectly match the MATK complete coding sequence from NCBI (U61326.1) (Fig 3.1.3.1 a-c). Therefore, they were also compared to all Calluna vulgaris MATK partial sequences on the NCBI database and the location of collection for all sequences was noted (Fig. 3.1.3.1.1a-c). Alignments showed that all sequences were very similar except between ~ base 783-807 (relative to the full coding sequence) and from from ~base 1148 (relative to the full coding sequence) to the end of the sequence (Fig. 3.1.3.1.1b). Two distinct sequence types were seen; there was the sequence common to the complete coding sequence (U61326.1), sequences from Portugal (HM850861.1) and Italy (HE966891, HE966890 and HE966889). The other sequence was common to samples from the UK (JN895888.1, JN895864.1 and JN894846.1), Denmark (KX821257, KX821258, KX821259 and KX821260) and collected samples from the UK, Northern Sweden and Norway (samples 250, 362, 221, 269, 178, 374, 342, 329, 289, 284, 238, 208, 182, 180, 179, 257, 309 and 342a). The specimen used by Kron (1997) to get a complete coding sequence which matched samples from Portugal and Italy, but not the UK or Scandinavia was vouchered as coming from the Royal Botanic Gardens Edinburgh. Upon querying this with the Royal Botanic Gardens Edinburgh we were directed to the record of this plant (Data.rbge.org.uk/living/19721433) where it was recorded as the cultivar "Rannoch" and not wildtype Calluna vulgaris.



Figure 3.1.3.1.1.a Clustal alignment of all Calluna vulgaris MATK nucleotide sequences available from NCBI at the time of writing with sequences from study samples. Sample 173 (previous identified as incorrect – section 3.2.2) has been removed from the analysis. The NCBI full coding sequence is only showing the section of sequence comparable to the other sequences, starting at base 391. Names are in the form genbank Code (NCBI sequences) or species code (study samples) Species Location (where known)

U61326.1\_Callunavulgaris\_completeCDS

HM850861.1\_Callunavulgaris\_Portugal

250 C.vulgaris Exmoor MATK

362 C.vulgaris Derby MATK

221 C.vulgaris BenMacdui MATK

269 C.vulgaris Portpatrick MATK 178 C.vulgaris Abisko MATK

329\_C.vulgaris\_Westwales\_MATK

289\_C.vulgaris\_Fylingdales\_MATK 284\_C.vulgaris\_GlenTrool\_MATK

238\_C.vulgaris\_Exmoor\_MATK

208\_C.vulgaris\_Yorkshire\_MATK

179\_C.vulgaris\_Teesdale\_MATK

374 C.vulgaris DerbyshireBleaklow MATK

342 C.vulgaris Breconbeacons MATK

182\_C.vulgaris\_Hedleyhopefell\_MATK

180\_C.vulgaris\_Hedleyhopefell\_MATK

257\_C.vulgaris\_NorwayGrødalen\_MATK 309\_C.vulgaris\_Yorkshire\_MATK

342a\_C.vulgaris\_Breconbeacons\_MATK

U61326.1\_Callunavulgaris\_completeCDS

HM850861.1\_Callunavulgaris\_Portugal

250 C.vulgaris Exmoor MATK

362 C.vulgaris Derby MATK

221 C.vulgaris BenMacdui MATK

269 C.vulgaris Portpatrick MATK

374\_C.vulgaris\_DerbyshireBleaklow\_MATK

342\_C.vulgaris\_Breconbeacons\_MATK

182\_C.vulgaris\_Hedleyhopefell\_MATK

180\_C.vulgaris\_Hedleyhopefell\_MATK

257\_C.vulgaris\_NorwayGrødalen\_MATK

342a\_C.vulgaris\_Breconbeacons\_MATK

329\_C.vulgaris\_Westwales\_MATK

289\_C.vulgaris\_Fylingdales\_MATK

284\_C.vulgaris\_GlenTrool\_MATK

238\_C.vulgaris\_Exmoor\_MATK

208\_C.vulgaris\_Yorkshire\_MATK

179\_C.vulgaris\_Teesdale\_MATK

309\_C.vulgaris\_Yorkshire\_MATK

178\_C.vulgaris\_Abisko\_MATK

KX821257.1\_Callunavulgaris\_Lodbjergfyr\_Denmark KX821258.1\_Callunavulgaris\_Lodbjergfyr\_Denmark 193 KX821259.1\_Callunavulgaris\_Lodbjergfyr\_Denmark 193 KX821260.1\_Callunavulgaris\_Lodbjergfyr\_Denmark 193 JN895888.1\_Callunavulgaris\_Breconshire\_UK 174 CAATCTTCTCATTTACGCTTAATATCTTCTGAAACTTTTCTAGAA AATCTTCTCATTTACCCTTAATATCTTCTCGAAACTTTTCTAGAAC JN895864.1\_Callunavulgaris\_Montgomeryshire\_UK 162 193 JN894846.1\_Callunavulgaris\_Glamorgan\_UK CAATCTTCTCATTTAC<mark>G</mark>CTTAATATCTTCT<mark>G</mark>AAACTTTTCTA<mark>G</mark>AAC CAATCTTCTCATTTACGCTTAATATCTTCTGAAACTTTTCTAGAAC 250\_C.vulgaris\_Exmoor\_MATK 288 362 C.vulgaris Derby MATK 298 CAATCTTCTCATTTAC<mark>G</mark>CTTAATATCTTCT<mark>G</mark>AAACTTTTCTA<mark>G</mark>AAC 221 C.vulgaris BenMacdui MATK 299 295 269 C.vulgaris Portpatrick MATK 276 178 C.vulgaris Abisko MATK 374 C.vulgaris DerbyshireBleaklow MATK 275 342 C.vulgaris Breconbeacons MATK 274 329 C.vulgaris Westwales MATK 294 289 C.vulgaris Fylingdales MATK 276 284 C.vulgaris GlenTrool MATK 293 238 C.vulgaris Exmoor MATK 281 208 C.vulgaris Yorkshire MATK 288 182 C.vulgaris Hedleyhopefell MATK 294 180\_C.vulgaris\_Hedleyhopefell\_MATK 294 179\_C.vulgaris\_Teesdale\_MATK 266 257\_C.vulgaris\_NorwayGrødalen\_MATK 274 309\_C.vulgaris\_Yorkshire\_MATK 266 342a C.vulgaris Breconbeacons MATK 259 U61326.1\_Callunavulgaris\_completeCDS 838 HM850861.1 Callunavulgaris Portugal HE966891.1 Callunavulgaris TriesteMt.Valerio Italy HE966890.1\_Callunavulgaris\_TriesteMt.Valerio\_ttaly 393 HE966889.1\_Callunavulgaris\_TriesteMt.Valerio\_ttaly KX821257.1\_Callunavulgaris\_Lodbjergfyr\_Denmark KX821258.1\_Callunavulgaris\_Lodbjergfyr\_Denmark KX821259.1\_Callunavulgaris\_Lodbjergfyr\_Denmark KX821260.1\_Callunavulgaris\_Lodbjergfyr\_Denmark JN895888.1\_Callunavulgaris\_Breconshire\_UK JN895864.1\_Callunavulgaris\_Montgomeryshire\_UK JN894846.1 Callunavuluaris Glamoruan UK 250 C.vulgaris Exmoor MATK 362 C.vulgaris Derby MATK 221 C.vulgaris BenMacdui MATK 269\_C.vulgaris\_Portpatrick\_MATK 178 C.vulgaris Abisko MATK 374 C.vulgaris DerbyshireBleaklow MATK 342 C.vulgaris Breconbeacons MATK 329 C.vulgaris Westwales MATK 289 C.vulgaris Fylingdales MATK 284 C.vulgaris GlenTrool MATK 238 C.vulgaris Exmoor MATK 208\_C.vulgaris\_Yorkshire\_MATK 182\_C.vulgaris\_Hedleyhopefell\_MATK 180\_C.vulgaris\_Hedleyhopefell\_MATK 179 C.vulgaris Teesdale MATK 257\_C.vulgaris\_NorwayGrødalen\_MATK 309\_C.vulgaris\_Yorkshire\_MATK 342a\_C.vulgaris\_Breconbeacons\_MATK



Figure 3.1.3.1.1 b. Clustal alignment of all Calluna vulgaris MATK nucleotide sequences available from NCBI at the time of writing with sequences from study samples. Sample 173 (previous identified as incorrect – section 3.2.2) has been removed from the analysis. The NCBI full coding sequence is only showing the section of sequence comparable to the other sequences, starting at base 391. Names are in the form genbank Code (NCBI sequences) or species code (study samples) Species Location (where known)

U61326.1\_Callunavulgaris\_completeCDS

HE966891.1\_Callunavulgaris\_TriesteMt.Valerio\_taly

HE966890.1 Callunavuluaris TriesteMt.Valerio Italy

HE966889.1 Callunavulgaris TriesteMt. Valerio Italy

HM850861.1\_Callunavulgaris\_Portugal

U61326.1_Callunavulgaris_completeCDS	987 COATTTTATGAGCTATCTTTCAAGTOTCCCATTACACCTTTTAATCOTACCCAATACAAAATCATACAAAATCATTTTAATCOTACAAAAACTTCCAATCCTAATACCCCAATTACTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCAATACCTCCCAATACCTCCCAATACCTCCCAATACCTCCCAATACCTCCCAATACCTCCCAATACCTCCCAATACCTCCCAATACCTCCCAATACCTCCCAATACCTCCCCAATACCTCCCAATACCTCCCAATACCTCCCAATACCTCCCCAATACCTCCCCCC	1135
HM850861.1_Callunavulgaris_Portugal	542 COATTITATOACCTATCTITCAACCTCTAACCCAATTACCCCAATTACTCCTACCCCAATCATTCATTCATACACCAATCATTCATTCTAATCCTAACCCCAATCATTCCAATCCCAATCATTCCTCTAACCCCAATCATTCCTCTAACCCCAATCATTCCTCTAACCCCCAATCATTCCTCTAACCCCCAATCATTCCTCTAACCCCCAATCATC	690
HE966891.1_Callunavulgaris_TriesteMt.Valerio_taly	542 COATTITATOACCTATCTITCAACCTCTAACCCATTACCCCTTTAATCCTACCCCACTCAAAACCACACACACACACACACACACACACACACACACA	690
HE966890.1_Callunavulgaris_TriesteMt.Valerio_ttaly	542 COATTITATOACCTATCTTTCAACTOCCAATTACACCTTTTAATCOTACCCAATCATCATACAAAATCATACAAAATTCATTTTAATACACAAAACTTCCAATCTTCCAATCCTACACCCCAATTACTCCAATCATTCCATCAT	690
HE966889.1_Callunavulgaris_TriesteMt.Valerio_ttaly	542 COATTITATOACCTATCTTTCAACTOTCCAATTACACCTTTTAATCOTACCCAATTACTACCAATCATACAAAATCATACAAAATTCATTTTAATACACAAAATCCTACTA	690
KX821257.1_Callunavulgaris_Lodbjergfyr_Denmark	491 COATTITATO A CITAT CITICAA O TO COATTACAO CITITAATOO TACOO AG TAAAAATO ATAO AAAATT CATTITTAATAO AO AATO CIAO TAAAAAO TI COATACTICIAATO CIAATO CIAAT	639
KX821258.1_Callunavulgaris_Lodbjergfyr_Denmark	401 COATTITATO ACCTATCITICAA CIGIC COATTACACCTITITAATOGIAC COATCATTO AAAAATCATACAAAAATCATTACAACACAATCATTACAAAAACTICCAATACCCCAATTACTCCAATCATTOGATCATTOCAACACTICCAATTACTCCCAATTACTCCAATTAC	639
KX821259.1_Callunavulgaris_Lodbjergfyr_Denmark	401 COATTITATO ACCTATCITICAA CIGIC COATTACACCTITITAATOGIAC COATTOCATO CAATOGIAC CAATOCIAA ACCTICAAAAACCICCAATACCCCAATACCAATOCIAATOCIAACACCICIAATOCIA	639
KX821260.1_Callunavulgaris_Lodbjergfyr_Denmark	491 COATTITATO A CITAT CITICAA O TO COATTACAO CITITAA TO TACAO CITITAA TO A TAO AAAATTACAACAI TA CITITAA TAO AAAATTACAACAI TA CITAA TAO AAAATTACAACAI TA CITAA TAO AAAATTACAACAI TA CITAA TAO AAAATTACAACAI TAA TAA AAAATTACAACAI TAA TAO AAAATTACAACAI TAA	639
JN895888.1_Callunavulgaris_Breconshire_UK	472 COATTITATOR CTATCITTCAAO TO TO COATTACAO CTITTAATOO TACGO AG TAAAAATO ATAGAAAATTCATTTTAATAGAGAAATO CTAO TAAAAAG TTCO ATACTC CAATGC CAATTACTC CA	620
JN895864.1_Callunavulgaris_Montgomeryshire_UK	480 CGATTTTATGAGCTATCTTTCAAGTGTGCGATTACAGCTTTTAATGGTACGGAGTAAAATGATAGAAAATTCATTTTAATAGAGAAATGCTAGTAAAAAGTTCGATACTCCAATTACTCCAATTACTGGATCATTGGTCAAAAATGATAGAAAATGCTAGAAAAGTTCGATACTCCAATTACTCCAATTACTGGATCATTGGATCATTG	608
JN894846.1_Callunavulgaris_Glamorgan_UK	401 COATTITATO A CITAT CITICAA O TO COATTACAO CITITAA TO TACAO CATTACAO CAATA CAAAA TO ATAO AAAAATTICATTITAA TAGA CAATO CITACAAO TO CAAATO CITACTIC CAATO CITACTIC CITACTIC CITACTIC CITACTIC CITACTIC CITACTIC CITACTIC CITACTIC CAATO CITACTIC	639
250 C.vulgaris Exmoor MATK	588 COATTITATOAC CTATCTATCAAGTOTOCOATTACAGCTTITAATOGTACOGAGTAAAATGATAGAAAATTCATTITAATAGAGAAAGCTCGATACTCCAATGCCAATTACTCCAATGCTAATGCTAAAGCTCTAAAG	734
362 C.vulgaris Derby MATK	598 COATTITATOAC CTAT CTAT CAAG TO COATTACAG CTITTAATOG TACOGAG TAAAATGATAGAAAATTCATTTTAATAGAGAAATC CTAGTAAAAG TT CGATACTCCAATGCCAATTACTCCAATGCTCAATGCTCAATGCCAATTACTCCAATGCTCAATGCTCAAAG	744
221 C.vulgaris BenMacdui MATK	597 CGATTITATGAG TATCTTT CAASTG TO CGATTACAG CTTTTAATGG TACGG AG TAAAATG TAGAAAATTCATTTTTAATGG GAAAAG TTCGATACTCTAATGC CAATTACTCCAATGATTGG TACGATGC TAGAAA	745
269 C.vulgaris Portpatrick MATK	593 CGATTITATGAG CTATCTTTCAAGTGTGCGATTACAGCTTTTAATGGTACGGAGTAAAATGATAGAAAATTCATTTTAATAGAGAAAGGTCGGTAGTACTCTAATGCCAATGCCAATGATTGGTCAATGGTCAATGCTAAA	741
178 C wilderis Abisko MATK	574 COATTEATOR TATEATOR TO TO COATTACAS CITETAATO TACAS CATTACATOR TAGAAATTCATATA AAAATTCATATATA AAAAATTCATATATA AAAAATTCATATATA AAAAATTCATATATA AAAAATTCATATATAT	722
374 C vulgaris DerbyshireBleaklow MATK	53 CRATTINA GAR CATE TTTCAAG TATE COATTACTOC CATTACTOC AND A COATTACTOC AN	721
342 C wildaris Breasheacons MATK	572 CONTINUE AND TATO TATO TATO TATA TAGA TATA TAGA TAGA	720
229 Condigana_Diecomeatoria_MATK		740
225_0.vulgaris_Westwates_MATK		790
265_0.vulgans_rynnguales_INATK		722
284_C.vulgans_Gien Inool_MATK		739
238_C.vulgans_Exmoor_MATK	5/9 CRATTITATE ACTA TOTT CAAS TO COATTACAS CITT TAATS TAATS TAAAA TOTTAATAGAAAA TI CATTITTAATAGACAAA TOTTAATAG CAATTACTCCAA TACTCCAA TACTCCAATS TOTAATAGAAAA	727
208_G.vulgans_Yorkshire_MATK	385 CGATTEATGACTATCTTCAAGTGTGCGATTACAGCTTTAATGGTACGGACAAAAATGATAGAAAAATTCATTETTAATAGAGAATGCTAGTAAAAGTTCGATCATTCCTAATGCTAATGCTAATGCTAATGCTAAAG	734
182_C.vulgaris_Hedleyhopefell_MATK	592 CGATTTTATGASCTATCTTTCAAGTGTGCGATTACAGCTTTTAATGGTACGGAGTAAAAATGATAGAAAATTCATTTTTAATAGAGAAATGCTAGTAAAAAGTTCGATACTCCAATGCCAATTACTCCAATGATCATTGGTACGGAGTAAAAAGTTCGATAGTAGAAAAGTTCGATAGTAGAAAAGTTCGATAGTAGAAAAGTTCGATGCCAATTACTCCAATGATCGATC	740
180_C.vulgaris_Hedleyhopefell_MATK	592 CGATTITATGAGCTATCTTTCAAGTGTGCGATTACAGCTTTTAATGGTACGGAGTAAAATGATAGAAAATTCATTTTTAATAGAGAATGCTAGTAAAAAGTTCGATAGCCAATTACTCCAATGATGGTCGATGGTCTAAAA	740
179_C.vulgaris_Teesdale_MATK	504 CGATITITATGASCTATCTITCAASCTGTGCGATTACASCTTTTAATGGTACGGAGTAAAAATGATAGAAAATTCATTTTTAATAGAGAAATGCTAGTAAAAAGTTCGATACTCTAATGCCGAATGCTATGGTACGATGGTACGATGGTACGATGGTACGATGGTACGATGGTACGATGGTACGATGGTACGATGGTACGATGGTAGGAGTAAAAGTTCATTGCCGATGGCTAGTAGAAAAGTTCGATGCCGAATGGCTAGTAGGAGTAGGATGGCTAGTAGAAAAGTTCGGATGCCGAATGGCTAGTAGGAGG	712
257_C.vulgaris_NorwayGrødalen_MATK	572 CGATTITATGAGCTATCTTTCAAGTGTGCGATTACAGCTTTTAATGGTACGGAGTAAAATGATAGAAAATTCATTTTTAATAGAGAATGCTAGTAAAAAGTTCGATAGCCAATTACTCCAATGATAGGATCATTGGATCATAGAAAAGTTCATTTTAATAGAGAAAGCTCGAATACTCTAATGCCAATTACTCCAATGATAGGATCATTGGATCATAGAAAAGTTCATTTTAATAGAGAAAGCTCGAATACTCTAATAGTCCAATTACTCCAATTACTCCAATTGGTCAAAG	720
309_C.vulgaris_Yorkshire_MATK	564 CGATTITATGAGCTATCTTTCAAGTGTGCGATTACAGCTTTTAATGGTACGGAGTAAAATGATAGAAAATTCATTTTTAATGGAGAATGCTAGTAAAAAGTTCGATACTCTAATGCTCCAATTACTCCAATGCTCTAAAG	712
342a C vulgaris Breconbeacons MATK	557 CGATTTTATGAGCTATCTTTCAAGTGTGCGATTACAGCTTTTAATGGTACGGAGTAAAATGATAGAAAATTCATTTTTAATAGAGAATGCTAGTAAAAAGTTCGATACTCTAATGCCAATTACTCCAATGATTGGATCATTGTCTAAAG	705
<u>-</u>		
U61326.1_Callunavulgaris_completeCDS	1138 COAAATTTTOTAACOTCTTAOGACATCCCOTTAOTAAOCCAOTCTGOOCCOATTTATCCOATTCTOATATTATTOACCOATTCOGOCCOTATATATAGAAAATATTTCTC	1243
U61326.1_Calluna vulgaris_complete CDS HM850861.1_Calluna vulgaris_Portugal	1136 COAAATTTTE TAACE TOTTAE GACATCCCE TTAE TAACCCA TOTEGE CCE ATTTATCCE ATTCTE ATATTATTE ACCE ATTCE GO CE TATATATAE AAATATTTCTC 601 CG AAATTTTE TAACE TOTTAE GACATCCCE TTAE TAAE CCAETCTE GACCE ATTCTE ATATTATTE ACCE ATTCE GO CE TATATATAE AAATATTTCTC	1243 798
U61326.1_Callunavulgaris_completeCDS HM850861.1_Callunavulgaris_Portugal HE966801.1_Callunavulgaris_TriesteMt.Valerio_taly	1136 DO AAATTITTO TAACOTOTTAGO ACATCOCOTTAO TAAGOCOAGTOTOGOGOCO ATTIATCOO ATTOTOGATATTATTO ACCOO ATTOGOGOCO TATATATAGAAATATTICTO 601 DO AAATTITTO TAACOTOTTAAGOACATCOCO TTAA TAAGOCAGIO TOGOGOCO ATTIATCO O ATTOTO ATTOTO ACCOO ATTOGOGO O TATATATAGAAATATTICTO 691 DO AAATTITTO TAACOTOTTAGO ACATCOCO TTAG TAAGOCCAGITO TOGOGOCO ATTOTO ATTOTO ATTOTO ACCOO ATTOGOGO O TATATATAGAAATATTICTO 691 DO AAATTITTO TAACOTOTTAGO ACATCOCO TTAGICCOO TOGOGOCO ATTIATCO O ATTOTO ATTOTO ACCOO ATTOGOGO O TATATATAGAAATATTICTO 691 DO AAATTITTO TAACOTOTTAGO ACATCOCO TTAGICAGITO TOGOGO CO ATTITOTO ATTOTO ATTOTO ACCOO ATTOGOGO CO TATATATAGAAATATTICTO	1243 798 745
UG1326.1_Callunavulgaris_completeCDS HMB50681.1_Callunavulgaris_Portugal HE966981.1_Callunavulgaris_TriesteNR.Valerio_kaly HE966980.1_Callunavulgaris_TriesteNR.Valerio_kaly	1136 Ε Ο ΑΑΑΤΤΙΤΤΟ ΤΑΑ C Ο ΤΟ ΤΤΑ Θ Ο ΑC ΑΤ C C C Ο ΤΤΑ Ο ΤΑΑΟ C C A O T O TO GO C C O ATT ΤΑΤ C C O ATT C TO A T A T TA T C O O C TA TA TA TA O A AA TA TITO T C 601 Ε Ο ΑΑΑΤΤΙΤΤΟ ΤΑΑ C Ο ΤΟ ΤΑ Θ Ο ΑC ATT C C C O TTA O TA O C C AO T O TO GO C C O ATT TA T C O O ATT C TO ATA TA TA O C O ATT A TA TA O A AA TA TITO T C 601 Ε Ο ΑΑΑΤΤΙΤΤΟ ΤΑΑ C Ο ΤΟ ΤΑ Θ Ο ΑΛΤ C C C O TTA O TA O C C AO T O TO GO C C O ATT TA TC O ATT C TO A C O C TA TA TA TA O A AATA TTT O T C 601 Ε Ο ΑΑΑΤΤΙΤΤΟ ΤΑΑ C Ο ΤΟ TA O G AC ATT C C O TTA O TA O C C AO T O TO GO C C O ATT TA TO C O ATT C O C O C TA TA TA TA O C A A T C C O TTA O TA O	1243 798 745 745
U61326.1_Calluna vulgaris_com/lete CDS HM85086.1_Calluna vulgaris_Portugal HE966891.1_Calluna vulgaris_Trieste Mt. Valerio_taly HE966891.1_Calluna vulgaris_Trieste Mt. Valerio_taly HE966891.2_Calluna vulgaris_Trieste Mt. Valerio_taly	1136 COAAATTTTTE TAACET CTTABE ACATCCCE TTAE TAAS CCASTCTGEGE CCEATTTATCCEATTCTEATATTATTE ACCEATTCEGEGEG TATATATAE AAAATATTTCTC 601 CEAAATTTTTE TAACET CTTAE GACATCCCE TTAE TAAS CCASTCTEGEG CCEATTTATCEATTCTE ATCATTE ACCEATTCEGEGEG TATATATAE AAATATTTCTC 601 CEAAATTTTTE TAACET CTTAE GACATCCCE TTAE TAAS CCASTCTEGEG CCEATTT 601 CEAAATTTTE TAACET CTTAE GACATCCCE TTAE TAAS CCASTCTEGEG CCEATTT 601 CEAAATTTTE TAACET CTTAE GACATCCCE TTAE TAAS CCASTCTEGEG CCEATTT 601 CEAAATTTTE TAACET CTTAE GACATCCCE TTAE TAAS CCASTCTEGEG CCEATTT.	1243 798 745 745 745 745
U61326.1.Calluna vulgaris_complete CDS HM950861.1_Calluna vulgaric, Pontugal HE 966891.1_Calluna vulgaris, Triestehlt. Valerio_taly HE 966890.1_Calluna vulgaris, Triestehlt. Valerio_taly HE 966899.1_Calluna vulgaris, Triestehlt. Valerio_taly K821257.1_Calluna vulgaris, Lodbjergfyr, Denmark	1136 DO AAATTITTO TAACOTOTTAGO ACATCOCOTTAO TAAO COAGTOTOGO CO ATTIATOCO ATTOTO ATTATTO ACCO ATTOGO GO CO ATTATATATA O AAATATTTOTO 601 DO AAATTITTO TAACOTOTTAGO ACATCOCO TTAO TAAO COAGTOTOGO CO GATTTATOCO ATTOTO ATATTATTO ACCO ATTOGO GO CO ATTATATATAGAAATATTTOTO 601 DO AAATTITTO TAACOTOTTAGO ACATCOCO TTAO TAAO COAGTOTOGO CO GATTT 601 DO AAATTITTO TAACOTOTTAGO ACATCOCO TTAO TAAO COAGTOTOGO CO GATTT 601 DO AAATTITTO TAACOTOTTAGO ACATCOCO TTAO TAAO COAGTOTOGO CO GATTT 601 DO AAATTITTO TAACOTOTTAGO ACATCOCO TTAO TAAO COAGTOTOGO CO GATTT 601 DO AAATTITTO TAACOTOTTAGO ACATCOCO TTAO TAAO COAGTOTOGO CO GATTT 604 DO GAAATTITTO TAACOTOTTAGO ACATCOCO TTAO TAAO COAGTOTOGO CO GATTT 605 ACATTITTO TAACOTOTTAGO ACATCOCO TTAO TAAO COAGTOTOGO CO GATTT 606 DO AAATTITTO TAACOTOTAGO TOTOCO TAO TAAO COAGTOTOGO CO GATTO	1243 798 745 745 745 745 747
U61326.1_Calluna vulgario_complete CDS HM50861.1_Calluna vulgario_Portugal HE 966891.1_Calluna vulgario_Portugal HE 966891.1_Calluna vulgario_TriestehR. Valerio_kaly HE 966889.1_Calluna vulgario_TriestehR. Valerio_kaly KX821257.1_Calluna vulgario_Lodgiergty_Denmark KX821257.1_Calluna vulgario_Lodgiergty_Denmark	1136 CO A A A TITTE GTA A COTO TTA OGA CATEC CONTAG TA A OCA OTO TO GO CCO ATTTA TCO A TITE TA CONTENT A TITO CONTA TA TA A OCA ATTTE TO CONTAGA A A ATTTE TO CONTAGA A ATTE TO CONTAGA A ATTER A	1243 798 745 745 745 747 747 747
U6 1326. 1. Calluna vulgario_complete CDS HM850861. 1. Calluna vulgario_Portugal HE 966890. 1. Calluna vulgario_Trieste Mt. Valerio_taly HE 966890. 1. Calluna vulgario_Trieste Mt. Valerio_taly HE 966890. 1. Calluna vulgario_Trieste Mt. Valerio_taly KX821257. 1. Calluna vulgario_Lodbjergfy_Denmark KX821258. 1. Calluna vulgario_Lodbjergfy_Denmark	1136 CO A A A TITTE TA A COTO TTA BO A CATOC CONTA STA SCC AO TO TO GOO CO A TITA TO CO A TITO TO A TA TITO TA A CONTO CONTA SCA A TA TITO TA A COTO TTA SO A A A TA TITO TA A CONTO CONTA SO A A A TA TITO TA A CONTO CONTA SO A A A TA TITO TA A CONTO CONTA SO A A A TA TITO TA A CONTO CONTA SO A A A TA TITO TA A CONTO CONTA SO A A A TA TITO TA A CONTO CONTA SO A A A TA TITO TA A CONTO CONTA SO A A A A TA TITO TA A CONTO CONTA SO A A A A A TA TITO TA A CONTO CONTA SO A A A A A TA TITO TA A CONTO CONTA SO A A A A TA TITO TA A CONTO CONTA SO A A A A A TA TITO TA A CONTO CONTA SO A A A A A A A A A A A A A A A A A A	1243 798 745 745 745 747 747 747 747
U61326.1.Calluna vulgaris_complete CDS HM55085.1.Calluna vulgaris_Portugal HE 966891.1_Calluna vulgaris_Portugal HE 966891.1_Calluna vulgaris_Triesteh&.Valerio_kaly HE 966899.1_Calluna vulgaris_Triesteh&.Valerio_kaly KX821255.1_Calluna vulgaris_Lodbjergty_Denmark KX821259.1_Calluna vulgaris_Lodbjergty_Denmark KX821259.1_Calluna vulgaris_Lodbjergty_Denmark KX821259.1_Calluna vulgaris_Lodbjergty_Denmark	1136 CGAAATTTTTGTAACGTOTTAGGACATCCCGTTAGTAAGCCAGTOTGGGCCGATTTATCCGATTCTGATATTATTGACCGATTCGGGCGTATATAGAAATATTTOTC 601 CGAAATTTTTGTAACGTOTTAGGACATCCCGTTAGTAAGCCAGTOTGGGCCGATTT 601 CGAAATTTTTGTAACGTOTTAGGACATCCCGTTAGTAAGCCAGTOTGGGCCGATTT 601 CGAAATTTTTGTAACGTOTTAGGACATCCCGTTAGTAAGCCAGTOTGGGCCGGATTT 604 CGAAATTTTTGTAACGTOTTAGGACATCCCGGTAGTAAGCCAGTOTGGGCCGGATTT 604 CGAAATTTTTGTAATGTGGTAGGTCATCCCACGAGTAAGCCGGTOTGGGCCGGCTTTATCAGATTCTGATATTATTGAACGATTCGGGCGTATATATGAAATCTTTGCG 604 CGAAATTTTGTAATGTGGTAGGTCATCCCACGAGTAAGCCGGTOTGGGCCGGCTTTATCAGATTCTGATATTATGAACGATTCGGGCGTATATATGAAATCTTTCGC 604 CGAAATTTTGTAATGTGGTAGGTCATCCCACGAGTAAGCCGGTOTGGGCCGGCTTTATCAGATTCTGATATTATGAACGATTCGGGCGTATATATGAAATCTTTCGC 604 CGAAATTTTGTAATGTGGTAGGTCATCCCACGAGTAAGCCGGTOTGGGCCGGTTTATCAGATTCTGATATTATGAACGATTCGGGCGTATATATGAAATCTTTCGC 604 CGAAATTTTGTAATGTGGTAGGTCATCCCACGAGTAAGCCGGTOTGGGCCGGTTTATCAGATTCTGATATTATGAACGATTCGGCGCTATATATGAAATCTTTCGC	1243 798 745 745 745 747 747 747 747 747
U61326.1_Calluna wulgaris_complete CDS HM850861.1_Calluna wulgaris_Portugal HE 966891.1_Calluna wulgaris_TriestehR: Valerio_taly HE 966899.1_Calluna wulgaris_TriestehR: Valerio_taly HE 966899.1_Calluna wulgaris_TriestehR: Valerio_taly KX821287.1_Calluna wulgaris_Lodbjergfyr_Denmark KX821289.1_Calluna wulgaris_Lodbjergfyr_Denmark KX821289.1_Calluna wulgaris_Lodbjergfyr_Denmark KX821280.1_Calluna wulgaris_Lodbjergfyr_Denmark KX821280.1_Calluna wulgaris_Lodbjergfyr_Denmark KX821280.1_Calluna wulgaris_Lodbjergfyr_Denmark	1136 CO AAATTTTT G TAACG T C TTAGG ACAT C C CG TTAG TAAG C CAG T C TG GG C CG ATTTAT C CO ATT C TG AT ATTATT TO AC CG ATT C GG G CG TAT AT AT AG AAATATTT C T C 601 CG AAATTTTT G TAACG T C TTAGG ACAT C C CG TTAG TAAG C CAG T C TG GG C CG ATTTAT C CG ATT C TG AT ATTATT G AC CG ATT C GG G CG TAT AT AT AG AAATATTT C T C 601 CG AAATTTTT G TAACG T C TTAGG ACAT C C CG TTAG TAAG C CAG T C TG GG C CG ATTTATT C GAT T C TG AT ATTATT G AC CG ATT C GG G CG TAT AT AT AG AAATATTT C T 601 CG AAATTTTTG TAACG T C TTAGG ACAT C C CG TTAG TAAG C CAG T C TG GG C CG ATTT 601 CG AAATTTTTG TAACG T C TTAGG ACAT C C CG TTAG TAAG C CAG T C TG GG C CG ATTT 601 CG AAATTTTTG TAACG T C TTAGG ACAT C C CG TTAG TAAG C CAG T C TG GG C CG ATTT 601 CG AAATTTTTG TAACG T C TTAGG ACAT C C CG TTAG TAAG C CAG T C TG GG C CG ATTT 601 CG AAATTTTTG TAACG T C TTAGG ACAT C C CG TTAG TAAG C CG C T TG GG C CG CT TTAT C AGATT C TG AT ATTATTG AAC GATT C TG CG C TTAT TAG AAAT C TTT C CG 601 CG AAATTTTTG TAACG T G G TAGG T CAC C CG G C T G GG C CG C T TTAT C AGATT C TG AT ATTATT G AAC GATT C G CG C T AT AT TAG AAAT C TTT C CG 600 CG AAATTTTG T AAT C T GG T AG G T CAC C CG G C T G G G C CG C T TTAT C AGATT C T CG AT ATTATT T G AAC G ATT C TG C T AT AT AG AAAT C TTT C CG 600 CG AAATTTTG T AAT G T GG T AG G T CAC CAG G T AG C C G G T C T T AT C AGATT C T CG AT ATTATT T G AAC G ATT C T T C C C 600 CG AAATTTTG T AAT G T GG T AG G T CAC AGAG T AG C C G G T C T G G C CG C T T AT AT AT T T G AAC G AT T C T T C C C 600 CG AAATTTTG T AAT G T GG T AG G T CAC AG G AT AG C C G G T C T T AT C AGATT C T C G AT AT T T T T T T T T T T C C C C T AT AT AT G T G T AG G T C C C C C AG G A T T T T C G C T T T T T C G C T T T T T	1243 798 745 745 745 747 747 747 747 747 728
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U61326.1_Calluna vulgaris_complete CDS MM850861.1_Calluna vulgaris_Portugal HE 966899.1_Calluna vulgaris_Portugal HE 966899.1_Calluna vulgaris_TriestehR: Valerio_taly HE 966899.1_Calluna vulgaris_TriestehR: Valerio_taly KX8212267.1_Calluna vulgaris_Lodbjergfy_Denmark KX8212269.1_Calluna vulgaris_Lodbjergfy_Denmark KX8212269.1_Calluna vulgaris_Lodbjergfy_Denmark KX821259.1_Calluna vulgaris_Lodbjergfy_Denmark KX821259.1_Calluna vulgaris_Lodbjergfy_Denmark KX821259.1_Calluna vulgaris_Breconshire_LK JN895988.1_Calluna vulgaris_Breconshire_LK JN895986.1_Calluna vulgaris_Glamoyan UK	1136 CO AAATTITTE TAACE TO TTABE ACAT CCCE TTAE TAAE CCAE TO TEGE CCE ATTITETCE ATATTATTE ACCE ATT CEGE CE TATATATE AAATATTTE TC 601 CE AAATTITTE TAACE TO TTAE GACAT CCCE TTAE TAAE CCAE TO TEGE CCE ATTITAT CCE ATTITE ACCE ATTITE ACCE ATTATATE AAAATATTTE TC 601 CE AAATTITTE TAACE TO TTAE GACAT CCCE TTAE TAAE CCAE TO TEGE CCE ATTITAT CE ATTATTE ACCE ATTICE CE ATTATATE AAAATATTTE TC 601 CE AAATTITTE TAACE TO TTAE GACAT CCCE TTAE TAAE CCAE TO TEGE CCE ATTIT 601 CE AAATTITTE TAACE TO TTAE GACAT CCCE TTAE TAAE CCAE TO TEGE CCE ATTIT 601 CE AAATTITTE TAACE TO TTAE GACAT CCCE TTAE TAAE CCAE TO TEGE CCE ATTIT 601 CE AAATTITTE TAACE TO TTAE GACAT CCCE TTAE TAAE CCAE TO TEGE CCE ATTIT 601 CE AAATTITTE TAACE TO TTAE GACAT CCCE TTAE TAAE CCAE TO TEGE CCE ATTIT 601 CE AAATTITTE TAATE TEG TAE GTAE TCCCAE BAE TAAE CCEE TO TEGE CCE ATTIT 600 CE AAATTITTE TAATE TEG TAE GTAE TCCAE BAE TAAE CCEE TO TEGE CCE OTTTAT CAE ATTOTE AATTATTE AACE ATT CEGE CE TATATATE AAAATC TTT CE C 600 CE AAATTITTE TAATE TEG TAE GTAE TCCAE CAE TAAE CCEE TO TEGE CCE OTTTAT CAE ATTOTE GATTATTATTAE AACE ATT CEGE CE TATATATE AAATC TTT CE C 600 CE GAAATTITTE TAATE TEG TAE GTAE TCCAE CAE TAAE CCEE TO TEGE CCE OTTTAT CAE ATTOTE GATTETE GATTE CEGE CE TATATATE GAACE CAT TO TEGE CCE TATATATE CAE ATTOTE CE ATTATTE AACE ATTO CEGE CE TATATATE AAATC TTT CE C 600 CE GAAATTITE TAATE TEG TAE GTAE TCCAE CAE TAAE CCEE TO TEGE CCE OTTTAT CAE ATTOTE GATTATTE GAACE GATT CEGE CE TATATATE GAACT TTT CE C 600 CE GAAATTITE TAATE TEG TAE GTAE TCCAE GAAE TAAE CCEE TO TTAT CAE ATTOTE GATTATTATE GAACE GATT CEGE CE TATATATE GAACT TTT CE C 600 CE GAAATTITE TAATE TEG TAE GTAE TCCAE GAAE TAAE CCEE TO TEGE CCE OTTTAT CAE ATTOTE GATTE TTAE GAAATC TTT CE C 600 CE GAAATTITE TAATE TEG TAE GTAE TCCAE GAAE TAAE CCEE TO TEGE CCE CO TTTAT CAE ATTOTE GAACT TTE GAAETTE TAATATE GAACT TTTCE C 600 CE GAAATTITE TAATE TEG TAAE TCCAE GAAE TAAE CCEE TO TEGE CCE CE TTATTE GAACT TTTE GAACT TTE GAAETTE TAATE GAAATC TTTCE C 600 CE GAAATTITE TAATE TEG TAAE TCCAE GAAE TAAE CCEE TO TEGE CCE CE CT	1243 798 745 745 747 747 747 747 728 716 747
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U61326.1_Calluna wilgaria_complete CDS HM850861.1_Calluna wilgaria_Portugal HE 966891.1_Calluna wilgaria_TriestehR: Valerio_taly HE 966891.1_Calluna wilgaria_TriestehR: Valerio_taly HE 966891.1_Calluna wilgaria_TriestehR: Valerio_taly KX821257.1_Calluna wilgaria_Lodbjergfy_Denmark KX821259.1_Calluna wilgaria_Lodbjergfy_Denmark KX821259.1_Calluna wilgaria_Lodbjergfy_Denmark KX821250.1_Calluna wilgaria_Lodbjergfy_Denmark KX821250.1_Calluna wilgaria_Lodbjergfy_Denmark KX821250.1_Calluna wilgaria_Lodbjergfy_Denmark JN895080.1_Calluna wilgaria_Lodbjergfy_Denmark JN895080.1_Calluna wilgaria_Lodbjergfy_Denmark JN895080.1_Calluna wilgaria_Lodbjergfy_Denmark JN895080.1_Calluna wilgaria_Lodbjergfy_Denmark JN895080.1_Calluna wilgaria_Montgomeryshire_UK JN89508.1_Calluna wilgaria_Montgomeryshire_UK JN89508.1_Calluna wilgaria_Baronothire_ZK JN89508.1_Calluna wilgaria_Baronothire_ZK JN89508.1_Calluna wilgaria_Baronothire_ZK JN89508.1_Calluna wilgaria_Baronothire_ZK JN89508.1_Calluna wilgaria_Baronothire_ZK	1136 CO A AA TITIT G TAA COTO TTAO O ACAT C C COTTAO TAAO C CAO TO TO GO C CO ATTITAT C CO ATT C TO ATTATTO AC CO ATT C CO ATTATTO AC CO ATTATTO CO COTTA ATTATTO AC CO ATTO CO COTTA ATTATTO ACCONTINUE CONTINUE	1243 798 745 745 747 747 747 747 747 747 728 716 747 828 831 846
U61326.1_Calluna wulgaris_complete CDS HM850861.1_Calluna wulgaris_Portugal HE 966891.1_Calluna wulgaris_Portugal HE 966891.1_Calluna wulgaris_Triesteftk. Valerio_taly HE 966899.1_Calluna wulgaris_Triesteftk. Valerio_taly KX821258.1_Calluna wulgaris_Lodbjergfy_Denmark KX821258.1_Calluna wulgaris_Breonshire_UK JN895868.1_Calluna wulgaris_Glamorgan_UK 250_C. wulgaris_Denty_MATK 251_C. wulgaris_Denty_MATK 251_C. wulgaris_Denty_MATK	1136 C G A A A TITTE G TA A C G TO TTA B G A C A T C C C G TTA G TA A G C C A G TO TG G G C C G A TTTA TC C G A TT C TG A TA TTA TTE A C C G A TT C G G C G A TTTA TA TA G A A A TA TTTO TC C G A TA TA TA G G C G TA TA TA TA G G A A TA TTTO TC C G A TTTA TC G A A TTTTE A C C G A TTTA TA G A A A TA TTTO TC C G A TA TA TA G G C G TA TA TA TA G G A C A TTTO TC C G TTA G G A C G C G A TTTA TC G G A TTTA TC G G A TTTA TC G G A TTTO TG G C C G A TTTA TC G G A TTTA TC G G A TTTA TA G G C G TA TA TA TA G A A A TA TTTO TC C G TTA G G A C G C G TA G C G G C G A TTTA TC G G C C G A TTTA TC G G A TTTO TG C G C C G A TTTA TC G G A TTTO TG C G C C G A TTTA TC G G A TTTO TG C G C C G A TTTA TC G G C C G A TTTA TC G G C C G A TTTA TC G G C C G TA G TA	1243 798 745 745 747 747 747 747 747 747 747 748 828 831 846 837
U61326.1_Calluna wilgaria_complete CDS MM850861.1_Calluna wilgaria_Portugal HE 966899.1_Calluna wilgaria_TriestehR. Valerio_taly HE 966899.1_Calluna wilgaria_TriestehR. Valerio_taly HE 966899.1_Calluna wilgaria_TriestehR. Valerio_taly KX821226.1_Calluna wilgaria_Lodbjergfy_Denmark KX821226.1_Calluna wilgaria_Lodbjergfy_Denmark KX821226.1_Calluna wilgaria_Lodbjergfy_Denmark KX821269.1_Calluna wilgaria_Lodbjergfy_Denmark KX821269.1_Calluna wilgaria_Lodbjergfy_Denmark KX821269.1_Calluna wilgaria_Breconshire_LK JN895886.1_Calluna wilgaria_Breconshire_LK JN895886.1_Calluna wilgaria_Breconshire_LK S10895486.1_Calluna wilgaria_Breconshire_LK S10895486.1_Calluna wilgaria_Breconshire_LK S20_C. wilgaria_Berkonsty_MATK S21_C. wilgaria_Portotok_MATK S28_C. wilgaria_Portotok_MATK	1136 CO AAATTITTE TAACE TO TTABE ACAT C CCE TTAE TAAE CCAE TO TEGE CCE ATTITE TO COATT C TO ATATTATTE ACCE ATT C BE ATATTATAE AAATATTTE TO TO COATTITE TAACE TO TTAE G ACAT C CCE TTAE TAAE CCAE TO TEGE CCE ATTITAT CCE ATTITE ACCE ATTICE G ATATTATE G AAATATTTE TO TO COATTIE COATT	1243 798 745 745 747 747 747 747 747 747 747 748 828 831 846 837 815
U61326.1.Calluna vulgaris_complete CDS HM850861.1.Calluna vulgaris_Portugal HE 966891.1.Calluna vulgaris_Portugal HE 966891.1.Calluna vulgaris_TriestehR. Valerio_kaly HE 966899.1.Calluna vulgaris_TriestehR. Valerio_kaly KX821257.1.Calluna vulgaris_Lodbjergfyr_Denmark KX821259.1.Calluna vulgaris_Lodbjergfyr_Denmark KX821259.1.Calluna vulgaris_Lodbjergfyr_Denmark KX821259.1.Calluna vulgaris_Lodbjergfyr_Denmark JK85808.1.Calluna vulgaris_Lodbjergfyr_Denmark KX821259.1.Calluna vulgaris_Lodbjergfyr_Denmark JK85808.1.Calluna vulgaris_Lodbjergfyr_Denmark JK85808.1.Calluna vulgaris_Lodbjergfyr_Denmark JK85808.1.Calluna vulgaris_Lodbjergfyr_Denmark JK85808.1.Calluna vulgaris_Lodbjergfyr_Denmark JK85808.1.Calluna vulgaris_Lodbjergfyr_Denmark JK85808.1.Calluna vulgaris_Lodbjergfyr_Denmark JK85808.1.Calluna vulgaris_Lodbjergfyr_Denmark JK85808.1.Calluna vulgaris_Lodbjergfyr_Denmark JK85808.1.Calluna vulgaris_Dedbjergfyr_Denmark JK85808.1.Calluna vulgaris_Dodbjergfyr_Denmark JK85808.1.Calluna vulgaris_Dodbjerg	1136 C G AAAT TITT G TAA C G T O TTAB G ACAT C C C G TTAB TAA C CCA T O TG G C C G ATT TAT C C G ATT C TG AT ATTATT G AC C G ATT C C G ATT AT AT A G AAAT ATT T O T C 601 C G AAAT TITT G TAA C G T O TTAB G ACAT C C C G TTAB TAA C CCA G T O TG G C C G ATT TAT C C G ATT C TG AT ATTATT G AC C G ATT C C G G C TAT AT AT A G AAAT ATT T O T C 601 C G AAAT TITT G TAA C G T O TTAB G ACAT C C C G TTAB TAA C CCA G T O TG G C C G ATT T 601 C G AAAT TITT G TAA C G T O TTAB G ACAT C C C G TTAB TAA C CCA G T O TG G C C G ATT T 601 C G AAAT TITT G TAA C G T O TTAB G ACAT C C C G TTAB TAA C CCA G T O TG G C C G ATT T 601 C G AAAT TITT G TAA C G T O TTAB G ACAT C C C G TTAB TAA C CCA G T O TG G G C C G ATT T 601 C G AAAT TITT G TAAT G T G G TAB G T CAT C C C A C G AG T C C G G C C G O TTAT T C AG ATT C T G AT ATTAT T G AAC G ATT C C G G C G T AT AT AT A G AAAT C T T T C C G 600 C G AAAT TITT G TAAT G T G T AG G T C AT C C A C G AG T A C C G G T O T G G G C C G O T T T AT C AG AT T C T G AT AT T T T T A AC C G AT T C G G C C G T AT AT AT A G AAAT C T T T C G C 600 C G AAAT TI T G T AAT G T G G T AG G T C AT C C A C G AG T A C C G G T O T G G G C C G T T T AT C AG AT T C T G AT AT T T T T T A AC G AT T C G G C C T AT AT AT A G AAAT C T T T C G C 600 C G AAAT TI T G T AAT G T G G T AG G T C AT C C A C G AG T AA C C G G T T T AT C AG AT T C T G AT AT T T T T T T T T T T T T T T T	1243 798 745 746 747 747 747 747 747 747 747 748 831 846 831 846 837 815 816
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Figure 3.1.3.1.1 c. Clustal alignment of all Calluna vulgaris MATK nucleotide sequences available from NCBI at the time of writing with sequences from study samples. Sample 173 (previous identified as incorrect – section 3.2.2) has been removed from the analysis. The NCBI full coding sequence is only showing the section of sequence comparable to the other sequences, starting at base 391. Names are in the form genbank Code (NCBI sequences) or species code (study samples)\_Species\_Location (where known)

# **3.2 Discussion**

# 3.2.1 CBF Sequences

# 3.2.1.1 CBF Amplification

There are several possibilities as to why CBF sequences could not be amplified from numerous species. One possible factor is DNA quality; one of the risks with samples from highly freezing tolerant species being DNA degradation in the -80 °C freezer. Many arctic species can survive when exposed to temperatures of -80 °C and below (Yamori et al., 2005, Stushnoff and Junttila, 1986), including several of the species sampled. Prior to acclimation, freezing tolerance is greatly reduced (Yamori et al., 2005). However, samples collected included species about which there are little to no data on their freezing tolerances and the impact of acclimation. Samples were collected in July at a point when late frosts were over and plants were most likely to have deacclimated. All DNA extracted showed single clear bands when run on an agarose gel, indicating that no DNA degradation had occurred and therefore -80 °C was sufficient to stop or greatly slow metabolic activity in the collected samples. Therefore, DNA degradation is unlikely to have resulted in the failure to isolate CBF sequences. Another possibility is that of contaminants inhibiting PCR: Arctic and alpine species have a propensity to contain high levels of phenolic compounds, likely due to the environmental pressures such as cold, UV and herbivory which can all increase plant phenolics (Semerdjieva et al., 2003, Rivero et al., 2001, Hartley and Firn, 1989), which can inhibit PCR reactions (Maltas et al., 2011, Paterson et al., 1993, Guillemaut and Maréchal-Drouard, 1992, Couch and Fritz, 1990). The KAPA3G Plant PCR Kit claims to contain a novel DNA polymerase with improved tolerance to PCR inhibitors such as polyphenolics and polysaccharides (KapaBiosystems, 2015). Many samples that previously did not produce any amplification with normal TaQ polymerase did indeed amplify with KAPA3G plant polymerase, indicating that PCR inhibitors were present in many DNA samples. However, *CBF* sequences still could not be amplified from numerous species indicating that either the inhibitor content might have been too high or that other factors were also affecting amplification.

Another possibility are the primer sequences themselves: The majority of collected samples did not have previously sequenced CBF genes or even members of the same genera with previously sequenced CBFs. Therefore, primers had to be designed based on *CBF* sequences from familial relatives of samples or, occasionally, a higher taxon level. Primers were designed to the 5' and 3' end of known CBF coding sequences. The 3' and 5' ends are not highly conserved, even between family members (e.g. Fig.2.5.1.1). The 5' end, to which the forward primers are designed, has not been identified as important in *CBF* function, hence mutations in this region are less likely to affect protein function and this region is, therefore, not likely to be subject to active selection to minimise mutations. The 3' terminus, to which reverse primers were designed, has been identified in likely having gene activation function in *CBF* proteins (Wang et al., 2005). However, it shows high functional redundancy and limited conservation (Wang et al., 2005), (primer design alignments – data not shown). This high variation in the 3' and 5' regions reduces the probability of having similar 3' and 5' CBF sequences between differing species (the probability decreasing further as species diverge). Therefore, primers designed to these ends have a decreasing probability of amplifying *CBF* sequences the further diverged the test species is from the species the primers were designed from. This likely explains the majority of failed PCR reactions.

# 3.2.1.2 CBF Sequence Quality

Several sequences failed quality control due to poor sequence data (data not included). There were several probable reasons for these failures, namely copy number, ploidy and heterozygosity. Typically, sequences failed due to multiple overlapping base peaks, the

pattern displayed being typical of several sequences overlapping (Personal Obs.). This is highly likely to be occurring given that different genes from the CBF family are often around the same size (e.g. Arabidopsis thaliana CBFs 1-4) and that the sample species are wild and therefore not necessarily homozygous (they may also have a large ploidy number). This is further supported by raw BLASTX (Altschul et al., 1990) data (data not shown) where the majority of sequences that failed quality control all still showed similarity to CBF sequences. Despite the inaccuracy of automatic base calling in these sequences it is likely that small sections of conserved identifiable regions, were sufficiently clear across the multiple overlapped sequences which the BLASTX (Altschul et al., 1990) search was able to detect. Those that did not show similarity to CBFs with a BLASTX search (Altschul et al., 1990) typically did not show any similarity to anything in the database. There are a couple of possibilities for the overlapping sequence effect in these cases. The overlapping sequences could be multiple *CBF* family genes that happen to differ slightly in size, due to insertions or deletions, but insufficiently different to be seen on a gel, this would result in misalignment of the conserved recognisable areas so raw data would not show these areas clearly. The multiple sequences could also be other similar size sections of non CBF family genes also amplified by the primers, but the likelihood of these other DNA sections being the same size as the expected CBF product is low. The multiple sequences could also consist of *CBF* gene relatives of similar size (such as other members of the AP2 family) or a CBF gene paralogue which has not required maintenance of the typical conserved regions due to taking on a different genetic function. It was thus expected that sequencing PCR products from wild samples with primers designed to amplify members of a gene family would produce overlapping sequences, as appears to have occurred. However, by sequencing straight from PCR products, samples with multiple sequences in these products could be identified for future isolation of sequences via cloning.

### **3.2.1.3** *CBF* Nucleotide Sequences

There is a great deal of variation in the nucleotide sequence of *CBF*s, isolated from various samples (Fig. 3.1.1.3.1 a-c, Fig. 3.1.1.2.1 a-b and Fig. 3.1.1.1.1). When these sequences are sorted via Clustal (Larkin et al., 2007) they form family groups (Fig. 3.1.1.3.1 a-c, Fig. 3.1.1.2.1 a-b and 3.1.1.1.1). Indicating that the difference in *CBF* nucleotide sequence has more to do with evolutionary relatedness of the species than freezing tolerance. This familial grouping also indicates that the various *Arabidopsis thaliana CBF*1-4 have more in common with one another and other Brassicaceae *CBF*s than *CBF*s from different families, indicating the divergence of *CBF* 1-4 occurred after the split of the Brassicaceae from the other families studied here, as supported by Xiong and Fei (2006).

It should be noted that some species identifications are tentative (those marked with a "?") and therefore their species identification is not certain, although the genus is highly likely to be correct. In some cases, this is due to the presence outside of the expected distribution of that plant. In others, slight morphological differences (possibly due to environmental stress or subspecies) hindered identification and several identifications were made upon vegetative tissue only further hindering identification at the species level.

#### **3.2.1.3.1 Brassicaceae**

Unlike Xiong and Fei, (2006) *CBF*1-3 of *Arabidopsis thaliana* did not group together separately from all other Brassicaceae. However, it should be noted that Xiong and Fei, (2006) only used the species *Brassica napus* and *Capsella bursa-pastoris*. The *Cardaminopsis arenosa* sequences from this study are interspersed between *Arabidopsis thaliana CBF*1, 2 and 3 showing greater similarity to individual *A. thaliana CBFs* that to other *CBF* sequences from the same species (Fig. 3.1.1.1), indicating that *CBF1-3* divergence occurred before the split of these two species. This fits with some

phylogenetic studies, suggesting *Cardaminopsis* is nested within *Arabidopsis* genus (Yang et al., 1999). Indeed *Cardaminopsis* has recently been moved to the *Arabidopsis* genus (Kew, 2013). It therefore seems likely that the split of *CBF*1, 2 and 3, and their differentiation, occurred within the *Arabidopsis* genus before speciation of *A. thaliana* from *Cardaminopsis arenosa* (aka. *Arabidopsis arenosa*).

*Cardaminopsis arenosa* also has a *CBF* sequence which is identical to one from *Capsella bursa-pastoris* and highly similar to one from *Arabis alpina* that is not seen in *Arabidopsis thaliana* (Fig. 3.1.1.1). Since *Arabidopsis thaliana* is fully genome sequenced, that it does not have this sequence is not due to a failure to isolate it. That this sequence is present in multiple genera, including *Arabidopsis* (to which *Cardaminopsis* is now asigned) but not *Arabidopsis thaliana* suggests that it was possibly an ancestral sequence which has been lost or heavily modified in *Arabidopsis thaliana*.

A sequence was isolated from *Barbarea vulgaris* which had high similarity to *Arabidopsis thaliana CBF*4. Both these sequences were distinctly different from all other sequences (Fig. 3.1.1.1). It is therefore likely that these two sequences share a common ancestor, likely branching before *A. thaliana CBF* 1-3 differentiated from one another, this is supported by Xiong and Fei, (2006) who found that *Arabidopsis thaliana CBF*4 differentiated before the split of *A. thaliana CBF* 1-3. It is therefore possible that, like *Arabidopsis thaliana CBF*4 (Haake et al., 2002), the *CBF* isolated from *Barbarea vulgaris* by primer pair 60 is induced by drought rather than cold, assuming that the change in regulation occurred early in the differentiation of *CBF*4 from *CBF*1-3.

The Brassicaceae family had more *CBF* sequences per species than all other families studied. However, this is likely to be an artefact of the ability to produce primers to already known *CBF* sequences, as this family has a great deal of genetic study performed on it as well as containing multiple crop species and the model plant species *Arabidopsis* 

*thaliana*. Eighteen primer pairs were produced for the Brassicaceae family, whereas only 1-2 primer pairs could be produced for the majority of the other families (for example Ericaceae only had one primer pair that could be designed for amplification of *CBF*).

#### **3.2.1.3.2** Ericaceae

There is less variance between sequences in the *Ericaceae* family than in the *Brassicaceae* family. This is likely due to 1 primer pair being used to isolate and amplify *CBF* as opposed to 18 primer pairs in Brassicaceae rather than being truly representative of less diversity within the Ericaceae.

Four subtly different, but highly similar, CBF sequences were isolated from Andromeda polifolia sample 43. This is unlikely to be due to different alleles as they would show as one singular band when run on a gel with overlapping sequence data resulting in degenerate bases. However, gene duplication in tandem could result in the different banding patterns seen but similar sequencing results; amplification may continue past the end of one *CBF* gene into another resulting in different reannealing patterns. This would explain the banding pattern seen namely a band of expected size (1 CBF strand + 1 CBF strand) a larger band (a 1  $\frac{1}{2}$  size band: 1 *CBF* strand + double *CBF* strand) and an even larger band (a 2X band: double CBF strand + double CBF strand) and possibly a fourth lager band (1 CBF strand + 3 CBF strands). Only bands between ~400-1500bp were sequenced so if more than 2-3 repeats existed, they would not have been sequenced, nor were they likely to be amplified due to a typical *CBF* sequence being  $\sim 600$  bp long and the probability of successful amplification dropping off as length increases. This would also explain why, when sequenced, all bands of different sizes gave sequence products of the same size and highly similar sequence. The occasional different base-pair would not disrupt sequencing and, given that typical sequence reads were less than 600bp long (the forward and reverse being amalgamated to form the full sequence), if sequences were sufficiently similar (as implied) the beginning of 1 CBF may have been combined with

the end of a second *CBF* in the tandem sequence. Tandem arrangement of multiple *CBF*s is a likely possibility as tandem alignment of varying copy number of *CBF* genes is common for example such as seen in *Arabidopsis thaliana* (Gilmour et al., 1998, Medina et al., 1999), *Medicago truncatula* (Tayeh et al., 2013) *Lolium perenne* (Xiong and Fei, 2006) *Prunus perscia* (Wisniewski et al., 2011) and *Triticum monococcum* (Miller et al., 2006). Frequent duplication of the *CBF* gene is also believed to have been a common occurrence as supported by the *CBF* phylogenetic study by Xiong and Fei, (2006). Greater *CBF* copy number has been linked with increased winter hardiness in barley and wheat cultivars (Knox et al., 2010, Wurschum et al., 2017) so multiple *CBF* copy numbers in highly freezing tolerant plants (as studied here) would be likely.

A similar situation as seen in *Andromeda polifolia* (namely multiple copy number of *CBF* in tandem) is likely to also be occurring in *Empetrum nigrum* as multiple banding patterns were also observed for this species with all bands giving similar sequence reads. If multiple copy number of *CBF* increases winter hardiness, as suggested by Knox et al., (2010) and Wurschum et al., (2017) this could also explain the association of tetraploids of *Empetrum nigrum* (often identified as *Empetrum nigrum subsp. hermaproditum*) with more cold environments (see section 5.2.6 "A note on *Empetrum nigrum* subspecies") as an increase in ploidy level would be a rapid way to increase *CBF* copy number and would certainly merit further study.

That the sequences from *Arctostaphylos alpina* and *Diapensia lapponica* were identical to one of the *Empetrum nigrum* sequences is highly suspicious, especially given that *Diapensia lapponica*, although closely related to the *Ericaaceae*, is not a member of the Ericaceae (included in the Ericaceae lineup here due to identical sequence). This is the same sequence as found in the *Calluna vulgaris* samples (removed and not shown) that showed 100 % identity to *Empetrum nigrum* MATK sequences. MATK sequences were not successfully checked from these two samples, so it is therefore possible that they
suffer from the same contamination as some of the *Calluna vulgaris* sequences and therefore the validity of their *CBF* sequence is highly suspect.

The high similarity in *CBF* sequence between the two *Vaccinium* species would indicate that the *CBF* gene sequenced has a recent common ancestor however differentiation has occurred between the species.

#### 3.2.1.3.3 All Other Species

There is a great deal of variance in nucleotide sequence between different species from different families. Given the variance seen within families (see section Ericaceae and Brassicaceae) this high variance across families is unsurprising.

The *Trifolium repens* sequence containing an 18 base insert not seen in any other species but seen across *Trifolium repens* samples from both Sweden and the UK indicates that this insert is not a recent mutation that has occurred and is specific to a local area, but that it has been retained by *T. repens* from differing locales alongside *CBF* sequence(s), in the same plant, without this insertion. This would indicate that, if it does not have functional activity, it is not a detrimental mutation. That the insert is 18 bases long (divisible by 3) indicates that it may be functional as no shift in reading frame occurs as a result. (This mutation is further discussed in the translated sequence section).

There has been no differentiation in the *CBF* sequence between *Fragaria vesca* from the UK and Sweden, indicating that no modification, to this particular sequence has occurred in response to differing environments.

Two highly distinct (both form one another, and other sequences) *CBF* sequences were isolated from *Salix herbacea*. Although distinct from *CBF* sequences isolated within this project a high similarity to *CBF* sequences from *Populus* species was found when MegaBLAST was performed (Morgulis et al., 2008). *Populus* is a highly studied genus within the *Salicaceae* of which *Salix herbacea* is another member. Unlike most other

families (where 1-2 was standard (section 2.5.1.1)) 7 primer pairs were able to be produced to attempt to amplify *CBF* from members of the *Salicaceae*. This is likely why two highly different sequences were able to be isolated from *Salix herbacea*, as with members of the Brassicaceae (see section 3.2.1.3.1), the greater number of primers is likely the cause of the isolation of very different *CBF* sequences from the same species rather than these species being the only ones with multiple highly differentiated *CBF* sequences.

All Matricaria recutita sequences, from the same sample, are very similar bar some degenerate bases and variation at the start and end of the sequence. This could either indicate that there are several CBF sequences with subtly different initial and terminal sequences (that result in different primer pairs binding) or that all primer pairs bound to the same sequence but there are some errors in the sequence read. Only the reverse sequence amplified by primer pair 5 produced a clear useable sequence read and this is also the sequence with apparent insertions in the sequence briefly changing the reading frame, it therefore seems likely that this is due to poor sequence reading towards the end of the reverse sequence (i.e. the start of the CBF sequence) rather than true insertions. However, the other two sequences read well in both the forward and reverse directions. Since all sequence chromatograms were manually checked for degenerate bases and correct basal calls, it seems more likely that there are at least two highly similar CBF sequences where different primers preferentially bind to one sequence over another. Given that the sequence amplified by primer pair 6 has the same sequence as that amplified by primer pair 17 but with extra degenerate bases (one base of which matches the other sequence amplified by primer pair 17) it is feasible that primer pair 6 is amplifying the same sequence as primer pair 17 alongside another similar but subtly different sequence.

*Leucanthemum vulgare*, had a *CBF* sequence very similar to that of sample *Matricaria* recutita. Tthis is unsurprising since these are closely related species and both members of the Anthemideae (within the Asteraceae family).

The was a large degree of failed amplification of *CBF* genes from DNA, only successfully amplified sequences which passed quality control are shown in Fig. 3.1.1.3.1 a-c, Fig. 3.1.1.2.1 a-b and Fig. 3.1.1.1.1. No successful amplification was achieved from all other species (Table 2.1.5). The large degree of failed amplification is likely due to the unavailability of sequences of closely related species from which to design primers. As evident from these line ups (Fig. 3.1.1.3.1 a-c, Fig. 3.1.1.2.1 a-b and Fig. 3.1.1.1.1) there is a great deal of variation in nucleotide sequence and therefore the design of primers to the beginning and end sequence of the CBF which can amplify non-closely related species was not possible. Primers which were used to attempt to isolate CBF sequences from species greater than family level always failed. That there is very little conservation between the beginning and end of the sequence indicates that these regions undergo a great deal of differentiation. If, as discussed in section 3.2.1.3.2, copying of CBF genes appears to be common, that would therefore result in more opportunities for variation to occur within the copied genes. Also, as previously discussed, where multiple primers were available different CBF sequences were often isolated and therefore that some families only had one primer pair available means that it is likely that other CBF sequences were not isolated.

#### **3.2.1.4 Translated CBF Sequences**

\*Note: as with the results section, comparisons (unless stated otherwise) are made against *Arabidopsis thaliana* CBF1. Therefore, the terms substitution, insertion or deletion are used simply relative to *Arabidopsis thaliana* CBF1; they do not have any evolutionary significance.\*

There are two known domains which predominately impact upon the activity of CBF. One of these is the COOH terminus consisting of clusters of hydrophobic residues forming loops, involved in activation activity with high functional redundancy (Wang et al., 2005). Of greater interest, due to the apparent lack of redundancy, is the COR binding domain consisting of; the AP2/ERF domain (common to all AP2 gene family members) (Sakuma et al., 2002) and the CBF flanking domains (specific to CBF); PKKP/RAGRxKFxETRHP (PKKPAGR) and DSAWR sequences (Canella et al., 2010). Single amino acid mutations in the COR binding domain can significantly impact on binding to the CRT/DRE element (Canella et al., 2010, Sakuma et al., 2002). Canella et al., (2010) tested multiple single amino acid mutations within the RKKFR motif (a region predicted to form an  $\alpha$ -helix) of the PKKPAGR sequence. K(8) to R (conserved) and A (non conserved), K(9) to R (conserved) and A (non conserved) and R(11) to S (non conserved); all had no significant effect on binding (Canella et al., 2010). Substitution of R(7) with either K (conserved) or S (non-conserved) significantly impaired binding. Substitution of F(10) with A or P (non-conserved) abolished binding and substitution with Y (conserved) greatly enhanced binding (Canella et al., 2010). Another amino acid that was found to be key to binding is V(14) in the AP2/ERF domain, a conserved substitution to A which prevents binding to the DRE/CRT element of COR genes (Sakuma et al., 2002). Therefore, naturally occurring amino acid substitutions within the CBF flanking sequences, and to a lesser extent the AP2/ERF domain, were of interest for their potential effect upon CBFs ability to bind, and therefore activate COR genes.

One natural substitution which was found in this study, was a substitution of one of the amino acid residues tested by Canella et al., (2010), namely R(11). Canella et al., (2010) artificially substituted this R with S (non conserved) which had no significant effect on binding. However, the naturally occurring substitution was a conserved substitution of R to K. This was found in all non-Brassicaceae, bar one of the *Salix herbacea* sequences

which also had R (Fig. 3.1.2.2). This substitution was therefore selected for further study. Other naturally occurring substitutions of interest were found in the DSAWR flanking region, which had not previously been tested for CRT/DRE binding efficiency. These substitutions (relative to *Arabidopsis thaliana* CBF1) are highlighted in Table 2.5.2.1. With the exception of R to Q (only seen in *Vaccinium vitis-idaea*) all substitutions in this region are conserved substitutions (Fig. 3.1.2.2).

#### **3.2.1.4.1 Full CBF Sequence Comparisons**

All translated sequences display the CBF flanking signatures (Fig. 3.1.2.2) indicating that all sequences amplified were CBF sequences and were not other AP2 proteins. Whilst the PKKPAGR sequence of *Matricaria recrutita* amplified by primer pair 5 does not match what is expected it is more likely that this is due to the aforementioned read problems which resulted in apparent base insertions (Section 3.2.1.3.3) rather than being an entirely different product.

The majority of difference seen between sequences is in the COOH terminus. Wang et al., (2005) found that there was high functional redundancy in the COOH region where a large number of mutations and disruption of several of the hydrophobic loops were required in order for any impact on activation of *CBF* target genes to occur. Therefore, high variance in this region and even large areas of deletions are unlikely to have a significant effect and similarity is more likely to indicate genetic relatedness than a relatedness in activity. Due to this high redundancy the COOH terminus was disregarded for further study.

Given the greater similarity of *Arabidopsis thaliana* CBF1-3 to one another than to other CBF sequences, and *A. thaliana* CBF4 having greater similarity to *A. thaliana* 1-3 than some other Brassicaceae sequences (namely: *Cardaminopsis arenosa* 66pp77 and *Arabis alpina* 69pp77 and *Capsella bursa-pastoris* 68pp75 ) and all other species sequences, no

assumptions about activity can be made based upon structural similarity to *A. thaliana* sequences.

#### **3.2.1.4.2 AP2 and CBF Signature Flanking Sequences**

The CBF signature sequences and AP2 domain (the sequence between the CBF signature sequences) was highly conserved across all species (Fig. 3.1.2.2). One exception is the six base insertion found in some CBF sequences from Trifolium repens. All Trifolium repens samples sequenced had a CBF sequence with this insertion as well as a CBF sequence without this insertion. This included samples from both Abisko, Sweden and the North Pennines, UK. This indicates that this CBF with an insertion is common and not a recent local adaptation. That this gene seems to be widespread indicates that it is of at least no negative influence upon *Trifolium repens*. It is unlikely that this insertion improves freezing tolerance as the LT50 of Trifolium repens is around -16 °C after acclimation (Svenning et al., 2001, Olsen et al., 2008) this is a much higher temperature than several other species studied here which do not have this insertion (Stushnoff and Junttila, 1986, Yamori et al., 2005). However, it is possible that this gene does have retained functionality given that the insertion maintains the reading frame. How this functionality is affected however is unknown. Binding tests (such as electrophoretic mobility shift assays as exemplified by (Canella et al., 2010)) would have to be performed, synthetic CBF genes for transient and stable transformation could also be produced and tested (as described in sections 2.5.2 and 2.8) to analyse the effect of this insertion. However, since this was unlikely to improve freezing tolerance (as previously discussed), and areas of greater interest were identified this was not studied further within this project.

V(14 - counting from the start of the AP2 domain) in the AP2/ERF domain previously identified as key for CBF binding to the DRE/CRT element of *COR* genes (Sakuma et al., 2002) was conserved across all sequences (Fig. 3.1.2.2).

That CBF4 in Arabidopsis thaliana has two unconserved amino acid substitutions in the AP2 & CBF signature sequences (an R instead of P, 4 amino acids into the CBF signature sequence and P instead of Q, 55 amino acids in) relative to CBF1-3 is likely a reflection of its evolutionary history; diverging from the progenitor of CBF1-3 prior to the differentiation of CBF1-3 (Xiong and Fei, 2006) (and as discussed in section 3.2.1.3.1). That the majority of sequences from all species had R (4) rather than P(4) and P(55) rather than Q(55) (Fig. 3.1.2.2) could indicate that R(4) and Q(55) is the ancestral state i.e. that P substituted R (4) and Q substituted P (55) in the progenitor of A. thaliana CBF1-3 after the split of the progenitor of A.thaliana CBF1-3 from CBF4. Therefore, Cardaminopsis arenosa 66pp66, pp69 and Capsella bursa-pastoris 68pp 71 and 68 sequences (all with the P(4) and Q(55) substitution) likely descended from the same progenitor as A. thaliana CBF1-3 after these substitutions occurred and that Cardaminopsis arenosa 66pp77 and Capsella bursa-pastoris 68pp75 and 76 descended from a different progenitor prior to these substitutions arising. (It should be noted that sequence data does not cover the 4<sup>th</sup> base of the CBF signature sequence for sequences Cardaminopsis arenosa 66pp67 and Capsella bursa-pastoris 68pp61. It is therefore unknown which amino acid is at this location). This evolutionary history is supported by the similarity in nucleotide sequences. However, these substitutions have likely occurred more than once as *Diapensia lapponica* 2pp22, Empetrum nigrum 5 TOPO(a-c) and Arctostaphylos alpina 3pp22 (Fig. 3.1.2.2) also have the P(4) rather than R(4) but not the Q(55) rather than P(55) substitution, the rest of the sequence (both amino acid and nucleotide) also shows greater similarity to other members of the Ericaceae than to any member of the Brassicaceae indicating a separate substitution event. Trifolium repens 77pp19, 58pp19, 94pp19 conversely has the

Q(55) substitution rather than the P (55) substitution but not the P(4) rather than R(4) substitution indicating a separate mutation event for p(55) to Q(55). The three *Trifolium* repens sequences with an insert in the AP2 domain 58pp13, 77pp13 and 94pp13 also displayed a substitution not seen in any other at the R(4) locus; a conserved (relative to R) substitution to K (Fig. 3.1.2.2). Multiple substitution events at this site suggests that either this is a locus of little import, where substitutions have little to no effect upon binding ability of CBF or, since the same substitutions occur multiple times, it is more likely that there is convergent evolution at this site and that these substitutions do confer a different binding behaviour or protein stability. Unfortunately, no comparison between the binding strength or stability of CBF4 vs CBF1-3 in A. thaliana has been undertaken by way of comparison. There is, however, no link between these substitutions and the freezing tolerance of the species with these mutations. The presence of these substitutions alongside CBFs without these substitutions within the same plant combined with the lack of a link between these substitutions and the freezing tolerance of the species studied, indicated a lack of role in altering freezing tolerance. Therefore, substitutions more likely to affect freezing tolerance were preferentially studied and these particular substitutions (described above) were not further investigated.

As well as the aforementioned P/R(4) site common substitutions, K(10) also seems prone to substitutions namely the unconserved substitution to I and V seen in some *Brassicaceae* and *Trifolium repens* sequences. This is in the RKKFR motif predicted to form an  $\alpha$ -helix (Canella et al., 2010) however conserved (R) and non-conserved (A) substitutions performed at this position in *Arabidopsis thaliana* CBF1 were previously found to have no significant effect on binding (Canella et al., 2010). It seems therefore likely these naturally found substitutions also have no effect upon binding.

That all species studied excluding some members of the Brassicaceae (including all *Arabidopsis thaliana* sequences), one of the *Salix herbacea* sequences and the

*Leucanthemum vulgare* sequence, have K rather than R at amino acid 12 is extremely interesting. Canella et al., (2010) performed a non-conserved substitution of this amino acid in *Arabidopsis thaliana* CBF1 which resulted in no change in binding. However, no conserved substitution, as observed, was tested. Given that substitution of the neighbouring F with a conserved amino acid greatly enhanced binding (Canella et al., 2010) it is possible that this conserved change could influence binding and therefore the level of activation of target genes. This substitution was therefore selected for further analysis and generation of a synthetic *Arabidopsis thaliana* CBF1 gene containing this substitution performed.

#### **3.2.1.4.3 The C-Terminus CBF Flanking Sequence**

The CBF flanking sequence (known as DSAWR due to the sequence in *Arabidopsis thaliana*) has not been previously studied with regards to how substitutions may affect binding. The majority of the sequence was highly conserved across all species. D, S and W were always conserved perhaps indicating that these amino acids cannot change without negative effects upon activity.

A was mostly conserved across all species, however two conserved amino acid substitutions were seen, to V (All Ericaceae, *Diapensia lapponica* and *Matricaria recrutita*) and L (*Leucanthemum vulgare*) (Fig. 3.1.2.2). The effect of this conserved substitution, if any, cannot be predicted, however given that conserved substitutions in the N-terminus AP2 flanking CBF signature sequence have been associated with increased binding (Canella et al., 2010) these substitutions were therefore investigated further via the generation of a synthetic *Arabidopsis thaliana CBF1* gene containing these substitutions.

R was conserved across all species except for *Vaccinium vitis-idaea* which had a nonconserved substitution of Q at this site. Previous studies of *Vaccinium vitis-idaea* with

this substitution found that this substitution was not present in Chinese samples of V. vitis*idaea* (Oakenfull et al., 2013). That it is present in both Swedish and UK samples (Fig. 3.1.2.2) indicates that this is a mutation that has been maintained across multiple related populations. Interestingly Oakenfull et al., (2013) found that V. vitis-idaea CBF (containing the Q substitution) had greatly reduced ability to activate COR genes in Arabidopsis thaliana, whereas the almost identical sequence of V. myrtillus could (Oakenfull et al., 2013). Further Vaccinium vitis-idaea is a highly freezing tolerant species, with no decrease in survival, once acclimated, at -80 °C (Yamori et al., 2005). However, the plant tested by Yamori, et al., (2005) to identify this tolerance was from Mt. Iwo, in the Yatsugatake mountains, Japan (2760 m above sea level) and the CBF amino acid sequence is unknown. Vaccinium vitis-idaea from Norway was found to be hardy (LST 66) to -35 °C in December months (Stushnoff and Junttila, 1986). It is likely that this sample does have the Q substation due to the proximity to Swedish samples. It is therefore possible that the Q substitution does reduce the efficacy of CBF, especially if it is assumed that the Japanese sample, similarly to the Chinese V. vitis-idaea does not contain this substitution. However, that other factors involved in freezing tolerance, such as upstream and downstream genes, other CBF genes, or other parts of the Vaccinium vitis-idaea sequence do result in a high freezing tolerance, even upon reduction in the activity of that particular CBF. Further, it should be noted that two different methods were used to assess freezing damage in *Vaccinium vitis-idaea*; Yamori et al., (2005) used the FDA staining method whereas Stushnoff and Junttila, (1986) used visual estimation and they are therefore they are not directly comparable. The effect of this Q substitution was therefore investigated further, both with the associated A -> V substitution and without, via the generation of a synthetic Arabidopsis thaliana CBF1 gene containing this substitution.

#### 3.2.2 MATK Sequences

There was a strong indication that all *Calluna vulgaris CBF* sequences isolated were in fact contaminated with *Empetrum nigrum* DNA, as surmised from the matching *CBF* sequences. Upon amplification of *MATK* from suspected contaminated DNA this was shown to be the case: the *MATK* sequence isolated from *Calluna vulgaris* DNA extracted prior to a certain date had 100 % identity to *Empetrum nigrum MATK* sequences and differed greatly from *MATK* sequences isolated from *Calluna vulgaris* DNA extracted after that date (Fig. 3.1.3.1). Therefore, all *CBF* sequences isolated from likely contaminated *Calluna vulgaris* samples were discarded. *CBF* could not be isolated from *Calluna vulgaris* DNA extracted after the suspected contamination date, which had accurate *MATK* sequences, further indicating that contamination had indeed occurred in the previous sample. It also seems likely that the two other samples with 100 % *CBF* sequence identity to *Empetrum nigrum (Diapensia lapponica* and *Arctostaphylos alpina)*, (Fig. 3.1.1.2.1) also sequenced around the same time as the *Calluna vulgaris* contaminated samples, but upon which *MATK* sequencing was not performed, were also contaminated and therefore should be disregarded from analysis.

The *Calluna vulgaris MATK* sequence from later DNA samples did not show 100 % identity to the GenBank *MATK* sequence (U61326.1) (Fig. 3.1.3.1) therefore these differences were investigated further and compared to more published *Calluna vulgaris* sequences. Upon comparison, two groupings of *MATK* sequences were found for *Calluna vulgaris* one common to samples from the UK, Denmark, Sweden and Norway and another from Portugal and Italy which matched the original reference; indicating two distinct and diverging populations. The original reference sequence, which matched sequences from Portugal and Italy was, however, recorded as being wildtype *Calluna vulgaris* coming from the Royal Botanic Gardens Edinburgh (Kron, 1997) this was

incorrectly recorded by the author and the specimen was not wildtype but was the cultivar "Rannoch" (RBG Edinburgh, 2018b). The similarity of the *MATK* sequence of the Rannoch cultivar to wild *Calluna vulgaris* from Portugal and Italy and dissimilarities to those from the UK and Scandinavia (Fig. 3.1.3.1) indicates that the cultivar has ancestry from *Calluna vulgaris* plants outside of the UK or Scandinavia, possibly from southern Europe. The more recent DNA extractions from *Calluna vulgaris* having 100 % match to other *Calluna vulgaris* MATK sequences from Scandinavia and the UK further confirmed that the recent DNA extractions from *Calluna vulgaris* were indeed from *Calluna vulgaris* samples and are not DNA contamination by another species.

The GenBank *Phyllodoce caerulea* sequence (U61318.1) had an extra G insertion not present in any other MATK sequences including the collected sample of *Phyllodoce caerulea* (Fig 3.1.3.1b). It therefore seems likely that this G insertion is due to a sequence read error, especially given that that it occurred immediately after another G strongly indicating that the automatic base calling software read one peak as two, and that this was not corrected by the author. Eight bases downstream of this insertion in the GenBank sequence (U61318.1) there is a deletion of T. It therefore seems likely this is also another error, possibly due to a slight misalignment in the base-calling program used. This miscalling can frequently occur, especially a misread of one large peak as two followed by the skipping of a later peak (pers. Obs.). Manual checking of all base calls on all sequences were made in this project to alleviate this risk (as well as checks for degenerate bases). Other differences between the GenBank sequence and collected sequence occur toward the end of the amplified fragment, these are substitutions rather than insertions and deletions so are more likely to be accurate. In both the MATK from collected *Phyllodoce caerulea* and the Genbank sample there are sections that differ from one another but match other MATK sequences (Fig. 3.1.3.1). Given that MATK sequences from plants from different locations can vary (see Calluna vulgaris MATK above) it is

possible that the Genbank sequence comes from a plant from a different location than the collected sample (Norway). Unfortunately this is unknown, as the Genbank sample was collected from a cultivated plant at the RBG Edinburgh (Kron, 1997) however no origin data on the plant exists (RBG Edinburgh, 2018a).

The 100 % identity between *Cassiope tetragona* from Abisko, Sweden, and the Genbank sequence (KC474423.1), from Canada (Fig. 3.1.3.1) indicates that these two populations have not greatly diverged.

The 100 % identity between all *Empetrum nigrum* sequences indicates very little divergence between these populations (Abisko, Canada and North America). Interestingly there is also 100 % identity with two plants identified as *Empetrum hermaphroditum* (now typically not recognised as a second species but as the subspecies *Empetrum nigrum subsp. hermaphroditum*). It is notoriously difficult to distinguish between the two subspecies (and the method of identification is also debated) (ee section: 5.2.6) so it is possible that these two specimens have been mis-identified (note: no attempt was made to distinguish between the two subspecies in samples collected in this project, see: section 5.2.6 for justification). Alternatively, these results might indicate that these are not separate subspecies, showing greater similarity to one another than different populations of the same species (*Calluna vulgaris*) do to one another (Fig. 3.1.3.1). For further discussion on *Empetrum nigrum* subspecies please see: Section 5.2.6.

### Chapter 4: Introducing Mutations into *Arabidopsis thaliana* CBF1 that Represent Common Polymorphisms Seen in Arctic and Alpine Plants and Testing for Effect upon Function

#### Aims and Objectives

The aim of this chapter was to test the polymorphisms identified in Chapter 3 to assess if they alter freezing tolerance. The main objectives were, therefore: to establish stable overexpressing transformed *Arabidopsis thaliana* lines with a range of levels of expression for each synthetic gene; to test the ability of these synthetic genes to induce *COR* gene expression; to assess the viability of the transient plasmid system provided by Nassuth et al., 2014 as a tool to compare the ability of these synthetic genes to bind and activate the CRT promoter motif.

#### **Hypotheses:**

- 9. The polymorphisms identified in Chapter 3 will alter the binding and/or activity of CBF when introduced into *Arabidopsis thaliana* CBF1.
- 10. The polymorphisms identified in Chapter 3 will alter the degree of *COR* gene expression, relative to wildtype *Arabidopsis thaliana* CBF1, when introduced into *Arabidopsis thaliana* CBF1 and stably transformed into *Arabidopsis thaliana*.
- 11. The polymorphisms identified in Chapter 3 infer greater freezing tolerance, relative to wildtype *Arabidopsis thaliana* CBF1, when introduced to *Arabidopsis thaliana* CBF1 and stably transformed into *Arabidopsis thaliana*.

#### **4.1 Results**

# 4.1.1 Transient Expression of *CBF1* Mutations in *Nicotiana* benthamiana

Synthetic *Arabidopsis CBF1* genes containing amino acid mutations based upon differences seen in wild plants (Table. 2.5.2.1 and section 3.2.1.4) were transiently expressed in *Nicotiana bethamiana* alongside reporter plasmids containing a CRT/DRE element (see Fig. 2.8.7.1 and methods section 2.8.10)

Results across all infiltration events were amalgamated, via further normalisation by setting the wildtype overexpressor for each experiment as 1 and expressing the other constructs as fold changes in CRT expression relative to wildtype (Fig. 4.1.1.1). This indicated very little difference in activation of CRT by the altered *CBF* genes DSAWQ, DSLWR, DSVWQ and DSVWR relative to *CBF1* wildtype overexpressor. The only *CBF* to demonstrate a change in CRT activation was the PKK synthetic gene which caused a decrease in CRT activation to an average of 0.24X that of the wildtype (Fig. 4.1.1.1).

There was a large variance in normalised photon count (indicating the degree of CRT activation – see section 2.8.10) across different infiltration experiments varying from 0.09 (Fig. 4.1.1.4) to 16.3 (Fig. 4.1.1.3) for wildtype *CBF* overexpressor. The other synthetic genes showed a similar variance with photon counts for individual experiments of the same order of magnitude as the corresponding wild type *CBF* overexpressor.

Different patterns were seen across different experiments. In the initial experiment (Fig. 4.1.1.2) DSAWQ showed a 2.0 fold increase in activation of CRT relative to wildtype (6.9 vs 3.5) with no overlap in standard error bars. DSVWQ and DSVWR also showed a possible 1.6 and 2.0 fold increase respectively compared to the *CBF* wildtype

overexpressor (5.5 and 6.9 vs 3.5) however standard error bars overlap those of the wild type overexpressor. DSLWR showed a decrease in activation to 0.085X that of the wild type overexpressor (0.3 vs 3.5) with clearly separated standard error bars.

In contrast to the increase seen in Fig. 4.1.1.2 in Fig 4.1.1.3 DSAWQ, DSVWQ and DSVWR all showed a decrease in CRT activation to 0.32X, 0.42X and 0.26X that of the *CBF* wild type overexpressor (5.2, 6.9 and 4.3 vs 16.3 respectively) with no overlap of error bars (Fig. 4.1.1.3). PKK also showed a decrease to 0.036X that of the *CBF* wild type overexpressor (0.05 vs 1.4) with no overlap of error bars which is consistent with results seen in Fig. 4.1.1.2. DSLWR also showed a possible 2.6X increase (3.6 vs 1.4) in contrast to Fig. 4.1.1.2 however the error bars overlapped (Fig. 4.1.1.3).

Again, a different pattern was seen in the third experiment (Fig. 4.1.1.4) very little difference was seen between DSAWQ and the wildtype overexpressor (0.09 vs 0.087) (Fig. 4.1.1.4). DSVWQ, DSVWR in concordance with (Fig. 4.1.1.3) but in contrast to (Fig. 4.1.1.2) showed a potential decrease in CRT activation to 0.86X and 0.78X that of the *CBF* wild type overexpressor (0.075, 0.068 vs 0.087 respectively) however all error bars overlap (Fig. 4.1.1.4). DSLWR also showed a decrease relative to the wild type overexpressor to 0.61X that of the wildtype (0.053 vs 0.087) in concordance with (Fig. 4.1.1.2) but in contrast to (Fig. 4.1.1.3) with no overlap of error bars (Fig. 4.1.1.4). PKK was consistent with (Fig. 4.1.1.2 and 4.1.1.3) and showed a decrease 0.55X that of the *CBF* wild type overexpressor (0.048 vs 0.087) with no overlap of error bars (Fig. 4.1.1.4).

The only *CBF* construct to show consistent activation behaviour across all experiments (Fig. 4.1.1.2, 4.1.1.3, 4.1.1.4) was PKK which consistently displayed a decrease relative to *CBF* wildtype with no overlap in error bars which is reflected in the amalgamated data (Fig 4.1.1.1).



Figure 5.1.1.1. Data from multiple transient expression experiments (see section 2.8.10), depicting fold increase in activation of CRT by various synthetic genes (Table 2.5.2.1) relative to *A. thaliana* wild type *CBF1* (*CBF* normalised to 1). Error bars denote standard error from the mean (n=3).



Figure 4.1.1.2. Data from a transient expression experiment (see section 2.8.10). Bars represent mean normalised photon count (see section 2.8.10 for explanation of normalisation) from the activation of CRT by various synthetic *CBF* genes (Table 2.5.2.1). Error bars denote standard error from the mean (n=3).



Figure 4.1.1.3. Data from a transient expression experiment (see section 2.8.10) performed across two consecutive days. Bars represent mean normalised photon count (see section 2.8.10 for explanation of normalisation) from the activation of CRT by various synthetic *CBF* genes (Table 2.5.2.1). Error bars denote standard error from the mean (n=3).



Figure 4.1.1.4. Data from a transient expression experiment (see section 2.8.10). Bars represent mean normalised photon count (see section 2.8.10 for explanation of normalisation) from the activation of CRT by various synthetic *CBF* genes (Table 2.5.2.1). Error bars denote standard error from the mean (n=3).

# 4.1.2 Overexpression of *CBF1* Constructs in *Arabidopsis* thaliana

#### 4.1.2.1 Establishment of Stable Transgenic Arabidopsis thaliana lines

Survival of transformant plants was low with approximately 56 % of all transformed seedlings dying before seed-set (Table 4.1.2.1.1). PKK transformants showed the highest death rate with only three viable seedlings growing from dipped seed and of those three two died before seed-set (Table 4.1.2.1.1). DSAWQ was the most prolific line with 43 seedlings, 25 of which died and 18 survived through to set seed. DSLWR had the greatest survival rate with only 50 % of plants dying (Table 4.1.2.1.1). Of those that survived to set seed, the amount of seed produced was highly variable (Table 4.1.2.1.2). Samples with low seed volumes were not taken forward for freezing assays, saving seed for expression analyses.

Table 4.1.2.1.1 Number of plants germinated and grown from transformed seed (total number of lines), of these the number that survived to set seed (Number of surviving lines) and the number of lines that died before setting seed (Number of deceased lines). The percentage of plants that died prior to seed set is also shown.

	Synthetic Gene	Total Number of	Number of	Number of	Percentage of	
Synthetic Gene	Code	Lines	Surviving Lines	Deceased Lines	Plants that Died	
DSAWQ	114	43	18	25	58	
DSLWR	122	12	6	6	50	
DSVWR	126	13	6	7	54	
WT	106	30	13	17	57	
DSVWQ	118	18	8	10	56	
РКК	110	3	1	2	67	
Total		119	52	67	56	

DSAWQ			DSLWR			Γ	DSVWR			WT						
~Volume of	Gene		1	~Volume of	Gene			ĥ	Volume of	Gene			~Volume of	Gene		
Seed (µl)	Code	Line		Seed (µl)	Code	Line		9	Seed (µl)	Code	Line		Seed (µl)	Code	Line	
5	114		5	50	122	2	1		40	126		1	200	106		4
300	114		7	5	122	2	3		200	126		4	30	106		7
100	114		8	180	122	2	4		100	126		5	100	106		8
5	114	1	4	50	122	2	7		200	126		8	150	106		9
100	114	1	5	100	122	2	11		250	126		9	0.5	106		10
200	114	1	7	100	122	2	12	L	150	126		10	80	106		12
150	114	1	9										40	106		14
100	114	2	0		DSVWQ					РКК			250	106		16
250	114	2	2	~Volume of	Gene			ſ	Volume of	Gene			5	106		19
250	114	2	3	Seed (µl)	Code	Line		5	Seed (µl)	Code	Line		70	106		21
100	114	2	4	5	118	3	2	L	125	110		3	40	106		22
150	114	2	5	125	118	3	4						250	106		23
400	114	2	6	500	118	8	5						0.5	106		25
3	114	2	8	200	118	3	8									
200	114	3	1	90	118	8	13									
300	114	3	2	85	118	3	14									
300	114	3	4	125	118	3	16									
500	114	3	5	300	118	3	18									
			_													

Table 4.1.2.1.2. Series of tables indicating approximate volume of seed harvested from each independent transgenic plant (the progenitor of a seed line). Tables separated by synthetic gene (see section 2.5.2 for full details on synthetic genes). Volumes  $\leq 30\mu$ l are highlighted in red.

### 4.1.2.2 Expression of *CBF1* and *COR* Genes in Transgenic *Arabidopsis thaliana* Lines

Fold increase in expression levels of the two different *COR* genes (*KIN2* and *LTI78*) relative to the fold increase of *CBF* expression was, overall, highly consistent within individual plant lines when comparing between the two *COR* genes *KIN2* (Fig. 4.1.2.2.1) and *LTI78* (Fig. 4.1.2.2.2). One exception was the extremely high expressing DSAWQ line (2057 fold increase in *CBF* expression) which had a 45 fold increase in *KIN2* expression (Fig. 4.1.2.2.1) which increased to a 110 fold increase in *LTI78* expression (Fig. 4.1.2.2.2). This resulted in this line having the highest expression of *LTI78* of all plants studied: whereas it was the 5<sup>th</sup> highest expresser of *KIN2*.

At high levels of *COR* gene expression (>7X) there was very little evidence of a pattern that might suggest one synthetic gene line expresses *COR* genes to a higher level relative to *CBF* expression than any other (Fig. 4.1.2.2.1 and Fig. 4.1.2.2.2). There was some slight indication that the overexpressor of the native wild-type *CBF1* may express *COR* genes to a greater level for a given increase in *CBF* expression however this was minimal (Fig. 4.1.2.2.1 and 4.1.2.2.2). There was no evidence of a linear relationship between increasing *CBF* expression and *COR* gene expression for any synthetic gene.

At low levels of *COR* gene expression <7X (see zoomed in sections of Fig. 4.1.2.2.1 and 4.1.2.2.2) there was a similar apparent random distribution between different synthetic genes. DSVWQ possibly showed a slight trend of lower *COR* gene expression relative to *CBF* expression level.

There were three examples of very low fold increase in *COR* gene expression associated with a high increase in *CBF* expression; DSVWQ 6x vs 85x, DSVWR 3x vs 96x and DSAWQ 3x vs 310x (Fig. 4.1.2.2.1 and 4.1.2.2.2).

There were several plant lines which show a fold decrease in *CBF* expression relative to the non-overexpressing wild-type plants but an increase in *COR* gene expression. Some lines from all synthetic genes exhibited this, with the exception of PKK. However, it should be noted there was only one line expressing this gene. Further, the DSVWQ lines and DSVWR lines show *COR* gene expression close to 1 (Fig. 4.1.2.2.1 and Fig. 4.1.2.2.2).



CBF Expression fold increase

Figure 4.1.2.2.1. Points indicate fold increase in *CBF* expression relative to wildtype (see section 2.7.5 for details) vs fold increase in *COR* gene *KIN2* expression relative to wildtype. Each sample consists of RNA extracted from multiple seedlings of the same genotype. Error bars denote standard error of the mean (n=3 technical repeats)

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CBF Expression fold increase

Figure 4.1.2.2.2. Points indicate fold increase in *CBF* expression relative to wildtype (see section 2.7.5 for details) vs fold increase in *COR* gene *LTI78* expression relative to wildtype. Each sample consists of RNA extracted from multiple seedlings of the same genotype. Error bars denote standard error of the mean (n=3 technical repeats)

## 4.1.2.3 Ability of Different *CBF1* Constructs to Activate *COR* Genes in Transgenic *Arabidopsis thaliana*

On average DSLWR had a greater fold increase in expression of *KIN2* and *LTI78* relative to *CBF* expression (indicating a greater activation ability) than all other synthetic genes (Fig. 4.1.2.3.1). For *KIN2* the error bars of DSLWR overlapped those of the wildtype *CBF1* overexpressor (Fig. 4.1.2.3.1) however for *LTI78* the error bars did not overlap with any other synthetic gene (Fig. 4.1.2.3.1). The other synthetic genes all had a similar degree of activation ability with overlapping error bars (Fig. 4.1.2.3.1).



Figure 4.1.2.3.1. Activation ability of various overexpressed *CBF* synthetic genes (see section 2.5.2) expressed as fold increase in *COR* gene (A) *KIN2* and (B) *LTI78* expression relative to wild-type/fold increase in *CBF* expression relative to wild-type. Error bars denote standard error of the mean, n=X above bars indicates number of independent transgenic plant lines successfully generated for that synthetic gene contributing qPCR data used for calculation of the mean.

### 4.1.2.4 Ability of Transgenic *Arabidopsis thaliana* to Survive Freezing in Relation to the Degree of *CBF1* and *COR* Gene Expession

When subjected to a sudden freezing shock of -7 °C for 24 h, the majority of plants died (Figs. 4.1.2.4.2, 4.1.2.4.3 & 4.1.2.4.4). However, several plants survived completely, whilst others showed partial survival. Those plants which had little to no negative response to the freezing shock had a compact dwarf phenotype. All plants with this phenotype survived (Figs. 4.1.2.4.2, 4.1.2.4.3 & 4.1.2.4.4). Whilst there were some small plants, such as a couple of examples of Wild-type CBF 1 overexpressor Line 7 ( "u" top row Fig 4.1.2.4.3a B&D and middle row 4.1.2.4.4a A&C) that did not survive, they did not show the compactness associated with the phenotype of the dwarf surviving plants (for example as seen with plant DSAWQ line25 ("n" Fig. 4.1.2.4.2c A&B)).

Those plants which survived were;

- Line DSAWQ 125 (n) where 2/5 plants demonstrated dwarfism and complete freezing survival (Fig 4.1.2.4.2b A,C top row and Fig. 4.1.2.4.2c A,B bottom row)
- Line *CBF* WT L21 (k) where 1/6 plants demonstrated high survival with partial dwarfism (Fig. 4.1.2.4.2b B,D bottom row)
- Line DSAWQ L24 (£) where 4/12 plants demonstrated dwarfism with high survival (Fig. 4.1.2.4.3b B,D central row, Fig. 4.1.2.4.4a B,D central row, Fig. 4.1.2.4.4b A,C top row and Fig. 4.1.2.4.4c A,B bottom row. A further plant showed no dwarfism and very little survival, with only the central meristem showing green Fig. 4.1.2.4.3c.
- Line DSAWQ L20 (x) where 2/12 plants demonstrated partial freezing survival. One plant had some green still visible in the central meristem (Fig. 4.1.2.4.4a B,D) and the other showed more green tissue in the central meristem (Fig. 4.1.2.4.4b A, C bottom row).

• Line DSAWQ (t) L23 where 1/12 plants demonstrated partial freezing survival with green tissue still seen in the central meristem (Fig. 4.1.2.4.4b B,D top row).

Those plant lines containing individuals which demonstrated full survival and dwarfism (DSAWQ L25, CBF WT L21 and DSAWQ L24) were also high expressers of both *CBF* and tested *COR* genes with a greater than 50 fold increase in CBF expression and greater than 20 fold increase in *COR* gene expression (Fig. 4.1.2.4.5 and Fig. 4.1.2.4.6). Two other plant lines, DSAWQ L15 and CBF WT L14 which also underwent visual freezing tests, also had a greater than 20-fold increase in both *COR* genes tested, however their *CBF* expression was lower than that of the survivors at 20X and 32X respectively (highlighted in yellow Fig. 4.1.2.4.5 and Fig. 4.1.2.4.6). These two lines demonstrated no freezing survival in the freezing assay tests (with 0/6 plants showing survival in both cases) and large healthy plants prior to freezing "e" and "j" (Fig. 4.1.2.4.2a-c)

The plants lines with some plants demonstrating partial survival, DSAWQ L20 and DSAWQ L23, had relatively low *CBF* and *COR* gene expression and were nested among numerous other plant lines which did not show any survival (Fig. 4.1.2.4.5 and Fig. 4.1.2.4.6)

The following observations were made when seeds were germinated for freezing assays. Numerous plates had variance in seedling size and this was especially evident in DSAWQ L24, which had a wide range of seedling size coupled with low germination. Examples from all sizes were taken to grow on for the freezing assays. CBF WT L7 had very low germination with extremely small plants and translucent cotyledons. Table 4.1.2.4.1. Letters/symbols correspond to those of figures 4.1.2.4.2, 4.1.2.4.3 and 4.1.2.4.4. OE Gene is the synthetic *CBF1* gene that the plant line has been transformed to overexpress (Table 2.5.2.1). No OE denotes that the plant has not been transformed i.e. is untransformed wildtype *Arabidopsis thaliana*. Line indicates the code given to each independent transgenic line and therefore which transgenic line corresponds with which letter/symbol from figures 4.1.2.4.2, 4.1.2.4.3 and 4.1.2.4.4.

Code	OE Gene	Line				
a	DSAWQ	134				
b	DSVWQ	114				
с	DSVWQ	113				
d	DSVWR	14				
e	DSAWQ	115				
f	РКК	134				
g	DSLWR	111				
h	no OE	Wildtype				
i	CBF1 WT	116				
j	CBF1 WT	114				
k	CBF1 WT	121				
1	DSVWR	15				
m	DSLWR	14				
n	DSAWQ	125				
0	DSLWR	L7				
р	DSAWQ	L32				
q	DSAWQ	L31				
r	DSAWQ	L8				
S	DSAWQ	L22				
t	DSAWQ	L23				
u	CBF1 WT	L7				
v	DSLWR	L1				
W	DSVWR	L1				
Х	DSAWQ	L20				
У	CBF1 WT	L12				
Z	no OE	WIldtype				
£	DSAWQ	L24				



Figure 4.1.2.4.2a. T1 generation of *Arabidopsis thaliana* plants transformed to constitutively overexpress synthetic *CBF1* lines (Table 2.5.2.1) (a-g, i-n) and wildtype *A. thaliana* plants (h). Before (A, B) and 3 days after (C, D) being subjected to a -7°C freezing shock (section 2.3.2). Letters correspond to the plant line studied (table 4.1.2.4.1).



Figure 4.1.2.4.2b. T1 generation of *Arabidopsis thaliana* plants transformed to constitutively overexpress synthetic *CBF1* lines (Table 2.5.2.1) (a-g, i-n) and wildtype *A. thaliana* plants (h) Before (A, B) and 3 days after (C, D) being subjected to a -7°C freezing shock (section 2.3.2). Letters correspond to the plant line studied (table 4.1.2.4.1).



Figure 4.1.2.4.2c. T1 generation of *Arabidopsis thaliana* plants transformed to constitutively overexpress synthetic *CBF1* lines (Table 2.5.2.1) (c-g, i-n) and wildtype *A. thaliana* plants (h) Before (A) and 3 days after (B) being subjected to a -7°C freezing shock (section 2.3.2). Letters correspond to the plant line studied (table 4.1.2.4.1).



Figure 4.1.2.4.3a. T1 generation of *Arabidopsis thaliana* plants transformed to constitutively overexpress synthetic *CBF1* lines (Table 2.5.2.1) (o-y, £) and wildtype *A. thaliana* plants (z) Before (A, B) and 3 days after (C, D) being subjected to a -7°C freezing shock (section 2.3.2). Letters correspond to the plant line studied (table 4.1.2.4.1).


Figure 4.1.2.4.3b. T1 generation of *Arabidopsis thaliana* plants transformed to constitutively overexpress synthetic *CBF1* lines (Table 2.5.2.1) (o-y, £) and wildtype *A. thaliana* plants (z) Before (A, B) and 3 days after (C, D) being subjected to a -7°C freezing shock (section 2.3.2). Letters correspond to the plant line studied (table 4.1.2.4.1).



Figure 4.1.2.4.3c. T1 generation of *Arabidopsis thaliana* plants transformed to constitutively overexpress synthetic *CBF1* lines (Table 2.5.2.1) (y, £) and wildtype *A. thaliana* plants (z) Before (A) and 3 days after (B) being subjected to a  $-7^{\circ}$ C freezing shock (section 2.3.2). Letters correspond to the plant line studied (table 4.1.2.4.1).



Figure 4.1.2.4.4a. T1 generation of *Arabidopsis thaliana* plants transformed to constitutively overexpress synthetic *CBF1* lines (Table 2.5.2.1) (o-y, £) and wildtype *A. thaliana* plants (z) Before (A, B) and 3 days after (C, D) being subjected to a -7°C freezing shock (section 2.3.2). Letters correspond to the plant line studied (table 4.1.2.4.1).



Figure 4.1.2.4.4b. T1 generation of *Arabidopsis thaliana* plants transformed to constitutively overexpress synthetic *CBF1* lines (Table 2.5.2.1) (o-y, £) and wildtype *A. thaliana* plants (z) Before (A, B) and 3 days after (C, D) being subjected to a -7°C freezing shock (section 2.3.2). Letters correspond to the plant line studied (table 4.1.2.4.1).



Figure 4.1.2.4.4c. T1 generation of *Arabidopsis thaliana* plants transformed to constitutively overexpress synthetic *CBF1* lines (Table 2.5.2.1) (v-y, £) and wildtype *A. thaliana* plants (z) Before (A) and 3 days after (B) being subjected to a  $-7^{\circ}$ C freezing shock (section 2.3.2). Letters correspond to the plant line studied (table 4.1.2.4.1).



#### CBF Expression fold increase

Figure 4.1.2.4.5. figure 4.1.2.2.1 depicting only those results from plants which were successfully grown for visual freezing assays. Plants which showed full or partial survival from freezing (see section 4.1.2.4) are labelled with their line name. Two other points of interest; those which did not show any survival in response to freezing are labelled with their line name and highlighted in yellow. Points indicate fold increase in *CBF* expression relative to wildtype (see section 2.7.5 for details) vs fold increase in *KIN2* expression relative to wildtype, each sample consists of RNA from multiple seedlings of the same genotype. Error bars denote standard error of the mean (n=3 technical repeats).



#### CBF Expression fold increase

Figure 4.1.2.4.6. figure 4.1.2.2.2 depicting only results from plants which were successfully grown on for visual freezing assays. Plants which showed full or partial survival from freezing (see section 4.1.2.4) are labelled with their line code. Two other points of interest; those which did not show any survival in response to freezing are labelled with their line name and highlighted in yellow. Points indicate fold increase in CBF expression relative to wildtype (see section 2.7.5 for details) vs fold increase in *LT178* relative to wildtype, each sample consists of RNA from multiple seedlings of the same genotype. Error bars denote standard error of the mean (n=3 technical repeats).

#### **4.2 Discussion**

# 4.2.1 Effect of *CBF1* Mutations Upon CRT/DRE Activation when Transiently Expressed in *Nicotiana benthamiana*

(See section 3.2.1.4 for discussion of the mutations performed in *CBF1* and their likely significance).

The only synthetic CBF to activate the CRT/DRE element with a consistent difference in expression level relative to wild-type was PKK (Table 2.5.2.1). This is reflected in the amalgamated data in being the only synthetic gene to show a difference from wildtype with no overlap of error bars (Fig. 4.1.1.1). The PKK substitution (R(11) to K) had greatly reduced activation of the CRT gene relative to wildtype (Fig. 4.1.1.1). Interestingly a previously unconserved (positively charged to neutral) substitution of this amino acid (R to S) had been shown to cause no significant effect upon CBF's ability to bind the CRT element (Canella et al., 2010), whereas R to K was highly conserved; both polar, positively charged and hydrophilic with a similar structure. Two possibilities for this reduced activation exist: either the R to K substitution greatly hinders binding to the CRT element, or it results in strong binding which is deleterious to the plant. It seems unlikely that the R to K substitution hinders binding, given that an un-conserved substitution does not affect binding (Canella et al., 2010). It has also been shown that Vaccinium myrtillis CBF (a sequence including the R to K substitution) is able to activate COR genes in Arabidopsis thaliana and synthetic CRT reporters in Nicotiana benthamiana (the same species as used in these experiments) (Oakenfull et al., 2013). It is also unlikely that all species sequenced bar the Brassicaceae have a greatly reduced ability of CBF to bind and activate COR genes, especially given their cold origin environments. It therefore seems more likely that the PKK substitution is deleterious. This is supported by the stable transformations of Arabidopsis thaliana where, after dipping, very few viable seeds were collected and of these successfully germinated individuals (three) only one individual survived to produce seed (Table. 4.1.2.1.1). Plants from this surviving line had a very low CBF expression (similar to that of wildtype) (Figs. 4.1.2.4.5 & 4.1.2.4.6). So, it is therefore possible that this remaining line is either an extremely low expressor (thereby limiting the potential deleterious effect) or not actually expressing the PKK synthetic gene at all. One possibility to explain the deleterious nature of the PKK substitution is that this conserved substitution is an extremely strong binder (as with the conserved F10 to Y substitution a performed by Canella et al, 2010). Although the CBF sequence of Nicotiana benthamiana is unknown, other members of the Solanaceae have the R(11) rather than K (uniport accession: Q8S9N5) as seen in members of the Brassicaceae (Fig. 3.1.2.2). Therefore, strong binding could result in a higher than usual activation of native COR genes leading to cell dormancy (in preparation for chilling and/or drought) or death from an extreme response. One possible example might be that a large increase in sugars, upregulated by CBF (Cook et al., 2004), could lead to cell toxicity or a high influx of water leading to cell rupture. High levels of activation of expression could also be activating native downregulators. 24 hours pass between infiltration and imaging so in this time expression of the CRT/DRE could be silenced. Alternatively, high levels of CBF protein, especially of those which have high affinity and bind strongly, could result in saturation and lead to non-specific binding to elements similar to the CRT/DRE (for example, other AP2 targets). This would result in the upregulation of numerous genes, some of which could be toxic to the cell in high concentrations or lead to the activation of programmed cell death, which other AP2 family transcription factors are involved in (Mase et al., 2013). It is known that various AP2 family members (of which CBF is itself a member) can bind to the binding elements of other members of the AP2 family with differing strengths (Agarwal et al., 2017). It is therefore possible that if the binding strength and quantity of CBF protein is changed it may bind to other AP2 binding promoters outside of the usual CRT/DRE.

An alternative possible explanation is that of squelching: whereby an excess of transcription factor binds to regulators of transcription off of the DNA, they then sequester these regulators preventing DNA transcription (Ptashne, 1988, Simon et al., 2015). This sequestration would be more severe in strong, stable binders. It is known that mediator is involved in the activation of COR genes (Targets of CBF) in Arabidopsis thaliana, with mutants in certain mediator subunits being unable to activate COR genes (Hemsley et al., 2014). It has been postulated that CBF binds directly with these subunits (Hemsley et al., 2014) therefore, it is possible that, due to an excess of CBF, CBF is binding to the mediator complex external to the DRE/CRT motif. CBF is able to bind to the CRT/DRE without mediator (Hemsley et al., 2014), recruiting mediator which regulates transcription via polymerase II. However, if the excess CBF has tightly bound free mediator, activation of genes with the CRT/DRE promoter (even with CBF bound) will be downregulated due to the reduced ability to recruit the mediator complex. It is even possible that the PKK mutation is involved with the recruitment and binding of mediator, hence the stronger response. This would also explain the poor survival of stable line transformation of the PKK mutant, as mediator is involved in the regulation of activation of numerous genes (Hemsley et al., 2014, Mathur et al., 2011).

All DSAWR constructs (DSAWQ, DSVWQ, DSVWR, DSLWR) caused different expression levels relative to wildtype for different experiments but this resulted in no difference relative to wildtype when data from the three experiments was amalgamated (Fig. 4.1.1.1). At the lowest levels (Fig. 4.1.1.4), with a normalised photon count of 0.08 for wildtype, all error bars (bar those of DSLWR) overlapped those of wildtype however the general trend was toward a decrease in activation (apart from DSAWQ which showed no difference). At medium/low expression levels (normalised photon count of 1.4 for

wildtype) DSLWR had increased activation of the CRT/DRE, however error bars overlapped that of wildtype (the other DSAWR constructs were not tested against this wildtype example) (Fig. 4.1.1.3 day 2). At medium expression levels ((fig 4.1.1.2) normalised photon count of 3.5 for wildtype) all, bar DSLWR, showed an increase in CRT/DRE activation although only DSAWQ did so without overlap of error bars. DSLWR showed a decrease with no overlap of error bars. At the highest expression levels (Fig. 4.1.1.3 day 1) (normalised photon count of 16.2 for wildtype) all DSAWR constructs (bar DSLWR which was not tested) showed a decrease in CRT/DRE activation. The overall trend is therefore: at low expression levels all DSAWR constructs activate CRT/DRE to a lesser degree than, or the same as, wildtype. At medium expression levels the activation is greater than wildtype and at the highest expression levels activation is once again lower. This trend is inferred from only three points and with error bars overlapping in some cases. However, that all examples follow this trend to some extent increases the likelihood of a genuine trend. Further repeats with differing levels would have to be performed to assess if this is a true trend. The explanation for this trend could be that all constructs are more potent than wildtype at activating the DRE/CRT as exemplified at medium expression levels. The reduced activation at higher levels of expression relative to the wildtype overexpressor could be due to numerous factors as discussed with the PKK mutation namely: downregulation via native genes, squelching, non-specific binding or high expression of COR genes leading to dormancy or death due to stronger binding or activation activity of the construct *CBF* genes.

At lower expression levels, activation is also lower in the construct *CBF* genes than the wildtype overexpressor. It is possible that, if the construct CBF's bind more strongly, they bind preferentially to the native CRT/DRE elements in *Nicotiana benthamiana* and do not disassociate as readily as the wildtype CBF therefore resulting in a reduced expression of the test gene. This is then overcome at medium expression levels resulting in higher

activation than the *CBF* wildtype overexpressor (Fig. 4.1.1.2 & 4.1.1.3). Assuming this pattern is not an artefact, DSLWR & DSAWQ are possibly the strongest binders or activators out of the DSAWR constructs (DSAWQ, DSVWQ, DSVWR, DSLWR), as this trend is shifted the furthest towards higher CRT/DRE activation at lower expression levels for these constructs (see Fig. 4.2.1.1 for conceptual diagram of trend shifts).

The transient expression experiments displayed a great deal of variability in results and large error bars that often overlapped (Fig. 4.1.1.2, 4.1.1.3 & 4.1.1.4), they were, therefore, deemed too variable for further exploration and thus stable lines were explored further instead. It would be interesting to perform *in vitro* studies upon the binding of these constructs to CRT/DRE elements, specifically electrophoretic mobility shift assays as exemplified by Canella et al., (2010). This could identify if the PKK substitution is capable of binding and if, as postulated, a strong binding effect is found. Similar tests could be performed upon all constructs. Other tests studying the health of transformed plants could also be performed such as chlorophyll fluorescence prior to and 24 h after introduction of the construct to identify the effect of the construct upon survival of the infiltrated leaves.



Figure 4.2.1.1. Conceptual diagram illustrating the two possible types of trend shift in fold change in *CBF* expression (relative to wildtype, set as 1) in A. DSAWQ, depicting a shift of curve to the left and B. DSLWR depicting a narrower spread resulting in earlier peak.

## 4.2.2 Effect of Stable Overexpression of *CBF1* Constructs in *Arabidopsis thaliana*

#### 4.2.2.1 Establishment of Stable Transformed Arabidopsis thaliana Lines

Problems were encountered with the development of stable transformed lines. Multiple transformation events were undertaken. However, survival to seed set was poor, especially in plants transformed with the PKK construct. Of those seeds successfully collected gemination rate was variable and especially poor in plants transformed with the PKK construct (Table 4.1.2.1.1). Survival of plants grown for bulking seed was also poor with an average of 56 % of plants dying before seed-set (Table 4.1.2.1.1). Possible explanations for poor survival and germination are discussed below.

Overexpression of *CBF*1 (the gene used for the wild-type overexpressing control and basis for the synthetic genes) results in increased expression of CBF1 target genes (Jaglo-Ottosen et al., 1998). One such target includes members the LEA protein family many of which have a CRT/DRE promoter element (Hundertmark and Hincha, 2008). Amongst other processes, members of this family are involved in is seed desiccation and maturation (Hundertmark and Hincha, 2008). It is therefore possible that overexpression of *CBF*, even at the flower bud infiltration stage, is causing premature seed maturation and desiccation resulting in infertile seeds and possibly bud termination leading to the observed low seed set.

*CBF*1 overexpression leads to an increase in expression of GA oxidase (Achard et al., 2008) which results in reduce accumulation of GA (Achard et al., 2008) which is involved in the breakdown of growth-repressing DELLA proteins (Achard et al., 2008). Among other activities, members of the DELLA protein subfamily repress germination (Tyler et

al., 2004). Therefore, *CBF*1 overexpressors, due to reduced GA levels, have higher DELLA protein levels and are consequently more likely to have repressed germination. Indeed inhibition of *CBF* expression has been indicated as a requirement to promote germination (Kendall et al., 2011). Therefore, the low germination rates of *CBF* overexpressors observed is to be expected, especially in those expressing to a high level or with high activation ability which would result in reduction of numbers of individual transgenic lines or even total loss of those lines.

The aforementioned reduced GA expression, resulting in reduced breakdown of DELLA proteins, leads to a dwarf phenotype (Achard et al., 2008). This phenotype was frequently observed in plants grown from transformed seed. As well as a dwarf phenotype the lower leaves frequently curled down at the edges and tightly hugged the substrate, with leaves higher in the rosette hugging the leaves below. This likely led to the formation of humid microclimates on the leaf underside possibly encouraging pathogen attack and resulting in further plant losses. Likewise, *CBF* overexpression is known to increase the concentration of various sugars (Cook et al., 2004) which could make the overexpressor more susceptible to pathogen attack and preferential targets for consumption by fly larvae, coupled with leaves closer to the growth substrate. Flies were observed around the plants during line establishment and appeared to show preference for the *CBF* overexpressor plants. Others overexpressing *CBF* in *Arabidopsis thaliana* have also reported this problem with flies (pers. com. Dr. R. Oakenfull). Increased susceptibility to pathogen and pest attack were observed (pers. obs.).

Dwarfing of plants led to smaller inflorescences which therefore produced less seed. Ga1 (involved in the GA biosynthesis pathway) knockout mutants also have sterile flowers (Tyler et al., 2004), therefore the reduced concentration of GA in *CBF* overexpressors

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(Achard et al., 2008) may also result in reduced flower fertility, leading to lower seed amounts (Table 4.1.2.1.2).

Despite observations of late flowering in plants overexpressing CBF1 (Achard et al., 2008, Gilmour et al., 2004). Early flowering was observed in these construct lines. It is possible that differing growth conditions or other environmental factor(s) (such as watering conditions, growth media etc) may alter the effect upon flowering time of CBF1 overexpressors. This is supported by previous CBF1 overexpressors from the same research group not displaying this delayed flowering phenotype ((Gilmour et al., 2004) in reference to Jaglo-Ottosen et al. (1998). Although not explicitly stated in Jaglo-Ottosen et al., (1998), Gilmour is also an author on this paper and it is therefore assumed her reference to this as a record of a lack of previous delayed flowering was an observation at the time). This indicates that other factors can affect the effect of CBF1 upon flowering time. It is also possible that different A. thaliana accessions have different flowering time responses to CBF1 overexpression; Columbia-0 was used in these experiments (early flowering phenotype), Jaglo-ottosen et al., 1998 used RLD (no change in flowering time), Gilmour et al., 2004 used Wassilewskija (late flowering) and Achard et al., 2008 used Landsberg erecta (late flowering). Indeed different accessions are known to have different freezing tolerances (Hannah et al., 2006, Cvetkovic et al., 2017) with Columbia-0 tolerating lower freezing temperatures after acclimation than Landsberg erecta (Hannah et al., 2006). Whereas, Wassilewskija tolerates lower temperatures than Col-0 after acclimation (Cvetkovic et al., 2017) (RLD was not tested) (Hannah et al., 2006, Cvetkovic et al., 2017). It has also been shown that exposure of Col-0 and Wassilewskija to cold (which would result in the upregulation of CBF (Gilmour et al., 1998)) can induce early bolting (Cvetkovic et al., 2017). However, this earlier bolting in response to prior cold exposure is not consistently seen each year (Cvetkovic et al., 2017) indicating that other factors also affect the time until bolting and whether cold exposure induces early bolting. It seems likely, therefore, that either some element of the growth conditions in this project and previous projects within the Knight group (pers. com.) promoted early flowering in *CBF*1 overexpressors, not seen in experiments from other labs and/or that it is due to the use of a different accession (Col-0). This early flowering resulted in greatly reduced seed numbers, or in some cases where the plant was very small, death likely due to insufficient energy resources prior to seed set. Plants transformed with the PKK mutation appeared to be more severely affected. This could indicate that the PKK mutation is highly damaging, possibly by strong binding to the CRT/DRE element (as discussed in section 4.2.1). This is supported by the only PKK line that survived having very low expression (Fig. 4.1.2.2.1 and 4.1.2.2.2). Therefore, it is possible that all higher expressors of the PKK mutation were not viable.

### 4.2.2.2 Levels of *CBF* and *COR* Gene Expression in Stable Transformed *Arabidopsis thaliana* Lines

*LTI78* (also known as *RD29A* or *COR78*) and *KIN2* (*COR6.6*) are both *COR* genes with the CRT/DRE regulatory promoter element (Yamaguchi-Shinozaki and Shinozaki, 1994, Nordin et al., 1993) and upregulated by *CBF*1 (Gilmour et al., 2004). Therefore, that the fold increase in expression in these genes, relative to wildtype, remains consistent between the two genes for each line is unsurprising (Fig. 4.1.2.2.2 and Fig. 4.1.2.2.1). The exception (the highest overexpressor of *CBF* - a DSAWQ line with a 2057 fold increase in *CBF* expression) had the highest expression of *LTI78* of all plants studied whereas it was the 5<sup>th</sup> highest expresser of *KIN2* (Fig. 4.1.2.2.2 and Fig. 4.1.2.2.1). This is unlikely to be a peculiarity of DSAWQ as other DSAWQ lines did not show this pattern (Fig. 4.1.2.2.2 and Fig. 4.1.2.2.1). Therefore, it seems likely that this response has something to do with the large overexpression of *CBF*. *LTI78* has 4 copies of the DRE/CRT element (Nordin et al., 1993) whereas *KIN2* has 1 (Wang et al., 1995).

Therefore, it is possible that, dependent upon the positioning, these extra DRE/CRT elements result in a higher expression of *LTI78*, under normal circumstances, than *KIN2*. It is therefore possible that upon reaching very high expression levels of *KIN2* another factor is downregulating *KIN2* expression, whereas the possibly much higher expressed *LTI78* may not have a down-regulatory gene that responds to *LTI78* concentrations or downstream responses. It is also possible that very high overexpression of *CBF* is activating other genes which, at high concentrations are influencing *KIN2* expression but not *LTI78*. It is not known exactly what LTI78 and KIN2 do in the plant, so a mechanism for this cannot be proposed.

All lines were compared to expression levels in wildtype, which was set arbitrarily as 1. Several lines have error bars which overlap 1 for both *CBF* and *KIN2* or *LTI78* expression. It is possible some lines were not successfully transformed, despite selection on hygromycin, as this weakens rather than directly kills the untransformed seedlings. An alternative is that only partial transformation may have occurred resulting in hygromycin resistance but not insertion of the synthetic CBF gene. Another alternative is that the gene may have inserted into an inactive section of the genome. Interestingly some lines show less than 1 for *CBF* expression (i.e. less than wild-type) even accounting for error bars, but higher than 1 for COR gene expression (KIN2 or LTI78). It would be expected that non-transformed plants or non-expressors would show the same profile as untransformed wildtype (i.e. 1). An example of this is a DSLWR line which has a fold change of 0.20 in *CBF* but 3.70 in *KIN2* with error bars not overlapping the fold change of 1 (Fig. 4.1.2.2.1). A line overexpressing wildtype CBF1 also has a CBF expression clearly less than 1 (0.18) with no overlap of error bars with 1 and a *COR* gene expression of either 1.00 (Fig. 4.1.2.2.2) or 1.88 (Fig. 4.1.2.2.1). This indicates that this is not a response due to the mutation in synthetic CBF genes, since the wildtype CBF1 overexpressors have a synthetic gene identical to their native CBF1. The same amount of RNA is used in qPCR so this cannot affect results. The time from sowing of the seed to RNA extraction is the same for all plants so in theory the seedlings should all be the same age. However, as previously discussed (section 4.2.2.1), *CBF* overexpression can result in dwarfing and delayed germination. Therefore, *CBF* overexpressing plants may be at a different growth stage to their wildtype control if there is a delay in germination and/or altered growth rate (e.g. dwarfing). This would result in a different ratio of different tissues which would result in different RNA expression. Another possible, but less likely, compounding factor is that although all plates were grown in the same growth cabinet where conditions should all be the same the exact location of the light strips and slight fluctuations in MS media firmness could also affect growth form and rate also affecting RNA expression. Therefore, as well as resulting in fold changes that appear less than wildtype, those which appear to be the same as wildtype may not necessarily be non-transformed or non-expressors.

From the scatter graphs there was no apparent difference or clear pattern that indicates any mutation results in greater *COR* gene expression for a relative level of *CBF* expression than any other (including wildtype) (Fig 4.1.2.2.1 and 4.1.2.2.2). However, when the ratio of fold increase in *COR* gene expression relative to wildtype over the fold increase in *CBF* expression relative to wildtype was taken (Fig. 4.1.2.3.1), DSLWR was shown to be expressing *COR* genes to a greater level for a relative level of *CBF* expression when compared to wildtype *CBF* overexpressors. This could indicate that the DSLWR mutation (from DSAWR -> DSLWR) more strongly activates *COR* genes, either via stronger binding or increased activity, which fits with the results seen from transient expression (see sections 4.1.1 and 4.2.1). However, there were only four independent lines expressing the DSLWR construct. These four independent lines are all low expressors, with a maximum *CBF* expression fold increase of 1.5 (Fig. 4.1.2.2.1 and 4.1.2.2.2) (which explains why a clear trend could not be seen from the scatter graphs

alone). One of these lines is also the aforementioned very low expressor of *CBF* with high COR gene expression. As discussed in transient expression (Section 4.2.1), expression levels of *CBF* to *COR* gene are not linear (neither are they logarithmic); at high levels of CBF expression relative COR gene expression drops, this can also be seen on the scatter graphs (Fig. 4.1.2.2.1 and 4.1.2.2.2). This tending towards a plateau, or even drop, in expression at higher levels would bring the averages down for all lines with higher expression. Therefore, DSLWR not having and high *CBF* expression examples, whereas there were high CBF expression samples for the wildtype CBF1 overexpressor, could mean that for DSLWR the average is not brought down by the higher expressors resulting in an apparent higher expression level. A way to address this problem would be to transform and establish more DSLWR lines, hopefully obtaining some high expressors, along with increased numbers of lines for other synthetic genes, including the PKK line. With a wide range of evenly distributed expression levels of *CBF* for each line, expression could be compared over different ranges of *CBF* expression as well as comparing the shift of any potential curves, as was the original intent. However, there are other problems which result in greater errors within the data to overcome first, including the problem of growth stage after a certain number of days. One potential way to address this is the application of GA to speed up germination and overcome growth inhibition of CBF overexpressors, however GA greatly alters the transcriptome (e.g. (Ju et al., 2018, Upadhyay et al., 2018, Xie et al., 2016)) so would likely interfere with results to an even greater degree. All plants including controls would therefore have to be subjected to GA treatment and it is unknown how this may alter relative results. Cotlydon size rather than number of days could be used to assess the growth stage of the seedling prior to harvesting, however the effect of number of days growing and slight fluctuations in environment and time of harvesting may also affect the transcriptome, therefore the best approach is to still use seedlings of the same age but to remain aware of potential

limitations e.g. via noting any morphological differences at that stage. Comparison with CBF1 overexpressors also helps to overcome this limitation to a degree with the exception that this limitation will be more pronounced for stronger binders/activators. Another limitation is that the data will include individuals within the lines which are not expressing i.e. non-transformed individuals. This is because RNA extraction was performed on T1 seedlings and this will be mixed population from a heterozygous parent so should include <sup>1</sup>/<sub>4</sub> homozygous transformed, <sup>1</sup>/<sub>2</sub> heterozygous and <sup>1</sup>/<sub>4</sub> homozygous untransformed (i.e. wildtype). However as discussed in (Section 4.2.2.1) seeds which are overexpressing CBF (i.e. the transformed homozygotes and heterozygotes) are less likely to germinate and/or have delayed germination, they are also more likely to be dwarf and slow growing with potentially less genetic material. The more potent the gene and the greater the expression the greater this problem will be. Since a small number of seedlings are taken for RNA extraction (~10) this variation and bias towards untransformed individuals could easily skew expression results. One way to address this problem would be to extract from a large number of seedlings, however this would not account for the cumulative effect of more potent expressors having a greater skew towards germination and growth of the <sup>1</sup>/<sub>4</sub> homozygous wildtype therefore altering the ratio of wildtype to non-wildtype. Therefore, the optimum way to address this problem would be the removal of the wildtype allele via the establishment of homozygous expressing lines.

## 4.2.2.3 Effect of Freezing Upon Transformed *Arabidopsis thaliana* lines and The Relation to *CBF* and *COR* Gene Expression

The only plants to completely survive the sudden freezing shock (at -7 °C) were dwarf plants, and all dwarf plants showed complete survival. These were lines 24 & 25 of DSAWQ and 21 of synthetic wild-type which all expressed *CBF* and *COR* genes to a relatively high level (Figs. 4.1.2.4.5 & 4.1.2.4.5). As previously discussed *CBF*1

overexpression leads to dwarfing (Achard et al., 2008) and *CBF*1 overexpression results in increased freezing tolerance relative to non-acclimated plants (Jaglo-Ottosen et al., 1998) therefore, that dwarf plants survived and that these were high CBF expressors is unsurprising. Not all plants from these lines survived nor were they dwarf (4/12, 2/5 and 1/6 for DSAWQ 24, 25 and wildtype OE 25 respectively were dwarf and survived). This is likely due to the mixed population of offspring from the heterozygous parent namely that the offspring, if there is a single insertion of the transgene, should consist of  $\frac{1}{4}$ homozygous transformed, <sup>1</sup>/<sub>2</sub> heterozygous and <sup>1</sup>/<sub>4</sub> homozygous untransformed (i.e. wildtype). Therefore, given random selection <sup>3</sup>/<sub>4</sub> of the offspring seedlings should be *CBF*1 overexpressors. This is not the case  $\frac{1}{4}$ ,  $\frac{2}{5}$  and  $\frac{1}{6}$  is seen. The imbalance seen is likely due to the aforementioned (section 4.2.2.1) inhibited and/or delayed germination and growth of CBF overexpressors as well as reduced survival. Indeed, it was noted that DSAWQ L24 had a highly variable range of seedling sizes and poor germination (Section 4.1.2.4). Whilst during selection an attempt was made to select a range of sizes of seedlings representing the proportions on the plate, this would not always be perfect, with larger plants giving the impression of a larger proportion. Also, if some seeds did not germinate ,their percentage was not counted with the smaller seedlings. Therefore, this would very likely skew the expected ratio of seedlings towards the untransformed individuals. This could also explain the lack of representation of two other lines with high expression of LTI78 and KIN2 (namely wildtype overexpressor line 14 and DSAWQ line 15 highlighted in yellow Fig. 4.1.2.4.5 & 4.1.2.4.6) for which no examples survived freezing, nor did they any examples with a dwarf phenotype. These lines did however have lower CBF expression than some lines which express LTI78 and KIN2 to a lesser degree (including members of the same construct – i.e. DSAWQ lines and wildtype overexpressing lines). It is therefore possible that, although LTI78 and KIN2 have been expressed to high levels in these lines, other *COR* genes may not be as highly activated; this is however, unlikely.

A couple of lines showed partial freezing tolerance; these were DSAWQ L20 and L23 (2/12 and 1/12 plants respectively). These plants were large, non-dwarfed plants and only showed partial green survival of the central meristem. These lines were both low expressors of CBF and COR genes (Fig. 4.1.2.4.5 & 4.1.2.4.6). That these were also DSAWQ lines is interesting (DSAWQ being the only line to show dwarfing and complete survival except for a wildtype overexpressor), this could indicate that the DSAWQ mutation induces greater freezing tolerance, even with lower apparent expression of CBF and COR genes (KIN2 and LTI78), perhaps via preferential upregulation of specific COR genes at the expense of others (such as KIN2 and LTI78), or that this line suffers particularly from the limitations discussed in section 4.2.2.2 resulting in an apparent lower expression via preferential selection of wildtype plants. However other low expressing DSAWQ lines were also tested and this improved freezing tolerance with no dwarfing was not seen (Fig. 4.1.2.4.5 & 4.1.2.4.6). It is possible that this is due to positioning in the freezer, in both cases the DSAWO 120 plants that showed partial survival were in corners of the trays (Fig 4.1.2.4.4a&b), and the DSAWQ 123 plant that showed partial survival was on an edge (Fig 4.1.2.4.4b). It is possible that, they were therefore furthest from the chilling element or close to a possible leak in the door seal and may have experienced marginally warmer temperatures than other plants.

Unfortunately, due to the limitations of this experiment and the high mortality rate, no conclusions can be fir mly drawn about the efficacy of various mutations relative to wildtype. The high mortality upon freezing resulted in survival of only very high expressing, dwarfed individuals. It is possible therefore that a higher temperature could perhaps distinguish between lower expressors. The plants were subjected to -7 °C for 24 h followed by 24 h at 5 °C then placed back in growing conditions and photographed after

3 days. Hannah et al., (2006) found that the LT50 for wildtype Col-0 plants grown in 16h photoperiod with a day/night temperature of 20 °C/18 °C was -5 °C for non-acclimated and -10 °C for acclimated plants. Therefore -7 °C should be a good test temperature. However, although these test plants were grown in the same photoperiod, they were transferred from a -20 °C growth room to a higher temperature growth room (~23 °C) due to refurbishment of the previous growth room. They also would not experience the cooler night time temperatures of the plants studied by Hannah et al., (2006). The plants were therefore accustomed to a higher temperature and the change to -7 °C was therefore a much greater relative drop than those tested by Hannah et al., (2006). There were also signs of stress in the test plants prior to freezing (especially evident in Figs. 4.1.2.4.4a-c in the form of leaf yellowing), likely due to higher temperatures. Therefore, future tests may give better results if plants were grown at a temperature of 20 °C day /18 °C night prior to freezing tests at -7 °C or, if higher growing temperatures were necessary, if higher freezing temperatures were also used.

# Chapter 5: Natural Variation in *Empetrum nigrum* and *Calluna vulgaris* from Differing Environments

#### Aims and Objectives

The aim of this chapter was to investigate whether species which grow across a wide environmental range have local genetic adaptations which may alter their freezing tolerance response. Therefore, the objectives were: to explore the possibility of establishing a model Arctic or alpine plant; to isolate and compare CBF sequences from a single species across a wide environmental range; to assess the possibility of using chlorophyll content as a proxy of damage for freezing tests on *Empetrum nigrum* and *Calluna vulgaris*; to adapt and assess the feasibility of using qPCR to study *CBF* expression kinetics in *Empetrum nigrum* from samples across a wide environmental gradient.

#### Hypotheses:

- 12. Polymorphisms exist within CBF sequences from the same species.
- 13. Polymorphisms in CBF within the same species are associated with the environmental conditions of the site of their collection.
- 14. CBF expression kinetics differ across the environmental range of a species.
- 15. Different morphologies observed in common garden grown cuttings of *Empetrum nigrum* and *Calluna vulgaris* are due to the influence of the environmental origin of the parent donor plant.

#### **5.1 Results**

#### 5.1.1 Establishment of Arctic/Alpine Model Plants

Growth of alpine plants in the attempt to establish a model plant for study (section 2.2.1) proved unsuccessful. Germination was unreliable, and many plants were lost due to mechanical and software failure of growth chambers. Of those that did survive, growth was slow combined with being slow to flower (and therefore produce seed) alongside a lack of consistency in flowering time. *Dryas octopetala* showed the most rapid flowering time of around 2 years after germination. *CBF* sequences could also not be isolated from these species. However, had other criteria been met, full genome sequencing would have been performed allowing for identification of *CBF* genes.

## 5.1.2 *CBF* Sequence Comparison from *Empetrum nigrum* Plants from Differing Environments

There was a high degree of conservation across all *Empetrum nigrum CBF* nucleotide sequences (Fig. 5.1.2.1a-f). Of the differences seen there was no apparent link between these and the location the sample was collected from (country or altitude or noted surroundings). These differences also are not linked (i.e. having one does not change the likelihood of having another).

Once translated, the sequences had even greater conservation (Fig. 5.1.2.2a-b). The key differences (only those seen in more than one sequence) were at amino acid 109 and 148 (using top sequence for count) (Fig. 5.1.2.2a-b). At amino acid 109 there was an unconserved substitution with some sequences having P, some L and some both (X) (Fig. 5.1.2.2a-b). At amino acid 148 another substitution occurs, some sequences have M, some T and some both (X), one sequence (158) has K rather than M or T (Fig. 5.1.2.2a-b). The

148 M substitution is always associated with 109 P. However, 109 P is not always associated with 148 M. There was no link between these alleles and the location the sample was collected from (country or altitude or noted surroundings).





Figure 5.1.2.1 a. Clustal alignment of *CBF* nucleotide sequences isolated from *Empetrum nigrum* samples. Sequences are ordered by Clustal sorting. Names are in the form "Species code\_Genus\_Species\_Site code\_Location" TOPO indicates that the sequence was isolated via TOPO cloning. Consensus bar shows degree of consensus between sequences, occupancy bar shows degree of nucleotide presence at that site.



Figure 5.1.2.1 b. Clustal alignment of *CBF* nucleotide sequences isolated from *Empetrum nigrum* samples. Sequences are ordered by Clustal sorting. Names are in the form "Species code\_Genus\_Species\_Site code\_Location" TOPO indicates that the sequence was isolated via TOPO cloning. Consensus bar shows degree of consensus between sequences, occupancy bar shows degree of nucleotide presence at that site.



Figure 5.1.2.1 c. Clustal alignment of *CBF* nucleotide sequences isolated from *Empetrum nigrum* samples. Sequences are ordered by Clustal sorting. Names are in the form "Species code\_Genus\_Species\_Site code\_Location" TOPO indicates that the sequence was isolated via TOPO cloning. Consensus bar shows degree of consensus between sequences, occupancy bar shows degree of nucleotide presence at that site.



Figure 5.1.2.1 d. Clustal alignment of *CBF* nucleotide sequences isolated from *Empetrum nigrum* samples. Sequences are ordered by Clustal sorting. Names are in the form "Species code\_Genus\_Species\_Site code\_Location" TOPO indicates that the sequence was isolated via TOPO cloning. Consensus bar shows degree of consensus between sequences, occupancy bar shows degree of nucleotide presence at that site.



Figure 5.1.2.1 e. Clustal alignment of *CBF* nucleotide sequences isolated from *Empetrum nigrum* samples. Sequences are ordered by Clustal sorting. Names are in the form "Species code\_Genus\_Species\_Site code\_Location" TOPO indicates that the sequence was isolated via TOPO cloning. Consensus bar shows degree of consensus between sequences, occupancy bar shows degree of nucleotide presence at that site.



Figure 5.1.2.1 f. Clustal alignment of *CBF* nucleotide sequences isolated from *Empetrum nigrum* samples. Sequences are ordered by Clustal sorting. Names are in the form "Species code\_Genus\_Species\_Site code\_Location" TOPO indicates that the sequence was isolated via TOPO cloning. Consensus bar shows degree of consensus between sequences, occupancy bar shows degree of nucleotide presence at that site.



Figure 5.2.1.2 a. Clustal alignment of translated CBF nucleotide sequences isolated from Empetrum nigrum. Sequences are ordered by Clustal sorting. Names are in the form "Sample code Genus Species Site code Location". TOPO indicates that the sequence was isolated via TOPO cloning. Conservation histogram indicates the degree of conservation of physicochemical properties between sequences, guality histogram indicates the likelihood of observing mutations at that point, consensus histogram shows degree of consensus between sequences and to the auto-calculated consensus below, occupancy bar shows degree of nucleotide presence at that site.



Figure 5.1.2.2 b. Clustal alignment of translated CBF nucleotide sequences isolated from *Empetrum nigrum*. Sequences are ordered by Clustal sorting. Names are in the form "Sample code\_Genus\_Species\_Site code\_Location". TOPO indicates that the sequence was isolated via TOPO cloning. Conservation histogram indicates the degree of conservation of physicochemical properties between sequences, quality histogram indicates the likelihood of observing mutations at that point, consensus histogram shows degree of consensus between sequences and to the auto-calculated consensus below, occupancy bar shows degree of nucleotide presence at that site.

#### 5.1.3 CBF Expression in Empetrum nigrum

#### 5.1.3.1 Identification, Design and Testing of Primers for use in qPCR on

#### cDNA from *Empetrum nigrum*

Table 5.1.3.1.1. Testing newly designed real time primers with *Empetrum nigrum* cDNA. Showing A. CT values and B. TM values from q-PCR for four different real-time primers sets. Two designed to amplify from previously isolated and sequenced *Empetrum nigrum CBF* gene (Section 5.1.2) CBF(1) (primer pair 166+167) and CBF(2) (primer pair 168+169) and two designed to amplify *RPB2I* gene RPB2Ia (primer pair 170 + 171) and RPB2Ib (primer pairs 172 +173) See 2.5.1.1. *Empetrum nigrum* sample 232 cDNA was used along side No Reverse transcriptase (NRT), and no template control (NTC) control.

A. CT Values				
	CBF(1) (166+167)	CBF(2) (168+169)	RPB2la (170 + 171)	RPB2lb (172+173)
cDNA	27.7923	29.1057	Undetermined	31.7811
cDNA	25.1645	28.6158	Undetermined	28.185
cDNA	24.8097	28.3155	Undetermined	27.0379
NRT Control	23.5356	28.4306	36	25.1799
NRT Control	23.5977	28.7515	30.7635	25.0653
NRT Control	23.8078	28	35.6571	25
NTC Control	38.5218	Undetermined	Undetermined	Undetermined
NTC Control	Undetermined	Undetermined	Undetermined	Undetermined
NTC Control	36	Undetermined	Undetermined	Undetermined
B. TM Value	S			
	CBE(1) (166+167)	CBE(2) (168+160)	PDB212 (170 ± 171)	DDB216 (172+172)
cDNA	CBF(1) (166+167)	CBF(2) (168+169)	RPB2Ia (170 + 171)	<b>RPB2Ib (172+173)</b>
cDNA	CBF(1) (166+167) 83.9 83.9	<b>CBF(2) (168+169)</b> 80.1 79 9	<b>RPB2Ia (170 + 171)</b> 76.9 77 9	RPB2Ib (172+173) 78.1 78 3
cDNA cDNA cDNA	CBF(1) (166+167) 83.9 83.9 84.2	CBF(2) (168+169) 80.1 79.9 79.9	RPB2Ia (170 + 171) 76.9 77.9 69.8	RPB2Ib (172+173) 78.1 78.3 78.3
cDNA cDNA cDNA NRT Control	CBF(1) (166+167) 83.9 83.9 84.2 83.9	CBF(2) (168+169) 80.1 79.9 79.9 79.9	RPB2la (170 + 171) 76.9 77.9 69.8 86.3	RPB2Ib (172+173) 78.1 78.3 78.3 77.9
cDNA cDNA cDNA NRT Control NRT Control	CBF(1) (166+167) 83.9 83.9 84.2 83.9 83.9 83.9	CBF(2) (168+169) 80.1 79.9 79.9 79.9 79.9 79.7	RPB2Ia (170 + 171) 76.9 77.9 69.8 86.3 77.4	RPB2Ib (172+173) 78.1 78.3 78.3 78.3 77.9 77.6
cDNA cDNA cDNA NRT Control NRT Control NRT Control	CBF(1) (166+167) 83.9 83.9 84.2 83.9 83.9 83.9 83.9 83.9	CBF(2) (168+169) 80.1 79.9 79.9 79.9 79.7 79.7 79.7	RPB2la (170 + 171) 76.9 77.9 69.8 86.3 77.4 77.6	RPB2Ib (172+173) 78.1 78.3 78.3 78.3 77.9 77.6 77.6 77.9
cDNA cDNA cDNA NRT Control NRT Control NRT Control NTC Control	CBF(1) (166+167) 83.9 83.9 84.2 83.9 83.9 83.9 83.9 77.9	CBF(2) (168+169) 80.1 79.9 79.9 79.9 79.7 79.7 79.7 70.7	RPB2Ia (170 + 171) 76.9 77.9 69.8 86.3 77.4 77.6 70.9	RPB2Ib (172+173) 78.1 78.3 78.3 78.3 77.9 77.6 77.6 69.8
cDNA cDNA cDNA NRT Control NRT Control NRT Control NTC Control	CBF(1) (166+167) 83.9 84.2 83.9 83.9 83.9 83.9 83.9 77.9 70.4	CBF(2) (168+169) 80.1 79.9 79.9 79.9 79.7 79.7 79.7 79.7 79	RPB2la (170 + 171) 76.9 77.9 69.8 86.3 77.4 77.6 70.9 74	RPB2Ib (172+173) 78.1 78.3 78.3 78.3 77.9 77.6 77.9 69.8 74.9
cDNA cDNA cDNA NRT Control NRT Control NRT Control NTC Control NTC Control	CBF(1) (166+167) 83.9 84.2 84.2 83.9 83.9 83.9 83.9 77.9 70.4 77.6	CBF(2) (168+169) 80.1 79.9 79.9 79.9 79.7 79.7 79.7 70.7 70.7	RPB2Ia (170 + 171) 76.9 77.9 69.8 86.3 77.4 77.6 70.9 74 70	RPB2Ib (172+173) 78.1 78.3 78.3 78.3 77.9 77.6 77.6 77.9 69.8 74.9 70

Initial tests were performed on the novel primers designed to amplify *CBF* and *RPB2I* from *Empetrum nigrum* RNA (Table 5.1.3.1.1). Between the two *CBF* primers CBF(1) had the highest TM which was furthest from the TMs of the *RPB2I* primers averaging 84 °C compared to 80 °C for CBF(2) with *RPB2I* average TMs being 72.5 °C for RPB2Ia and 78.2 °C for RPB2Ib. There was a great deal of variation in the TM of RPB2Ia with a
maximum difference of 8.1 °C vs a maximum difference of 0.2 °C for RPB2Ib, 0.3 °C for CBF(1) and 0.2 °C for CBF(2). TM values indicate the temperature at which 50 % of the cDNA will disassociate and become single stranded allowing primer binding and amplification. When comparing between two genes (housekeeping and test gene) similar TM values are desirable so that amplification occurs at a similar temperature.

Some amplification was seen in the no template control for CBF(1) primers however the CT values (the number of cycles at which a set amount of amplification occurs) were very high and close to the maximum number of cycles (40), a slightly higher CT target value would have also have lead to these being registered as undetermined (Table 5.1.3.1.1).

Amplification in the no reverse transcriptase control (a control to test for amplification from genomic DNA contamination that was not removed during RNA isolation rather than amplification from cDNA) was greater than from the cDNA when using CBF(1) primers, averaging 23.6470 cycles to reach the set amount of amplification vs 26.9222 cycles for the cDNA. CBF(2) however showed close to equal amplification between the no reverse transcriptase control and amplification from cDNA averaging 28.3940 for the control and 28.6790 for cDNA. Again a great deal of variation was seen between the repeats for CBF(1) primers with a maximum difference of 2.9826 cycles vs a maximum difference of only 0.7902 cycles for CBF(2) primers (Table 5.1.3.1.1).

RPB2Ia primers showed no amplification from the cDNA and minimal amplification from the no reverse transcriptase control. RPB2Ib primers did amplify from cDNA however they showed great variation in CT values with a maximum difference of 4.7432 cycles. The no reverse transcriptase also showed greater amplification than the cDNA averaging 25.0817 cycles vs 29.0013 cycles for cDNA (Table 5.1.3.1.1). Primer pairs CBF(2) and RPB2Ib were therefore selected as the primers to use for further experimentation.

# 5.1.3.2 Identification of the Optimal *Empetrum nigrum* cDNA Dilution for qPCR

A steady increase in both CT and TM value was seen (Fig. 5.1.3.2.1) with increasing dilutions of cDNA for both primer pairs. Variability in results, as indicated by error bars, increased from the 1:50 dilution for CBF primers and from 1:100 for RPB2I.

The dilution factor of 1:20 was, therefore, selected for future real-time experiments.



Figure 5.1.3.2.1. Showing CT values (blue) and TM values (orange) for several dilutions of Empetrum nigrum sample 232 cDNA, for both CBF primers (Pale) and RPB2I primers (Dark). Error bars show standard deviation of the mean of three technical repeats.

# 5.1.3.3 Effect of chilling upon CBF Expression in Empetrum nigrum

Expression of *CBF* decreases (0.18X that of fresh tissue) after being clipped from the plant and submerged in ice for 3 hours with no overlap of error bars (Fig 5.1.3.3.1).



Figure 5.1.3.3.1. Showing fold change in *CBF* expression in *Empetrum nigrum* plant 310 (nr. Hutton-le-hole, Yorkshire, England 159m altitude) between RNA extracted from tissue freshly cut vs RNA from tissue cut from the plant and subjected to burial in ice for 3 h. RNA from fresh tissue is normalised to 1. Error bars show standard error of three technical repeats.

# 5.1.3.4 Kinetics of *CBF* Expression in Response to Chilling in *Empetrum nigrum* Between Plants Originating From Different Environments

# 5.1.3.4.1 qPCR Amplification Irregularities

Amplification irregularities occurred with the timecourse at 2 h from both sample 232 and 251 (highlighted in red; Fig. 5.1.3.4.1.1). There is apparently extremely rapid amplification of 232 2hCBF (within the second cycle) and similar rapid amplification from around cycle 13 of 251 2hCBF and RPB2I. A repeat of this experiment with a new dilution of both 2hsamples produced similar irregularities (Fig. 5.1.3.4.1.2) however the extremely rapid amplification of 232 2hwithin the second cycle is with the housekeeping RPB2Ib primers on this occasion and 251 2h*CBF* showing rapid amplification then drops off.



Figure 5.1.3.4.1.1 Realtime amplification plot 1 corresponding with Fig. 5.1.3.4.2.1. Showing amplification of CBF and RPB2I per PCR cycle (X axis). Each coloured line represents one well. Each reaction is repeated across three wells. Unusual amplification patterns are highlighted.



Figure 5.1.3.4.1.2 Realtime amplification plot 2 corresponding with Fig. 5.1.3.4.2.2. Showing amplification of CBF and RPB2I per PCR cycle (X axis). Each coloured line represents one well. Each reaction is repeated across three wells. Unusual amplification patterns are highlighted.

#### 5.1.3.4.2 Results

Several results were discounted, namely 232 2h (from both Fig. 5.1.3.4.2.1 & Fig. 5.1.3.4.2.2) 251 0 hfrom Fig. 5.1.3.4.2.1 and 251 2 h from Fig. 5.1.3.4.2.2 due to all results being undetermined. Likewise 251 2 h from Fig. 5.1.3.4.2.2, despite showing results for 3 repeats, was also discounted due to the irregularities mentioned in sections 5.1.3.4.1 & 5.2.3.4.1.

For both runs expression of *CBF* in sample 232 showed a general trend of decreasing then gradually rising and reaching basal levels of expression between 8 and 14 hours then increasing above the basal 0 h reading from 14 h.

The complete lack of a 0 h result for sample 251 in the first run and only 1 example in the second run made comparisons with its 0 h timepoint difficult. However, the general trend across both runs showed an increase from 5 to 8 h which rapidly dropped back down by 14 hours and continuing to drop by 24 hours.

Although based on only one sample for 0 h (for sample 251), it appears that sample 232 had a higher basal expression level (at 0 h) of *CBF* with sample 251 only showing 0.17x the level of expression of 232 at 0 h (Fig. 5.1.3.4.2.2). It is not known if sample 251 would also show a decrease in *CBF* expression prior to an increase, however its maximal expression is a greater total level than sample 232, also given the possible lower basal *CBF* expression level of sample 251 the fold increase is much greater at 17.7x vs 2.8x (Fig. 5.1.3.4.2.2).



Figure 5.1.3.4.2.1. Showing change in *CBF* expression in response to chilling at 5 °C (see section 2.3.1), 0 h sampled immediately before chilling at 16 °C. Two *Empetrum nigrum* plants were used; 232 (Ben Macdui, Cairngorms, Scotland 1100m altitude) and 251 (Dunkery Beacon, Exmoor, England 476m altitude). 232 0 h sample set to an arbitrary value of 1. Bars show the mean of 3 technical repeats, however in some cases 1 or more result was undetermined so stats were not applied. n=x indicates number of technical repeats that were used in the calculation of the mean of the corresponding bar (i.e. were not undetermined). For timepoints 232 2 h and 251 0 h all results were undetermined.



Figure 5.1.3.4.2.2. Showing change in CBF expression in response to chilling at 5 °C (see section 2.3.1), 0 h sampled immediately before chilling at 16 °C. Two *Empetrum nigrum* plants were used; 232 (Ben Macdui, Cairngorms, Scotland 1100m altitude) and 251 (Dunkery Beacon, Exmoor, England 476m altitude). 232 0 h sample set to an arbitrary value of 1. Bars show the mean of 3 technical repeats, however in some cases 1 or more result was undetermined so stats were not applied. n=x indicates number of technical repeats that were used in the calculation of the mean of the corresponding bar (i.e. were not undetermined). For timepoints 232 2 h and 251 2 h all results were undetermined. Numbers on bars from sample 251 show fold change from the 251 0 h timepoint.

# 5.1.4 Changes in Chlorophyll Content Of *Empetrum nigrum* and *Calluna vulgaris* in Response to Freezing

The freezing shocks of -20 °C and -80 °C cause an apparent reduction in chlorophyll content (of chlorophyll a, b and total chlorophyll) relative to the 16 °C treated samples in *Calluna vulgaris* sample 208 (Fig. 5.1.4.1). However, a significant difference (p<0.05, as assessed by a 1-way ANOVA followed by Tukey's post hoc test) was only seen between the fresh cut 16 °C sample and -20 °C treated sample for chlorophyll a, b and total chlorophyll and between the fresh cut 16 °C sample and -80 °C treated sample for chlorophyll a. There was, however, no significant (P<0.05) difference between either the fresh cut tissue and the 102 hours at 16 °C tissue, nor any significant (P<0.05) difference between the -20 °C or -80 °C treatments for chlorophyll a, b or total chlorophyll. There is also no significant (P<0.05) difference between the 102 hour 16 °C cut tissue and the -20 °C and -80 °C treatments for chlorophyll a and b and total chlorophyll content.

A different trend, relative to that seen for *Calluna vulgaris* sample 208, was seen for *Empetrum nigrum* sample 232 (fig. 5.1.4.1.) For chlorophyll a, b and total chlorophyll; the 102 hours at 16 °C tissue had a significant (p<0.05) (Chlorophyll b: 102 hours at 16 °C vs -80 °C) or highly significant (P<0.01) (all other samples) greater chlorophyll content than the fresh cut tissue or freezing shocked tissue. There was also a significantly (P<0.05) greater chlorophyll a content in the -80 °C treated tissue relative to both the -20 °C and fresh cut 16 °C treatments, but this is not seen for chlorophyll b or total chlorophyll. There was no significant difference between fresh cut tissue and the -20 °C treatment for any chlorophyll content.



Sample

Figure 5.1.4.1 showing  $\mu$ g of chlorophyll A (A), B (B) and Total A+B (C) per g tissue from two living plants of *Calluna vulgaris* (208) and *Empetrum nigrum* (232) from cuttings collected from Creag Dubh and Ben Macdui, Cairngorms, Scotland, UK respectively (see table 2.1.1.1) after four different treatments. 16°C Fresh - Tissue collected from the plant at the same time as the other samples and immediately frozen in liquid nitrogen and stored at -80°C prior to extraction, 16°C – clipped tissue spent 102 hours at 16°C prior to extraction, -20°C – clipped tissue spent 24 hours at -20°C and 78 hours at 16°C prior to extraction, -80°C - clipped tissue spent 24 hours at 16°C prior to extraction. Error bars show standard error of the mean (n=2 for *Calluna vulgaris* 208 16°C and n=3 for all other samples). Letters above bars indicate a significant difference (1 way ANOVA and post hoc Tukey's test) between that bar and the bar with the matching letter for the same species and chlorophyll type. \* = p<0.05 \*\*= p<0.01.

# 5.1.5 The Association of the Environmental Origin of the Parent Donor Plant with the Morpholoy of Common Garden Grown Cuttings of *Empetrum nigrum* and *Calluna vulgaris*

### 5.1.5.1 Empetrum nigrum

*Empetrum nigrum* had a highly significant (p<0.01) strong negative correlation (-0.8 coefficient) between compactness and longitude (Table 5.1.5.3.1 & Fig. 5.1.5.3.3) indicating that the further west that the parent donor plant came from the greater the likelihood that the sample would be compact. There was also a highly significant (P<0.01) positive (0.5) correlation between compactness and latitude (Table 5.1.5.3.1 & Fig. 5.1.5.3.3) indicating that the further north the parent donor plant came from the greater the likelihood that the sample would be compact. Likewise, there was a highly significant (P<0.01) significant (P<0.01) strong positive (0.7) correlation between compactness and altitude (Table 5.1.5.3.1 & Fig. 5.1.5.3.1 & Fig. 5.1.5.3.3.) indicating that the higher the altitude that the parent donor plant came from the more compact the sample was likely to be. The one sample from Galloway: Portpatrick appears as an outlier in both the longitude and compactness correlation (Fig. 5.1.5.3.3).

The correlation tests checking for sampling location bias in *Empetrum nigrum* indicated no association between latitude and altitude (Table 5.1.5.3.1 & Fig. 5.1.5.3.4.). However, some areas have a larger range of altitudes than others (which is unsurprising given that some areas of the UK are more mountainous than others). There was a highly significant (P<0.01) strong negative correlation (-0.7) between longitude and altitude (Table 5.1.5.3.1 & Fig. 5.1.5.3.4.) indicating that samples that came from the greatest altitude were also the most westerly. The Galloway: Portpatrick sample was, again, a clear outlier in this case, being the most westerly and the lowest altitude (Fig. 5.1.5.3.4).

also a highly significant (p<0.01) negative correlation (-0.4) between longitude and latitude, indicating that the more northernly the site the greater the likelihood that it was more westerly (Table 5.1.5.3.1 & Fig. 5.1.5.3.4).

### 5.1.5.2 Calluna vulgaris

For Calluna vulgaris visually the correlations appeared less strong than those seen for Empetrum nigrum (Fig. 5.1.5.3.5 & Fig. 5.1.5.3.3). However, Calluna vulgaris had a greater number of samples (n=68 vs n=37 for Empetrum nigrum) increasing the robustness of the spearman's rank tests. There was a highly significant (P<0.01) negative correlation (-0.4) between compactness and longitude (Fig. 5.1.5.3.5 & Table 5.1.5.3.1) indicating, as with Empetrum nigrum, that the further west that the parent donor plant came from the greater the likelihood that the sample would be compact. This correlation (-0.4) was weaker than seen in *Empetrum nigrum* samples (-0.8) (Table 5.1.5.3.1). The Yorkshire samples (further east) seem to be the predominant cause of this weakened trend with several very compact individuals (Fig. 5.1.5.3.5). There was a highly significant (P<0.01) positive (0.5) correlation between compactness and latitude (Fig. 5.1.5.3.5 & Table 5.1.5.3.1) indicating that the further north the parent donor plant came from the greater the likelihood that the sample would be compact. This was identical with the same rank coefficient (0.5) to the pattern seen in *Empetrum nigrum* (Table 5.1.5.3.1). However, there is a greater range in compactness at each latitude in Calluna vulagris (Fig. 5.1.5.3.5 & Fig. 5.1.5.3.3). There was a highly significant (P<0.01) negative correlation (-0.4) between altitude and compactness (Fig. 5.1.5.3.5 & Table 5.1.5.3.1) indicating that there is a greater probability of more compact plants from lower altitudes. This is the opposite of what was seen for *Empetrum nigrum* which had a strong positive correlation (0.7) (Fig. 5.1.5.3.5 & Fig. 5.1.5.3.3 & Table 5.1.5.3.1.)

The correlation tests checking for sampling location bias showed no significant trend between latitude and altitude as with *Empetrum nigrum* (Table 5.1.5.3.1 & Fig. 5.1.5.3.6). There was also no significant correlation between longitude and altitude (Table 5.1.5.3.1 & Fig. 5.1.5.3.6), unlike *Empetrum nigrum* which had a strong negative correlation (Table 5.1.5.3.1). However, as with *Empetrum nigrum*, there was a highly significant (P<0.01) negative correlation (-0.5) between longitude and latitude (Table 5.1.5.3.1 & Fig. 5.1.5.3.6) again, indicating that the more northernly the site the greater the likelihood that it was further west.

Table 5.1.5.3.1. Spearman's coefficients and p-values comparing correlation of compactness with longitude, latitude and altitude and location parameters with one another (longitude, latitude and altitude).

	Empetrum nigrum			Calluna vulgaris		
			Significance:			Significance:
	Spearman's		*= P≤ 0.05	Spearman's		*= P≤ 0.05
	Rank		**= P≤ 0.01	Rank		**= P≤ 0.01
	Coefficient	p value	***= P≤ 0.001	Coefficient	p value	***= P≤ 0.001
Compactness						
& Longitude	-0.756771	5.99x10 <sup>-8</sup>	***	-0.4357726	2.04x10 <sup>-4</sup>	***
Compactness						
& Latitude	0.4805234	2.61x10-3	**	0.4800531	3.43x10-5	***
Compactness						
& Altitude	0.6875309	2.59x10 <sup>-6</sup>	***	-0.3655773	2.17x10-₃	**
Latitude &						
Altitude	0.2202466	0.19		-0.1410738	0.25	
Longitude &						
Altitude	-0.679706	6.88x10 <sup>-6</sup>	***	0.2221947	0.069	
Longitude &						
Latitude	-0.4397819	6.91x10 <sup>-3</sup>	**	-0.4670459	5.94x10⁻⁵	***



Figure 5.1.3.2. Depicting locations of parent donor plants from which cuttings were collected and grown on. Cairngorms (greens) consisting of: Ben Macdui (Sites 59, 61-63, 68 & 70 table 2.1.5), Braemar (Sites 44-46, 50 & 52 table 2.1.5) and Craig Dubh (Sites 53-57 table 2.1.5). Derby (blues) consisting of: Beely Moor (Sites 144-153 table 2.1.5) and Bleaklow (Sites 154-161 table 2.1.5). Galloway (pinks) consisting of: Portpatrick (Sites 89-99 table 2.1.5) and Glentrool (Sites 100-104, 106 table 2.1.5). Pennines (reds) consisting of: Hedlyhope Fell (Sites 40-42 table 2.1.5) and Nenthead (Site 43 table 2.1.5). Wales (grey) consisting of Brecon Beacons (Sites 132-139 & 143 table 2.1.5). Yorkshire (purple) consisting of: Moors 1 (Sites 111, 113 & 114 table 2.1.5), Moors 2 (Sites 115-126 table 2.1.5) and Fylingdales (Sites 107-110 table 2.1.5). Exmoor (oranges) consisting of: Exmoor (Sites 71 & 73 table 2.1.5) and Dunkery Beacon (Sites 78-81 & 83-86 table 2.1.5).

Outline map: Contains OS data © Crown copyright and database right 2018.



Figure 5.1.5.3.3. Average (n=3) observed compactness of common garden grown *Empetrum nigrum* plants grown from cuttings taken from wild plants from various sites across the UK vs. location (Degree Decimal (DD) Latitude, Longitude and Altitude (m)) of parent donor plant. Compactness ranges from a score of 1 for very leggy samples with very little/no branching, long stems, widely spaced leaves and thin stems to 10 for highly compact samples with frequent branching, short branches, closely bunched leaves and thick stems.



Figure 5.1.5.3.4. Location distribution data for parent donor plant of common garden grown cuttings of *Empetrum nigrum* used for scoring compactness. Each of the three factors (Degree Decimal (DD) Latitude, Longitude and Altitude (m)) are plotted against one another to check for location bias based correlations.



Figure 5.1.5.3.5. Average (n=3) observed compactness of common garden grown *Calluna vulgaris* plants grown from cuttings taken from wild plants from various sites across the UK vs. location (Degree Decimal (DD) Latitude, Longitude and Altitude (m)) of parent donor plant. Compactness ranges from a score of 1 for very leggy samples with very little/no branching, long stems, widely spaced leaves and thin stems to 10 for highly compact samples with frequent branching, short branches, closely bunched leaves and thick stems.



Figure 5.1.5.3.6. Location distribution data for parent donor plant of common garden grown cuttings of *Calluna vulgaris* used for scoring compactness. Each of the three factors (Degree Decimal (DD) Latitude, Longitude and Altitude (m)) are plotted against one another to check for location bias based correlations.

# **5.2 Discussion**

# **5.2.1 Potential Model Plants**

Attempts at growing model plants from seed were unsuccessful; growth and flowering was too slow to be of productive use as a model plant in the timescale of the PhD. Therefore, further exploration of this avenue was discontinued. However, of the species studied *Dryas octopetala* showed the greatest potential, growing well from seed and flowering after 2 years. It could thus be further explored as a potential species of interest for the establishment of a model plant from high altitude and northern distribution.

*Calluna vulgaris* grew well from cuttings and flowered readily (continuously under ideal conditions (pers. obs)), which resulted in seed set and easy growth from this seed. Although not displaying as high an altitudinal or latitudinal distribution as the original model species candidates, *Calluna vulgaris* may, however, prove a good target for establishment of a model moorland shrub species. *Calluna vulgaris* specifically may be of interest for further study, especially given its dominant distribution in moorland habitats and commercial value for moorland game. That *MATK* sequences could be isolated from *Calluna vulgaris* despite the inability to isolate *CBF* indicates that lack of DNA purity is not the cause of this failed *CBF* isolation and therefore *Calluna vulgaris* could be a target for full genome sequencing and possible establishment as a model species.

# 5.2.2 The CBF sequence of Empetrum nigrum

The *Empetrum nigrum* samples sequenced came from a wide range of environments and altitudes (Table. 2.1.5a-x) which represent an array of different maximum freezing temperatures and likelihood of freezing events (Met-Office, 1981-2010). It was therefore predicted that there might be differences in maximal freezing tolerance and onset of

acclimation in *Empetrum nigrum* plants which experience different maxima and timing of freezing events. One possible mechanism of any difference could be via the transcription factor CBF which is involved in acclimation and acquisition of freezing tolerance (Jaglo-Ottosen et al., 1998, Gilmour et al., 1998, Thomashow et al., 2001). Changes to the protein structure of the CBF sequence could alter protein stability, binding and/or activation of *COR* genes (the targets of CBF) altering freezing tolerance. Once compared there were, however, very few differences between the nucleotide, and hence translated, sequences of CBF in *Empetrum nigrum* from a wide variety of environments (Fig. 5.1.2.2a-b).

The key differences (those seen in more than one sequence) were at amino acid 109 (P or L) and 148 (M or T, one sequence had K). The first difference falls within the AP2 region, the latter within the COOH terminus. The COOH terminus has a high degree of functional redundancy and even large changes in this sequence do not affect binding or activity (Wang et al., 2005). It is therefore unlikely that the variance at site 148 has any effect on the functionality of the CBF. The mutation in the AP2 region may affect CBF binding as key amino acids for binding have been identified in the AP2 region previously (Sakuma et al., 2002) however this position is not one that has been previously identified. There was no link between location and the presence of one amino acid at this position over another and numerous samples were degenerate for this amino acid at that base (i.e. had a degenerate codon in the nucleotide sequence, which resulted in P and L or M and T from the same sample, seen as X in the translated sequence (Fig. 5.1.2.2a-b)). Therefore, this suggests that these are different alleles which are both present throughout the species, with some homozygous and heterozygous individuals existing in the populations studied, and is therefore unlikely to alter CBF binding. That 148(M) is always associated with 109 (P), but not the other-way-around, suggests that the M substitution arose from a sequence with 109 (P).

Given the high overall conservation and the ubiquity of the few changes seen, it does not appear that changes to the CBF sequence have occurred in response to differing environments. It is possible that there is no variance in freezing tolerance in *Empetrum* nigrum between different environments, all reaching maximal freezing tolerance once acclimated. However, given that CBF expression has a negative effect upon growth (Achard et al., 2008, Suo et al., 2012, Liu et al., 1998, Gilmour et al., 2000, Pino et al., 2007), unless a bypass for this growth retardation has evolved, it seems unlikely that all plants from differing environments, and therefore differing freezing risk, would respond in the same manner to cold events (i.e. if a plant which frequently experiences cold without freezing activates CBF, as readily as one which does not, it will suffer with frequently reduced growth). It is possible that other CBF genes are involved; Only one gene was isolated here but many plants have more than one CBF gene (Medina et al., 1999, Xiong and Fei, 2006, Skinner et al., 2005, Xiao et al., 2008, Miller et al., 2006). Whilst it is also possible that the COR genes (targets of CBF) have changed to alter freezing tolerance, this is unlikely due to the large number of COR genes and their individual effects upon freezing tolerance. For example, constitutive overexpression of COR15A increases freezing tolerance of chloroplasts and protoplasts in A. thaliana (Artus et al., 1996). However this is at the expense of reduced survival at higher (however still freezing) temperatures (Artus et al., 1996). Whereas, expression of multiple COR genes via overexpression of CBF1 increases freezing tolerance to a greater degree than COR15A alone (Jaglo-Ottosen et al., 1998). Therefore, changes to COR genes would have to occur over multiple COR genes to affect all aspects of increased freezing tolerance, and control of this tolerance would still be required (provided by CBF). Therefore, it seems more likely CBF would change. However, given that there is no change to CBF protein itself it is more likely that the control of CBF has altered. This could be via changes in the promoter sequence (Zarka et al., 2003) or upstream regulators of CBF such as ICE1, a

positive transcription factor that regulates CBF transcription (Chinnusamy et al., 2003). Therefore, the individual freezing tolerances of plants from different locations were investigated, to identify if different freezing tolerances are associated with different locations, alongside *CBF* expression studies to identify any changes in extent and timing of *CBF* expression responses to cold based upon the location of origin of the parent plant (of common garden grown cutting plants).

# 5.2.3 CBF expression in Empetrum nigrum

# 5.2.3.1 Suitability of Primers Designed for qPCR

No previous research utilising qPCR has been reported on *Empetrum nigrum*. Therefore, several methodological steps (optimised for *Arabidopsis thaliana*) had to be re-optimised.

qPCR requires primers which are specific to the gene of interest and, ideally, perfectly match the sequence. Therefore, the sequence data for *CBF* gathered in this project for *Empetrum nigrum* was used for primer design. There is no full genome data for *Empetrum nigrum* and therefore the possibility of priming elsewhere in the genome could not be ascertained in any other way than performing PCR itself. As no previous research utilising qPCR has been reported for *Empetrum nigrum*, there were no known and tested primers for housekeeping genes (i.e. genes whose expression remains constant to normalise the change in expression of the test gene against), nor had any likely housekeeping gene candidates been previously identified. After searching the NCBI database (NCBI, 2018) for sequenced *Empetrum nigrum* genes which could be of use as housekeeping genes, only *RPB2*I was identified as a potential candidate. *RPB2I* was selected as it was the only nuclear gene sequence found covering an exon that was not ribosomal RNA or likely to respond to stress. Two pairs of primers for each gene (*CBF* and *RPB2I*) were then designed and tested. Upon testing, primers *CBF*(2) were chosen over *CBF*(1) due to

having a TM closer to the housekeeping genes, less variation in CT values between repeats and the amplification from the genomic control (NRT) relative to the cDNA being lower than seen for *CBF*(1). The TM value is the temperature at which 50 % of the primer DNA disassociates; therefore having similar TM values is advantageous. The CT value is the number of cycles taken to reach a set amount of amplification product; less variation between matching reactions indicates more reliable reactions, although this variation could be due to pipetting error. The higher amplification in the genomic control may indicate that CBF(1) was priming elsewhere in the genome. RPB2Ib was chosen over RPB2Ia because primers RPB2Ia did not amplify from cDNA and had highly variable TM values indicating a failure to bind the site of interest. This could possibly be due to non-specific binding at multiple sites within the genome, and/or the formation of primer dimers and/or that the sequence of *RPB2I* in these collected samples was not the same as for the sample used. The *Empetrum nigrum* specimen from which *RPB2*I was amplified was from Washington, USA (Goetsch et al., 2005) whereas the test samples used were from across the UK. Further it is also not stated which subspecies of Empetrum nigrum was used by Goetsch et al., (2005) to obtain the RPB2I gene in the NCBI database. It is therefore possible that the UK specimens have a different RPB2I gene sequence. However, given that the MATK sequences for samples of *Empetrum nigrum* from across the world (including north America and the UK) and from alleged different subspecies are identical (the existence of subspecies is also in question: see section: 5.2.6) and that the RPB2I exons encode a vital protein (RNA polymerase subunit 2) the probability of differing *RPB2I* exons is greatly reduced. However, this inability to amplify meant that *RPB2*a primers could not be used for amplification of the housekeeping gene. There was higher amplification from the genomic control than from the cDNA with primers *RPB2*Ib which indicated that the primers were priming elsewhere in the genome. However, since no other potential housekeeping genes were sequenced at the time of study and no other viable primer design sites were present in the *RPB2*I sequence primers *RPB2*Ib were used. In all cases the genomic control (NRT: RNA with no reverse transcriptase added when making cDNA – so no cDNA is formed) had lower CT values (i.e. a set amount of product reached with fewer cycles) than the cDNA samples. Similar CT values would be expected if there was preferential binding to the genomic DNA (as there would also be genomic contamination in the cDNA test – as it was amplified from the same RNA as the genomic tests). It therefore seemed possible that in the absence of high levels of target sequence (genomic control) primers were binding to less specific sites which would not occur, or be greatly reduced, in the presence of appropriate target (cDNA) and therefore these primers were taken forward for use.

### 5.2.3.2 Optimal dilution of *Empetrum nigrum* cDNA for qPCR

A 1:20 dilution of cDNA is the standard used in the Knight lab for qPCR on *Arabidopsis thaliana* cDNA. However, due to using a species not previously studied via qPCR (*Empetrum nigrum*) a variety of dilutions were tested to find the optimum dilution and test for the presence of inhibitors. The steady increase seen in CT value as starting material is diluted is as expected (since less target sequence, the limiting factor, means a longer time is taken to reach a certain amount of product). The regularity of this increase indicates that the RNA is unlikely to contain inhibitors which would otherwise show low amplification at high concentrations (as the concentration of inhibitor would be higher) and greater amplification at lower concentrations, as the inhibitor is diluted. A 1:50 dilution was selected as optimum due to a reasonable amplification rate, limited variation between results (i.e. small error bars) and a larger dilution therefore maximising usage of cDNA and hence RNA (which was vital due to low RNA yields).

### 5.2.3.3 The Effect of Chilling upon CBF Expression in Empetrum nigrum

An apparent decrease in *CBF* expression was seen upon subjecting tissue to chilling in ice for 3 h. This is contrary to what is typically seen for the *CBF* response in *Arabidopsis* thaliana (Gilmour et al., 1998, Medina et al., 1999). Assuming that the decrease seen is real and not an artefact, it is possible that this CBF gene is a negative regulator of other *CBF* genes and therefore downregulated in response to cold to allow for rapid increase in activation of COR genes by other CBF genes. Negative regulators of other CBF genes have been proposed in Arabidopsis thaliana, namely that CBF2 is a negative regulator of CBF1 and 3 (Novillo et al., 2004). It is unknown how many CBF genes are present in *Empetrum nigrum*; only one gene was isolated using the method described in sections 2.5-2.8. However, this does not exclude the possibility of multiple CBF genes in *Empetrum nigrum*, merely a failure to isolate them. There are other possible causes of this apparent reduction in expression. It is possible that this apparent decrease could be due to the tissue being cut from the plant for 3 hours whilst submerged in ice whereas the ambient sample was taken straight from the plant and submerged in liquid nitrogen. Removal from the plant could result in changes in the priority of expression, preparation for potential freezing being of lesser importance than severance from roots. It is known that *Empetrum nigrum* roots easily from cuttings (section 2.2.2.2), therefore it is possible that priority RNA expression is given to wounding responses and preparing for tissue regeneration and rooting. Also, whilst regulation of RPB2 (the gene chosen as a housekeeping gene for Empetrum nigrum) does not change in response to cold in Arabidopsis thaliana (Kilian et al., 2007, Winter et al., 2007, TAIR, 2018), it should be noted that expression of *RPB2* is slightly upregulated in in response to wounding in A. thaliana (Kilian et al., 2007, Winter et al., 2007, TAIR, 2018). A. thaliana has a very different growth habit and lifecycle to Empetrum nigrum and does not root easily from cuttings (a form of wounding), unlike E. nigrum. Therefore, the degree of this change in

expression of RPB2 may be different in *E. nigrum* relative to *A. thaliana*. If expression of *RPB2*I is upregulated slightly or to an even greater degree than seen in *Arabidopsis thaliana* in response to wounding this could result in an apparent decrease in *CBF* expression, due to an increase in the background reading which is assumed to remain constant. Another possibility, or potentially compounding factor, is that in response to cold there could be an increase in metabolites that act as inhibitors to qPCR in the plant, such as phenolics and polysaccharides (Schrader et al., 2012), whilst inhibitors were not a problem for RNA from fresh tissue, likely having been successfully removed in the extraction steps, they could overload the removal process at high concentrations resulting in inhibitors in the extracted RNA. This inhibition would increase the apparent CT value, however the baseline housekeeping gene would also have an increase CT value as a result of inhibition, therefore inhibition is unlikely to be the sole cause.

Another possibility for the apparent decrease is the effect of circadian rhythm. The fresh sample was placed immediately into liquid nitrogen whereas the sample exposed to the ice treatment was cut at the same time then spent 3 hours on ice. The basal level of *CBFs* and the level to which *CBFs* can be induced is known to fluctuate with the circadian clock in *Arabidopsis thaliana* (Fowler et al., 2005); the three hour difference could therefore result in different basal levels. For example, the fresh tissue could have been taken at a time where there is a higher basal *CBF* level, therefore *CBF* may be induced in the 3hour iced sample relative to the basal level 3 h later (which may be significantly lower). This seems unlikely however as it would be expected that ice should still induce *CBF* to a higher level than even the highest basal level. Another possibility is that 3 hours is insufficient time for *CBF* to be induced, either due to a general slow response or being less responsive for the time of day. For *Arabidopsis thaliana CBF* transcripts increase rapidly within 15 mins and continue to do so for up to the next 2 h, although levels then drop (yet remain higher than plants not exposed to cold) (Gilmour et al., 1998, Medina et

al., 1999), it therefore seems likely that an increase would be seen in *Emperum nigrum* after 3 hours. However, it is possible, that due to *Empetrum nigrum* coming from a colder natural environment, where cold periods are frequent, combined with a lower basal freezing tolerance prior to acclimation (approx.. -15 °C) (Yamori et al., 2005) relative to *Arabidopsis thaliana* (-4 °C to -8 °C) (Hannah et al., 2006), that a much slower induction of *CBF*s occurs in *Empetrum nigrum*. This could be because induction of genes involved with acclimation for freezing are not required until temperatures below -15 °C are expected. Cool temperatures are likely frequently experienced and so longer periods of cold may be required to upregulate *CBF*.

In order to investigate the change of expression over time a timecourse was performed with set sampling intervals. The whole plant was also cooled to avoid potential issues caused by prolonged periods separated from the plant.

# 5.2.3.4 Kinetics of *CBF* Expression in Response to Chilling in *Empetrum nigrum* Between Plants Originating From Different Environments

#### 5.2.3.4.1 Irregular Amplification

Whilst the qPCR amplification plots for the majority of samples and timepoints (Figs.5.1.3.4.1.1 & 5.1.3.4.1.2) display standard amplification patterns, the 2 h timepoint for both sample 232 and 251 exhibit abnormal amplification. This abnormal amplification takes the form of an apparently extremely rapid amplification of both the test gene (*CBF*) and the housekeeping gene (*RPB2I*) in sample 232 and rapid then failed amplification in sample 251 (Figs .5.1.3.4.1.1 & 5.1.3.4.1.2). A dilution error (namely a higher concentration) would result in apparent rapid amplification of both the test and housekeeping gene as seen in sample 232 due to higher initial template. However, this possibility was excluded by re-running the qPCR with a new cDNA dilution of this sample and the same anomaly still occurred (Fig. 5.1.3.4.1.2). Likewise, the re-run also

removed the possibility of contamination at the plate preparation stage (e.g. too much reporter probe), and also a mastermix was always used during preparation of both plates which significantly reduced the likelihood of this situation occurring initially. That abnormal amplification is seen for both samples at the 2 h timepoint and across re-runs, suggests this is due to something that occurred at this timepoint. A high increase in CBF would explain the rapid increase in CBF read but not the corresponding increase in the housekeeping *RPB2*I gene which should not change in expression. It is possible that RPB2I is not a true housekeeping gene and is also being upregulated, although RPB2 has previously been found to be suitable as a housekeeping gene in parthenogenic fungi (Jacob et al., 2012) and does not respond to cold in Arabidopsis thaliana (Kilian et al., 2007, Winter et al., 2007, TAIR, 2018) it does not exclude the possibility that expression does change in Empetrum nigrum however the probability is reduced. It is also possible that something else is being upregulated in the genome (alongside CBF) which the RPB2Ib primers are also priming to. This is supported by the primer tests (see section 5.2.3.1) which indicated the possibility of the RPB2Ib primers priming elsewhere in the genome. If expression of this/these other sites to which RPB2Ib primers are priming to are upregulated after 2 h this would explain the increase in *RPB2I* expression. It is also possible the plants are not responding to the chilling but to some other environmental factor that may have occurred at the 2 h timepoint. The growth chambers are constantly monitored and no record of an unexpected change in the environment was recorded. However, it is possible that a very brief sudden change would not be recorded due to the infrequency of the sampling by the monitor. The likelihood of this resulting in a change in RPB2I expression seems unlikely, given extreme transience of such an event and the general stability of expression *RPB2*I in response to stresses in *A. thaliana* (Kilian et al., 2007, Winter et al., 2007, TAIR, 2018). The abnormal readings could alternatively, or additionally, be the cause of an accumulation of inhibitors such as phenolics or polysaccharides (Schrader et al., 2012). This is unlikely to be a direct response, however, since inhibitors, inhibit qPCR rather than resulting in an apparent increase. One possibility is that inhibitors, via the inhibition of amplification, are resulting in the promotion of the formation of primer dimers; if the target sequence is sequestered due to inhibitors primer dimers may be more likely to form. SYBR green binds non-specifically to any dsDNA (Giglio et al., 2003, ThermoFisher, 2018b), disassociating upon DNA melting. Therefore, it will bind to primer dimers giving a false fluorescence response, which could explain the apparent rapid increase in product (which is in fact primer dimers) in both the test (*CBF*) and housekeeping (*RPB2*I) genes. Whilst low target sequence could also result in primer dimers it would not explain the apparent increase in *RPB2*I which should remain constant. However, evidence of primer dimers should appear on the dissociation curves (ThermoFisher, 2018a) and this was not seen.

#### 5.2.3.4.2 Kinetics of CBF Expression

There was very little amplification of *CBF* in the 0 h timepoint for sample 251 (Exmoor), resulting in several results being undetermined (undetermined samples being those that do not reach the designated amount of product within 40 cycles), those which were not undetermined had a very high CT values. This indicates that either there was some problem with amplification, possibly due to contaminants in the RNA (for example inhibitors such as phenolics or polysaccharides) or that there was very little to no basal level of *CBF* expression in sample 251. The latter possibility is plausible; 16 °C, with light cycles mimicking summer, is not a temperature and season in which a low altitude and southernly Exmoor sample (sample 251: Dunkery Beacon, Exmoor, England 476m altitude) is likely to experience a freezing event (Met-Office, 1981-2010) therefore there may indeed be no or very little basal level of *CBF* expression. Sample 232 however, being from a northernly and high altitude location (Ben Macdui, Cairngorms, Scotland 1100m altitude), would possibly experience freezing events in summer (and was collected from

above snow lie in July) where sudden changes in temperature are likely common. For example on the  $30^{\text{th}}$  September 2015 the lower altitude (339m) met-office recording station at Braemar recorded a maximum temperature of 24 °C and a minimum of -1.3 °C (Met-Office, 2016). Therefore sample 231 having a higher basal expression level of *CBF* expression relative to 251 might be to be expected.

Both samples show a pattern of an initial drop in *CBF* expression upon chilling, followed by an increase in expression. In sample 251 (Exmoor) the CBF expression drops back down between 8 and 14 h, whereas sample 232 (Cairngorms) does not appear to decrease after 24 hours. CBF expression in Arabidopsis thaliana starts to increase after ~15 minutes and peaks at around 2 h before dropping back down (Gilmour et al., 1998, Medina et al., 1999). Based on these expression patterns it would appear that the response in Empetrum nigrum is much slower peaking at around 14-24 h for the Cairngorms sample (232) and around 8 h for the Exmoor sample (251). It should be noted, however, that it is not known what happens between 15 minutes and the 5 h timepoint and there are still some amplification issues after 5 h (as exemplified by n<3 (Figs. 5.1.3.4.2.1 & 5.3.4.2.2)). As discussed in section 5.2.3.4.1 readings within these timepoints are not reliable due to interference with the qPCR meaning the fluorescence signal cannot be correctly read. It is therefore possible that there is an initial early increase in CBF expression which is not seen in these results. Multiple peaks in *CBF* expression has been reported in Arabidopsis thaliana in response to chilling, a second peak occurring around 2-3 h (depending on CBF gene and plant accession) (McKhann et al., 2008). It is therefore possible that the missing data may cover an initial peak in *CBF* expression and that the observed decrease followed by an increase is a later peak. Assuming, that the apparent slower than A. thaliana response is correct this slower response could be due to the higher basal freezing tolerance of *Empetrum nigrum* (approx. -15 °C) (Yamori et al., 2005) over Arabidopsis thaliana (-4 °C to -8 °C) (Hannah et al., 2006). The risk due to a further drop

in temperature is therefore of less risk to *Empetrum nigrum* than to *Arabidopsis thaliana*, therefore a slower response allows for assessment of the temperature drop to identify if the new temperature is consistent or merely a fluctuation. This would also explain the apparent slower response in the Cairngorms sample than the Exmoor sample. A prolonged period of 5 °C in Exmoor is more likely to indicate the onset of winter than a prolonged period of 5 °C at hgh elevation in the Cairngorm mountains where drops to 5 °C would be common even in summer. For example, the lower altitude (339m) Braemar weather station records a minimum close to 5 °C or lower almost every month (Met-Office, 1959-2018), this higher altitude sample (1100m) would therefore regularly experience 5 °C. The Cairngorms sample also has an apparent higher basal level expression on *CBF*. If this is actually the case (see problems discussed earlier) it is possible that, prior to acclimation, it is more freezing tolerant than the Exmoor sample, therefore providing another reason for the lack of requirement to respond as quickly as the Exmoor sample. That the expression of *CBF* in the Cairngorms sample does not drop after 24 h is also interesting and there are several possibilities to explain this. One is that it is a slow and steady response relying on a slower and longer expression of CBF to achieve high freezing tolerance rather than an initial high increase in expression of CBF, allowing for easy and rapid reversal should the cold period not persist. Another possibility is that because the 24hour timepoint is 2 hours after the lights switch on in the chambers, this would cause a slight warming effect, it is known that CBF1-3 expression also increases during de-acclimation in Arabidospsis thaliana (Zuther et al., 2015), and in warm spells in acclimated overwintering birch (Welling and Palva, 2008). However, the temperature fluctuation would be very minor (a degree or two) so this seems unlikely. Another possibility is that the maintained high CBF expression after 24hours is a new basal CBF expression level for the Cairngorms sample and this is higher after exposure to cold than the Exmoor sample: Although *CBF* expression decreases after an initial peak the overall level usually remains higher than before acclimation in *Arabidopsis thaliana* (McKhann et al., 2008) and the degree of increase is dependent on the accession, with more freezing tolerant accessions tending towards a greater increase (McKhann et al., 2008). It has also been shown that a maintained high level of *CBF* is associated with increased freezing tolerance, with a more freezing tolerant winter wheat maintaining a higher *CBF* expression level upon exposure to cold than a less freezing tolerant Spring wheat variety (Kume et al., 2005). Indeed, the winter wheat variety still maintained a high *CBF* transcript level (and still some evidence of still increasing expression) after 63 days in the cold whereas the transcript level for a spring wheat variety started to decrease after 21 days (after a steady increase up to that point) (Kume et al., 2005). This long-term increase *CBF* expression level in wheat varieties was not apparent upon a 24hour scale, with both varieties appearing to decrease in *CBF* expression by 24 hours (Kume et al., 2005). Therefore, it is highly possible that, if studied over a much longer time scale, a different pattern of *CBF* expression may be seen for the *Empetrum nigrum* samples.

Another possible explanation for the apparent expression pattern is that the *CBF* sequenced could be a negative regulator of other *CBFs*. It has been proposed that *CBF*2 regulates expression of *CBF*1 and 3 in *Arabidopsis thaliana* (Novillo et al., 2004). The Cairngorms sample may have a consistent higher basal level of such a regulator as it would be subject to regular temperature drops which could result in energy loss if constant acclimation and declamation occurred. It would also be important at high altitude and latitude to maximise the growing period therefore continuing to grow at low temperatures whereas this would be of less import to a low altitude and latitude plant where there are more, longer and more optimal growing degree days. The initial apparent decrease in *CBF* expression in both samples could be the removal of the negative regulator in response to the rapid temperature drop, therefore allowing upregulation of the other *CBFs* to cold acclimate. The negative regulating *CBF* then increases to a high level to cease

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upregulation of the other *CBF*s. The relatively low altitude and latitude Exmoor sample then reduces expression of this regulator again to continue at the current, new level of acclimation. The high altitude sample, upon sensing no further drop in temperature, may continue with a high level of negative regulation both in preparation for removal of the negative regulation to rapidly increase freezing tolerance and acclimate in response to a further decrease in temperature but also to inhibit dormancy and maintain metabolic and growth activities at the current lower temperature.

Due to high variability in results and amplification issues no reliable conclusions could be drawn from the study. Even between repeats with the same cDNA samples, variation in the pattern was seen indicating high error and amplification issues. Potential causes include the initial RNA isolation step where genomic contamination could be present as indicated in section 5.2.3.1. The RNA could also be contaminated with inhibitors. Although the dilution tests indicated no presence of inhibitors at ambient temperatures it is possible that inhibitors, such as polysaccharides (Schrader et al., 2012) would accumulate in response to CBF expression, as seen in Arabidopsis thaliana (Cook et al., 2004) and therefore timepoints with higher expression of *CBF* may also have inhibitors present. Primer issues seem the most likely cause (as identified in section 5.2.3.1 and 5.2.3.4.1). It is possible that the housekeeping primers were non-specific and the CBFprimers may also have been relatively non-specific. However, this specificity issue cannot be addressed by the creation of new primers, as no alternative sequences are known to design the primers to. RNA transcript level could also be assessed via other methods such as RNA Gel-Blot Hybridization Analysis as described in Gilmour et al., (1998) and Fowler et al., (2005), however there is a loss of finer detail using this method rather than qPCR. The ideal solution would be to fully genome sequence *Empetrum nigrum* to identify all potential CBF genes and design of specific primers for each. This would overcome the non-specificity issue and would also allow for the analysis of all CBF

sequence responses which could aid in the identification of whether the CBF assessed in this timecourse was a negative regulator of possible other CBF sequences or not. Full genome sequencing would also allow for the identification of multiple potential housekeeping genes which could be further tested, such as via RNAseq, and the optimum (or a selection of several) housekeeping gene(s) could then be used to improve robustness of the data and baseline readings. The possible issues with inhibitors or extremely high transcript levels could also be assessed and addressed by further dilution tests at multiple timepoints. Once the qPCR technical issues were addressed, further biological complications could be assessed. Repeat timecourses could be run to identify what happens at the 2 hour timepoint that is missing from this experiment due to the issues described above. The issue of circadian cycling could be assessed via a timecourse without chilling to assess how the baseline expression of *CBF* cycles throughout the day, which could then be compared to the same timepoints after a chilling event. More frequent earlier timepoints could also be taken to assess early changes in *CBF* expression and if multiple peaks in expression is seen. Later timepoints, over days and weeks exposed to the new cold temperature, could assess if the increased CBF expression is truly a new basal level or a slow increase in CBF expression response, and comparisons could be made between the length of maintenance and degree of CBF expression between plants from different environmental origins. Freezing assays on plants from different locales could also be performed to assess if the basal level of freezing tolerance differs between samples from different locales and if this correlates with the basal CBF expression level. More timepoints and/or freezing assays would however require the generation of more tissue, plants would have to be grown on to a larger size or multiple cuttings taken in order to have multiple clone plants to test. The possibility of delayed dormancy in plants which are more prone to cold periods outside of winter such as 232 could also be assessed

by infra-red gas analysis (IRGA) to assess the change in photosynthesis upon chilling relative to plants from warmer climates.

Despite inherent unreliability of results and resultant difficulty in interpretation, the preliminary data does, however, suggest that there may be a difference in regulation and timing of *CBF* expression between plants collected from differing locations. Therefore, further investigation to assess if this is consistently the case and follows a specific pattern and, if so, the mechanism for this difference, is warranted.

# 5.2.4 The use of Chlorophyll Content to Assess Freezing Tolerance in *Empetrum nigrum* and *Calluna vulgaris*

# 5.2.4.1 Calluna vulgaris

The lack of any significant difference between the chlorophyll content of fresh cut *Calluna vulgaris* tissue and tissue excised and stored at 16 °C (Fig. 5.1.4.1) may suggest that any effect on chlorophyll content due to time cut from the plant was minimal. Therefore, the time spent cut from the plant during and after thawing of the test samples could be discounted as an influencing factor. However, significant (p<0.05) differences in chlorophyll content between tissue exposed to freezing temperatures and those at 16 °C only occur between the frozen and fresh tissue and not between the frozen and the cut and stored at 16 °C tissue (Fig 5.1.4.1). This indicates that the length of time cut from the plant does have an effect. This effect, however, cannot be the sole cause of the significant difference between the 16 °C fresh tissue and the frozen tissue was purely a factor of the time the frozen tissue spent excised from the plant, there should also be a significant difference in chlorophyll content between the 16 °C fresh tissue and the frozen tissue and the 16 °C excised and stored tissue; which does not occur (Fig. 5.1.4.1). Additionally, given that
cooler temperatures should at the very least slow, if not stop, degradation, if the observed difference between fresh cut 16 °C tissue and frozen tissue was purely due to the time spent cut from the plant by the frozen tissue, it would be expected that the chlorophyll content of the frozen tissue would be higher than that of the tissue cut and stored at 16 °C. However, the data indicates a trend (although not significant) towards less chlorophyll content in the frozen tissue relative to the cut and stored at 16 °C tissue (Fig. 5.1.4.1). This would suggest that time cut from the plant is not solely responsible for the significantly reduced chlorophyll content in the frozen tissue relative to the fresh tissue, however, time cut from the plant does have enough of an effect to add sufficient variance to the chlorophyll content to remove any significant effect of damage by freezing. A further compounding factor is that the 16 °C excised and stored tissue control favours an artificially reduced difference between it and the frozen tissue: This is because during the 24 hours at 16 °C, whilst the frozen test samples are at -20 or -80 °C, the metabolism at 16 °C is not slowed, as it is in the frozen samples. Therefore, damage as a result of being cut from the plant, will be more severe in the excised 16 °C sample relative to the frozen samples. Despite this limitation the possibility of the length of time excised from the plant having an effect (even whilst frozen) does have to be accounted for by running this comparison as no studies have been performed upon the metabolic activity of these species at these temperatures; therefore, it is unknown if degradation may occur whilst frozen. Given the general, yet non-significant, trend of reduced chlorophyll content in samples exposed to freezing temperatures, relative to the excised and stored at 16°C tissue, and that standard error bars between frozen tissue and tissue not exposed to freezing do not overlap, it seems likely that with more replicates of multiple tissue fragments (for this experiment three chlorophyll extractions were performed on one or two plant fragments) this variance may be minimised resulting in a significant difference between tissue excised and stored at 16 °C and frozen tissue.

There was no difference in chlorophyll content of *Calluna vulgaris* samples between -20 °C and -80 °C (Fig. 5.1.4.1), indicating that both temperatures are equally damaging. Given the reduced chlorophyll content, therefore implying reduced plant health, of samples exposed to freezing shocks of -20 °C and -80 °C relative to the 16 °C samples (Fig. 5.1.4.1), it seems likely that this sample is not constitutively freezing tolerant (at least to -20 °C). The sample comes from an altitude between 500 and 600m above sea level and records show that temperatures of -20 °C are occasionally reached in winter at the nearby station, Aviemore (228m above sea level) (WeatherOnline, 1999-2018), with occasional summer freezing temperatures (WeatherOnline, 1999-2018) and a high variability in summer temperature. For example a weather station in Braemar (also nearby and 327m above sea level) recorded the UK highest and lowest temperature maxima and minima on the same day in September 2015 (24 °C and - 0.3 °C) (Met-Office, 2016). Either these summer freezing temperatures are sufficiently rare that although minimal damage may occur, the risk outweighs the benefit of increased growth through a lack of constitutive freezing tolerance (Achard et al., 2008, Suo et al., 2012, Liu et al., 1998, Gilmour et al., 2000, Pino et al., 2007) or, more likely, there is a basal freezing tolerance conferred for mild freezing temperatures (greater than the -20 °C tested). This basal level is either then increased upon acclimation signals for more extreme freezing events or regular low temperatures maintain acclimation. However, once plants are in controlled warm environments this basal level is lost. Previous studies indicate that Calluna vulgaris is freezing tolerant to around -15 °C in the wild, during months they are likely to be acclimated (April and October) (Sæbø et al., 2001, Caporn et al., 1994) which would explain the inability to tolerate temperatures below -15 °C prior to acclimation and support the evidence that the tissue was killed at both -20 °C and -80 °C. However, it seems unlikely that all Calluna vulgaris plants are incapable of tolerating temperatures below -15 °C after acclimation, given that the parent plant donor plant likely experienced

temperatures below -20 °C in its natural environment (WeatherOnline, 1999-2018, Met-Office, 1959-2018). Comparisons between freezing tolerance (such as via electrolyte leakage assays) before and after acclimation for *Calluna vulgaris*, coupled with realtime results of *CBF* expression (if *CBF* could be isolated from *Calluna vulgaris* via another method such as full genome sequencing) may, therefore, isolate differences between plants with differing environmental origins.

#### 5.2.4.2 Empetrum nigrum

The significantly higher chlorophyll content in the cut tissue stored at 16 °C relative to the freshly cut tissue (Fig. 5.1.4.1) would indicate that the fresh tissue has potentially undergone a damaging incident not experienced by the tissue stored at 16 °C. Since all tissue samples were cut at the same time, this cannot be explained by any stress experienced by the plant prior to the collection of fresh tissue, but post collection of the stored at 16 °C tissue. The fresh cut tissue was immediately frozen in liquid nitrogen (~-196 °C) prior to transfer and storage at -80 °C. It was then re-transferred to liquid nitrogen immediately prior to freeze drying. Empetrum nigrum has thick, fleshy and waxy leaves, it is therefore possible that damage occurred whilst warming, due to prior freezing, during the 24 hour freeze drying process if sublimation did not reliably occur. The 16 °C stored tissue may not have experienced as much damage as it would have already undergone some desiccation from having spent time excised from plant. Therefore, it would undergo less freezing damage when stored in the -80 °C due to less available water to result in ice formation and it would also be quicker to desiccate when freeze-dried due to being partially desiccated prior to being freeze-dried. Another possibility is that the prior desiccation may have resulted in increased freezing tolerance as it is known from other species that there are shared pathways between drought and freezing acclimation; one inferring tolerance to the other (Haake et al., 2002). It is also been shown that acclimated *Empetrum nigrum* can survive at -80 °C (Yamori et al., 2005), therefore, the tissue stored at 16 °C may have been acclimated and as a result less damaged by freezing at -80 °C and immersion in liquid nitrogen.

Another and potentially compounding possible factor was that -80 °C may be insufficient to completely cease all metabolic activity, no studies have been performed on the metabolic activity of *Empetrum nigrum* at -80 °C however *Empetrum nigrum* is able to survive at -80 °C after acclimation (Yamori et al., 2005). Therefore, it is possible that the "fresh" tissue, after undergoing potentially severe freezing damage in liquid nitrogen, may have started to degrade at -80 °C. Therefore, the fresh sample stored at -80 °C (for 4 days longer than all other samples) may have undergone degradation after damaging freezing stress in liquid nitrogen whereas the sample stored at 16 °C was immediately transferred to -80 °C (with no liquid nitrogen step) and stored for two days prior to being placed in liquid nitrogen for freeze drying.

Other explanations or compounding factors include that tissue age may influence chlorophyll content, although young tissue was preferentially selected it is possible that tissue of a different age, with a differencing level of chlorophyll content, was used for the fresh sample. However, the most likely explanation is that the fresh sample (containing a higher water content due to lack of 72 hours at 16 °C which would result in partial desiccation in the other samples) did not undergo full desiccation during freeze drying, therefore when weighed prior to grinding water would still be present adding to the apparent "dry" weight therefore reducing the apparent chlorophyll content per gram of dry weight result.

The chlorophyll a content of tissue exposed to -80 °C was significantly higher than for the -20 °C (P <0.01) and 16 °C fresh tissue (P< 0.05) however this is not seen for chlorophyll b or total (a+b) chlorophyll (Fig. 5.1.4.1). That there is significantly more

chlorophyll a but not b may be a reflection of the scale, namely that there is more of a than b so slight variations in readings have a greater effect on the error of chlorophyll b. The significantly higher chlorophyll a content in the -80 °C sample could therefore imply that the -20 °C and 16 °C samples have undergone greater damage. A possible explanation as to why the 16  $^{\circ}$ C and -20  $^{\circ}$ C tissue may have showed signs of greater damage than the -80 °C sample could be that -20 °C does not entirely stop metabolic activity. Therefore, being removed from the plant has initiated degradation and chlorophyll loss to the 16  $^{\circ}$ C fresh and -20 °C tissue but not the -80 °C treated tissue where metabolic activity has ceased. Alternatively, If the -20 °C and 16 °C fresh samples had a different initial chlorophyll a:b ratio than the -80 °C treated sample this could explain the significant difference in chlorophyll a but not b or total chlorophyll. Differences in chlorophyll a:b ratios could be due to shading by other parts of the plant, as shading is known to increase chlorophyll b relative to a (Shao et al., 2014, Muhidin et al., 2018) or differing tissue ages (Day et al., 1996). Total chlorophyll loss, however, remains constant across all three treatments (Fig. 5.1.4.1). Given the significant, and larger, decrease in both chlorophyll a and b at -80 °C and -20 °C relative to the 16 °C excised tissue and the aforementioned probable decrease in apparent chlorophyll content in the 16 °C fresh tissue; it is more likely that both the -20 °C and -80 °C treated tissue samples have been severely damaged. Therefore, the apparent variation in chlorophyll a is more likely due to the aforementioned possible differences in tissue and initial chlorophyll a:b ratios as opposed to greater survival at -80 °C.

Despite compounding factors and an unusual fresh tissue result, these results do indicate, however, that the plant sample studied (232) is not constitutively acclimated to withstand freezing shocks of -20 °C; sample 232 was a cutting from a plant found 1100m above sea level where freezing events would be common (and was collected above patches of mountain snow lie in July (personal obs.)). For example, the nearby lower altitude (339m)

Braemar weather station records a minimum close to 5 °C or lower almost every month (Met-Office, 1959-2018), therefore the plant at 1100 m above sea level would likely experience much lower and freezing temperatures all year round. Yamori et al., (2005) found that *Empetrum nigrum* can withstand ~-15 °C prior to acclimation, indicating that basal freezing tolerance is high and therefore increased freezing tolerance is not required except in response to consistent low or freezing temperatures. It is therefore probable that -20 °C and -80 °C killed the non-acclimated tissue samples and the variation seen is due to other factors (discussed above).

Another way of assessing freezing tolerance *Empetrum nigrum* may be via electrolyte leakage freezing assays. However given the ability to tolerate extremely low tempertures, especially after acclimation (Yamori et al., 2005) precision temperature drops within the desired range -10 °C to -90 °C would be difficult to achieve. An alternative possibility would be to investigate the effect of mild freezing on photosynthetic activity and plant health upon recovery, to identify if any difference in tolerance and rate of recovery is seen between plants from different locations. This could be assessed via methods such as chlorophyll fluorescence and infra red gas analysis (IRGA).

# 5.2.5 The Association of the Environmental Origin of the Parent Donor Plant with the Morpholoy of Common Garden Grown Cuttings of *Empetrum nigrum* and *Calluna vulgaris*

Different plants taken from cuttings from wild plants displayed a variety of different morphologies when grown in common conditions, implying that there is a genetic influence upon the morphology. Possible causes of these differences include: Genetic selection favouring certain morphologies under certain environmental conditions; genetic drift within the populations due to spatial separation of populations due to fragmented environments (both manmade and natural); epigenetic traits carried across in the cuttings i.e. cutting grown plants carried over a genetic memory, and therefore activation of genes appropriate for their previous environment. These genetic differences could also be either inherent (i.e. always the case e.g. compact plants came from cuttings from compact donor parent plants) or plastic with the morphology changing relative to the parent donor plant due to the change of environment in which the cuttings were grown relative to the environment of origin (i.e. plants from different environments may alter their morphologies in different ways in response to the change of environment given their previous environment, e.g. the common garden temperature may be, on average, warmer or cooler than previous environment, nitrogen status may be more or less than previous environment, different light levels etc).

The presence of significant correlations in the controls; longitude with altitude and longitude with latitude for *Empetrum nigrum* and longitude with latitude for *Calluna vulgaris* means that these factors cannot be isolated from one another to analyse their effect upon morphology. Other environmental factors not recorded which may also correlate in some way could also have an impact upon morphology. One factor, for example, could be related to the samples from Galloway (the west-coast of Scotland) frequently being outliers as well as being the furthest west and lowest altitude samples. For example these locations, being on the west coast, are also from the wettest location (although not substantially wetter than the Cairngorms) (Met-Office, 1981-2010) and the majority of Galloway: Portpatrick samples were collected on the beach edge where they would experience salt stress. There was only one example of *Empetrum nigrum* that could be found in Galloway, this was an individual extremely large plant on a coastal beach path under a large overhang, no nearby plants could be found. Therefore, as well as the problems associated with inferring a pattern with only one example from a location it also seems likely that this particular example may not be from the area and a recent invader,

potentially via seed spread by users of the coastal path. The correlation of longitude with altitude does not occur in *Calluna vulgaris*. Samples of *Empetrum nigrum* and *Calluna vulgaris* were collected from the same sites wherever possible so the cause of no correlation in *Calluna vulgaris* is not due to different collection site locations but due to the much higher number of samples collected from low altitude in Galloway (furthest west site) where *Empetrum nigrum* was not present in abundance. If the Galloway samples were removed from the analysis, *Calluna vulgaris* would show a similar correlation between longitude and altitude.

These problems are further compounded in *Empetrum nigrum* by the samples from the Cairngorms: they are both the most northernly and highest altitude plants with the largest representation from the various locations. They are also the only specimens with compact forms (i.e. greater than 5.5). If the Cairngorms group were removed from the scatter plots the positive trend between compactness and latitude would be lost, in contrast a slight negative trend may be seen (Fig. 5.1.5.3.3). This indicates that it is unlikely that latitude has as significant effect upon morphology as shown by the statistical tests. If the Cairngorms samples were removed from the longitude scatter graphs, however, a similar negative correlation pattern would still be seen. Given that longitude and latitude are themselves negatively correlated with one another the negative correlation of one factor with compactness could be driving the other towards an apparent negative correlation. Given the more consistent negative correlation of longitude with compactness (i.e. not altering upon addition/removal of Cairngorms samples) it seems likely that longitude will be impacting upon the apparent negative correlation (upon removal of the cairngorms samples) of compactness with latitude. However, given the current data this cannot be tested. The strong correlation of altitude with compactness appears to be the most reliable correlation: If the Cairngorms samples are removed a positive trend between altitude and compactness is still present. If the Cairngorms data points are analysed in isolation of all

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other samples the positive trend is still seen and this eliminates the compounding factors of latitude and longitude. It therefore seems probable that given sufficient adjustment to collection methods to reduce compounding factors (see later) this correlation may still be seen. However, no reliable conclusions can be drawn with the current limitations.

Arctic and alpine plants have a dwarf and frequently compact growth form (D. Billings, 1973) so a correlation in compactness with altitude is not unexpected. However, this compact for or dwarfism is typically associated with separate species rather than within the same species. Well known examples include trees such as the prostrate willow Salix herbacea and cushion plants such as Silene acaulis which have taller, non-dwarf, counterparts in the same genus at lower latitudes and altitudes. The dwarfism of the majority of arctic and alpine plants is clearly genetic, as they are frequently grown in low altitude and latitude gardens as ornamental plants, with the dwarf morphology continuing through future lowland-grown generations. The genetic cause of this dwarfism is unknown, and indeed may not be a single cause for all species. Dwarf Arabidopsis thaliana can be found on swiss mountain slopes (Luo et al., 2015) and these plants have greater fitness in alpine locations than non-dwarf A. thaliana (Luo et al., 2015). Upon investigation Luo et al., (2015) found that the cause of the dwarfism in two dwarf accessions (of 12) was a single nucleotide deletion in the gene encoding GA 20-oxidase (GA5) (an enzyme in the GA<sub>3</sub> pathway). However this does not indicate that this is the causative factor for all species and indeed was not found in other dwarf accessions within the species (Luo et al., 2015), 10 of 12 accessions remaining unexplained, including the highest altitude accession. Overexpression of CBF genes is associated with dwarfism (Achard et al., 2008, Suo et al., 2012) and since this infers constitutive freezing tolerance (Knight and Knight, 2012) as well as inferring the advantages of compact dwarfism in these environments (such as remaining near the boundary layer, limiting damage from wind and ice, producing own microclimate and taking advantage of microclimates) would

also protect against freezing all year round, assuming the same mechanism of freezing tolerance is utilised by these plants. However arctic and alpine plants do acclimate (Yamori et al., 2005, Stushnoff and Junttila, 1986) so are not constitutively acclimated, though upon acclimation they are able to tolerate temperatures much lower than plants typically studied for CBF overexpression, such as Arabidopsis thaliana and Glycine max (Achard et al., 2008, Suo et al., 2012, Hannah et al., 2006). The basal level of freezing tolerance is also higher (tolerating lower temperatures) than the basal level of typically studied A. thaliana and crop species (Yamori et al., 2005, Stushnoff and Junttila, 1986, Odasz, 1990, Jaglo-Ottosen et al., 1998, Hannah et al., 2006, Staggenborg et al., 1996). This indicates that either a secondary mechanism is involved or there is a greater expression of *CBF* or a more potent form with a basal level of expression all year round which increases to a much higher level upon acclimation. Whilst greater expression or more potent CBF genes would be an interesting explanation for arctic and alpine dwarfism this has not been studied and preliminary tests on a high altitude and latitude Empetrum nigrum vs a low altitude and latitude Empetrum nigrum are inconclusive (see section 5.2.3). It is however possible that the sample from a higher altitude and latitude does have a higher basal expression of CBF (See sections 5.1.3.4.2 and 5.2.3.4.2). However, expression results are highly unreliable and further study would be needed.

The greater number of *Calluna vulgaris* plants increased the robustness of the Spearman's rank correlation tests but confounding factors, predominantly related to collection site, are still a problem. A weaker correlation between compactness and longitude was seen for *Calluna vulgaris* (-0.4 vs -0.8 for *Empetrum nigrum*) the correlation in this case appears less strong due to several compact samples from Yorkshire. Rather than another environmental aspect of Yorkshire having an effect upon morphology weakening the apparent trend, it seems more probable that this apparent negative trend may be a symptom of a very small number of plants from several sites in the longitude range

between the Cairngorms and Yorkshire (North Pennies (n=3), Wales (n=3) and Derbyshire (n=6)) which score with a less compact morphology. Given the large range of compactness scores at each longitude where there are a larger number of samples it seems likely that the score range when only a small number of samples were taken will not be representative of the true range. The positive trend between compactness and latitude is similarly unreliable, as well as being unable to distinguish affects due to longitude vs latitude (due to the correlation of the two variables), this trend is due to the higher compactness levels of the two Scottish samples (Galloway and The Cairngorms). These locations have previously been identified as unusual, in that the Cairngorm samples are from the highest altitude far surpassing that of any other site and not having any low altitude examples. Therefore, the effect of altitude in this case cannot be discounted. Both Galloway and the Cairngorms also receive higher rainfall than all other sample sites (Met-Office, 1981-2010) and Galloway samples are also typical outliers with the lowest altitude and no high altitude examples. Galloway samples were also collected from on, or immediately adjacent to, the beach (Portpatrick) or from under woodland (Glentrool) so other environmental factors are of especially high risk in this case (such as shade or salt adaptations) therefore the reliability of this apparent correlation is in question. The apparent negative correlation (-0.4) between altitude and compactness is also in question. This relatively weak correlation seems primarily driven by the low altitude Galloway samples which all display a much more compact morphology relative to other sites. If these samples were removed this trend would be substantially weakened. Unlike in *Empetrum nigrum*, neither a positive nor negative trend is immediately apparent from the scatter plot when looking only at the Cairngorms samples thereby removing the majority of other influencing environmental factors. It therefore seems likely that this correlation between altitude and compactness is not reliable and that other environmental factors are causing this correlation.

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It should also be noted that compactness ranks between *Calluna vulgaris* and *Empetrum nigrum* are not directly comparable. Criteria for compactness were subtly different; a score of 1 was used for the most leggy plant and 10 for the most compact, this range varied between the two species. Leggy *Calluna vulgaris* is still quite bushy whereas leggy *Empetrum nigrum* was typically one or two long continuous creeping stems. *Empetrum nigrum* seemed much more plastic in its morphology, therefore the range of morphologies between 1 and 10 for *Empetrum nigrum* was much larger than the range between 1 and 10 for *Calluna vulgaris*.

Some of the aforementioned limitations and confounding factors preventing clear analysis could be overcome via a comprehensive and strategically planned collection. The samples were not collected with this analysis in mind, sample locations were chosen to cover large sections of the UK in a limited window of time (with two collection days at each site, 3 in the Cairngorms). These sites were also chosen for convenience, differing maxima and timing of freezing events (Met-Office, 1981-2010) and where there were known records of both *Calluna vulgaris* and *Empetrum nigrum* plants growing (BSBI, n.d.). Numerous plants also died prior to study due to regular failures of the growth chambers greatly reducing plant numbers. The effect of latitude vs compactness and altitude vs compactness is of the most interest for this study, due to increased severity and frequency of freezing events with increasing latitude (Met-Office, 1981-2010) and altitude (Met-Office, 2017). It is harder to limit environmental effects across a latitudinal gradient and it is limited by the geology of the study area, however the robustness of this study could still be greatly improved by collecting numerous samples across multiple latitudinal gradients thus limiting longitudinal effects. Altitude had the strongest evidence for a possible correlation in *Empetrum nigrum* and would be of the greatest interest for further study. The indication that a correlation can be seen across an altitudinal gradient at one site (Fig 5.1.5.3.3: Cairngorms) indicates that multiple studies limited to one altitudinal gradient each may be possible, which would limit other potential environmental factors. Numerous altitudinal gradients on both slope sides, to account for other potential differing conditions on different faces, could be analysed both individually (i.e. 1 mountain and facing) grouped (all mountains of one facing) and all together (i.e. all mountains and facings). The factor of longitude and latitude would be essentially removed (due to being on too small a scale to cause an effect) and environmental differences would be largely removed. Altitude would still tend to have compounding factors such as soil quality and depth generally decreasing as higher altitudes are reached along with a change in the degree of wind exposure however microclimates would also have an effect and would alter not with the altitudinal gradient but with topology. The possible effect of ploidy level on morphology (see 5.2.6 A Note on *Empetrum nigrum* subspecies) could also be addressed in such a study and it would be interesting to compare distribution, ploidy level and flower morphology with overall plant morphology.

### 5.2.6 A Note on *Empetrum nigrum* Subspecies

It should be noted that a potential compounding factor in the analysis of *Empetrum nigrum* samples is that there are two subspecies of *Empetrum nigrum* (*subsp. nigrum and subsp. hermaphroditum*). These two subspecies are almost identical. Debate surrounds the defining characteristics between the two species and is not recent (e.g. (Bell and Tallis, 1973)). Initial description of (at the time described as a new species) indicated that *E. nigrum subsp. hermaproditum* was a tetraploid (whereas *E. nigrum subsp. nigrum* was diploid) with hermaphroditic flowers (Hagerup, 1927). Traditional identification resources indicate that the flower morphology is the key identifying factor; *E. nigrum subsp. nigrum* is dioecious whereas *E. nigrum subsp. hermaproditum* is hermaphroditic (Rich et al., 1992). However (Suda et al., 2004) found some examples of diploid plants with hermaphroditic flowers (the archetypical feature for *Empetrum nigrum subsp.*).

*hermaproditum*) in the Czech republic. Diploid plants with hermaphroditic flowers have also been identified within the UK (Blackburn, 1938). Whilst all tetraploids studied by Suda et al., (2004) had hermaphroditic flowers, sterile triploid individuals were also observed (Suda et al., 2004). Both those identified morphologically as E. nigrum subsp. hermaphroditum and subsp. nigrum covered a wide range of overlapping altitudes and environments; the predominate separating feature being different mountain ranges (Suda et al., 2004). Similar overlapping ranges have been reported elsewhere using ploidy level as the defining characteristic between the two species (Suda, 2002). Traditional identification guides also refer to "atypical" individuals when morphologically identifying between the two subspecies (Rich et al., 1992) and that the ranges of the two subspecies overlap (BSBI et al., n.d., Rich et al., 1992). It can therefore be argued that these two subspecies cannot be reliably identified between using flower morphology or range. Mention of differing growth morphology has been made between the two alleged subspecies, namely that *Empetrum nigrum subsp. nigrum* has a propensity to be more sprawling whereas Empetrum nigrum subsp. hermaphroditum is typically more compact (Rich et al., 1992). However, no mention of how these subspecies were originally identified prior to making this observation is made, it is unlikely to be ploidy level given that the source is an identification guide, leaving either flower morphology or location which as previously discussed are not reliable indicators. This study also found a continuing gradient of morphologies with no clear differentiation into two groups (Fig. 5.1.5.3.3). If altitude and latitude does affect morphology (see sections 5.1.5 & 5.2.5), the apparent more compact form *Empetrum nigrum subsp. hermaprodiitum* may have more to do with its association with high altitude and latitude locations rather than distinguishing features of two subspecies. Ploidy level would, therefore, have to be taken as the defining characteristic, as triploids have been reported as sterile and no evidence of backcrossing has been found (Suda, 2002, Suda et al., 2004) however this was a limited

study and does not exclude the possibility of backcrossing. The majority of identification will likely still rely on morphology therefore previous identifications of one subspecies over another cannot be taken as reliable. MATK sequencing suggests no difference between the two subspecies (Fig. 3.1.3.1a-c) however it is unknown how those species were identified (by ploidy, flower morphology or distribution). Interestingly in other species which have both diploids and tetraploids, the diploid is often the more cold tolerant and found in glacial refugia (with the tetraploid found in previously glaciated areas) (Parisod et al., 2010). Whilst the references for other species seem accurate in Parisod et al.'s (2010) review these authors also claim that the tetraploid of Empetrum nigrum is more cold tolerant referencing Elvebakk and Spjelkavik, (1995) in support of this. Unfortunately, Elvebakk and Spjelkavik (1995) merely discuss the presence and distribution of *Empetrum nigrum subsp. hermaphroditum* in Svalbard, using flower morphology as the identifying factor, yet no study of ploidy level is made (Elvebakk and Spjelkavik, 1995). It seems likely Parisod et al., assumed that all *Empetrum nigrum subsp.* hermaphroditum are hermaphroditic and tetraploid, however as earlier discussed this is not the case. Phlogenetic trees based on multiple plastid genes found examples of both tetraploid and diploid *Empetrum nigrum* in two clades (Popp et al., 2011). The separate ploidy levels did not separate out with some samples of *Emeptrum nigrum* (both diploid and tetraploid) showing greater similarity to a subclade of *Emeptrum rubrum* than to other *Empetrum nigrum* (both diploid and tetraploid) (Popp et al., 2011). It therefore seems likely that multiple ploidy events have occurred within Empetrum nigrum and therefore that ploidy level cannot be reliably used as subspecies identification either. There was no discussion of flower morphology associated with the phylogenetic tree (Popp et al., 2011), however it seems likely as discussed earlier, that flower morphology is not necessarily a reliable indicator either. It is therefore possible that these two subspecies are indeed not subspecies, but represent plasticity within the genus. Therefore, for the

purpose of this study no attempt to distinguish between the two alleged subspecies was made. However, the possible effect of polyploidy upon cold and freezing tolerance in *Empetrum nigrum* would be of interest for further study.

### Chapter 6: General Discussion

Although none of the amino acid mutations within CBF1 were conclusively demonstrated as influencing freezing tolerance, several paths for further investigation into these amino acid changes were identified. Continued investigation into single amino acid changes to native CBFs is a route of interest for crop breeding because single amino acid mutations are possible to achieve without use of standard genetic modification. Therefore, if a single amino acid mutation is found to beneficially alter freezing tolerance, conventional breeding techniques (which includes mutagenic breeding) could be used and the mutation screened for via marker assisted selection thereby avoiding restrictions imposed with genetic modification. Simple amino acid changes are, therefore, preferential to the introduction of entirely new CBF genes. Since the results gathered with single amino acid changes were inconclusive further study would be beneficial. Constitutive overexpression of CBFs containing these mutations resulted in the same dwarfing and reduced yield issues as seen with overexpression of native Arabidopsis thaliana CBF genes (Achard et al., 2008, Gilmour et al., 2000, Liu et al., 1998). Replacement of the native promoter with a constitutive promoter or the addition of a constitutive promoter to native CBFs would be nigh impossible using traditional (non GM) breeding methods due to the large changes that would need to be made. Therefore, on two fronts, CBF overexpression is only of interest as a diagnostic tool and not as a solution for improving crop plants. The behaviour of mutated CBFs under the control of standard CBF1 promoters would therefore be of interest and bypass the growth problems associated with overexpression (see section 4.2.2.1). The effect of these mutations, if any, could then be studied both prior and post acclimation. In this way it could be assessed if these mutations result in any change in the basal pre-acclimation freezing/cold tolerance and if there is any effect upon plant growth, as well as assessing any change in maximal freezing tolerance upon acclimation. Increased basal tolerance would be advantageous to crops in providing protection against early or late frosts and, possibly, midwinter de-acclimation. An increase in maximal freezing tolerance would allow for growth in locations where winter conditions are too cold for the crop to currently be grown or where extreme winter freezing events are likely to increase. Therefore, either, or both, of these changes would prove desirable crop traits. However, there is a risk that any increased basal tolerance may result in similar, but milder, unwanted effects as seen in overexpressors; namely dwarfing and reduced yield. Therefore, if the *CBF* mutations proved to be more potent and alter basal freezing tolerance, further study would therefore be required to assess if growth limitations occur and whether benefit to the crop outweighs that of any potential loss in yield.

The first step to assess the possibility of single amino acid changes altering either maximal (upon acclimation) and/or basal (pre-acclimation) freezing tolerance would be to further investigate the binding and potency of CBF proteins containing these mutations. Binding ability could be assessed via in vitro electrophoretic mobility shift assays as exemplified by Canella et al., (2010) and potency could be assessed via improved realtime expression methods as discussed in section 4.2.2. If more potent or stronger binders were found they could be introduced either additionally to the native CBFs (i.e. as per sections 2.5.2 and 2.8 but with a normal promoter as opposed to a constitutive 35S promoter) or by modifying the native *CBF1* via site directed mutagenesis (genome editing). Modification of the native CBF1 by site directed mutagenesis would be preferential as it would not result in the introduction of additional CBF genes (as with normal transformation) and therefore would not alter the *CBF* copy number (see below). Changes in freezing tolerance, both pre- and post acclimation, could then be compared alongside assessments, under non-acclimated conditions, on the effect, if any, upon health and yield (as discussed above). Other cold regulated native CBF genes could also be mutated (Arabidopsis thaliana CBF2 & CBF3) as it is possible that different mutations may affect different *CBF* genes differentially or result in different degrees of effect.

One possible method of increasing freezing tolerance worthy of further investigation is that of increasing CBF copy number. Greater CBF copy number has been associated with increased winter hardiness in barley and wheat cultivars (Knox et al., 2010, Wurschum et al., 2017). There is also a possibility that tetraploidy (therefore containing twice the gene copy number of diploid plants) in *Empetrum nigrum* is associated with environments that experience lower temperatures than the diploid plants (see section 5.2.6). It would be extremely interesting to artificially change the CBF copy number (under normal promoters) in Arabidopsis thaliana and assess the change, if any, upon maximal and basal freezing tolerance. There are a variety of methods which could be used to explore this possibility. To explore the possibility of the effect of polyploidy upon freezing tolerance a complete genome duplication event could be initiated via methods such as the application of colchicine (Nebel, 1937). Another study, purely looking at the increase in CBF genes, could also be performed using stable transformation (as per sections 2.5.2 and 2.8). Different numbers of tandem copies per gene insert could be used to insert tandem repeats of varying copy number into the genome. Alternatively, a variety of different strains of agrobacterium, with different virulence, could be used to induce varying numbers of multiple transformation events. The number of transformation events per line could then be calculated. CBF copies would not be in tandem using this method instead they would be scattered through the genome. These transformed plants could then be assessed as per section 4.1.2. Depending on the results the effect of tandem vs scattered increased copy number could also be assessed. Polyploidy and tandem duplication (as opposed to a scattered increase in copy number) are the most likely genetic alterations that could be induced in crops without resorting to genetic modification and therefore of greater interest.

Another route of exploration is the effect of differing *CBF* expression kinetics. The ability to alter the kinetics of *CBF* expression in crops would be of great interest, potentially

being able to change the rate of acclimation, change freezing tolerance levels, alter the time to reach maximal freezing tolerance, alter responses to freeze thaw cycles and alter the timing of deacclimation. Changing the rate or responsiveness of CBF expression and therefore the rate of acclimation would allow for breeding of crops with more rapid responses to low temperatures (in areas where low temperatures are likely to indicate freezing) or slower responses (where low temperature fluctuations are regular without freezing). Changes to the basal freezing tolerance levels could protect against unexpected cold/freezing events that would otherwise be damaging e.g. (Brammer, 2018, News Staff, 2012, Ewing, 2013, The Local, 2017, Park, 2018). Whereas changes to the maximal freezing tolerance would protect against lower freezing temperatures in winter. This could allow growth of crops in new colder areas or continued growth of crops in areas where temperatures in winter more likely to experience extremes as a result of climate change (such as currently experienced in the eastern US (Cohen et al., 2018, Massey and Samuel, 2018)). The degree of acclimation could potentially also be able to be altered, introducing levels of acclimation between the unacclimated basal level and the maximal freezing tolerance level, these intermediate levels would be of great advantage especially during autumn/spring where early and late frosts are of great risk to crops e.g. (Brammer, 2018, News Staff, 2012, Ewing, 2013, The Local, 2017, Park, 2018) but do not result in freezing to the same degree as midwinter temperatures. An intermediate acclimation level would therefore be able to tolerate milder freezing temperatures whilst potentially still taking advantage of growing degree days during this period. If these differing levels of acclimation occur in wild populations they are most likely to be found in plants which need to maximise use of growing degree days whilst being prepared for freezing temperatures (such as in arctic and alpine environments) and, as per this study, are the most promising candidates for investigation. Response to freeze-thaw cycles and longer warm periods is also of interest due to the risk of deacclimation. The kinetics of CBF expression has been found to be involved in maintaining freezing tolerance during freezethaw cycles in birch (Welling and Palva, 2008) Therefore, the ability to prevent crops from deacclimating whilst risk of freezing persists would be extremely advantageous. Likewise altering the rate of deccalimation could result in prolonged freezing tolerance where severe late freezing events are a risk, or during prolonged periods of warm winter temperatures where return to freezing temperatures can decimate crops e.g. Strassmann, (2017).

An excellent route of investigation of differing CBF kinetics would be via the species *Empetrum nigrum* and *Calluna vulgaris* as preliminarily studied within this report. These species occupy a wide range of environments, providing the potential to be able to identify differences, within a species (thereby reducing the occurrence of mutations due to divergence), which may alter *CBF* expression kinetics. This study provided preliminary data suggesting that Empetrum nigrum plants from different environments of origin have identical CBF sequences but differing CBF kinetics. As well as Empetrum nigrum and Calluna vulgaris potentially being useful to identify differences which alter CBF expression kinetics which may prove of use for crops, these species are also worthy of investigation in their own right. Both of these species are dominant ground cover species in subarctic and moorland environments, so further understanding of how these populations are composed and how climate change may affect their distribution and survival is of utmost importance. This understanding could also help guide peatland restoration and reintroduction programs. As well as being of high environmental value they are also of commercial and cultural value: Calluna vulgaris is vital for grouse moorlands providing a primary diet for red grouse and is an iconic moorland species the loss of which would affect tourism. Empetrum nigrum is a minor crop in its own right and is also grazed by reindeer herds (Inga and Öje, 2012) which are vital for the livelihoods of indigenous reindeer herders such as the Sami (Heikkilä, 2002). There is,

therefore, a strong case for full genome sequencing of both these species. Full genome sequencing would allow for identification of all CBF genes; a limitation of the project project is the inability to assess if other CBF genes are present and if so how many are present, their sequence and how their expression changes. Additionally, quantitative transcriptome sequencing could also be performed which would then allow for identification of which stimuli these CBF genes respond to e.g. cold like Arabidopsis thaliana CBF1-3 (Gilmour et al., 1998) or drought as with A. thaliana CBF4 (Haake et al., 2002). Genome sequencing would also overcome some of the problems discussed in section 5.2.3 and 3.2.1.1 and allow for easier and more complete further study of these species. Once sequences are known for one member of the species primers could be designed to isolate genes of interest from multiple members of that species and compare these genes for the possibility of regional differences. Having a full genome sequence would also make the study of the CBF promoters (of unknown sequence) possible. Calluna vulgaris CBF sequences could also be isolated and the species studied as with Empetrum nigrum. The first step would be to first optimise the qPCR, which has a far greater chance of success with a full genome sequence (Section 5.2.3) and perform expression analyses on all CBF sequences on multiple samples to check for differences in expression kinetics. Long (weeks-months) time courses could also be performed to determine how expression varies over a prolonged period as discussed in section 5.2.3. The sequence and relative positioning of the promoters of these samples could then be studied and compared between samples showing different expression kinetics. If sequence differences were present their effect and significance could be investigated in vivo in Arabidopsis thaliana as were performed with the CBF sequence differences found in this project. Full genome sequencing on multiple samples of the same species from different locations could also allow for the comparison of CBF copy number (as discussed above). Other genes involved in cold acclimation could also be investigated and isolated from multiple samples following full genome sequencing. A prime candidate gene for further study would be ICE1; a transcription factor involved in the upregulation of *CBF* (Chinnusamy et al., 2003).

In conclusion, CBF continues to be a worthwhile route of continued investigation for analysing and altering acclimation and freezing tolerance. Likely routes of further study have been proposed. Amino acid modifications of interest are highlighted along with suggested methods of further analysis. The copy number of *CBF* and the effect of ploidy is also a possible factor that effects freezing tolerance and acclimation and deserves further investigation; suggested methodology is described above. *CBF* expression kinetics are highlighted as being of interest. Continued exploration of *CBF* expression kinetic differences within a species is warranted to elucidate the origin of these differing kinetics and their effect upon freezing tolerance and acclimation. *Empetrum nigrum* is identified as a key species ideally suited for such studies.

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