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**Historical dynamics of Leopard Seal (*Hydrurga leptonyx*) and
Southern Elephant Seal (*Mirounga leonina*) populations in the
Southern Ocean**

by

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This thesis is submitted in candidature for the degree of

Master of Science

DECLARATION

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DEDICATION

I dedicate this work to my family, my teachers, and my friends, the people who encouraged me to fight for my dreams.

"Nothing is too wonderful to be true, if it be consistent with the laws of nature."

-Michael Faraday

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ABSTRACT

The history of the Pinnipeds living in Antarctic waters has been shaped by periods of climatic changes and anthropogenic impact which have affected their population dynamics. In this study, molecular genetic data were used to test hypotheses about the impact of environmental change and the mechanisms generating changes in the population dynamics of two Southern Ocean Pinniped species. The genetic diversity of contemporary Southern Elephant Seal populations was investigated in order to assess the demographic history and the degree of connectivity between the only continental colony and the three closest island colonies. For Leopard Seals, ancient DNA in comparison with modern samples provided even greater resolution on how the dynamics of a population changed in the Antarctic region through time and how this could be related to climate change. The Leopard Seal data provided an estimate of the whole mitochondrial genome mutation rate that was higher than previous phylogenetic estimates, but consistent with other estimates incorporating ancient DNA (including that calculated earlier for the Southern Elephant Seal). The Leopard Seal showed an expansion that occurred from 7,500 to 2,500 YBP, overlapping with two major periods of climatic change. For the Southern Elephant Seal, all sub-Antarctic island colonies could be considered as a single population, whereas the mainland population (the Argentinean colony) was genetically differentiated from the island colonies and had a significantly lower effective population size. The divergence of the continental colony from the island colonies occurred during the Holocene. Each species showed transitional changes during the Holocene, but while the Leopard Seal population expanded, the Southern Elephant Seal populations diverged, founding a new colony on the mainland. The broader implications for understanding historical biogeography in marine systems are discussed.

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CHAPTER 1. GENERAL INTRODUCTION

1.1 Natural processes in the dynamics of populations

1.1.1 *Structure of populations*

In order to have a greater understanding of the structure of natural populations, two concepts are necessary: the demographic structure and the genetic structure. In the case of demographic structure, this can be defined by all those processes like the rate of births, dispersal, death, the mating system and life history (Slatkin 1994), which can be considered a challenge for research when collecting this sort of information from a non-model population. Thus, most of the studies regarding demographic histories of populations have been mainly conducted on humans and model species (Pyhäjärvi *et al.* 2007). Population dynamics is the branch of life sciences responsible for studying the demographic structure of natural populations as a dynamic system. For instance, if the numbers of individuals in a closed population is increased by births, and decreased by deaths, or if the population is not closed then immigration and emigration have to be considered in the calculation (May & McLean 2007). In other words, the lack of balance between births and deaths might affect the population trends over time; if births exceed deaths, the population will tend to increase and vice versa.

The genetic structure, on the other hand, could be roughly defined as the genetic patterns of individuals inside a population given by the set of frequencies of different alleles, and the differences in these patterns when comparing subpopulations (Jacquard 1974). One of the key concepts to measure the degree of genetic structure in populations is the genetic diversity, which is the primary source of evolutionary change. Genetic diversity allows the species to adapt to

different scenarios of environmental change, have a better response to threats such as predators, or create resistance to disease; therefore, it is strongly related to the evolutionary potential of a species (Allendorf & Luikart 2007). Nevertheless, the demographic history should be considered to achieve a better understanding of the dynamic structure of a population.

Early genetic studies excluded the demographic history of a population because in many cases the demographic history was unknown and these had a limited number of loci to compare (Pyhäjärvi *et al.* 2007), whereas modern genetic studies have provided large amounts of DNA sequences among different taxa where the demographic history is important or even critical in order to interpret such genetic data. The genetic structure and genetic diversity are both influenced by the demographic structure, and also by genetic processes such as selection, recombination, migration, mutation, and drift (Slatkin 1994). Thus, by examining these genetic processes, it is possible to better explain the demographic history and genetic connectivity of a species (Guerrero *et al.* 2015). Thanks to contemporary studies on nucleotide diversity of multiple loci, it is easier to resolve the evolutionary history of a species. Hence, changes in the whole genome are driven by demographic events (losing haplotypes by sampling or acquiring them by migration), whereas natural selection influences different regions of the genome (Pyhäjärvi *et al.* 2007). The sum of changes in the genomes of a population leads to evolutionary changes in species.

1.1.2 Evolutionary theory and population genetics

Evolutionary biology has been mainly concerned with the development of a general theory capable of predicting the life-history traits most likely to evolve under different ecological

scenarios in an accurate way (Travis 1994). In this matter, the theory of population genetics is probably the most studied theory in evolutionary biology, providing the essential components to explain evolution as the theory that we know today (Ridley 1993). The discipline of population genetics deals with the laws postulated by Gregor Mendel and other relevant genetic principles that affect populations, including the study of the various forces that result in evolutionary changes through time (Hartl & Clark 1997), and tries to understand two main related variables: gene frequency and genotype frequency. The gene frequency is the proportion of alleles at a given locus in the population, whereas genotype frequency is defined as the proportion of individuals with each genotype (Ridley 1993). These variables are studied to understand better the genetic basis of evolution. Finally, an important feature that makes population genetics different to many other disciplines in biology is that it is theoretical rather than observational or experimental (Gillespie 1998). For this reason, it has been possible to create interdisciplinary branches in science to address different questions that might be answered with the help of population genetics theory.

1.1.3 Evolutionary forces and change in genetic variation

The theory of population genetics can contribute to explaining evolution, but it is also necessary to understand several processes that interact in the dynamics of populations. These processes in conjunction comprise natural selection, gene flow, genetic drift, and mutation, which are the evolutionary forces that determine gene frequencies (Ridley 1993). The mutation element is maybe the most important source of new genetic variation. Any change in the genetic material by this means will be heritable, that said, mutations happen very slowly, usually around

10^{-4} to 10^{-6} mutations per gene per generation (Hartl & Clark 1997). Then, migration resulting in gene flow is another source of adding genetic variation through the movement of organisms and transferring alleles of genes among different populations. In other words, gene flow is a way of holding subpopulations together genetically, setting a limit to the genetic divergence that is taking place (Hartl & Clark 1997), and can change allele frequencies (Ouborg *et al.* 2010).

Alternatively, when gametes from both parents are combined to conceive an offspring, there is a random element that will affect the next generation which can result in changes of allele frequency that do not vary in any known way by this sampling process (Hartl & Clark 1997). This evolutionary effect is known as random genetic drift, and it is more relevant for the evolutionary potential of a species when the effective population size (N_e) is small (Ouborg *et al.* 2010). The last of the forces is the widely studied natural selection, which enables to alleles that enhance survival and reproduction to rise gradually in frequency through time. With every generation that passes, those alleles that do not help the species to persist will tend to disappear, and the population will be more fitting to survive and reproduce, leading to adaptation (Hartl & Clark 1997).

Summarising the dynamics of these evolutionary forces; the genetic diversity is increased by mutation, acquired by migrants, reduced by natural selection, and lost by sampling in small populations due to genetic drift (Frankham *et al.* 2004). The combination of several complex processes allows these forces to change the pattern of gene frequencies in populations or the new arrangement of previously existing patterns of variation within genomes or among subpopulations (Hartl & Clark 1997). When talking about populations, the level of genetic diversity can be affected by various factors. For instance, the effective population size (N_e),

which is fundamental to determine how fast genetic drift is depleting the genetic diversity (Frankham *et al.* 2002); if the N_e is small, the genetic diversity will be lost faster and vice versa. In this matter, the effective population size can be explained as the number of breeding individuals in an ideal population showing the same amount of dispersion of allele frequencies under random genetic drift or inbreeding as the population being considered (Ouborg *et al.* 2010).

1.1.4 Inbreeding depression

The removal of any trait that influence the performance or fitness in an organism (functional traits), occurs usually by a recessive effect and showing their consequences only when both alleles are homozygotes, therefore, when homozygosis increases through mating between close relatives, fitness is likely to be reduced (Amos & Balmford 2001). In other words, a loss of fitness is occurs as a consequence of inbred crossing (compared to the offspring of an outcross), which is referred to as inbreeding depression (Frankham *et al.* 2004; Ouborg *et al.* 2010). The major consequence of this effect is a decline in the population, caused by a rise in mating between relatives. This increase in relatedness brings a reduction in fecundity success and reduced survival of inbred descendants (Amos & Balmford 2001).

The relevance of inbreeding depression in a demographic and genetic context is led by the following: genetic stochasticity encompasses the deleterious consequences of inbreeding, reduction of genetic diversity and mutational accumulation on species, and changing the birth and death balance towards a reduction in population size (Frankham *et al.* 2004). When the genetic diversity is reduced due to high levels of homozygosity, it also reduces the possibilities

of populations to adapt to changing environments by natural selection (Gillespie 1998). This pattern of reduced population size, inbreeding, loss of genetic diversity, and the consequent disappearance of the species, is an effect known as “extinction vortex” (Frankham *et al.* 2004), the ultimate consequence of the inbreeding depression by a reduction in population size.

1.1.5 Genetics and conservation principles

These days, many conservation studies are including the genetic field to define the status of a determined species (Hu *et al.* 2010; Karamanlidis *et al.* 2012; Sugimoto *et al.* 2014). As an example, when little genetic diversity is detected in a species or population, several problems can arise as a consequence, such as a propensity to diseases, reduced evolutionary potential and mutational meltdown to mention some (Amos & Balmford 2001). Some of the factors that typically lead to such decline of species and increase the risk of extinctions are habitat loss, over-exploitation, introduced species, pollution; and at small population sizes, additional random factors like demographic, environmental, genetic and catastrophic events (Frankham *et al.* 2004). Probably, the major disadvantage of a population with reduced variability is the lack of ability to react to sudden changes in the environment (Amos & Balmford 2001).

In this matter, the sub-discipline of conservation genetics uses genetic theory and other knowledge of biology to generate information that might be useful when trying to reduce the risk of extinction in species that are threatened (Frankham *et al.* 2004). This sort of research into natural populations is valuable when describing the genetic health of a species. Thus, information can be generated to create conservation units, and proper plans can be made concerning the management of a species (Guerrero *et al.* 2015). Even when studying some non-

threatened species, these provide knowledge about the basis of conservation, and helping to prevent the population decline of stable populations. At last, conservation genetics brings a wider perspective on processes related to small population size and fragmentation of habitats (Ouborg *et al.* 2010).

1.1.6 Environmental changes and past demographic events

At this time, in scientific literature and the general public perception, climate change has become an important topic due to the impact on ecological and biological systems on a global scale (Prost *et al.* 2010). In principle, the climate has a major influence on the metabolic rates of living organisms, which contribute significantly to whether a species is prone to resist, disappear, or move from a particular place (May & McLean 2007). Therefore, a good understanding of the consequences of climate change upon species and at a population level is needed, to assess and predict potential ecological scenarios (Prost *et al.* 2010). As environmental alterations can drastically influence the demographic patterns of a species, this last one affects the associations between different loci. Consequently, the non-random relation of alleles at different loci, or linkage disequilibrium, rises during a great reduction in population size (bottleneck effect), or as a result of admixture (Pyhäjärvi *et al.* 2007). Usually, species that have been domesticated or populations that have gone through a bottleneck event during colonisation are expected to suffer a reduction in nucleotide diversity (Nei *et al.* 1975). Almost all natural populations are likely to have gone through changes in N_e during their history, which is evident in their DNA sequences and such changes can be detectable through contemporary statistical models (Pyhäjärvi *et al.*

2007). However, these demographic events have to be very severe to be detected by any statistic (Depaulis *et al.* 2003).

1.2 Molecular tools to resolve evolutionary questions

1.2.1 Some approaches on molecular ecology

Using what is known so far about genetics and evolutionary theory, molecular biology has developed the tools to investigate at a finer scale the mechanisms that shaped the contemporary populations. Sometimes, these “molecular tools” work as a support for other disciplines in biological sciences, such as taxonomy, biomedicine, phylogeography, and genetic engineering. In the case of phylogeography, this approach allows researchers to infer colonisation histories, positions of populations or species by tracking the patterns of molecular markers (Ouborg *et al.* 2010). Furthermore, molecular biology can be used to generate insights into historical evolutionary forces affecting a species, especially when the morphological variation is absent or biogeographic history is unknown (Morrone & Crisci 1995). The fusion of these fields is known as molecular biogeography, and it allows a way to answer evolutionary questions about the distribution of genetic variation based on morphological variation or historical influences (Weisrock & Janzen 2000).

Another way to make measurements of the relationship among descendants over extended periods of time is by using a “molecular clock”. This method establishes the time of separation between species taking into account the number of mutations accumulated along genomic DNA, and is measured as elapsed evolutionary time (Barnes & Dupre 2008). The original idea behind the concept of a molecular clock was first stated by Zuckerkandl & Pauling

(1965), saying that ‘point mutations will both occur and accumulate in a clock-like way and the amount of differences between DNA molecules will act as a function of time since evolutionary separation’. However, the molecular clock by itself cannot assign precise dates of divergence; to achieve this is necessary to calibrate against fossil records which bring independent evidence about times (Benton & Donoghue 2007). This concept shows the relationship between genotypic variation, where changes constantly occur, and the change in the phenotype which we classically call evolution (Woese 1987).

Even though molecular clocks are widely applied and have predicted results recognised to be of primary importance, there are several problems perceived about their use, for example, it is hard to find a strict clock-like behaviour. Non-random (selected) sequence changes accumulate among the randomly introduced changes, which artificially add phylogenetic distances, and leading to differences in the rates at which the various positions in a sequence tend to change among other technical problems (Woese 1987). These considerations make molecular clocks difficult to implement, and despite the potentially valuable contributions, these studies have been a subject of controversy. Originally, the molecular clock was useful to calculate the divergence time between closely related species, limiting its resolution and the accuracy of the separation. Recently, the molecular clocks have been improved due to the inclusion of ancient DNA (aDNA), allowing a direct calibration of the mutational rate within the same species, making calculations about divergence time of populations more reliable.

1.2.2 Mitochondrial genome and ancient DNA

In most of the vertebrate species the genomic DNA that can be found in the mitochondria is inherited from the mother with minimal parental leakage, the implication of this is that genomes are passed on to the next generation almost unchanged by recombination derived from the father (Barnes & Dupre 2008). Each cell has thousands of copies of the mitochondrial genome (Wiesner *et al.* 1992), making it very abundant in each sample and therefore, a convenient marker for aDNA and elusive species such as marine mammals (Foote *et al.* 2012). Due to this abundance of genetic material in the mitochondrion, it is useful in ancient DNA studies where the abundance and quality of the total DNA have been depleted.

The studies that use aDNA are trying to answer questions regarding past population dynamics since it permits the direct comparison of DNA sequences from population spaced by hundreds of generations (Prost *et al.* 2010). These studies have been greatly enhanced by the improvement of sequencing capacity in recent years, enabling a more reliable reconstruction of temporal demographic histories on a determined time scale. The advance of technology in this field has allowed the sequencing of millions of copies of the remaining aDNA molecules, that were fortunately preserved in rare natural circumstances or by museum specimens (Shapiro & Hofreiter 2012). The potential of aDNA resides in the combinations of ancient and modern samples to resolve phylogeographic studies (Scheel *et al.* 2014), and in order to reveal biotic responses, reconstructed demographic changes can be correlated with climatic events from other sources of independent evidence (Prost *et al.* 2010).

1.2.3 Advances in sequencing technologies

The sequencing of nucleic acid is a method which tries to determine the exact number and order of base pairs in the DNA or RNA molecules, which has increased exponentially in its use in the past decades, becoming more available to research (Grada & Weinbrecht 2013; Shapiro *et al.* 2013). In 1975, Edward Sanger developed the chain termination method (Sanger sequencing), which became the primary sequencing technology (first generation) for almost three decades (Sanger *et al.* 1977) being implemented as the nuclear technology for commercial and laboratory applications (Liu *et al.* 2012). The Human Genome Project was the first major attempt into sequencing a whole human genome using Sanger sequencing, which took around 13 years and \$3 billion to be completed (Pettersson *et al.* 2009; Grada & Weinbrecht 2013). Shortly after the completion of this project, Life Sciences launched the 454 sequencer in 2005, allowing high-throughput sequencing at a low cost compared with Sanger's method. The following years, Genome Analyzer (Solexa) and SOLiD (ABI), became the most used sequencer systems in Next Generation Sequencing (NGS) (Liu *et al.* 2012).

Presently, NGS technologies are opening new opportunities for research in different areas, since it has improved in precision and throughput, and have enabled the sequencing of entire genomes more easily (Lander *et al.* 2001; Walker *et al.* 2013). This advance in technology has brought some revolutionary changes in some branches of evolutionary biology and conservation genetics. These changes enabled unprecedentedly sequencing of genomes and subsets of genomes from many individuals, but processing a high number of samples is still expensive enough to limit research to projects where funding opportunities are relatively substantial (Pettersson *et al.* 2009; Fumagalli *et al.* 2014). With the continuing improvement of

NGS, the accessibility of this tool would increase for research institutions in countries all around the world since it will be more cost effective, leading to progress in genomics and other areas (Liu *et al.* 2012; Snyder *et al.* 2015). Due to the current advance in sequencing, now is a critical time to explore the limitations and advantages of applying genomic tools to conservation problems (Allendorf *et al.* 2010).

Furthermore, the advance in sequencing technologies has enabled the transition of some areas like conservation genetics to “conservation genomics” (Ouborg *et al.* 2010), given that it allows the use of genome technology as a standard practice by processing more sequences, at a higher rate and for accessible costs (Simon *et al.* 2009). The NGS allowed the use of Single Nucleotide Polymorphisms (SNPs), discovered by ‘genome sampling’ methods, covering greater sections of the genome than the traditionally used microsatellites or AFLPS, and are used lately to get a more accurate representation of the genetic variation at individual and population level (Ouborg *et al.* 2010).

Another important techniques that were developed thanks to NGS are the genome-wide association studies (GWAS), which are unravelling the genetic basis of phenotypic variation, having considerable ecological relevance, and are permitting the identification of loci under selection (Stapley *et al.* 2010). These methods also lead to inferences about demography, genetic structure, gene flow, population history, and inbreeding, with a higher resolution than traditional sequencing (Ouborg *et al.* 2010; Ekblom & Galindo 2011). Additionally, NGS also facilitate molecular ecologists to work on gene regulation, Transcriptome profiling, and epigenetics (Simon *et al.* 2009). Finally, one extensive involvement of genomic technologies in conservation

could be the accurate monitoring of the changes in allele frequencies to assess the effect of natural selection, genetic drift, and hybridization in wild populations (Allendorf *et al.* 2010).

The growth of NGS studies in the following years will probably be focused on the history of selection, genetic architecture, and the gene regulation, and trying to relate this to conservation rather than only focusing on detecting signatures of selection (Ekblom & Galindo 2011). Genomic studies like the assessment of inbreeding and pedigrees based on various markers to identify regions for local adaptation or outbreeding might benefit the management of natural populations and solve conservation issues (Allendorf *et al.* 2010). Even when genomic techniques are not fundamental or suitable to all conservation studies, genomics is having a great impact in addressing several challenges regarding critically endangered populations to monitoring gene flow and genetic drift to great contiguous populations (Allendorf *et al.* 2010; McMahon *et al.* 2014; Grueber 2015). Moreover, ecological studies are receiving a major aid from NGS technology, due to the small amount of genetic sample that is required for some analysis. In consequence, this technology is more approachable for studies of endangered species or to recover aDNA from preserved organisms to provide reliable information to compare with modern populations (Ekblom & Galindo 2011).

Moore's law is usually used to describe the growth in the number of transistors in an integrated circuit which has doubled approximately every two years since 1975 (Schaller 1997). This law can be applied to sequencing technology where current technological advancements are increasing the throughput even more, to the extent of analysing sequence-based expressions at individual cellular level (Simon *et al.* 2009). For this reason, bioinformatics is a fundamental part when dealing with genomic methodologies, given that is the primary tool to manage the large

amounts of output data, and which is adapting to every change in gathering techniques (Allendorf *et al.* 2010). At the same time, the organisms being sequenced are increasing exponentially; thus a vast amount of genetic data is being processed worldwide every day (Liu *et al.* 2012). If technology continues evolving at this rate, storage and sharing systems will need to be improved as well, given that current servers might not be enough to bare the massive storage of genetic data (Ekblom & Galindo 2011).

1.3 Southern Pinnipeds and the Antarctic ecosystems

The present work investigates genetic patterns of two key species of the Southern Oceans, the Leopard Seal (*Hydrurga leptonyx*) and the Southern Elephant Seal (*Mirounga leonina*). Firstly, it is important to understand some of characteristics and relationships of the group that they belong to, the Pinnipeds. The members of this group are 33 extant species belonging to the order Carnivora (King 1983), and are generally separated into three families: Otariidae with 14 species (Sea Lions and Fur Seals), Odobenidae, where the Walrus is the only member, and Phocidae, or commonly called ‘true Seals’ with 18 species (Sarich 1969; King 1983; Fulton & Strobeck 2010). This classification has been very controversial and obscure, and for many years, evolutionary systematists debated about the origin of this clade (Riedman 1990; Arnason *et al.* 1995; Fulton & Strobeck 2010). In recent years, has been accepted that Pinnipeds form a monophyletic clade with respect to carnivores, which is separated in the three families mentioned above (Nyakatura & Bininda-Emonds 2012).

The Phocidae is the most diverse and well-distributed group of the Pinnipeds, and their members are the best adapted to marine life (Davies 1958). Within the family Phocidae there are

two accepted subfamilies, the Northern Hemisphere Seals (Phocinae) and the Southern Hemisphere Seals (Monachinae) (Davis *et al.* 2004). According to the fossil record, at least five genera have appeared by the middle Miocene, and two of the present subfamilies (Phocinae and Monachinae) were distinguishable (Davies 1958). Moreover, a phylogenetic analysis by Arnason *et al.* (1995), suggests different evolutionary branches from northern and southern Phocids derived from a common ancestor related to the Monk Seals. However, because of the lack of fossil evidence, it is complicated to define whether the southern Phocids colonised the Southern Hemisphere on one or more dispersals from ancestral species living in lower latitudes.

The Monachinae ('Southern' Seals) split from the Phocinae 15 million years ago on the eastern coast of North America (Fulton & Strobeck 2010) and most of the members of the group live in cold waters (phagophilic) off the South Pole. The only species that do not live in cold waters are the Monk Seals (*Monachus spp*) and the Northern Elephant Seals (*Mirounga angustirostris*) inhabiting tropical and temperate waters respectively, and these species are found in the Northern Hemisphere (Riedman 1990; Davis *et al.* 2004; Fulton & Strobeck 2010). Even when contemporary taxonomy includes all Southern Hemisphere Seals inside the Monachinae, the recognition of the sub-division of Antarctic Seals (Lobodontini), Elephant Seals (Miroungini), and Monk Seals (Monachini), has to be tested (Davis *et al.* 2004). Knowing the evolutionary history of this group will help to understand better the current distribution of different populations and the demographic status of the species studied in this thesis, allowing better interpretations that could apply to other Pinnipeds living in the Southern Hemisphere.

The Miroungini tribe comprises the Southern Elephant Seal (*M. leonina*) and the Northern Elephant Seals (*M. angustirostris*) (Davis *et al.* 2004; Fulton and Strobeck 2010). On

the other hand, the Lobodontini tribe includes the Leopard Seal (*H. leptonyx*), the Ross Seal (*Ommatophoca rossii*), the Weddell Seal (*Leptonychotes weddellii*), and the Crabeater Seal (*Lobodon carcinophagus*) (Davis *et al.* 2004; Fulton and Strobeck 2010). The Elephant Seals arose in the Southern Hemisphere, where the existing species, *M. leonina*, has succeeded in colonizing most of the anti-Boreal zone, whereas a later spread to the north must have taken place in the Pleistocene glacial age allowing the establishment of the Northern Elephant Seal (*M. angustirostris*) after having been cut off from the Southern Elephant Seals due to the rewarming of the seas (Davies 1958). In the other hand, the members of Lobodontini have each been placed in a separate genus, though they are probably derived from adaptive radiation from one group (Davies 1958). Bayesian estimations of divergence times have been performed in Phocids using BEAST v.1.4.8. (Drummond & Rambaut 2007), suggesting that tribes Lobodontini (Antarctic Seals) and Miroungini (Elephant Seals) are estimated to have diverged in the eastern Atlantic 7 millions of years ago (Ma) and a single Lobodontini dispersal to Antarctica occurred shortly afterwards (Fulton & Strobeck 2010). These Bayesian analyses are very useful when trying to explain the evolutionary history of a group of species, or when enough samples are available, the demographic history of a population.

The Pinnipeds inhabiting the Southern Ocean are six species that represent each a different genus. The Southern Elephant Seal (*M. leonina*), the Leopard Seal (*H. leptonyx*), the Crabeater Seal (*L. carcinophagus*), the Weddell Seal (*L. weddellii*), the Ross Seal (*O. rossii*), and the Antarctic fur Seal (*Arctocephalus gazelle*) (Laws 1984). Each of the four species of Antarctic Phocids, in addition to Antarctic fur Seals and Southern Elephant Seals, occupy a distinctive position in the Antarctic ecosystem (Riedman 1990). The four species of ice-breeding seals that

usually live in the pack-ice region are the Leopard Seal, the Weddell Seal, the Crabeater Seal, and the Ross Seal; meanwhile, the Southern Elephant Seal can be found in northern regions on sub-Antarctic islands (Siniff 1991).

It can be the case that two or more species feed on the same food resource when they are geographically separated, but utilise different food resources when their ranges overlap (Riedman 1990). The four Antarctic phagophilic species are to some extent separated geographically, occupying different ecological niches (Davies 1958). The Leopard Seal has the most extensive distribution of the Antarctic Phocids, and its range overlaps with that of the Weddell Seals near the Antarctic continent and over the shelf feeding on different preys (Riedman 1990). Although, the Weddell Seal lives farther south than any other mammal, breathing holes in the ice, and feeding principally on fish, squid, and other invertebrates (Davies 1958). On the other hand, the Leopard Seal ranges from the ice edge northward to the sub-Antarctic islands and feeds mainly on Antarctic krill (*Euphausia superba*) and warm-blooded prey like Adélie penguins (*Pygoscelis adeliae*) and young seals (Davies 1958; Riedman 1990). However, the most abundant species of seal is by far the Crabeater Seal, probably because it has specialised in consuming Antarctic Krill, taking advantage of the abundance of this prey species (Siniff 1991). The last of the Antarctic Seals is the Ross Seal, which current distribution overlaps with the same habitat as the Crabeater Seal, but the Ross Seal feeds on different prey like squid and fish (Riedman 1990). Given that the Leopard Seals feeds on a wide variety of prey and its distribution sometimes overlap with the other phagophilic seals, its study might be of particular interest when trying to explain the demographic status of Antarctic Pinnipeds and their response towards historical changes in the environment.

The other two, Pinnipeds inhabiting the Southern Ocean, the Southern Elephant Seal and sub-Antarctic fur Seal, were driven near to extinction by intensive sealing in the latter part of the 1800s (Bonner 1982). These two seals can be found living together north of the pack ice where the Elephant Seal feeds on fish and squid, and the fur Seals consume primarily krill (Riedman 1990). On the other hand, the four species of Antarctic Seals that inhabit the sea ice region have not been exploited extensively, and thus are often considered unaffected by the influence of humanity (Bonner 1982). This might be due to the fact that it is not economically feasible to maintain an industry on the harvest of this species, probably due to the difficulties of operating in such habitat and the high costs that this represents (Bonner 1982; Siniff 1991).

Even when the four ice-breeding Seals have not been impacted intensively by humans as the Elephant Seals, some of their competitors have been exploited extensively. For example, most populations of large whales dropped due commercial whaling (Siniff 1991). The removal of a top predator in a fragile ecosystem like the Antarctic Ocean can represent a huge readjustment in the food webs affecting several species simultaneously (Pace *et al.* 1999). Nevertheless, this area is recovering from a previous exploitation of large mammals which feed on the same prey as the pack ice Seals, and thus, it seems sure that competition for food is probably increasing (Siniff 1991). Moreover, some key species that inhabit this region show very high sensitivity to minuscule increases in temperature, which could cause drastic changes in their population size or even the extinction of one or several species (Meredith & King 2005). For these reasons, the ecological role of the Leopard Seal becomes more important with the reduction in the abundance of its competitors.

Some regions of the planet are highly sensitive to variation in temperature and are prone to drastic changes in the landscape in a very short period. Such is the case of the Western Antarctic Peninsula (WAP), which has been associated with cryospheric impacts since the 1950's, due to an increase of 3C° in the atmosphere (Meredith & King 2005). The latter induces the production of krill given that they are highly dependent on the temperatures of this region. As Antarctic krill is a key species for several predators in the Southern Ocean food web, and its known dependence on the temperature, the WAP is crucial in breeding and nursing for this species which have significant ecological implications (Meredith & King 2005). The Leopard Seal occupies a high trophic level in the Antarctic ecosystem, which means that it can feed upon many vertebrate and invertebrate species like Antarctic krill (Siniff 1991). Therefore, any change or alteration in the ecosystem should be translated into changes in their population dynamics.

Seals are large mammals, for which population dynamics are difficult to understand given that they live for extended periods, limiting the potential for long-term study (Siniff 1991). The Southern Elephant Seal and the Leopard Seal are the biggest and the second biggest seals living in the southern circumpolar waters (Riedman 1990), and even when both species are placed in different tribes, they form a monophyletic clade between Lobodontini and Miroungini (Davis *et al.* 2004). Moreover, these species have been the subject of study in many ecological, phylogenetic, and population genetics studies (Slade *et al.* 1998; Hoelzel *et al.* 2001; Davis *et al.* 2008), this due the abundant hypotheses on Pinniped relationships based on morphological traits, making this group absorbing to include in molecular studies (Arnason *et al.* 1995). However, additional studies are needed using newer technologies to have a better perspective on the population dynamics of these Southern Phocids. Given that the Southern Elephant Seal and the

Leopard Seals present differences in behaviour, occupy different trophic levels, and their distributions barely overlap, studying the demographic history of both species through genomic methods will provide a broader perspective about the responses of southern Pinnipeds towards climate change.

The focus of the present study is on the demographic histories of both Leopard Seals and Southern Elephant Seal, using Bayesian approaches. In the case of the Leopard Seal in the present study, ancient and modern DNA is being analysed to determine the mutational rate of its mitochondrial genome, in order to make reliable estimations of their historical population size. This mutation rate could be potentially used in other closely related Pinnipeds, having significant implications for increasing the knowledge of Seals in the Antarctic.

Similarly, several modern populations of Elephant Seals are investigated in this thesis to determine the effective population size in different islands, as well as their genetic structure, comparing whole mitochondrial genomes to have a higher resolution of the historical dynamics of Antarctic Phocids than in previous studies. By studying the genetic structure, genetic diversity, genetic flow, and historical effective population size of some colonies of Southern Elephant Seals, it is possible to explain their demographic history in the context of environmental changes and assess the status of the South Atlantic Ocean populations. For instance, if a colony presents very low genetic diversity in comparison with the other colonies, this will have lower chances to adapt to environmental changes due a low evolutionary potential (Allendorf & Luikart 2007).

1.4 Thesis outline and objectives

1.4.1 Justification

The history of the seals living in Antarctic waters has been shaped by periods of climatic changes and anthropogenic impact which have resulted in their changing distribution and population size. In the specific case of the Leopard Seals, human impact has affected its conservation, distribution and population size in a non-systematic way; while in the Elephant Seals, hunting reduced the population size drastically over the last 150 years. Such reduction could lead to a drastic change in its genetic diversity due to the bottleneck effect; moreover, the current differences between continental and island preferences for breeding can change the genetic structure of these populations. For these reasons it is important to investigate the genetic diversity of modern Elephant Seals, to assess gene flow among populations, population dynamics and the degree of structure. At the same time, studies using ancient DNA in comparison with modern samples of Leopard Seals will provide a greater resolution as to how the dynamics of the populations have changed in the Antarctic region and how this can be related to climate and anthropogenic disturbances. Given that the Leopard Seal is considered a generalist top predator that feeds on a wide variety of species (including Antarctic Krill, which is highly dependent of sea ice); any change in the environment will affect directly the population size of Leopard Seals in the Southern Ocean.

Therefore, studying the population genetics of Leopard Seals and Southern Elephant Seals could provide a wider perspective on the population dynamics of Phocid Seals inhabiting the Southern Hemisphere. Studies have shown that changes in global temperature can modify

substantially the conditions in the Antarctic ice shelf and sea ice (Meredith & King 2005; Spence *et al.* 2014), which may imply migration or decline of populations. Thus, using molecular methods like NGS, it is possible to find a correlation between historical climate changes, human impact, and the population dynamics of the species reflected in the variation of population size and distribution through time. The purpose of this study is to investigate the historical demographic changes of Southern Elephant Seals and Leopard Seals by using genomic approaches, comparing these changes to historical environmental alterations, and trying to explain these results in an ecological and biogeographic context.

1.4.2 Thesis objectives

1) To assess ancient and modern DNA data from Leopard Seals to allow a direct calibration of a mutational rate and use it to calculate the historical N_e throughout time.

2) To test the genetic diversity of several geographical populations of modern Elephant Seals, assess the structure among populations, and the levels of connectivity between them.

3) To use genetic data and coalescence methods to determine the historical population dynamics of each species, trying to explain the results in the context of environmental change.

4) To test the following hypotheses:

A). Given the natural history of the Southern Elephant Seal (*M. leonina*) and Leopard Seal (*H. leptonyx*), environmental alterations will be reflected in changes in population size and distribution, based on analysis of the mitochondrial genome of both species in different geographic locations and through time.

B). As *H. leptonyx* is a top predator that feeds in a broad variety of prey, significant environmental alterations will be reflected as changes in N_e and haplotypic frequencies of the entire mitochondrial genome, which will be directly correlated to the climatic history of the Antarctic ecosystem.

C). As traditional molecular clocks have been calibrated against fossil records of close relatives in Pinnipeds, the use of ancient and modern DNA samples of *H. leptonyx* will allow an “internal calibration”, which will result in a different mutation rate compared with previous studies for this species. The studies that calculate mutation rates by using aDNA have shown a tendency to generate much faster mutation rates than those studies using fossil records.

D). The different breeding colonies of *M. leonina* will show different tendencies in population growth, genetic diversity, and genetic connectivity depending on the local resources and environmental conditions; subsequently, such trends will be reflected in the mitochondrial genome of modern organisms, and will be correlated with the major environmental changes that might affect the breeding colonies in the South Atlantic Ocean.

F). Given that the Argentinean population of *M. leonina* is the only continental population that has been reported as a breeding colony, marked differences in genetic diversity, N_e , and structure will be evident in comparison with the other three sub-Antarctic islands.

CHAPTER 2. ANCIENT AND MODERN POPULATION DYNAMICS OF LEOPARD SEAL (*Hydrurga leptonyx*).

2.1 Introduction

2.1.1 Ecology, status, and characteristics of Leopard Seals

The Leopard Seal has the most extensive distribution of the Antarctic Phocids, ranging from the ice edge northward to the sub-Antarctic islands and feeding in a wide variety of species (Davies 1958; Riedman 1990). Moreover, understanding the ecology and overall situation of this species is crucial, because its predatory pressure is significant at several trophic levels, which can help to understand better the ecosystem's dynamics in the Southern Ocean (Siniff & Stone 1985).

Early surveys calculated the total population size of this species at around 300,000 individuals (Erickson & Hanson 1990), but later estimations reduced this number down to 35,500 (Southwell *et al.* 2012). Moreover, no evidence has been found for population structure among different geographic locations (Davis *et al.* 2008). This species is an apex predator feeding on species at several trophic positions; these include krill (50% of diet), fish (9%), penguins (20%), cephalopods (6%) and other Pinniped (15%) such as the Antarctic fur Seal (Laws 1977; Lowry *et al.* 1988; Vera *et al.* 2004).

Leopard Seal can consume between 5% and 6% of its total weight daily (Siniff & Stone 1985), however, given that Leopard Seals are generalists and opportunistic, its dietary preferences and quantities may vary by the seasons, locations and the availability of resources. It has been suggested that during the spring in some areas, the primary prey resource are penguins, while during the winter, crustaceans like Antarctic krill (*Euphausia superba*) are the leading

resource, reflecting its role as a generalist top predator (Casaux *et al.* 2009). Nevertheless, observations of shallow dives in pups contradicted the previous suggestion that Antarctic Krill is the main food resource during the winter season, due to krill being found at greater depths at this season (Kuhn *et al.* 2006). The previous statement could suggest that the prey diversity and flexibility also vary during the different stages of its lifespan.

The predation rate of Leopard Seal on mesopredator species such as penguins, other seals and their prey (krill and icefish), has been investigated by Forcada *et al.* (2009). These authors used mathematical models to quantify the impact of fisheries on rare apex marine predators. These results suggest that even when targeted fisheries do not compete directly with Leopard Seal for food resources, the fishing pressure may impact directly on the mesopredators that the Leopard Seals depredate. Nevertheless, this interaction is problematic to address due to the evasive behaviour of top predators, their broad geographic distribution, mobility, complex life history, seasonality, and shift of habitat (Forcada *et al.* 2009).

Observations made by Casaux *et al.* (2009) at Danco Coast, Antarctic Peninsula, suggested a preference for some non-abundant prey species and attributed this as a mechanism to reduce the inter-specific competitions for food, thus, partitioning the use of the feeding area with other seals such as Weddell Seals and Antarctic fur seals (*Arctocephalus gazelle*; Casaux *et al.* 2009). Furthermore, the Leopard Seals have become an important regulatory element in the abundance of Antarctic fur seals in specific parts of the world like Livingston Island. A study in 2004 reports the increment in the abundance of Leopard Seals and their depredation strategies during the breeding season, causing the decrease of Antarctic fur seals in comparison with data

from previous decades (Vera *et al.* 2004). These kind of studies has documented the regulatory element that this top predator exerts on its prey species.

The Leopard Seal is the most widely distributed of the Seals in the Antarctic pack ice, sub-Antarctic islands and sub-tropical areas, and they exhibit age segregation depending on availability of food resources, season, and shape of the landscape, such as the extent of sea ice (King 1983; Bester & Roux 1986). Between early November and late December, it is the season when Leopard Seals usually breed in the sea ice, but it can be flexible from early October to early January depending on the environmental conditions (Southwell *et al.* 2003). The seasonal movements of individuals apparently from south to north is a response to the changes in the pack ice extent (Bester & Roux 1986).

The Leopard Seals share similarities in their reproductive cycle with other Antarctic Seals, such as the influence of the seasonal distribution of prey items on their reproductive strategy (Siniff 1991). Nevertheless, the ability of Leopard Seals to utilise many different prey species allows a more flexible breeding season compared with other Antarctic Seals (Siniff & Stone 1985). Taking this into account, it is very likely that if change in climate affects the Leopard Seals, it also would affect the other Antarctic Seals in a similar way given that they share some ecological and adaptive features.

The Leopard Seal and the other members of the family Phocidae have various adaptations to an aquatic marine habitat, such as an engrossed skin, thick blubber layer, and adapted sebaceous glands (Gray *et al.* 2006). During winter 2002, Kuhn *et al.* (2006) opportunistically measured the behaviour at sea and the diving physiology of a young Leopard Seal in the Antarctic Peninsula, and they determined the body oxygen storage, the aerobic dive limit, and

observed shallow diving behaviour in comparison with other Antarctic Phocids. They concluded that the haul-out behaviour is inversely proportional to how cold the wind is, affecting its mobility. They also noted differences related to age and spatial distribution; older seals tend to disperse further than younger seals, probably related to increased aggression between older seals (Rogers & Bryden 1997).

There are some reports of errant Leopard Seals in the north coastal part of Argentina which were in a poor physical condition and died due dietary stress. Rodriguez *et al.* (2003) suggest that this is related to their feeding strategies. The annual cycle of the species in combination with competition for food during the winter may force immature seals to move into Sub-Antarctic areas near South America, and there are transported by the Malvinas-Falkland current to coastal areas in Argentina. Similarly, on the coast of Chile, there have been other cases of Leopard Seals travelling to the northern limits of their distribution, possibly driven by the availability of resources. Vargas *et al.* (2009), reviewed these cases where most of the Seals were found in poor health condition due to inanition, presence of diseases, and attack of other species or human aggression. They conclude that frequency of these cases can be a potential indicator of environmental changes because the temperature is one of the most important variables in the ecosystems dynamics (Vargas *et al.* 2009).

Given its behaviour, reproductive strategies, wide distribution, and adaptations to the environment, the Leopard Seal population could be hypothesised to be a single panmictic population, which has been supported by molecular evidence (e.g. Davis *et al.* 2008). When investigating some aspects of its life history, this is hindered by its inaccessibility to some areas, great extension of its distribution, and the costs of continuous and significant sampling methods,

while the use of molecular techniques allow researchers to know about the demographic history, using fewer samples and with a non-invasive approach. Because of its generalist behaviour and role as an apex predator in the Antarctic ecosystem, the present study will use molecular markers and coalescent analyses to investigate the demographic changes in Leopard Seals in the context of environmental changes over time.

2.1.2 *The Ross Sea Area*

Understanding how species are distributed among habitats depending on their abilities to get certain resources in a region, is fundamental to preserve an ecosystem (Ballard *et al.* 2012). However, the lack of information about the abundance at a local level, and the inaccessibility to the pack-ice make difficult to quantify the trophic interactions and its significance in this ecosystem (Southwell *et al.* 2008).

The Southern Ocean is located south of 50° S, which plays a crucial role in the marine currents systems and the biogeochemical cycles of marine nutrients (Arrigo *et al.* 2008). In the past, the Ross Sea was affected by seawards fluctuations of outlet glaciers on the Antarctic Ice Sheet, by some changes on alpine glacial at a local level, and by inland advances of the grounded Ross Ice Shelf; thereby, the present conditions of the Ross Sea reflect the interactions of these previous dynamic systems (Chinn 1981).

The diversity of diatoms found in the sediments of marine glacial from the Ross Sea, varies considerably depending on the geographic location in the ice shelf, which indicates different rates of ice retreat, marine currents, biogenic sediment production and preservation (Anderson *et al.* 2014). Besides, the glaciers outside of the ice sheet have remained relatively

unchanged since the most recent glacial period in North America (Wisconsin glaciation), compared with these glaciers from the Ross Ice Shelf (Chinn 1981), which can be constrained by marine and terrestrial data (Anderson *et al.* 2014). However, given that Antarctic ice sheets, glaciers, and ice shelves are changed very rapidly by climate, it is harder to interpret the climate of the Holocene in this area (Chinn 1981). Regions with high productivity like the Ross Sea work as a key mechanism, facilitating the flow of CO₂ from the atmosphere into the Southern Ocean. Photosynthesis produced by phytoplankton operates on the surface reducing the partial pressure of CO₂ in water, thereby, establishing a gradient between atmosphere and the sea (Arrigo *et al.* 2008).

Even when the Ross Sea is one of the most studied regions and the largest continental shelf ecosystem of the Antarctic continent, it only represents around 2% of the total extension of the Southern Ocean (Smith *et al.* 2007). The limits of the Ross Sea at the continental shelf are located at 72-78°S and 170°E to 158°W and is considered a pristine marine ecosystem, contrasting with northern impacted regions of the Southern Ocean (Ainley *et al.* 2006). It is naturally isolated from human populations and is under the Antarctic Treaty, which protects its coastal habitat and several Antarctic Specially Protected Areas (Halpern *et al.* 2008). Additionally, the Ross Sea has the conditions that allow the co-existence of several species that are considered predators, such as birds, whales, large fish, and seals, which are placed in an upper trophic level (Ainley 2010).

The high primary production in the Ross Sea compared with other regions might be contributing to the richness of apex predators and mesopredators (Ballard *et al.* 2012), and it is similar to the Weddell Sea regarding productivity (Arrigo *et al.* 2008). The whole continental

shelf is used by mesopredators, which permits these species to settle colonies in this region (Ballard *et al.* 2012). The four species of Antarctic pack ice seals, all occupy the Ross sea region at some point during the year and their differences in life histories allow them to use various components of the ecosystem in different ways (Ainley 2010).

The reciprocal effect between prey and predator altering abundance, productivity, or biomass of a population (or trophic level), is defined as a “trophic cascade” and the Ross Sea is prone to this effect due to its sensitivity to subtle environmental changes (Pace *et al.* 1999). Therefore, top predators such as the Leopard Seal, have a significant influence in this neritic system and the stability of their prey’s population (Ainley *et al.* 2006).

The Leopard Seal is often found in coastal areas of the Ross Sea during summer, where it preys upon species like young crabeater and Weddell Seals, penguins, krill, fish and during the winter, they move out into the pack ice north of the Ross Sea, probably searching for other resources (Ainley 2010). In the marine ecosystems, the trophic cascades formerly linked to top predators are not evident in modern times, attributing to the overfishing and consequent loss of upper trophic levels (Pace *et al.* 1999). This highlights the importance of studying the population dynamics of Leopard Seals on this kind of habitats, to understand better one of the last pristine ecosystems.

The coasts around the Ross Sea region represent an ideal location to take ancient samples of Antarctic wildlife. For instance, near to Victoria Land Coast is an area with high availability of mummified organisms that have been lying for hundreds of years (Hall *et al.* 2006; de Bruyn *et al.* 2009; Parks *et al.* 2015). Ancient DNA samples, like sub-fossil bones or mummified skin, are preserved over extended periods of time thanks to the cold temperature of these high-latitude

sites (Foote *et al.* 2012), therefore, it is critical in the rate of success when sequencing the DNA fragments of paleogenomics projects (Rizzi *et al.* 2012).

It has been argued that even at very low temperatures and under ideal conditions, DNA is not able to survive more than a million years, therefore, impossible to be amplified (Willerslev & Cooper 2005). The availability of these ancient samples represents an excellent opportunity to investigate directly the demographic history of Leopard Seals through genomic methodologies, which could be interpreted according to information gathered about environmental change and life history of the species.

2.1.3 Genetic studies relevant to the Antarctic ecosystems

The use of molecular tools allows researchers to investigate the evolutionary processes and dynamics of a given species in the context of environmental change. For example, for a scarce population of Weddell Seals inhabiting the isolated White Island in the Ross Sea, cytonuclear disequilibrium suggested that they are the direct descendant of a group that founded the colony on that island during a brief retreat in the sea ice. This retreat allowed them the access to White Island around 1950's, which is consistent with historical records, and with the results of Gelatt *et al.* (2010), who predicted a decrease in the population and a posterior inbreeding depression that will threaten the survival of the population. This provides evidence for the effects of natural isolation in seals and such studies have the potential to assess the effect of isolation in similar species (Gelatt *et al.* 2010).

The most important study on the genetic variability of Leopard Seals was carried out by Davis *et al.* (2008) where they tested the genetic structure of the four species of ice-breeding

Seals associated with their geographic distribution and ecological factors. In the mentioned study, 150 samples of Leopard Seals were obtained from six geographical regions using 14 different microsatellite loci, but they concluded that there was not enough genetic structure among geographic zones to statistically support different populations (Davis *et al.* 2008). This result contradicted their hypothesis that Leopard Seals might be the most likely of the four studied species to exhibit population structure caused by differences in Leopard Seal vocalisations between geographically distant locations (Thomas & Golladay 1995). These differences in vocalisation have been discussed for other phocid populations (Le Boeuf & Petrinovich 1974; Van Parijs *et al.* 1999), which could be the result of little interaction between distant subpopulations.

Mitochondrial DNA is a very recurrent marker used in aDNA studies (Foote *et al.* 2012), given that a small piece of skin or muscle has hundreds of copies (Wiesner *et al.* 1992) to extract enough mtDNA and sequence it successfully. Ancient DNA can help to resolve phylogenetic reconstructions by combining ancient and modern samples, as in the case of the Caribbean Monk Seal, where samples were collected from museums. In that study by Scheel *et al.* (2014), the phylogenetic analysis of *cytb* sequences showed a closer relationship between Caribbean and Hawaiian monk Seals, while Mediterranean Monk Seal fell outside that lineage, hence the proposal for a new genus corresponding to the New world species.

The improvement of aDNA research, has the potential to shed light on historical demography of a species and patterns of genetic structure among populations (Cooper & Wayne 1998), but keeping samples free of contamination from modern sources and deterioration, is problematic (Austin *et al.* 1997). However, regions around the Ross Sea (e.g. Victoria Land

Coast) present conditions to preserve the remaining of death organisms with minimal degradation during hundreds of years, allowing the mummified remains of Antarctic fauna like penguins (Lambert *et al.* 2002; Subramanian *et al.* 2009; Parks *et al.* 2015) and Antarctic seals (Dort 1975; Hall *et al.* 2006; de Bruyn *et al.* 2009) to be available today.

Ancient DNA studies and coalescent methods have improved the estimations of historical demographic patterns, which are useful when comparing climate records and the response of species between the past and modern variations (Hoelzel 2010). A study by de Bruyn *et al.* (2009) used ancient DNA extracted from mummified Southern Elephant Seal skins from a now extinct colony that inhabited the Ross Sea to reconstruct its demographic history. They compared the mtDNA haplotypes of the HVRI of most modern colonies and the extinct Antarctic colony, on which concluded that the Antarctic breeding colony was founded by individuals migrating from Macquarie Island during a warm period that caused a retreat in the sea ice from the Ross Sea Embayment around 7500-8000 Years Before the Present (YBP). Further to the colonisation of this new habitat, this colony expanded rapidly and isolated from other colonies, though a significant part of the genetic diversity that was created by that initial expansion was removed when the ice returned and the population declined. In a similar approach, aDNA studies that include historical in samples of Leopard Seals to compare them with modern samples could provide an opportunity for a better understanding of its population dynamics. Moreover, the change of these dynamics in the Antarctic region can be related to climate change and anthropogenic disturbances, helping to predict better future consequences in the ecosystem caused by environmental alterations.

A recent study carried out by Parks, *et al.* (2015) about the population genomics of Adélie Penguins (*Pygoscelis adeliae*), highlighted the advantages of using NGS at recovering ancient genomes and shedding light on the evolutionary and the demographic history of this species. They estimated a mutation rate of $7.0E^{-8}$ substitutions per site per year (S/S/Yr) for the full mitochondrial genome and concluded a coalescent age for the populations around 101,000 years with a divergence time of 53,000 years between the central lineages. This availability of ancient and modern samples of Antarctic species at the Ross Sea represents an ideal location to investigate the evolutionary changes and genomic patterns of these populations over time (Parks *et al.* 2015). In the same area, preserved samples of Leopard Seals are available which could be used in research with a similar approach. The evolutionary rate of nucleotide sequences has been mostly calculated from comparative methodologies between different living species and calibrating a rate against geological estimates of splitting time (Cann *et al.* 1987). However, these approaches are limited and cannot determine intra-specific rates given that calibration points are spaced by large timescales (Subramanian *et al.* 2009). If radiocarbon dating determines the ages of several ancient samples of the same species, then, sequencing their genomes would be useful to establish internal calibration points to estimate the genomic mutation rate within that species (Orlando *et al.* 2015).

Population genomics studies focused on wild population have the potential to benefit significantly to aDNA research in general. Currently, the most of the aDNA studies have been using short regions of the nuclear and mitochondrial genomes, however, in taxa where a large number of well-preserved samples are available, a more extensive sampling would allow direct testing of hypotheses regarding the natural history of a species (Ramakrishnan & Hadly 2009).

Nevertheless, larger sections of the genome can be identified and selected for sequencing and be assembled through a method called “target enrichment”, which allows pulling the particular regions to be amplified in order to increase the number of copies of those regions from ancient samples (Parks *et al.* 2015). This method is allowing the sequencing of targeted sequences which is crucial when working with ancient DNA. For these reasons, and given the availability of ancient and modern samples of Leopard Seals, the present study is trying to elucidate the demographic history of Leopard Seals and assess this in the context of environmental changes in the ecosystem.

2.2 Chapter outline and Aims

2.2.1 *Justification*

Demographic studies of Leopard Seals are very challenging due their unpredictable habits, high mobility, wide distribution, and the extreme conditions of the habitat for human activities. However, molecular methods have been proved useful for investigating historical dynamics of remote non-model populations, using low impact sampling methods (e.g. biopsies) in elusive species. Now NGS provide the opportunity to recover entire genomes from small amounts of tissue, facilitating the analysis of contemporary and ancient DNA. The Ross Sea is considered pristine and near to Victoria Land Coast is an ideal region to find well-preserved mummified samples of Leopard Seals that lived thousands of years ago, as well as contemporary samples from living Seals. Comparing the complete mitochondrial genome of modern and ancient samples will embody novel information about the evolutionary history of this species, allowing directly calculating a mutation rate through coalescent methods and estimating the effective population size of this species as it changed through time. The results of this chapter will be interpreted in the context of temporal biotic and abiotic changes in the Antarctic.

2.2.2 Objectives of the chapter

1) Sequencing the whole mitochondrial genome from modern and ancient DNA of Leopard Seals inhabiting the Ross Sea and compare their genetic diversity.

2) Use of coalescent methods to determine the historical population dynamics of Leopard Seals and explain the results in the context of environmental change.

2.2.3 Hypothesis

Given the trophic position of the Leopard Seal (*Hydrurga leptonyx*), their historical population dynamics will be directly correlated to important environmental alterations through the climatic history of the Antarctic ecosystem.

2.3 Materials and Methods

2.3.1 *Collection of modern and ancient samples*

A total of 25 ancient samples from mummified Leopard Seals were collected during expeditions to the Ross Sea, Antarctica. The Seals were identified in the field when possible, collecting a small fragment of skin or bone and taking the coordinates for future references. The ancient samples were collected and dated by Brenda Hall from the University of Maine, Emily Brault and Paul Koch both from the University of California, Santa Cruz. The radiocarbon dating was carried out following the work of de Bruyn, *et al.* (2009). The present study included five modern samples from the Ross Sea and seven samples from Bird Island (Figure 2.1). In total, 12 modern samples of Leopard Seals were provided by the Department of Biological Sciences, University of Alberta, Edmonton. A list of sampling sites and locations is provided in Table S1.

2.3.2 *DNA extraction*

For modern DNA samples, a small portion of the tissue from approximately 0.5 cm³ was finely chopped using a scalpel. and incubated overnight at 37°C in digestion buffer (50 Mm Tris pH 7.5, 1Mm EDTA, 100 Mm NaCL, 1 % w/v SDS) with 50 µl proteinase K (10mg/ml) (Milligan 1998). The DNA was then extracted using a standard phenol: chloroform extraction (Sambrook *et al.* 1989). The presence and quality of genomic DNA were then tested by viewing results on 1.2 % agarose gels which were run for 30 minutes alongside a 1 Kb DNA ladder.

For ancient DNA, a series of modifications were performed compared with modern DNA methodology, following the recommendations of Fulton (2012), and using a different laboratory

entirely isolated from any modern DNA to avoid cross contamination. A hand-held drill with disposable abrasive discs was used to cut little pieces from bone and mummified skin samples. Firstly, the surfaces of the samples were abraded thoroughly using the disposable discs and drill to remove contaminated DNA. Secondly, some small pieces of tissue were extracted (up to ~1 cm³), and deposited in a Mixer Mill MM 200 to grind it into a fine powder. Precautions were taken between the handling of each individual sample to avoid cross contamination, soaking all the drill parts in 10% bleach, exposing them to UV light, and changing blade each time that handling a new sample.

The DNA extraction was carried out following the recommendations of Barnet & Larson (2012) and Rohland (2012). The enzymatic digestion was carried out by adding 0.5 ml of digestion buffer (0.425 M EDTA pH 8, 0.5% sodium dodecyl sulphate, 0.05 M tris, pH 8.5) and 50 µl of proteinase K (20 mg/ml) to the powder of each sample. The samples with extraction buffer were placed in a Stuart Rotator SB2 inside an incubator at 50°C to provide mixing for 24 hours. Finally, QIAamp DNA Mini Kit was used to extract the ancient DNA following the manufacturer's guidelines.

2.3.3 DNA Shearing

For modern DNA samples and before the library construction, it is necessary to shear the modern DNA into small fragments of 200-600 bp using a Covaris S220 Focused-ultrasonicator following the instructions of the manufacturer. The ancient DNA is naturally fragmented, and it does not need to be sheared.

2.3.4 *Library construction*

A series of modifications were made in order to construct the libraries based on methods suggested in Briggs & Heyn (2012) and Knapp *et al.* (2012), which can be separated into several stages depending if working with modern or ancient DNA; an end-repair (only ancient DNA), adapter ligation, adapter fill-in, an initial amplification of ancient DNA, and a final amplification and extension of the adapters.

Because ancient DNA is prone to cytosine deamination which can lead to miscoding errors, an end-repair is needed to remove the excess of uracil using 10x NEB Buffer 2, T4 Polynucleotide Kinase (10U/ul), and USER enzyme (1U/ul) (Briggs and Heyn 2012). The next step is an adapter attachment, which will help to differentiate each sample by allocating specific adapters P5 (barcode), a universal adapter P7, 10X T4 Ligase Buffer, 50% PEG-4000, and T4 Ligase (5U/ul). These universal adapters attach to non-specific sequences and take part in the replication of the sequences. For the adapter fill-in, MyOne C1 Streptavidin beads are used during several washes to remove extra adapters not attached to the DNA as suggested by Knapp *et al.* (2012) and using Thermopol buffer (10x), dNTPs, and BST polymerase (8U/ μ l). The initial amplification for ancient samples consists of adding specific primer P7 (index) to each sample separately, followed by the addition of a "master mix" containing a universal primer P5, 10x Thermopol buffer, dNTP mix, and AmpliTaq Gold DNA Polymerase. The amplification was carried out using a PCR machine and following the cycling conditions stated in Table 2.1.

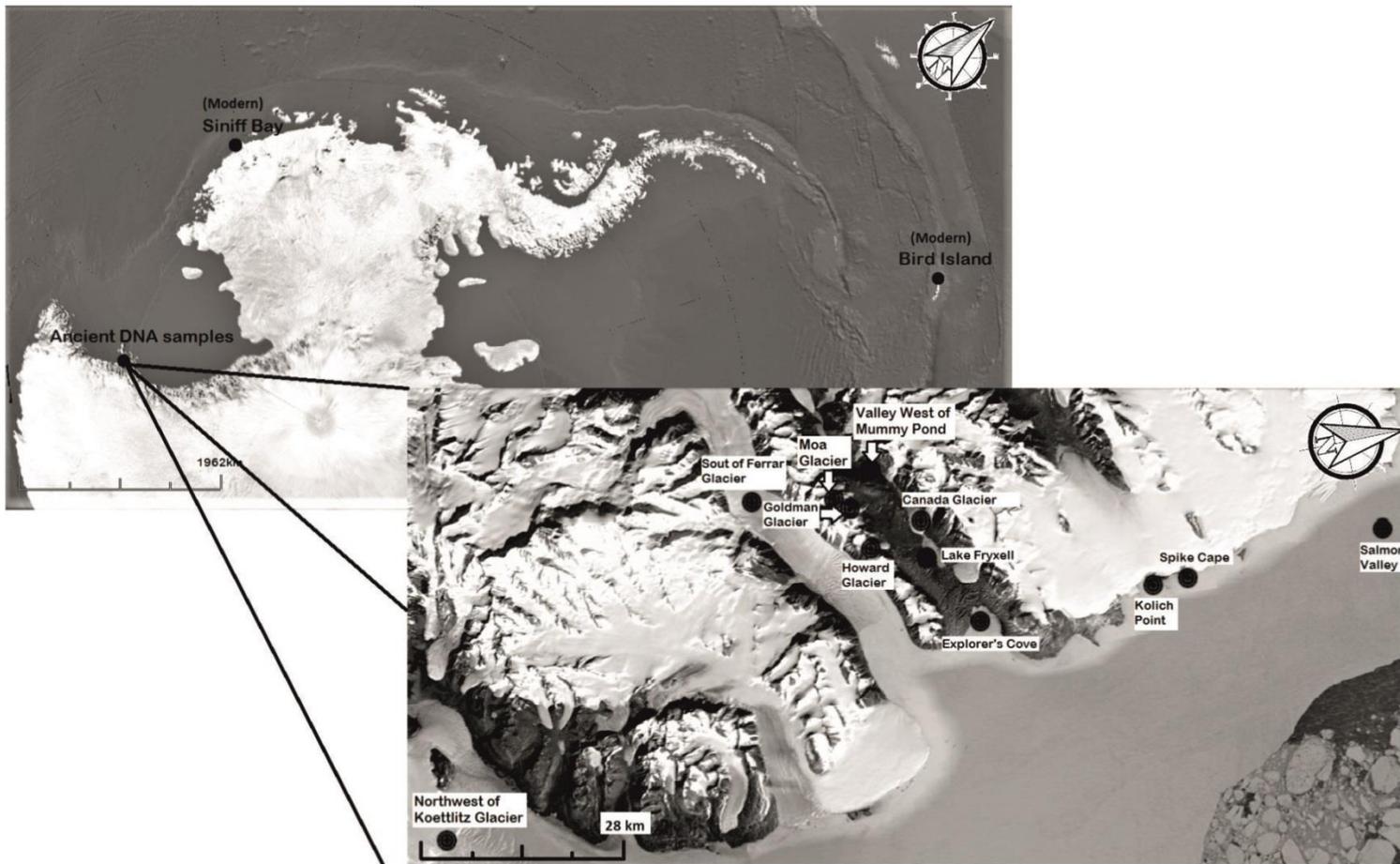


Figure 2.1. Study area and sampling localities. The area near to Victoria Land Coast is amplified to have a better view of the localities where the ancient samples were collected.

Table 2.1 PCR program for Initial amplification of ancient DNA.

Temperature	Time	Cycles
95°C	12 min	
95°C	30 seg	X12
60°C	30 seg	X12
72°C	1 min	X12
72°C	3 min	

After this stage, the remaining steps can be carried out in the modern laboratory, given that all samples now have a particular combination of adapter and index which should not be repeated for samples included in the same sequencing lane. For this study, a combination of 8 barcodes and 12 indexes were used, allowing 96 different combinations per lane of the sequencer platform HiSeq 2500®.

The last stage of library preparation is the final library amplification, which will be slightly different for ancient and modern DNA. In the case of modern DNA, a specific primer P7 (index) will be added to each sample separately, followed by the addition of a universal primer P5 and 2x Phusion Master Mix. Given that the ancient DNA was previously amplified and a unique primer P7 has been added, the universal primers P5 and P7 were used, in addition to 2x Phusion Master Mix per sample and following the PCR conditions as shown in Table 2.2.

Each library should have a unique combination of adapters and indexes P5 and P7 which will allow identifying individual samples after sequencing by using bioinformatics tools.

Table 2.2. PCR thermal conditions for final amplification of libraries.

Temperature	Time	Cycles
95°C	12 min	
94°C	30 seg	X10-20
58°C	30 seg	X10-20
72°C	1 min	X10-20
72°C	10 min	
10°C	Hold	

2.3.5 Target enrichment

In order to generate larger data sets for multiple regions of the mitochondrial genome, bait molecules were used to select target regions from the DNA libraries for sequencing. Mybaits kit with a custom library of biotinylated single-stranded RNA designed specifically to capture mitochondrial DNA of seals was used. The approach used is based on the work of Gnirke *et al.* (2009) and Horn (2012). A Kapa Library Quantification Kit was used to quantify the concentrations of the samples to combine all the samples at equal concentrations into a single pool of 10 nM.

2.3.6 Sequencing

The pooled samples of modern and ancient DNA with specific indexes and barcodes were sent to the DNA Sequencing and Fragment Analysis Facility located in the School of Biological and Biomedical Sciences at Durham University (DBS Genomics). The samples were run on the Illumina HiSeq 2500 sequencer platform, and the output data was received in the form of raw FASTQ.

2.3.7 *Bioinformatics*

The raw files were put through filtering pipelines to remove adapter and index sequences. The first step after that is the demultiplexing, where the raw files have to be split into smaller files according to the different barcodes and indexes that were allocated in each of the samples. For this purpose, the program Stacks was used, allowing the generation of two FASTQ files per sample which correspond to paired-end reads (Forward and Reverse) and containing all the sequences for that barcode/index combination (Catchen *et al.* 2013). The program Bowtie 2 was required in order to align the sequences of both FASTQ files, using a reference genome to reconstruct the mitochondrial genome of each individual into a SAM (Sequence Alignment Map) format (Langmead & Salzberg 2012). The SAM file was converted to a BAM format using the software SAMTOOLS to visualisation the genomic sequences. A quality filter removes reads which are unsuitable for downstream analysis.

2.3.8 *Consensus sequences and cleaning*

The SAM files for individual sequences were run in the software Geneious® 8.1.7 to create a consensus sequence of the mitochondrial genome. Subsequently, sequences were visualised and revised looking for ambiguities in base pairs or shallow reads lower than 4X, in which case it was given the code “N” with the value of complete ambiguity. Only the consensus sequences with coverage above 90 percent were selected to align all the genomes to each other in order to check the length of the final sequences and trim them. Geneious® 8.1.7 allows exporting datasets in different output formats, which is very useful when using different software for statistical analysis.

2.3.9 Estimating substitution rate and historical population size

The software BEAST v1.8.3 is a robust and customizable Bayesian method that employs Markov Chain Monte Carlo (MCMC) simulation analyses for demographic patterns and substitution parameters (Drummond & Rambaut 2007). It requires input files to be generated by BEAUTi version 1.4.2 from a NEXUS file format. BEAST v1.8.3 software was used to obtain direct substitution rate estimates using ancient DNA dated samples and modern DNA sequences to explore past demographic changes through the Bayesian Skyline Plot (BSP). This analysis gathers credible genealogies and estimates a substitution rate by the use of historical samples with calibrated ages, and the demographic history of the sampled population. The datasets used for this analysis were ancient and modern samples combined, to estimate a substitution rate from temporally spaced sequence data, obtained by radiocarbon dating. Even though such dates will have an associated error, they can provide enough information to calibrate and estimate a rate of evolution, and presumably more accurate than an external calibration point (Drummond *et al.* 2002; Ho & Larson 2006).

To generate credibility intervals that represent the coalescent model and phylogenetic uncertainty, three independent MCMC were run for 50,000,000 generations, sampled 5,000 generations. The initial 10% of the MCMC was discarded as burn-in, and LogCombiner was used to combine these three independent runs and then was analysed in Tracer v1.4. The substitution model chosen for this analysis was Hasegawa, Kishino and Yano (HKY) and applying a strict molecular clock model (Hasegawa *et al.* 1985).

2.3.10 Population genetics statistics

Standard diversity indices, molecular diversity indices, Neutrality tests (Tajima's D and Fu's Fs), and mismatch distribution were calculated using Arlequin 3.5.2.2 (Excoffier & Lischer 2010). The neutrality tests determined whether sequences are evolving randomly, as expected under the neutral theory, or if they are affected by alternative mechanisms such as selection, gene flow, demographic expansion or decline. A population that has experienced any of these alternate mechanisms will result in a rejection of the null hypothesis of neutrality (Tajima 1989; Fu 1997). These tests can, therefore, identify the effects of demographic changes. Additionally, Minimum Spanning Network and Neighbour Joining were used to create a network using PopART (<http://popart.otago.ac.nz>), in order to group the haplotypes and show the number of evolutionary steps between them (Bandelt *et al.* 1999).

2.4 Results

2.4.1 *Samples, libraries, and genome coverage*

25 ancient indexed libraries were prepared from mummified samples collected from the Ross Sea area and C¹⁴ dated from 200 to 1400 YBP, using the methods described above for aDNA extraction and sequencing. In a similar way, 12 modern indexed libraries were prepared in total, six from the Ross Sea and six from Bird Island. A reference genome of Leopard Seal (GenBank ID: NC_008425) was used in order to reconstruct the genomes of individual samples, and compare the percentage of coverage aligned with it. In the case of the ancient specimens, coverage ranging from 0.05 % to 95% was obtained, where only 15 had coverage above 90 %.

On the other hand, all modern samples were above 90 % coverage, compared with the reference genome. The ends of all modern sample mitogenomes were trimmed to have equal lengths across all sequences, resulting in sequences of 16174 bp representing 97.42 % of the reference genome (16602 bp long).

For the ancient samples, the number of samples was reduced down to 15 in order to include only those with the highest number of informative sites (>90% coverage). After checking and editing the sequences from ambiguities, one of the sequences that showed long regions with many "N" values was excluded. The final dataset to be included in the analysis consisted of 14 ancient samples, six modern samples from the Ross Sea, and six modern samples from Bird Island, making a total of 26 of 16174 bp long for both modern and ancient samples (Table 2.3)

Table 2.3. Information of the samples used in the study

ID	Sample name	¹⁴ C age	Coverage (%)	Locality
704-504	ECA 12-14	1450	0.5	The Ross Sea*
703-501	ECB 12-18	1640	29.7	The Ross Sea*
703-502	ECC 12-26	Not dated	14.8	The Ross Sea*
709-502	ECC 12-60	1370	97.7	Ross Sea
705-501	HGA 12-03	1650	89.3	Ross Sea
705-502	HGA 12-28	1240	90.7	Ross Sea
705-505	HGA 12-30	1230	98.7	Ross Sea
705-506	HGA 12-33	1260	95.2	Ross Sea
709-501	HGA 12-45	1430	97.8	Ross Sea
708-508	HGB 12-48	1910	98.4	Ross Sea
704-503	HGC 12-01	1240	93.1	Ross Sea
712-506	HGC 12-17	Not dated	6.2	The Ross Sea*
712-508	LBA 13-01	1120	99.1	The Ross Sea*
702-503	LBA 13-25	5950	24.9	The Ross Sea*
702-506	LBB 13-18	2690	95.9	Ross Sea
703-505	LBB 13-48	3630	44.9	The Ross Sea*
701-503	LBC 13-30 AFFSP	Not dated	14.9	The Ross Sea*
701-504	LBC 13-40A	Not dated	19.4	The Ross Sea*
701-507	LBC 13-40Be	Not dated	53.7	The Ross Sea*
701-508	LBC 13-48	1150	99.5	Ross Sea
703-506	LBC 13-50	1400	89.6	The Ross Sea*
704-507	MVA 13-13	1560	52.8	Ross Sea
701-501	MVA 13-19 AFFSP	2102	98.1	Ross Sea
701-502	MVA 13-26 AFFSP	2113	97.6	Ross Sea
704-508	MVB 13-16	1510	98.2	Ross Sea
MLS17-b	MLS17-b		>95	Bird Island
MLS7	MLS7		>95	Ross Sea
MLS8	MLS8		>95	Ross Sea
MLS9	MLS9		>95	Ross Sea
MLS10	MLS10		>95	Ross Sea
MLS11	MLS11	Modern	>95	Ross Sea
MLS12-b	MLS12-b		>95	Bird Island
MLS4	MLS4		>95	Ross Sea
MLS13-b	MLS13-b		>95	Bird Island
MLS16-b	MLS16-b		>95	Bird Island
MLS6	MLS6		>95	Ross Sea
MLS14-b	MLS14-b		>95	Bird Island

*Not included in the analysis

2.4.2 *Phylogenetic network*

In order to visualise better the haplotype frequencies and the number of evolutionary steps, a Minimum Spanning Network was implemented. The network showed that most of the haplotypes are unique except for one shared between 701-501_818 and 706-508_mod. Moreover, no clear distinction was shown between ages, or between locations (Figure 2.2).

2.4.3 *Population genetic statistics*

Tajima's D and Fu's F_s was calculated in order to have an insight into the demographic trend of the population, resulting in values of $D=-1.96128$ ($p=0.00900$), and $F_s=0.83657$ ($p=0.54700$) respectively. Fu's F_s value yielded no evidence of expansion within the population, whereas a negative value in Tajima's D suggested a rapid population expansion or selective sweep. A summary of the population genetics and demographic statistics is given in Table 2.4. In order to detect signals of demographic and spatial expansion, a mismatch distribution approach was used, but only weak signals for expansion were observed suggesting a long-term stable population (Figures 2.3 and Figure 2.4).

2.4.4 *Mutation Rate and Bayesian Skyline Plots*

Bayesian methods were used in order to calibrate modern samples against estimated radiocarbon ages from ancient samples, producing an overall mitochondrial mutation rate of $3.13E^{-7}$ S/S/yr and a Higher Posterior Distribution Interval (HPDI) of $2.58E^{-7}$ - $2.69E^{-7}$ S/S/yr. The HPDI were rather narrow, and there is a well-resolved peak in the sampling

distribution graph (Figure 2.5). The BSP estimated the timing and the magnitude of the female N_e , indicated an expansion around 7000-6000 YBP (Figure 2.6).

Table 2.4. Population genetic statistics of ancient and modern samples of the leopard seals.

Statistic	Value	CI/P-Value
N	26	
S	434	
h	25	
Nucleotide Diversity (π)	0.01723	+/- 0.008482
Haplotype diversity	0.9969	+/- 0.0117
Fu's (P-value)	0.83657	P-value= -0.547
Tajima's D(P-value)	-1.96128	P-value= -0.014
Average number of bp differences	278.3323	+/- 122.989215

N = Number of individuals; S : segregating sites; h :number of haplotypes

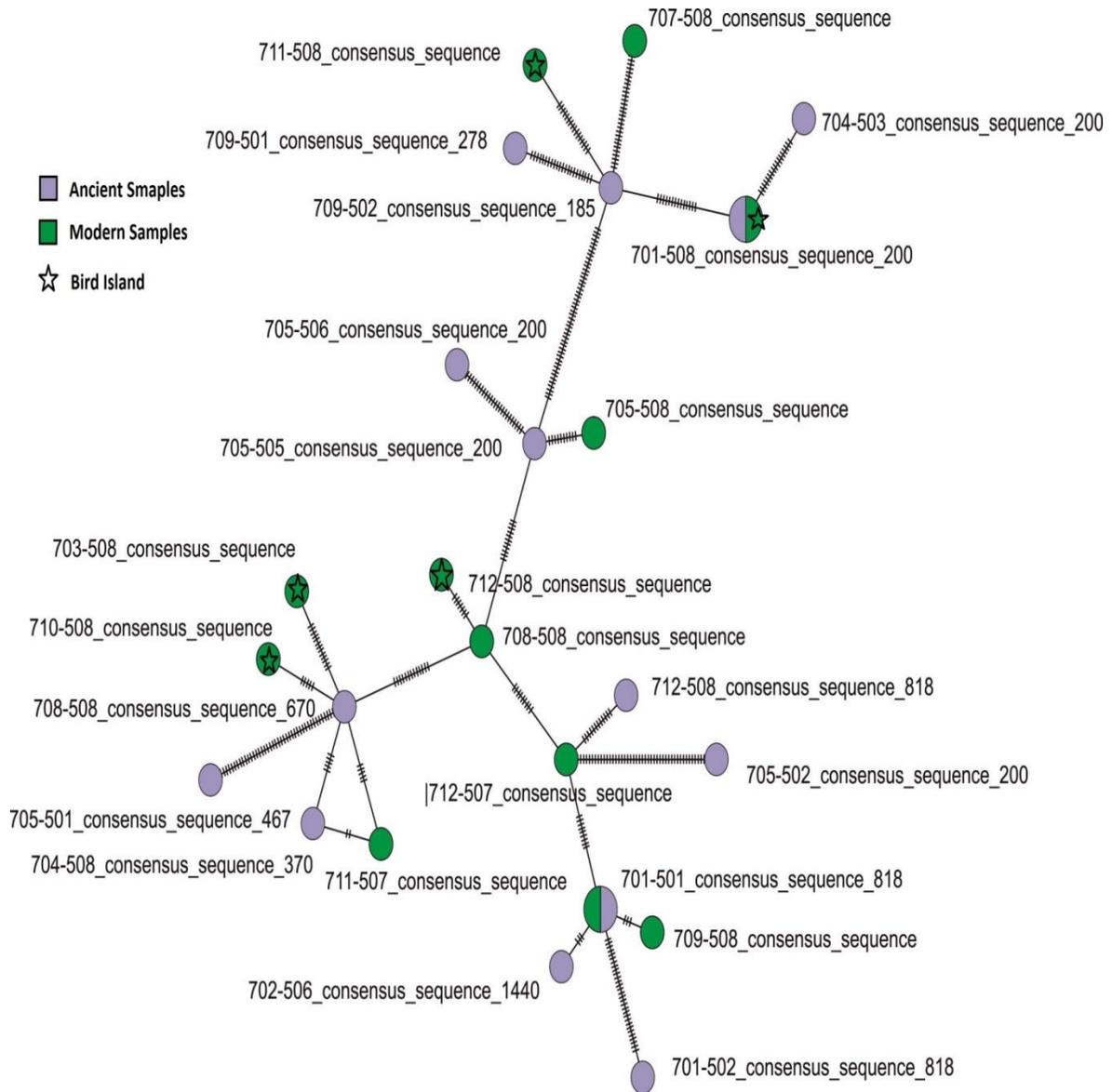


Figure 2.2. Minimum Spanning Network, showing all the haplotypes from the dataset. The transversal lines between haplotypes represent the number of evolutionary steps.

Mismatch distribution (spatial expansion)
ancient-modern

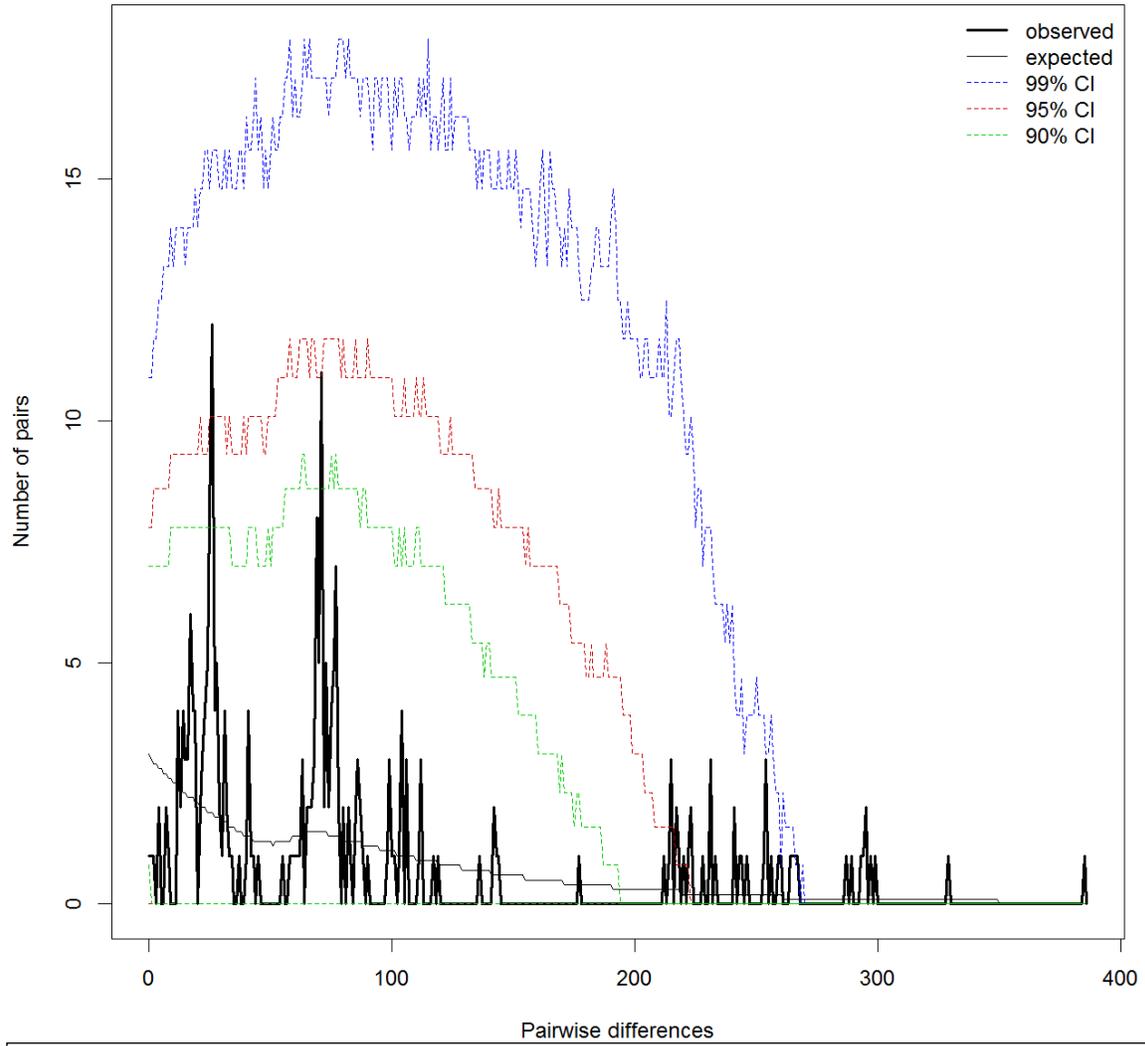


Figure 2.3. Mismatch distribution for spatial expansion.

Mismatch distribution (demographic expansion)
ancient-modern

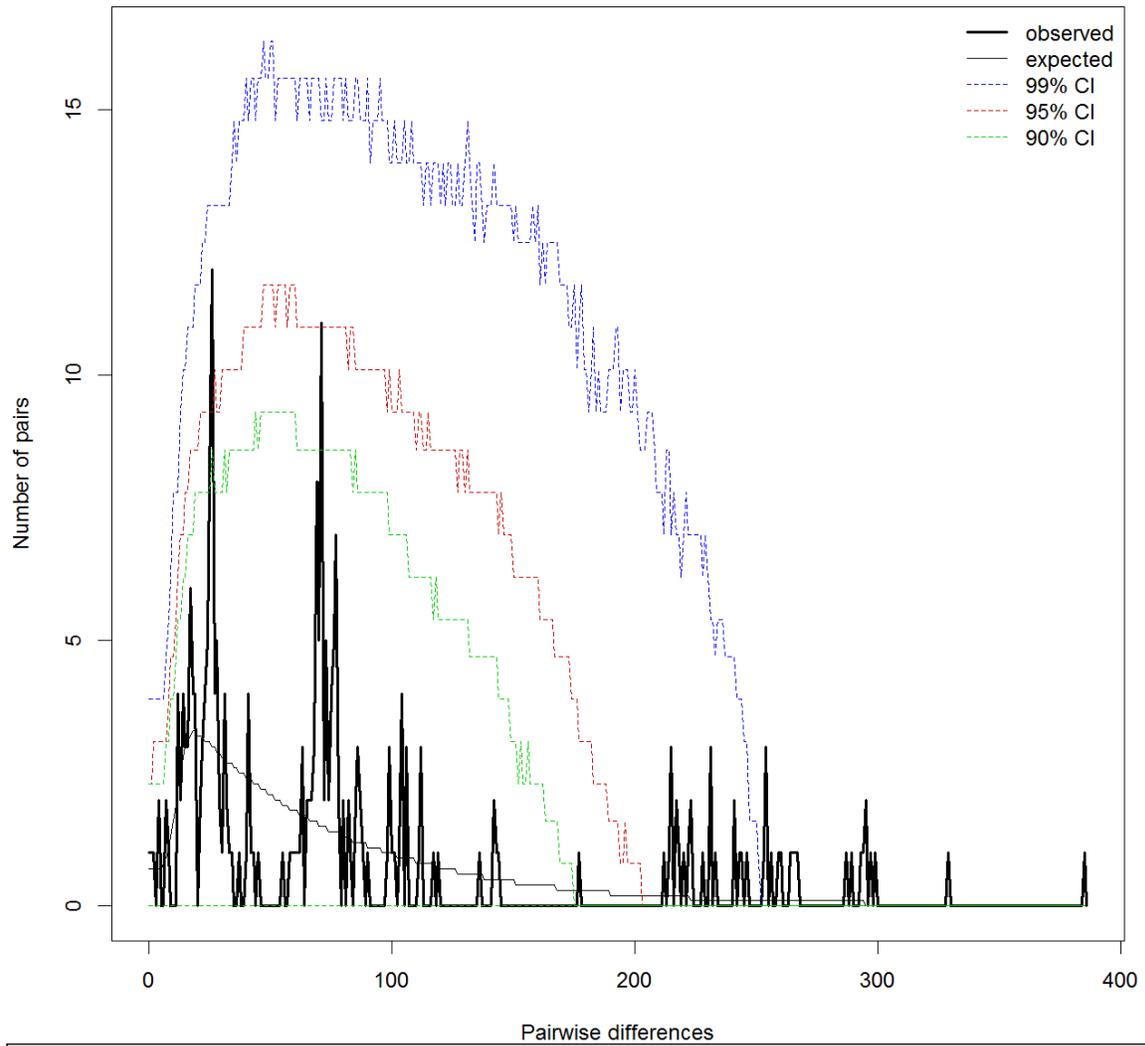


Figure 2.4. Mismatch distribution for demographic expansion.

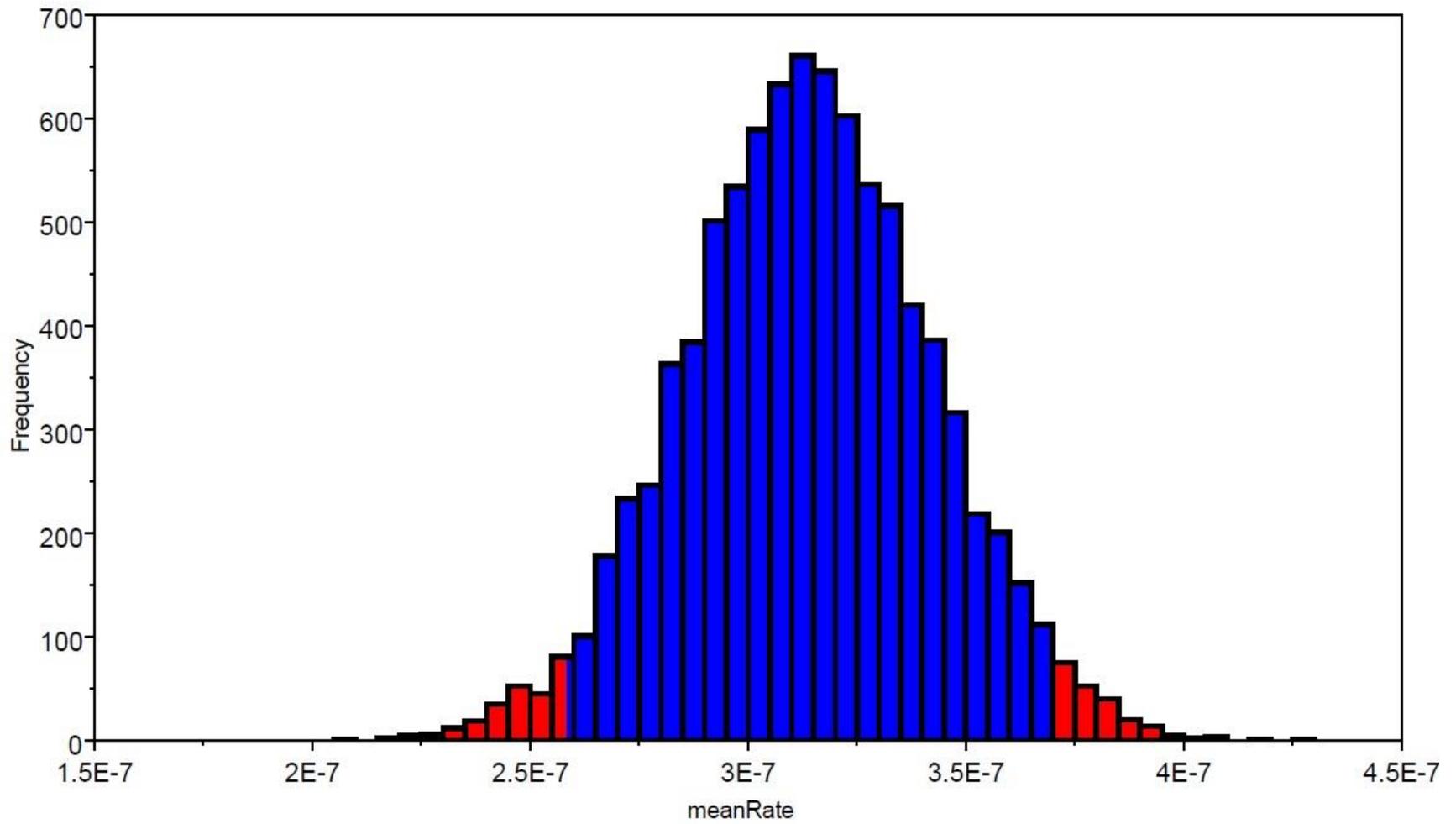


Figure 2.5. Higher Posterior Distribution for the mean mutation rate and intervals.

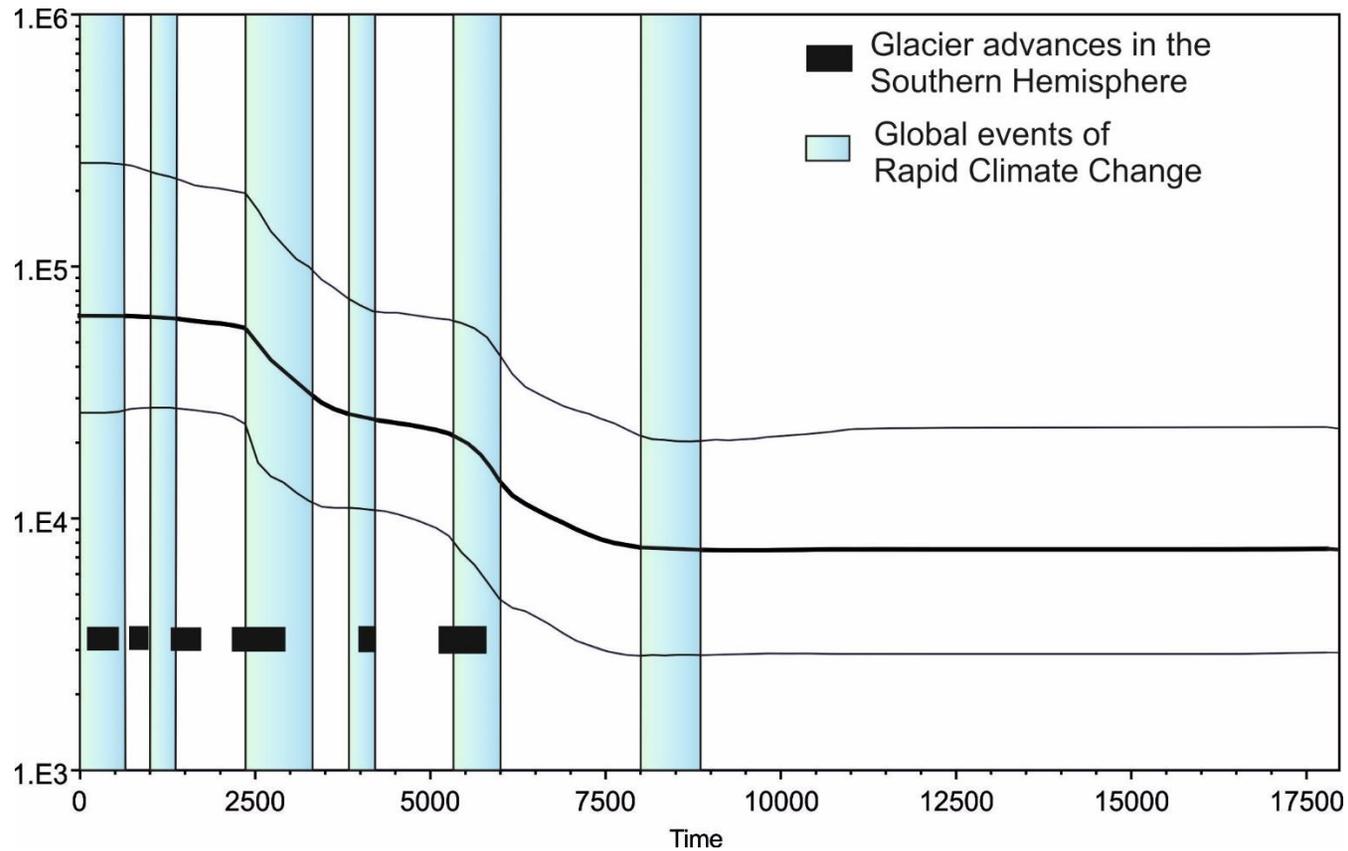


Figure 2.6. Bayesian Skyline Plot. Effective population size is shown in the Y-axis and years before the present in the X-axis. The black squares represent the periods of Glacier advances in the Southern Hemisphere. The blue bars represent periods of relatively fast climate change towards colder conditions globally (Mayewski *et al.* 2004).

2.5 Discussion

Given the poor condition of ancient DNA for some samples, sequences were too scarce to include them in the analysis, whereas all the modern samples obtained coverage near 100%. However, retaining 60% of the ancient sequences was deemed sufficient for this study, and previous studies using ancient DNA have been able to calibrate a molecular clock with fewer samples (Vila et al. 2001; Freitas et al. 2003; Huynen et al. 2003; Rohland et al. 2005; Barnes et al. 2007; Dalen et al. 2007). Furthermore, a study by Molak et al. (2013) tested the accuracy of calculating mutation rates with different datasets of ancient and modern samples and concluded that the estimated rates did not differ substantially until the threshold of six samples was reached. When working with ancient DNA, it is challenging to find a proper strategy to balance the number of individuals to be included, and the number of loci sequenced in order to produce the most robust and informative datasets within economic constraints (Parks *et al.* 2015). The present study aimed to include only samples above 90 % coverage compared to a reference genome in order to have only high-quality samples with the maximum number of informative sites as possible.

Ancient DNA has been used progressively since recent years in order to estimate molecular timescales, mainly in studies to find substitution rates and the demographic history (Molak *et al.* 2013). Furthermore, the inclusion of genomic data is a significant advance for ancient DNA studies. Most of the research using historical samples has been limited to very short regions of the mitochondrial or nuclear genome (Ramakrishnan & Hadly 2009), whereas, the strengths of a population genomics studies compared to a population genetics studies are usually two times greater (Parks *et al.* 2015). As an

example, studies on Adélie penguin have shown that using aDNA to calibrate molecular clocks result in a much faster mutation rate with narrower confidence intervals than using fossil record (Lambert *et al.* 2002; Shields & Wilson 1987; Subramanian *et al.* 2009). In a similar way, the main findings of the present studies show a much faster mutation rate compared with previous studies that used fossil records to calibrate a molecular clock. Finally, the BSP showed a significant rise in the effective population size during rapid climate change events in the Holocene, and most of the ancient haplotypes were shared between ancient and Modern samples regardless of the collection site.

The mutation rate calculated in the present study for the whole mitochondrial genome was $3.13E^{-7}$ S/S/yr (HPDI $2.58E^{-7}$, $3.69E^{-7}$), which seems feasible when compared with other studies using whole mitogenomes to calibrate a mutational rate (Table 2.5). The presented results indicate that the Leopard Seal’s mitogenome evolves at a higher rate than the genome of the Equus group, Adélie penguins or humans, but at a lower rate than Woolly Mammoth.

Table 2.5. Comparison mitochondrial mutation rate in different vertebrates

Species	Study	Mean mutation rate (S/S/yr)	95% HPD Lower	95% HPD Upper
Leopard Seal	Present Study	3.13E-07	2.58E-07	3.69E-07
Human	Fu et al., 2014	2.53E-08	1.76E-08	3.23E-08
Woolly Mammoth	Barnes et al., 2007	1.48E-06	1.37E-06	1.77E-05
Adélie Penguin	Subramanian et al., 2009	5.40E-08	3.10E-08	9.00E-08
Equus group	Orlando et al., 2013	1.02E-09	2.03 E-9	6.79E-10

In previous studies, a mutation rate has been estimated for Antarctic seals (Slade 1998; de Bruyn *et al.* 2009), whereas this is the first study where an internal calibration has

been made for the Leopard Seal in particular. A study by Slade (1998), determined a phylogenetically derived mutation rate for the mitochondrial HVRI sequence of $7.5E^{-8}$ S/S/yr using fossil records of some ice-breeding seals and Elephant Seals. If the whole mitochondrial genome evolves 10.4 times slower than the HVRI as suggested by Quinn (1992), the Antarctic seals' mitogenome would evolve at a rate of $7.21E^{-9}$ S/S/yr. A study by de Bruyn *et al.* (2009), calculated a substitution rate of $9.80E^{-7}$ S/S/yr (HPDI $1.67E^{-9}$, $2.06E^{-6}$) for the HVRI using ancient DNA samples to calibrate the internal nodes of this species directly.

The results of de Bruyn *et al.* (2009) represent a significantly higher rate than the phylogenetically estimated rate from Slade *et al.* (1998), and this agrees with several works using ancient DNA that suggest faster evolutionary rates when using more recent dates to calibrate molecular clocks (Vila *et al.* 2001; Freitas *et al.* 2003; Huynen *et al.* 2003; Rohland *et al.* 2005; Barnes *et al.* 2007; Dalen *et al.* 2007). In the present study, the results also follow this tendency of higher mutation rates when using younger calibration nodes, suggesting that the mitochondrial genome in Leopard Seals is evolving approximately 14 times faster than previous phylogenetic estimates. Conventionally, the main problem with mutation rates is the difficulty to estimate it and are likely have broad confidence limits, leading to large uncertainties when estimating effective population size and the time since population expansion (Curtis *et al.* 2009). The results of the present study show an HPDI for mitochondrial mutation rate considerably narrower than previous estimates for other vertebrates.

The Bayesian Skyline Plot in this study suggests that a period of sharp population growth started around 7,500 YBP, then making a semi-plateau around 5,500-3,500 YBP, and then started increasing rapidly again until it stopped around 2,000 YBP. Effective population size has remained with no significant changes since ~2,000 YBP until present times. This increase in population size started when the effective population size was around 8,000 females at 7500 YBP, growing over a period of 5000 years to reach an effective population size of females of 60,000 in present times. These estimates are within a credible range in comparison with census data on population size. For example, Erickson & Hanson (1990) calculated the total population size at around 300,000 individuals, and later on, Southwell *et al.* (2012) estimated around 35000. Nevertheless, underestimation by these surveys has been debated.

The effective population size is typically a fraction of the census population size (e.g. ~1/10 from a well-cited meta-analysis; Frankham *et al.* 2014), though that relationship is not precisely known for this species. One possible explanation for an increase in population size since ~8000 YBP would be climatic variations that changed the dynamic of the ecosystem, impacting directly in the abundances in this top predator. The timing is coincident with the founding of an Elephant Seal colony in the Ross Sea at a time of climate warming (de Bruyn *et al.* 2009), and the subsequent expansion of that population.

On a global scale, a warm period in the around 10,000- 5,000 YBP was followed by a cooling period that lasted from 5,000 YBP to late Holocene, and finishing in the Little Ice Age 200 YBP, the coldest temperatures of the Holocene (Marcott *et al.* 2013). However, these changes in the temperature varied slightly or dramatically, in different regions of the

planet. Previous studies have remarked that glaciers expanded in different regions during the second half of the Holocene (Wanner *et al.* 2008), calling this period “neoglacial” which followed warm period (Hypsithermal) between 9,000 and 5,700 YBP, and culminating in the return of the ice between 5,400 and 4,900 YBP (Porter 2000). In the Southern Hemisphere (30°S to 90°S), temperatures dropped ~0.4°C gradually between 11,000 to 7,000 YBP (with a small warming period around 8,000 YBP), followed by relatively constant temperatures except for some possible significant variation along the past 2500 years (Marcott *et al.* 2013).

In the Southern Hemisphere, in particular, the major Rapid Climatic Change (RCC) events occurred between 6,000–5,000 and 3,500– 2,500 YBP, agreeing with records that suggest a decline in solar output at these periods, and coinciding with the major glacial advances (Mayewski *et al.* 2004). Some ice-core data from the Ross Sea during the Holocene indicated high productivity lasting from 6200 to 3000 YBP, according to diatom records (Cunningham *et al.* 1999). The effect of climate change on seals has been documented in the establishment of a colony of Elephant Seals in the Ross Sea ~8000 YBP until it finally disappeared around 1000 YBP. These changes suggested that temperature was warm enough to allow this expansion during at least two long periods; the first one from 1000 to 2300 YBP and the second one from 4000 to 6000 YBP (Hall *et al.* 2006).

These studies represent a substantial evidence of the dynamic climate of the Holocene, which allows the comparison of these climate reconstructions with the demographic data in the present study. Considering such climatic reconstruction in addition to the present results could be correlated to a cold period with an increase in Ne, whereas

warm periods would be representative of a stable population. The BSP shows an increase in population at 7,500-5,500 YBP and 3,500-2,500 YBP, agreeing with the longest periods of glacier advances. On the other hand, before 7,500 and around 5,500-3,500 YBP the population remained static when the temperature was warmer. From 2,500 YBP to the present, the climate was very variable, and culminating in a cold period 1,000 YBP, though the BSP shows a stable population.

Leopard Seals use to breed on sea ice (Southwell *et al.* 2003), so it is possible that the increasing of N_e in the BSP is directly correlated with the abundance of the sea ice. If this hypothesis is correct, then the other ice-breeding seals might be prone to such changes depending on the presence of sea ice. Simultaneously, the reduction of this top predator (and mesopredators) would bring changes on the entire trophic web in the Antarctic ecosystem.

Alternatively, in a study by Etourneau *et al.* (2013) changes in ice presence, water temperature, and primary productivity since 9000 YBP were documented in the WAP, using a sedimentary core, employing a combination of two biomarkers for temperature, and micropaleontological data for primary production. This climatic reconstruction suggests the opposite to what previous authors proposed (Mayewski *et al.* 2004; Hall *et al.* 2006; de Bruyn *et al.* 2009). This alternative climatic reconstruction suggests that local climate went cold, and extension of the sea-ice season lasted longer in the WAP than in other regions of the Southern Ocean, prompting accelerated diatom growth during 7000-3800 YBP. On the other hand, the last part of the Holocene (since 2100 YBP) presented warmer and reduced local primary productivity, which could be related to shorter growing season compared to

the first half of the Holocene. The ecological relevance of this climate reconstruction is that, the WAP region is considered very important habitat for Antarctic krill for breeding, and given that it is the primary food resource for seabirds, fish and marine mammals, it is considered a key species in the Southern Ocean; and also, it feeds on phytoplankton depending greatly on the physical environment (Meredith & King 2005; Parmesan 2006).

If this alternative climatic reconstruction is true, these changes in temperature and primary productivity could help to explain the increase in effective population size of Leopard Seals by the availability of resources. Since the Antarctic system is characterised by being very sensitive to changes in the food web, the reduction in primary productivity might cause a reduction in N_e of higher trophic levels and producing a “cascade effect”. These changes in productivity and temperature would then concur with the two major rapid growths in the BSP and would suggest that colder temperatures and advances in sea-ice cover represent better conditions for Leopard Seals promoting increased population size.

Regardless of which climatic reconstruction is used to compare the presented results, both scenarios suggest substantial changes in temperature at periods of significant changes in N_e according to the BSP, which stands out the sensitivity of this species to variation in climate. Many species of birds, plants, and marine vertebrates have been shown to decline in response to climate change, mainly because the extent of the sea-ice, which might induce a trophic cascade in this system (Parmesan 2006). Since 1976, records indicate a progressive decline in the sea-ice extent that apparently has an adverse effect on the abundance of ice algae; thus declines in krill as well (Atkinson *et al.* 2004). Nowadays, the Leopard Seal is under the category of “least concern” according to the IUCN Red List

of Threatened Species (Hückstädt 2015), which seems to be corroborated by the BSP (~60,000 Ne). Nevertheless, the effects of a contemporary and rapid warming in the Antarctic ecosystem are unpredictable, and it should not be ignored.

The haplotype network illustrates the differences in diversity between ancient and modern samples, but there is no evident pattern in the clustering of the haplotypes. The genetic diversity in Leopard Seals was very high given that only two samples were sharing one haplotype, which means that most of the samples represent different genetic variability. Both nucleotide and genetic diversity indices observed in this study are a reflection of working with large sequences, reducing the probabilities of sampling two individuals with the same haplotype. However, the nucleotide diversity found in this study is very high compared to a bottleneck effect of other Pinnipeds like the Northern Elephant Seal, which was estimated around $\pi=0.0066$ (Hoelzel *et al.* 1993). Tajima's D test supported expansion, whereas the Fu's F_s gave signals of a stable population. The mismatch distribution also showed signals of a stable population over time even when the BSP and Tajima's D suggested population growth, given that the observed data varies considerably in comparison with the expected trend in the graph (Figure 2.4). An explanation for these results could be that Ne is large even historically as observed in the BSP, and even when there is an expansion, this is not strong enough to be detected by Fu's F_s or the mismatch distribution. Nevertheless, the demographic trend of Leopard Seals proposed in the present study represents new information about this species which should be investigated deeply given the little information about its genetic composition.

2.6 Conclusions

This is the first study that sequenced entire mitochondrial genomes of ancient and modern Leopard Seals, in order to investigate more about its evolutionary and demographic history. The mutation rate estimated in this work, represents a much higher rate than traditionally phylogenetically estimated rates for Antarctic seals, evolving approximately 14 times faster, and it is similar to rates reported in previous analyses using ancient DNA of vertebrates. Much smaller error intervals are observed in the estimation of the mutation rate, in comparison to those with external calibration nodes. These smaller error intervals represent more accurate estimations when using this rate to calculate effective population sizes and divergence times, which can be crucial when trying to assess the conservation of a species.

No evidence of a sudden demographic expansion was found even when the BSP showed an increase on N_e . An explanation for these results could be that N_e is large even historically as observed in the BSP, and even when there is an expansion, this is not strong enough to be detected by Fu's F_s or the mismatch distribution. The presented results suggest that an expansion occurred from 7,500 to 2,500 YBP, overlapping with two major climatic changes, which might provide the conditions resources for this species to proliferate. In this period, the effective population size arose from 8,000 to 60,000 females, after that, the population growth stopped and remained without significant changes since then.

The increase in N_e at 7,500-5,500 YBP and 3,500-2,500 YBP overlaps with the longest periods of glacier advances. On the other hand, before 7,500 YBP and around 5,500-3,500 YBP the population remained static when the temperature was warmer. From 2,500 YBP to the present, the climate was very variable, and culminating in a cold period 1,000 YBP, though the BSP shows a stable population in this period. A possible explanation is that Leopard Seals breed in the sea ice, so it is directly proportional to the increase in the population. The abundance of Antarctic krill is dependent on the physical environment like the extension of the sea-ice (Atkinson *et al.* 2004; Meredith & King 2005; Parmesan 2006), and it is known that Leopard Seals spend winter around the Ross Sea waters searching for resources like krill and other species that feed on krill as well (Ainley 2010). Consequently, changes in the sea ice should affect the abundance of other ice-breeding Seals, and thus, changing most of the trophic levels in the Antarctic ecosystem due to the removal of a top predator and mesopredators.

Overall, the present study has provided novel information about evolutionary events that have shaped the demographic history of Leopard Seals during the Holocene, and how these respond to climate changes. Even when this study shows that the Leopard Seal population is not threatened ($\sim 60,000 N_e$ nowadays according to the BSP), the effects of a contemporary and rapid warming in the Antarctic ecosystem could cause a severe reduction of the population.

Additionally, even when human activities do not compete directly with Leopard Seals for food resources, fisheries may impact directly on the mesopredators that the Leopard Seals depredate, therefore, the human impact should not be discarded in future

scenarios of demographic change. Past climatic changes have affected the biology of many species around the world; thus this kind of studies might help to predict future responses of Antarctic species, which could be a critical aid to long-term conservation and management of such sensitive habitats.

CHAPTER 3. MODERN POPULATION GENOMICS AND DEMOGRAPHIC DYNAMICS OF SOUTHERN ELEPHANT SEALS (*Mirounga leonina*) POPULATIONS IN THE SOUTH ATLANTIC OCEAN.

3.1 Introduction

3.1.1 *The Elephant Seals*

Early taxonomists studying the Phocidae group defined four subfamilies: the Cystophorinae (hooded and Elephant Seals), Lobodontinae (Antarctic Seals), Monachinae (monk Seals), and the Phocinae (other Northern Hemisphere Seals; Davis *et al.* 2004). These studies were based on morphological features and placed the Northern Elephant (*M. angustirostris*), the Southern Elephant Seal (*M. leonina*), and the Hooded Seal (*Cystophora cristata*) into the Cystophorinae family (Bladdernose Seals; Davies 1958). In a study carried out by King (1966), the elephant seals and hooded seals were separated because of the few morphological characters used in previous studies to define the group were likely convergent, whereas the differences between the two genera were numerous. Consequently, the hooded Seal and Elephant Seals were placed into the tribes Cystophorini and Miroungini, within the Phocinae and Monachinae, respectively. This last classification was supported by molecular studies carried by Arnason *et al.* (2006).

The evolutionary history of Elephant Seals is likely to have an origin in the Southern Hemisphere with a later expansion into the Northern Hemisphere during the Pleistocene glacial age via the west coast of South America and then separated into two independent populations due to a rewarming of the seas (Davies 1958). These movements according to Davies (1958) originated the two extant species of the genera *Mirounga*, the

northern and Southern Elephant Seals. However, Briggs & Morejohn (1976) presented a different hypothesis, arguing the relative primitiveness of the Northern Elephant Seal and that fossil records in California are inconsistent with a southern origin, and proposed a tropical ancestor that entered the Pacific through the Central America Seaway. The interpretation of this hypothesis is that the northern species is the older of the two, and the southern species evolved directly from the Northern Elephant Seal or shared a common ancestor (Laws 1994). Molecular estimations of divergence (around 4 MY; Arnason *et al.* 2006) may be consistent with this scenario, suggesting speciation as a consequence of the formation of Isthmus of Panama and a disruption in an ancestral population of the two extant species. Molecular phylogenies also support the monophyletic relationship between these two species (Davis *et al.* 2004).

3.1.2 Ecology of Southern Elephant Seals

The Southern Elephant Seal is the biggest of the Pinnipeds, the adult male of this species usually weigh between 1500 and 3000 kg, with maximum weights reaching 3700 kg (Ling & Bryden 1981). The adult Elephant Seals are remarkably sexually dimorphic, males can reach a size ten times larger than females and also having distinctive secondary sexual characteristics; a thick skin on the sides and underside the neck and an enlarged proboscis that is absent in females (Laws 1994). This species has a circumpolar range, but populations are concentrated on and around sub-Antarctic islands lying near the Antarctic Convergence and one continental colony in Peninsula Valdes, Argentina (Ling & Bryden

1992). Their broad breeding range from Peninsula Valdes, Patagonia, to King George Island covers c. 20° latitude (Bornemann *et al.* 2000).

The Southern Elephant Seal is a major consumer of fish and squid (McCann & Rothery 1988), having a diet based on approximately 75% Cephalopods and 25% Fish (Laws 1977). Further to this, no strong differences in prey choice were found between sexes at King George Island, South Shetland Islands (Daneri *et al.* 2000), however the population in Argentina exhibit differences in prey choice and trophic level between females and males, as well as among subgroups of males (Lewis *et al.* 2006). The large populations, wide distribution and high energy demands of Elephant Seals play an important role in the dynamics of their marine food resources, mainly squid and fish (Bornemann *et al.* 2000).

The Sea-ice extent is one recognized determinant of primary production in the Antarctic region of the Southern Ocean (Loeb *et al.* 1997; Nicol *et al.* 2000), consequently, changes on the sea-ice could be translated into changes in the entire food chain over time (McMahon *et al.* 2003). The species has a circumpolar range, but typically inhabits beach and tussock areas on sub-Antarctic islands, and they may encounter ice and snow in the southernmost part of their range and on the Antarctic continent itself (Ling & Bryden 1992). The disappearance of a population in the Ross Sea before Antarctic sealing indicates that humans did not induce abandonment of the Victorian Land Coast but the result of environmental change (Hall *et al.* 2006), suggesting a high sensitivity of this species to change due to climatic and ecological changes.

3.1.3 Global Status

Southern Elephant Seal populations in 1990 was 664,000 individuals divided in three or four main stocks, South Georgia (SGES) and Peninsula Valdes (ASES) with 60% of the global population, Kerguelen Isles with 28%, and Macquarie Island with 12% (Laws 1994). Southern Elephant Seals have been exploited in many parts of their range since the early nineteenth century, primarily for the oil produced from their blubber (Ling & Bryden 1992). Sealing has led to the decrease in population size drastically from 1820 to 1906 hunting more than a million seals during this period (McCann & Rothery 1988; Laws 1994). Hunting ceased in most areas by 1906, and around the 1950's most of the populations were thought to have recovered, except on South Georgia where the oil industry based on this species continued until 1964 (Bonner 1958; McCann & Rothery 1988). However, recent declines have occurred mainly in the Indian and Pacific Ocean, while sites in South Atlantic including South Georgia remain apparently stable or increasing (Boyd *et al.* 1996). It is inferred that the decrease in the Pacific and the Indian Ocean between 1950 and 1990 was driven principally by resource limitation and more predation pressure in the Southern Ocean (McMahon *et al.* 2003).

3.1.4 South Atlantic population

South Georgia is one of the biggest breeding colonies of SES in the world. In a survey by McCann & Rothery (1988), they counted 87,711 females and 10,260 males, with an annual pup production of 102,000, and no significant change in population size from 1951 to 1985. The latest survey in South Georgia Islands was done by Boyd *et al.* (1996),

estimating an abundance of 113,444 breeding females, which was combined with information from previous surveys that supports the idea that the total population size has remained static during the past 45 years. They hypothesised that that the lack of any change in population size might be linked to a limited availability of high-quality breeding habitat.

A continental colony in Argentina located at Peninsula Valdes has shown a major recovery in numbers and a census report in the 1990s. Lewis *et al.* (1998) suggested that this may have been the only colony that was recovering. This colony increased from 7,455 in 1982 to 9,636 breeding females in 1990, and if pups are included this number rises to the order of 19,000 (Campagna & Lewis 1992). The Argentinean colony is the largest northernmost colony of the species, and its expansion may be explained by the availability of food resources, sandy beaches, and lack of competitors (Campagna *et al.* 1993).

Traditionally, the Elephant Seals that inhabit Peninsula Valdes and South Georgia have been considered as a single population representing the South Atlantic Ocean. However, genetic studies based on both mitochondrial and nuclear DNA (Hoelzel *et al.* 1993; Slade *et al.* 1998; Hoelzel *et al.* 2001; Fabiani *et al.* 2003) indicate that these two locations are significantly differentiated. The Argentina population has very low genetic variation in comparison with South Georgia population which suggests that the mainland population could be founded by as few as only one sub-Antarctic matriline followed by little or no migration between the two populations (Hoelzel *et al.* 1993).

A study by Galimberti & Sanvito (2000), reported 1,827 individuals in Seal Lion Island which is located south of the Falkland Islands, which is a small population size compared with the other sub-Antarctic colonies. In a later study by Galimberti *et al.* (2001),

they tested the viability of that population and concluded that even when it does not appear to be at immediate risk of extinction, the loss of this population should be taken into account to avoid local reduction of biodiversity. Moreover, the importance of this island has been suggested as a possible role as a gene flow conduit between South Georgia and Peninsula Valdes populations (Hoelzel *et al.* 1993).

Another colony exists on Elephant Island (EI), South Shetland Islands, in which a study in 1971 estimated a population size of around 6,000 individuals. They registered a male that was tagged in South Georgia between 1957-1965, which suggested the migration of individuals between South Georgia and Elephant Island (Hunt 1973). Human activities have had an impact on Seals inhabiting nearby King George Island, causing population decline (Harris 1991), possibly associated with a reduction of the availability of squid in this continental shelf (Daneri *et al.* 2000). A study by Bornemann *et al.* (2000) on King George Island, which represents the southernmost breeding colony of this species in the Antarctic, shows that the foraging habitats of female Elephant Seals are closely associated with the sea ice zone. They tracked 13 females for two months and found evidence of movement of individuals between King George Island, Elephant Island, and South Georgia. More details about locations and population size of the South Atlantic colonies are available at Figure 3.1 and Table S2.

3.1.5 Genetic studies in Southern Elephant Seals

Slade *et al.* (1998) investigated genetic variation among three main populations of SES on sub-Antarctic islands (South Atlantic, South Indian, and South Pacific oceans), and

a smaller continental population at Peninsula Valdes, Argentina using mtDNA and nDNA, and found significant population structure. They also were able to calculate a mutation rate of $7.5E^{-8}$ (substitutions per site per year) for the HVRI based on fossil calibrations. This rate was used to estimate a divergence time of 270,000 YBP between South Georgia and Peninsula Valdes with an effective population size of 30,000 and 3,000 individuals respectively. Although Peninsula Valdes and South Georgia are in the same oceanic region, there is an order of magnitude greater divergence between these two populations than between separate oceanic populations (Slade *et al.* 1998).

Fabiani *et al.* (2003) compared the hypervariable sequence of the mtDNA control region among populations from Falkland Islands, Elephant Island, South Georgia, Peninsula Valdes, Heard Island, and Macquarie Island, resulting in a Maximum Parsimonious tree that shows little structure for most of the islands, excepting the well-supported lineages for Macquarie Island and Peninsula Valdes. Furthermore, evidence of gene flow among populations from Macquarie Island to SLI was found in a unique long-range dispersal event for a migrant male that resulted in a significant number of paternities (Fabiani *et al.* 2003). In a later study by Fabiani *et al.* (2004), paternal success at Sea Lion Island was investigated using both behavioural measures and genetic markers. They found that the average success of harem-holding males at Sea Lion Island (SLI) is significantly higher than both the Northern Elephant Seal and the nearby Southern Elephant Seal population at Peninsula Valdes, Argentina.

The stability and migration of an Elephant Seal colony are strongly related to climate, and such is the case of a breeding colony that existed proximate to the Ross Ice

Shelf during the Holocene. Thanks to the discovery of mummified remains in this locality, Hall *et al.* (2006) proposed that this colony was viable due a warming period beginning around 8000 YBP followed by a drastic drop in temperature around 1000 YBP, followed by the disappearance of this breeding colony (Hall *et al.* 2006). After that, de Bruyn *et al.* (2009) sampled and sequenced the HVR1 of all the main extant populations and the extinct population on Victoria Land Coast (VLC), concluding that individuals came initially from MQ, with some of them returning there once the VLC habitat was lost due the return of the ice 7000 years later. Their results indicated that a new habitat was quickly exploited by SES and that the founded population was isolated from the distant source population. These results suggested that future adaptive radiation might happen in a small timescale when the conditions are optimal, but this potential can be rapidly lost as well (de Bruyn *et al.* 2009).

Subsequently, de Bruyn *et al.* (2014) investigated the same ancient dataset by applying comparative Bayesian computational analysis. They found that the substantial increase in population genetic diversity of hundreds of generations could be explained by rapid population growth and sustained large population size. They also suggested that environmental change might provide the right conditions for adaptive evolution by a sharp increase in population size in a relatively brief timescale; thus, affecting multiple phenotypic traits (de Bruyn *et al.* 2014). Moreover, Hoelzel *et al.* (1993) compared South Georgia (SGES) and Peninsula Valdes (ASES) populations, finding 23 control region mtDNA haplotypes with average sequence difference of 2.3% between populations. These results suggested a limited degree of mixing, and finding small genetic variation in ASES compared to SGES suggested a historical contraction of the ASES population. The ASES

population had only three haplotypes, each differentiated by a single base-pair substitution, indicating monophyletic origin or, in other words, a single surviving matriline of the historic bottleneck representing 0.67% (Hoelzel *et al.* 1993), which based on more recent estimates of mutation rate would require 7,000 years to occur (see Corrigan *et al.* 2016).

The most recent study on the demography and genetic structure of the SES populations were carried by Corrigan *et al.* (2016) based on 15 microsatellite DNA loci and mtDNA, and who used the mtDNA mutational rate proposed by de Bruyn *et al.* (2009) in order to determine demographic parameters. They found that MQ and ASES were the colonies with the lowest genetic diversity, whereas comparisons between SGES, EI, and SLI resulted in the lowest fixation index (F_{ST}) values, suggesting a very low degree of structure between these colonies. Additionally, they agree with previous studies (Fabiani *et al.* 2003; de Bruyn *et al.* 2009) in considering ASES an isolated population and grouping all the other South Atlantic colonies together as a single population.

Earlier studies compared mtDNA regions of 200-600 bp (Hoelzel *et al.* 1993; Slade *et al.* 1998; Fabiani *et al.* 2003). In the present study, the use of NGS allows the sequencing of the whole mitochondrial genome of SES from four geographically proximate breeding populations to provide higher resolution of their genetic diversity and the degree of structure of these South Atlantic Ocean populations. Additionally, estimated mutation rates can be used to calculate the historical population size, divergence time, and the number of migrants for each of the tested populations, providing a better understanding of the demographic history of this species.

3.2 Chapter outline and aims

3.2.1 *Objectives of the Chapter*

1) Sequencing the whole mitochondrial genome of modern Elephant Seals inhabiting four breeding colonies in the southernmost limits of the Atlantic Ocean, and assess their genetic structure.

2) Use of coalescent methods to determine the historical population dynamics of Southern Elephant Seals and consider the results in the context of environmental change.

3.2.2 *Hypothesis*

The different breeding colonies of Southern Elephant Seals show different tendencies in population growth and genetic connectivity depending on the local resources and environmental conditions. Such tendencies will be reflected in the mitochondrial genome of modern organisms and correlated with the major environmental changes that might affect the breeding colonies in the South Atlantic Ocean.

3.3 Materials and Methods

3.3.1 *Collection of samples*

A total of 69 samples from Southern Elephant Seals from four different breeding colonies were collected by several expeditions on previous studies from collaborators (Hoelzel *et al.* 1993; Slade *et al.* 1998; Fabiani *et al.* 2003). Tissue samples were collected from the hind flippers of seals and preserved in the field in 100% EtOH. 15 samples were used from Elephant Island, 15 samples from Seal Lion Island, 15 samples from South Georgia, and 24 samples from Peninsula Valdes, Argentina (Figure 3.1).

3.3.2 *Laboratory methods and bioinformatics.*

For DNA extractions, library constructions, target enrichment, and demultiplexing, and reconstruction of genomes see section 2.3.2 to 2.3.9. These sections contain the methods utilised in the analysis of samples from Southern Elephant Seals excluding the sections that indicate specifically the use of ancient samples only.

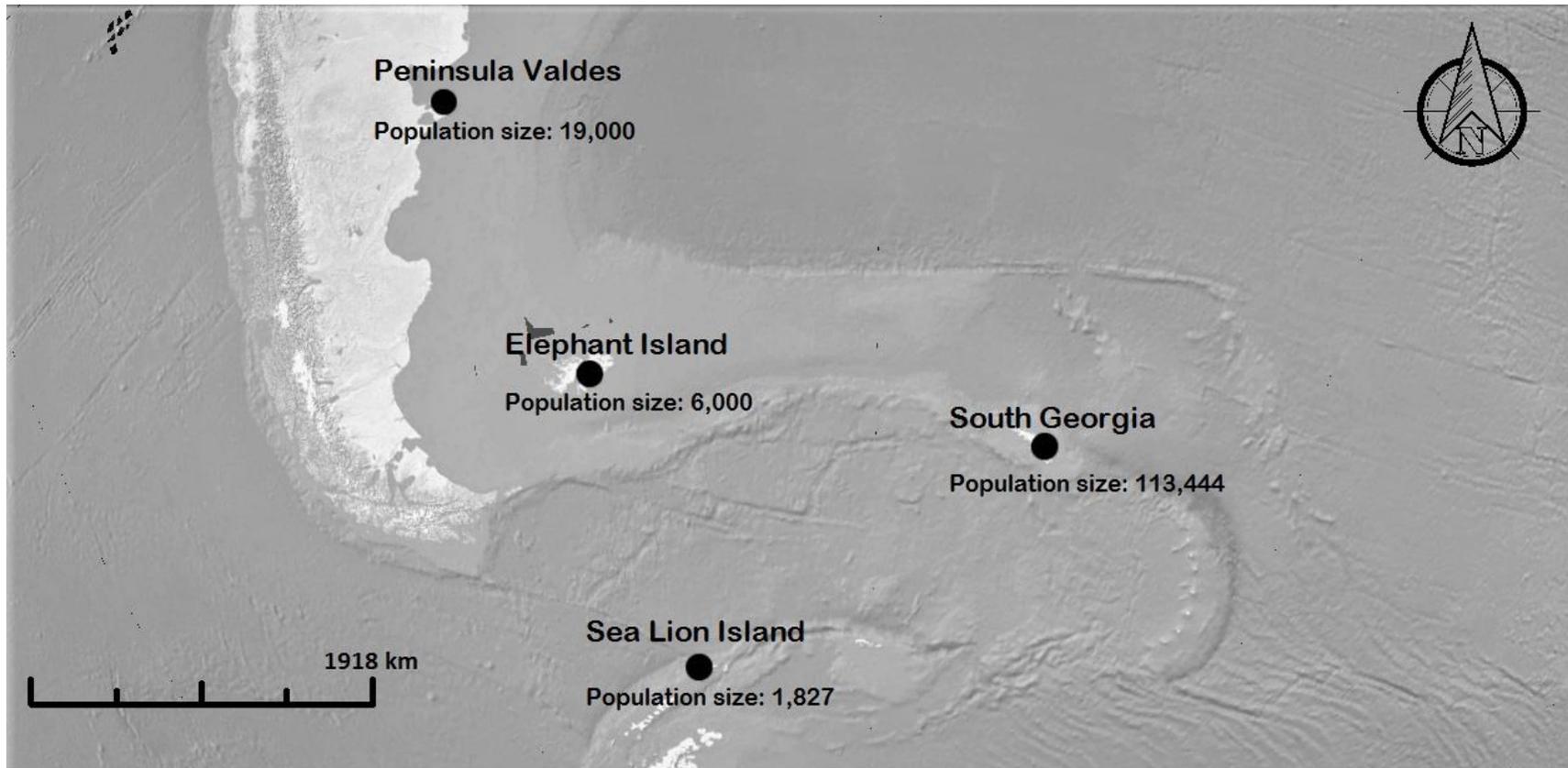


Figure 3.1. Geographic locations of the colonies sampled in the present study. Population size calculated by previous studies is provided for each colony.

3.3.3 *Estimating historical population size and other demographic parameters*

The program BEAUTi version 1.4.2 was used to set the appropriated parameters to use in the Bayesian analysis and to generate an input file to be used in the software BEAST v1.8.3. In order to estimate the historical population size, a strict molecular clock was used with a substitution rate of $4.9379 \text{ E}^{-7} \text{ S/S/yr}$ (95 HPDI 2.63 E^{-7} to $7.12 \text{ E}^{-7} \text{ S/S/yr}$) for the entire mitochondrial genome. This mutation rate was calculated by Welch *et al.* (in prep), using a dataset of ancient and modern samples of Southern Elephant Seals, to calibrate the internal nodes from temporally spaced sequence data, and setting sensitive priors on BEAUTi 1.4.2 for each sample based on radiocarbon dating.

The accuracy of BEAST when determining effective population size depends on the availability of strong and informative data about population history. Using the information from several surveys, the lower and upper limits for effective population size were set to explore past demographic changes through the generation of a BSP. Three independent MCMC samples per alignment were run for 50,000,000 generations and sampled every 5,000 generations after the initial 10% were discarded as burn-in. These three independent samples were combined using LogCombiner and analysed in Tracer v1.4. to provide more confidence that represent the coalescent model and phylogenetic uncertainty, and to produce the final BEAST results. A strict molecular clock model was applied, and the substitution model chosen was Hasegawa, Kishino and Yano (HKY) (Hasegawa *et al.* 1985). Only the parameters with an effective sample size (ESS) higher than 200 were taken into account.

Maximum Likelihood estimates of demographic parameters were obtained using the software MDIV, which determines divergence times, migration rates, and recent common ancestry under the finite sites model (HKY) and assuming no recombination (Nielsen & Wakeley 2001). This model assumes that an ancestral population splits into two descendant populations with gene flow possibly continuing between the divergent populations using Bayesian coalescent methods to integrate all possible genealogies through MCMC simulations. The program also estimates demographic parameters such as θ (theta) of the ancestral and two descendant populations scaled by mutation rate (μ) ($\theta = 4N_e\mu$); gene flow rates per gene copy per generation; and time (t) since population divergence from an ancestral population. The MDIV model is under the assumption of selective neutrality, that the two populations being tested are each other's closest relatives, and random sampling from a panmictic population (Nielsen & Wakeley 2001). For this analysis, all the possible combinations were tested between populations and running each pair by triplicates with a Markov Chain of 50 million generations after discarding 10% burn in.

3.3.4 Population genetics statistics

Standard diversity indices, molecular diversity indices, Neutrality tests (Tajima's D and Fu's Fs), fixation index (F_{ST}), and mismatch distributions were calculated using Arlequin 3.5.2.2 (Excoffier & Lischer 2010). The Analysis of Molecular Variance (AMOVA) was used to derive hierarchical F_{ST} and Φ_{ST} values among individuals and breeding colonies, incorporating both haplotype frequencies and the number of nucleotide differences between each pair of haplotypes.

The pairwise F_{ST} (conventional F-statistics: haplotype frequencies only, $\alpha=0.5$) and Φ_{ST} (distance method: Tamura-Nei, $\alpha=0.5$) were also calculated between different colonies using 10000 permutations. Φ_{ST} is different from F_{ST} given that it considers both haplotype frequencies and the number of nucleotide differences between each pair of haplotypes; which makes it more suitable for complex and variable sequences (Excoffier *et al.* 1992).

The neutrality tests (Tajima's D and Fu's F_s) were used to know if sequences are evolving randomly (neutral theory), or if alternative mechanisms like selection, drastic demographic shifts, or gene flow, are affecting the population. A population that has suffered a drastic change in population size will result in a rejection of the null hypothesis of neutrality, presenting great negative values (Tajima 1989; Fu 1997). Mismatch distributions were also used to estimate possible events of expansion. Additionally, Minimum Spanning and Neighbour Joining networks were used to create phylogenetic reconstructions using PopART (<http://popart.otago.ac.nz>), to group the haplotypes and show the number of evolutionary steps between them (Bandelt *et al.* 1999).

Finally, several phylogenetic trees were created to know the relationship between haplotypes of different populations and looking for consistent topologies. For this task, MrBayes (Ronquist *et al.* 2012) was used to calculate a Bayesian Inference of Phylogeny to build a phylogenetic tree, while PAUP* (Swofford & Sullivan 2009) was used to generate Neighbour Joining and Maximum Parsimony trees.

3.4 Results

3.4.1 Samples, libraries, and genome coverage

69 indexed libraries were produced from modern organisms collected from different Sub-Antarctic islands and a continental colony in Argentina. Using a reference genome of Southern Elephant Seal (GenBank ID: NC_008422) in order to reconstruct the genomes of individual samples, and compare the percentage of coverage aligned with it. Given that all the samples of Elephant Seals had good quality, most of the sequences obtained had above 90 % coverage compared to the reference genome. The ends of all the mitochondrial genomes were trimmed to have equal lengths across all the sequences, resulting in sequences of 16163 bp representing 95.09 % of the reference genome (16970 bp long).

After checking and editing the sequences all the sequences, two sequences were excluded, one from ASES and one from SLI that showed significant regions of the genome with many "N" characters with the value of total ambiguity. The final dataset to include into the statistical analysis consisted of 15 individuals from Elephant Island, 14 from Sea Lion Island, 15 from South Georgia, and 23 from Península Valdés, Argentina; making a total of 67 mitochondrial genomes.

3.4.2 Phylogenetic network

The phylogenies of the samples were reconstructed for the entire dataset to visualise the relationship between colonies, haplotype frequencies, and the number of evolutionary steps between haplotypes. Median Joining and Minimum Spanning Networks were

generated in the software PopArt, based on 16163 bp sequences from the full dataset. The networks show the Argentine colony as a well-resolved group that does not share any haplotype with the other colonies. The most similar haplotype to Argentinian samples is one haplotype from Elephant Island with 24 mutational steps and located in a basal section of the network. The other three colonies did not show evident segregation in the distribution of the haplotypes, and several individuals from different colonies shared identical sequences.

Most of the samples from this study remained with moderate distances to each other and great reticulation at the centre of the network, whereas three haplotypes presented distances around 40 mutational steps close to the central section of the network. The first distant haplotype formed a branch grouping eight haplotypes; the second one formed a branch that contains five haplotypes; the third was represented by only one haplotype (Figure 3.2).

3.4.3 Phylogenetic Trees

Phylogenetic trees were calculated for the whole dataset of samples to investigate which haplotypes or colonies were more closely related. These trees were consistent in showing a very well resolved group in the Argentinean colony, which was already evident in the phylogenetic network. In the phylogenetic trees, it is notable that the closest group of haplotypes belong to individuals from SGES and EI, but none of them is from SLI. Lineages including samples from SLI branch closer to the basal node (Figure 3.3, 3.4, & 3.5).

3.4.4 Population genetic statistics

Tajima's D and Fu's F_s were calculated in order to have an insight into the demographic trends in each of the colonies of this study (Table 3.1). Under assumptions of neutrality, negative values indicate a signature of population expansion. None of the D 's or F_s 's values were significant (Table 3.1). However, Elephant Island had higher nucleotide diversity, followed by South Georgia, and Sea Lion Island. A summary of the population genetics and demographic statistics is given in Table 3.1. In order to further assess signals of demographic and spatial expansion, a mismatch distribution approach was used, but weak signals of expansion were observed in all four populations suggesting long-term stable populations (Figure S1, S2, S3, S4, S5, S6, S7, and S8).

The genetic differentiation between populations was calculated and tested with F_{ST} and Φ_{ST} for the full dataset using ARLEQUIN 3.5 (Table 3.2). The pairwise F_{ST} and Φ_{ST} between different colonies were calculated using 10000 permutations shown in Table 3.3, and the associated P-value for each of these combinations are shown in Table 3.4. The average Φ_{ST} value for all populations demonstrated a very high degree of structure between populations according to Wright (1978) and suggesting that 31.78% of the variation is generated among populations, whereas the 68.22% of the genetic variation is found within the populations (Table 3.2). On the other hand, F_{ST} values showed a very low degree of structure, with 2.44% of the variation generated among populations, whereas 97.56 % of the genetic variation was found within the populations (Table 3.3).

Moreover, pairwise Φ_{ST} showed different levels of structure for specific population pairs. The highest degree of structure was observed between ASES and each of the other

colonies (Table 3.4 & Figure 3.6). In the island colonies, none of the comparisons was significant (Table 3.6). The haplotype distant matrix for the full dataset can be found in Supplementary materials (Figure S9). Similarly, pairwise F_{ST} also showed differences between specific population pairs. Nevertheless, this F_{ST} values suggested less degree of genetic structure between island colonies (Table 3.5), and all comparisons were significant (Table 3.7).

A graphic representation of the molecular distances within and between populations is available in Supplementary materials (Figure S10).

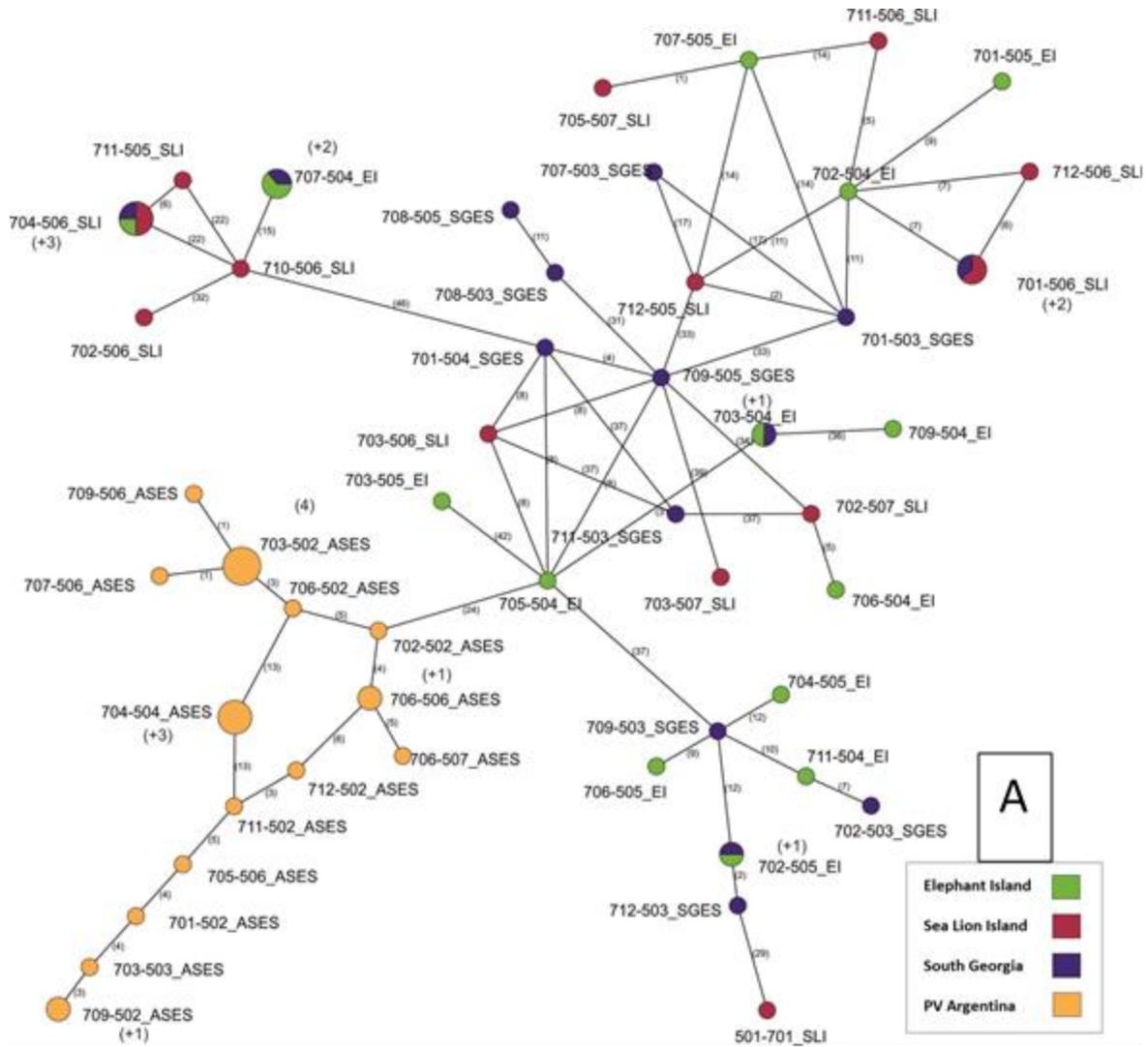


Figure 3.2. Networks of phylogenetic relationships among the four sampled colonies mitochondrial genomes haplotypes. (A) Minimum Spanning Network. (B) Median Joining Network. The size of the circle indicates relative frequency of the haplotype, the colors correspond to different colonies, and the numbers indicated in brackets refers to the number of differences between haplotypes (continues).

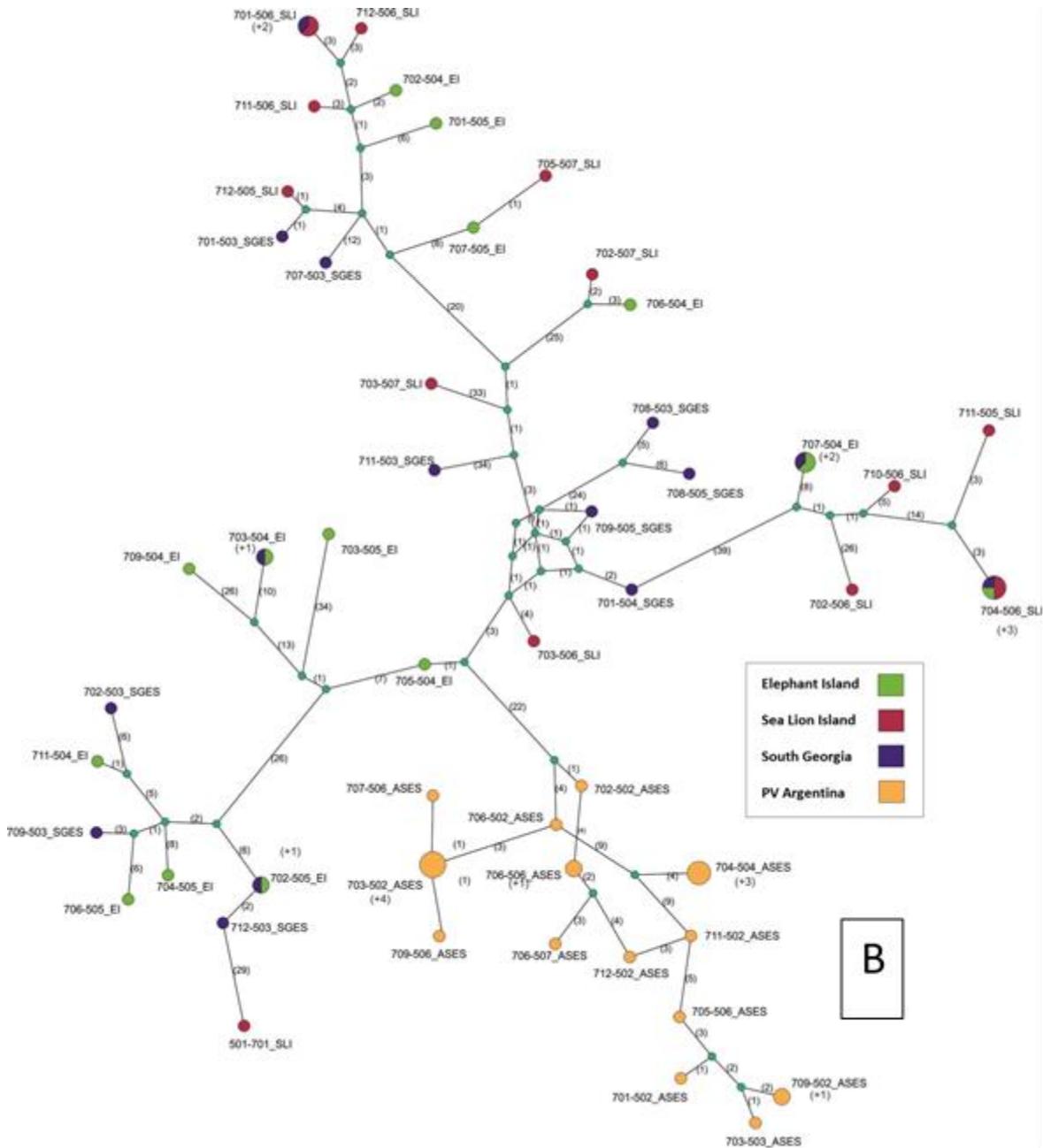


Figure 3.2. Networks of phylogenetic relationships among the four sampled colonies mitochondrial genomes haplotypes. (A) Minimum Spanning Network. (B) Median Joining Network. The size of the circle indicates relative frequency of the haplotype, the colors correspond to different colonies, and the numbers indicated in brackets refers to the number of differences between haplotypes.

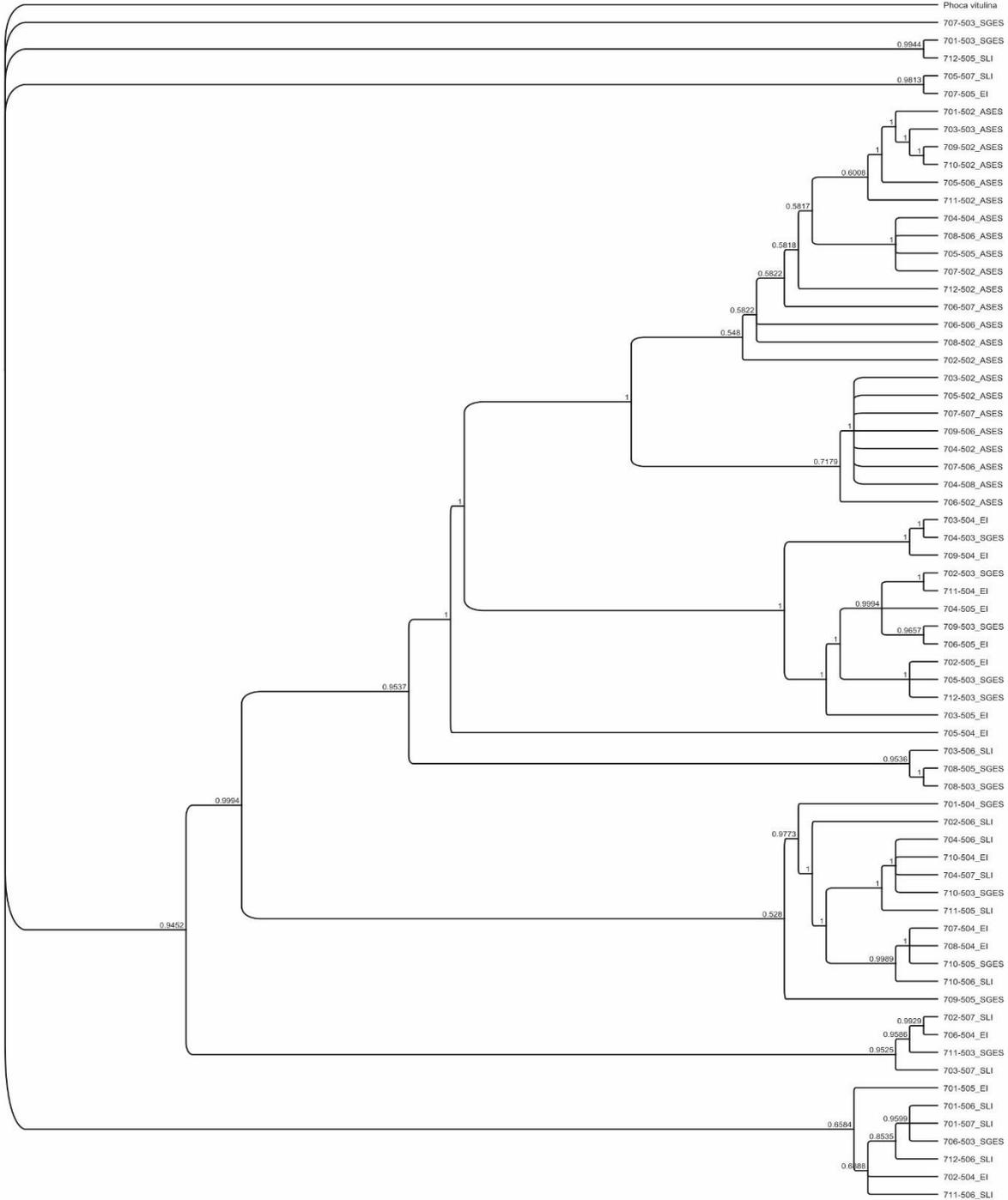


Figure 3.3. Bayesian inference in phylogeny tree calculated by MrBayes. *Phoca vitulina* (Root) has been added as an outgroup to show the clustering of mitochondrial genomes from the different colonies of Southern Elephant seals in the South Atlantic Ocean. Nodes are labelled with the support of posterior probabilities.

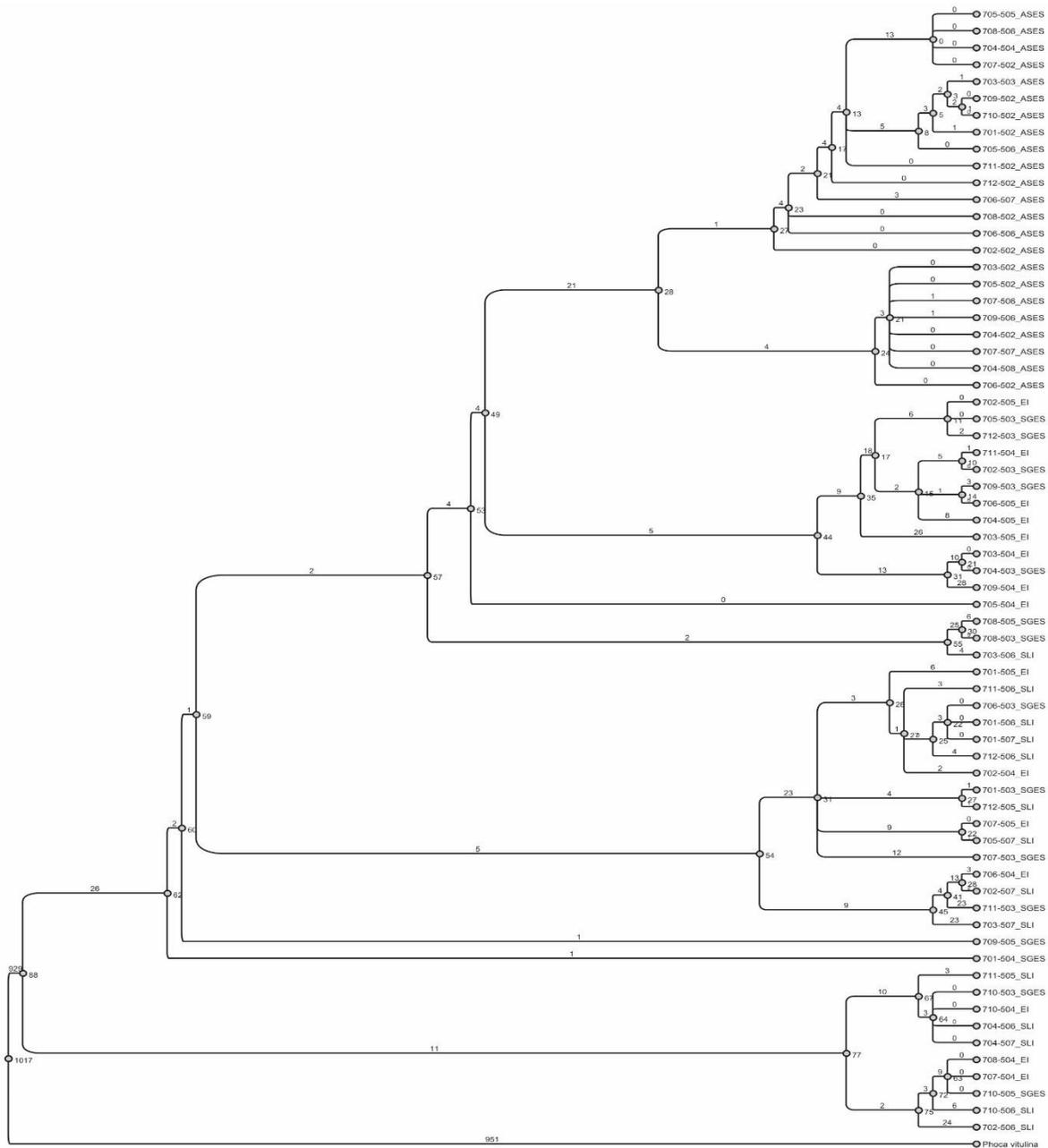


Figure 3.4. Maximum Parsimony Tree generated by the program PAUP*. *Phoca vitulina* (Root) has been added as an outgroup to show the clustering of mitochondrial genomes from the different colonies of Southern Elephant seals in the South Atlantic Ocean. Numbers above the branches indicate the substitution/site. Values next to the nodes indicate the node height.

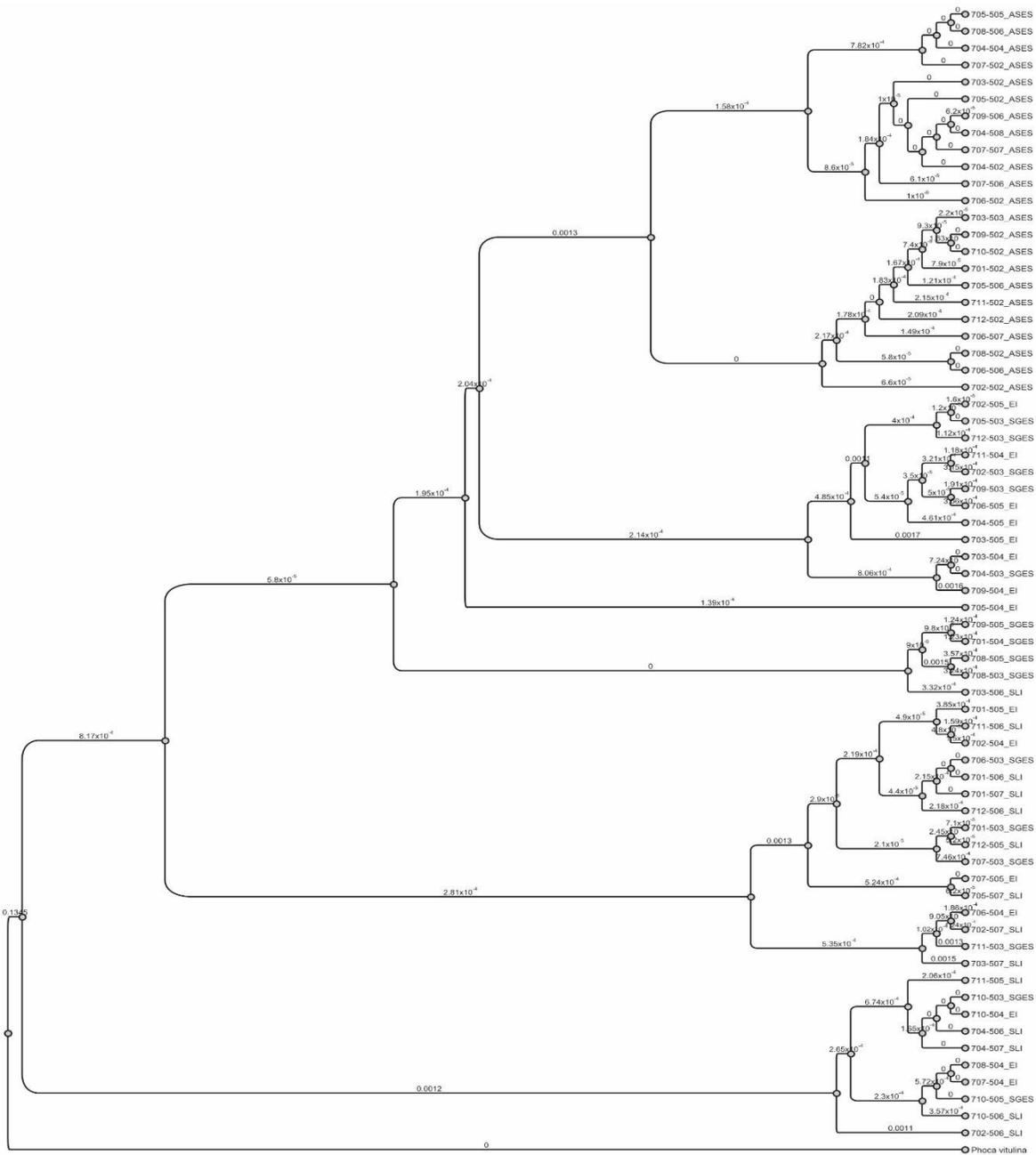


Figure 3.5. Neighbour Joining tree calculated by PAUP*. *Phoca vitulina* (Root) has been added as an outgroup to show the clustering of mitochondrial genomes from the different colonies of Southern Elephant seals in the South Atlantic Ocean. Numbers above the branches indicate the substitution/site. Values next to the nodes indicate the node height.

Table 3.1. Population genetic summary and demographic statistics for the different populations

	<i>N</i>	<i>S</i>	<i>h</i>	<i>Nucleotide Diversity</i>	<i>Genetic Diversity</i>	<i>Fu's Fs</i>	<i>Tajima's D</i>	<i>Pairwise differences</i>	<i>Harpending's Raggedness index</i>
ASES	23	39	15	0.000908 +/- 0.000470	0.9447 +/- 0.0303	-0.22693 P-value=0.483	1.4956 P-value=0.956	14.675078 +/- 6.818744	0.02708994 P-value=0.25930
SGES	15	245	15	0.0038 +/- 0.001950	1 +/- 0.0243	-1.26062 P-value=0.174	-0.83639 P-value=0.198	61.481512 +/- 28.139078	0.02176871 P-value=0.48420
SLI	14	178	12	0.003516 +/- 0.001816	0.978 +/- 0.0345	1.87242 P-value =0.787	0.04546 P-value=0.56	56.881279 +/- 26.175370	0.03526144 P-value=0.17310
EI	15	262	14	0.004182 +/- 0.002144	0.9905 +/- 0.0281	0.4531 P-value=0.504	-0.73377 P-value=0.243	67.612402 +/- 30.912056	0.03165533 P-value=0.11270

N= Number of individuals; S: segregation sites; h:number of haplotypes

Table 3.2. AMOVA design and results with average Φ_{ST} for all populations

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	3	593.375	10.61453 Va	31.78
Within populations	63	1435.382	22.78384 Vb	68.22
Total	66	2028.756	33.3986	
Fixation Index	Φ_{ST} :	0.31782		

Table 3.3. AMOVA design and results with average F_{ST} for all populations

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	3	2.064	0.01220 Va	2.44
Within populations	63	30.682	0.48701 Vb	97.56
Total	66	32.746	0.49921	
Fixation Index	F_{ST} :	0.02444		

Table 3.4. Population Pairwise Φ_{ST} .

	ASES	SGES	SLI	EI
ASES	0			
SGES	0.47736	0		
SLI	0.57031	0.06879	0	
EI	0.46292	-0.04097	0.06975	0

Table 3.5. Population Pairwise F_{ST} .

	ASES	SGES	SLI	EI
ASES	0			
SGES	0.02848	0		
SLI	0.03929	0.01093	0	
EI	0.03313	0.00476	0.01572	0

Table 3.6. P-Values corresponding to the pairwise Φ_{ST} among each pair of populations.

	ASES	SGES	SLI	EI
ASES	0			
SGES	0.00000+-0.0000	0		
SLI	0.00000+-0.0000	0.05306+-0.0024	0	
EI	0.00000+-0.0000	0.95842+-0.0021	0.06277+-0.0026	0

Table 3.7. P-Values associated to the pairwise F_{ST} among each pair of populations.

	ASES	SGES	SLI	EI
ASES	0			
SGES	0.01792+-0.0014	0		
SLI	0.00762+-0.0009	0.04297+-0.0020	0	
EI	0.00980+-0.0009	0.47698+-0.0046	0.04821+-0.0022	0

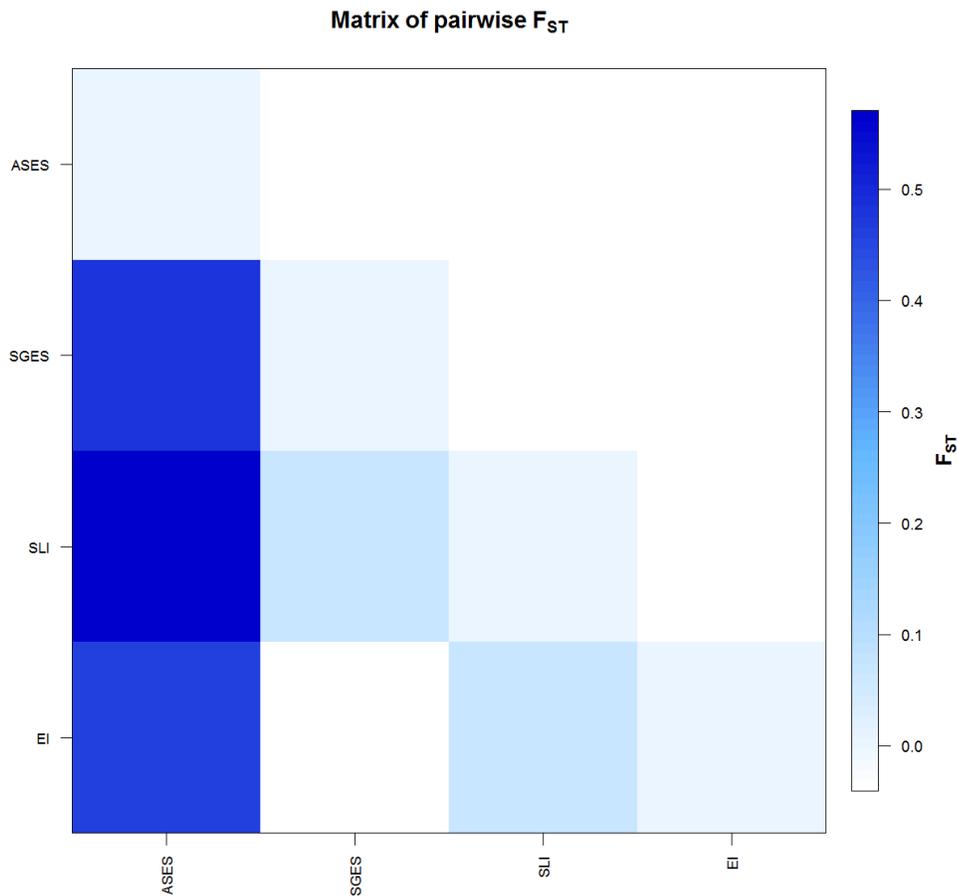


Figure 3.6. Graphic representation of the Φ_{ST} values between specific populations.

3.4.5 Bayesian methods and MDIV

Bayesian methods were used in order to calculate the historical effective population size of the different populations using a substitution rate of 4.9379 E^{-7} S/S/yr (95 HPDI 2.63 E^{-7} to 7.12 E^{-7} ; after Welch, in prep). The BSP projected both the timing and magnitude of effective (female) population size change (N_{ef}), for each of the populations.

Península de Valdés showed the demographic history of the past 600 years as a stable population with no change in effective population size until the past 100 years where it dropped from around 1500 to 800 N_{ef} (though the confidence limits were broad; Figure 7). The BSPs for South Georgia, SLI and Elephant Island were all similar, showing a stable population the last 3,000-4,000 years in the range of 10,000-20,000 (Figures 3.8-3.10). Small trends were all within the range of the confidence limits.

In addition to the BSP, the software MDIV by Nielsen & Wakeley (2001) was used, in order to calculate migration (m), female effective population size (N_{ef}), and divergence time (t). A summary of these parameters for all possible combinations is given in Table 3.8. When estimating theta, the posterior distributions for all populations showed a distinct peak (Figure 3.11). However, m and t posterior distributions presented a peak shape only for those colonies compared with ASES, suggesting that between the other islands migration is very high and divergence time very recent (Figure 3.12 & 3.13).

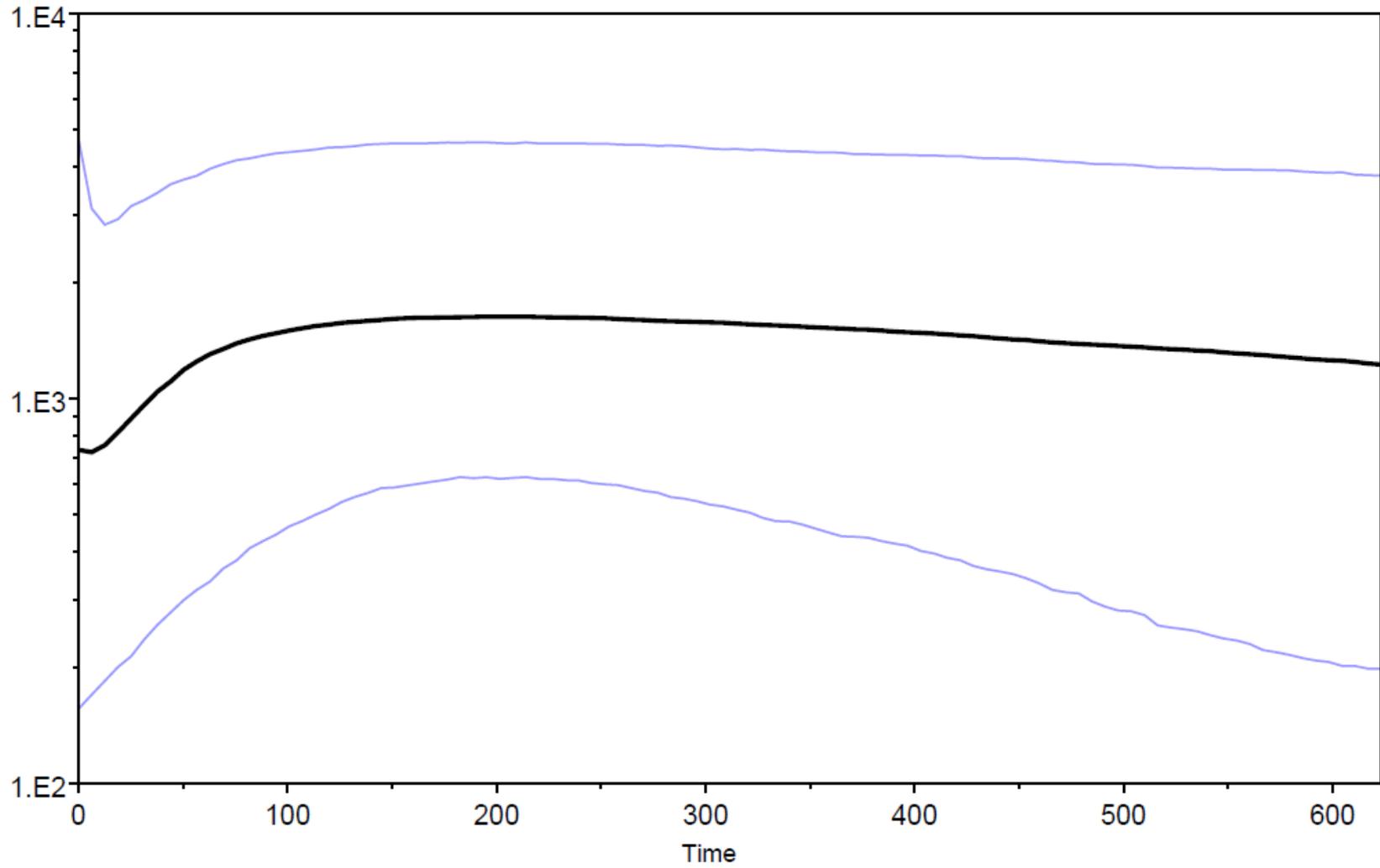


Figure 3.7. BSP showing the change on the effective population size through time for Península Valdés colony.

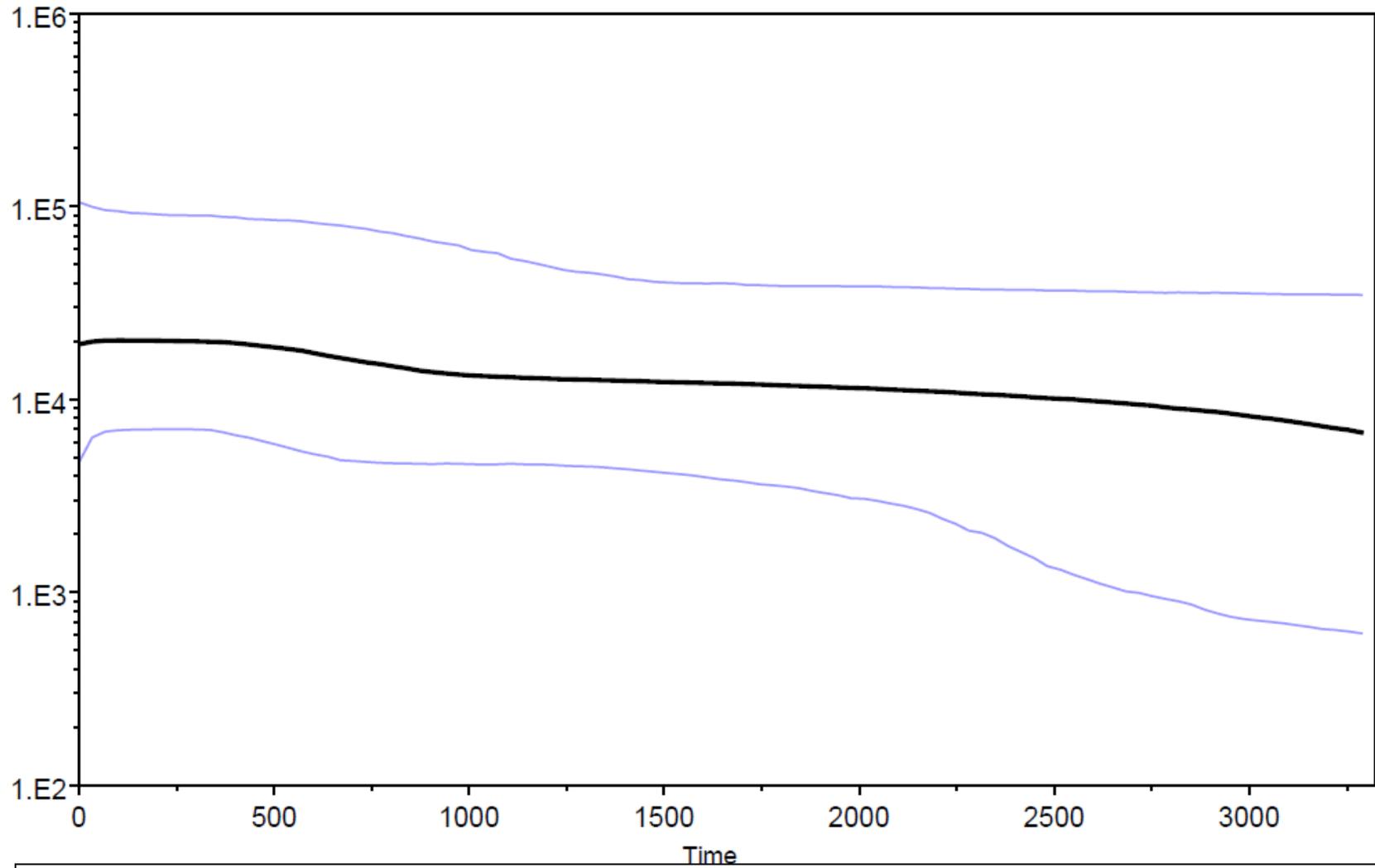


Figure 3.8. BSP showing the change on the effective population size through time for Elephant Island

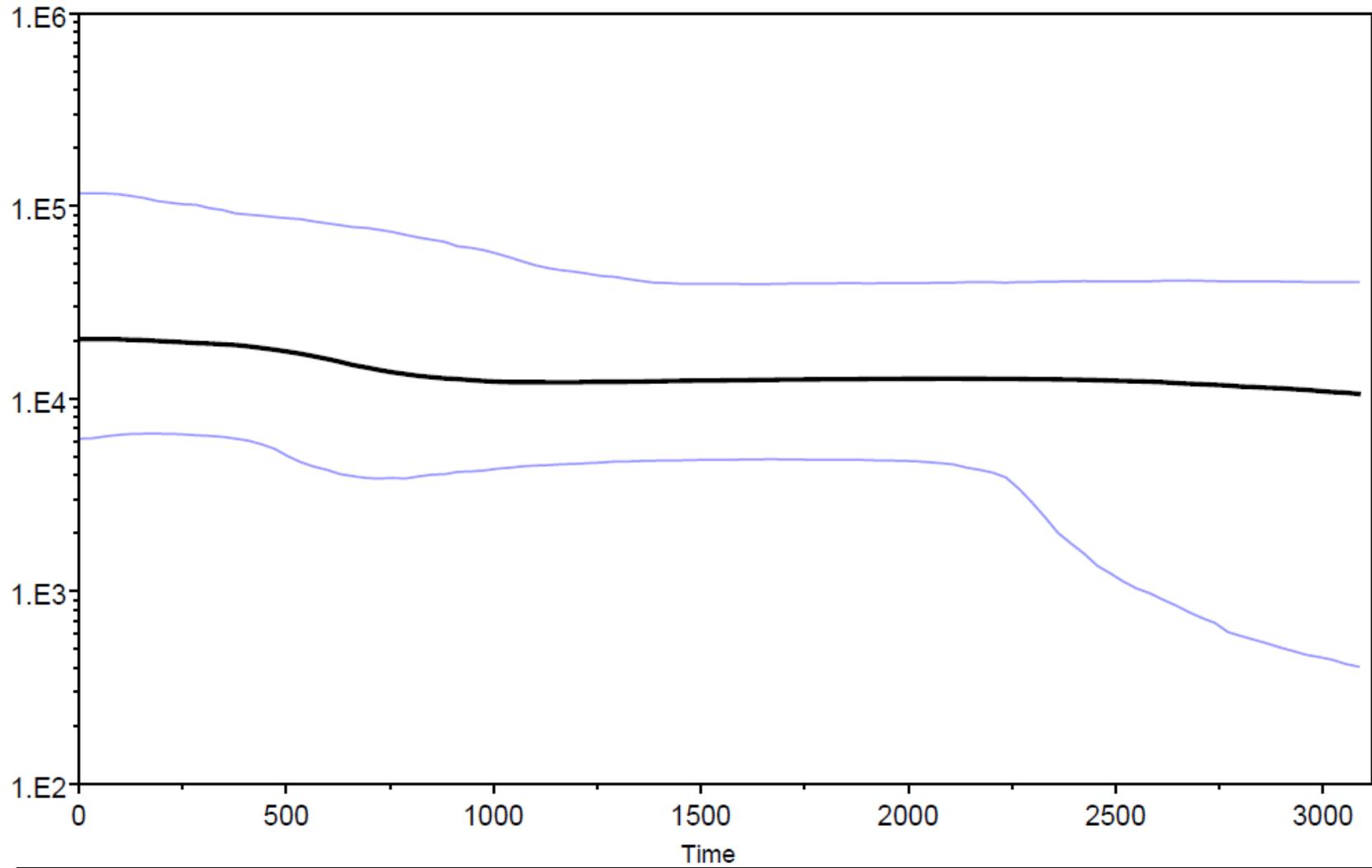


Figure 3.9. BSP showing the change on the effective population size through time for South Georgia

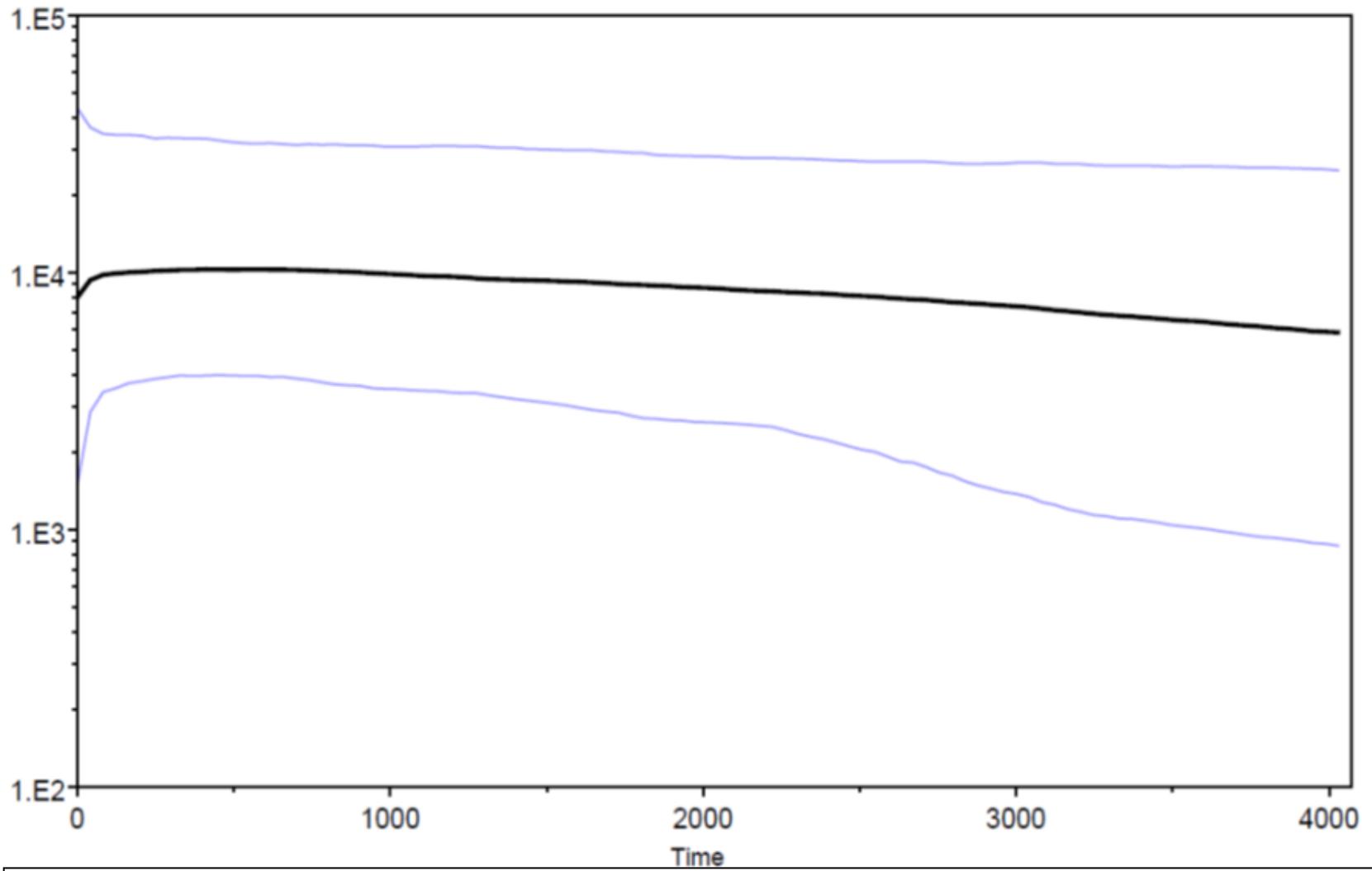


Figure 3.10. BSP showing the change on the effective population size through time for Seal Lion Island

Table 3.8. Maximum likelihood estimates of MDIV model parameters and their respective demographic conversions for the different combinations of populations. The statistical parameters in italics (*t*, *m* and θ) are estimated by a given mutation rate (μ). The demographic parameters (Ne, m, and t) were calculated according to the recommendations of Nielsen & Wakeley (2007), where: Ne=effective population size; t= divergence time in generations; m= average number of migrants per generation.

Populations	Generation time	Theta (θ)	<i>m</i>	<i>t</i>	Ne	m	t
ASES vs. EI	4 years	63.39	0.26	0.62	991.46 (491.56-1485.56)	0.000131 (0-0.000494)	1229.41 (18.90-3627.73)
	8 years	(31.80-94.98)	(-0.947-1.467)	(0.019-1.221)	495.73 (248.68-742.78)	0.000262 (0-0.000988)	614.71 (9.45-1813.87)
ASES vs. SGES	4 years	60 (37.539-82.461)	0.32	0.36	938.43 (587.13-1289.74)	0.000590	675.67 (0-2220.94)
	8 years		(-0.882-1.522)	(-0.141-0.861)	469.23 (293.57-644.87)	0.000341 (0-0.0118)	337.84 (0-1110.47)
ASES vs. SLI	4 years	56.92	0.2	1.06	890.27 (476.73-1303.81)	0.0001123 (0-0.000373)	1887.36 (310.83-4678.05)
	8 years	(30.48-83.36)	(-0.573-0.973)	(0.326-1.794)	445.13 (238.36-651.90)	0.000225 (0-0.000746)	943.68 (155.41-2339.027)
EI vs. SLI	4 years	90 (54.941-125.059)	>30	<0.01	1407.66 (859.31-1956.01)	∞	0
	8 years				703.83 (429.66-978.03)		
EI vs. SGES	4 years	82.92	>30	<0.01	1296.92 (803.21-1790.64)	∞	0
	8 years	(51.35-114.48)			648.46 (401.61-895.32)		
SGES vs. SLI	4 years	90.06	>30	<0.01	1408.60 (955.63-1861.57)	∞	0
	8 years	(61.10-119.02)			704.30 (477.81-930.74)		

Note: Two values of demographic parameters (Ne, m, and t) are given based on different generation times (4 & 8 years).

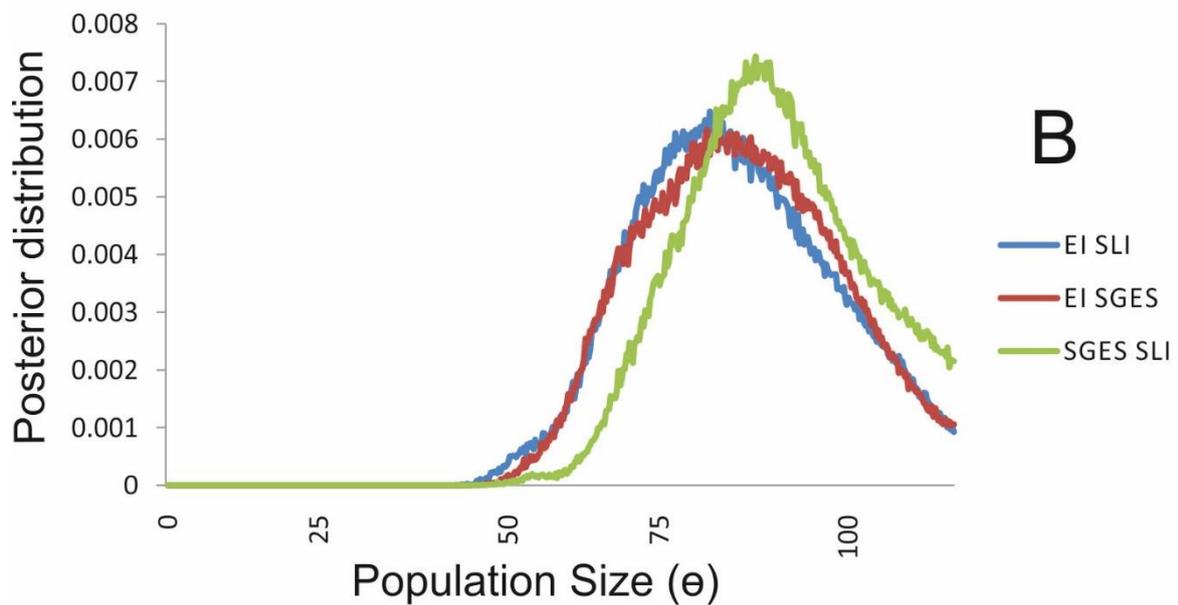
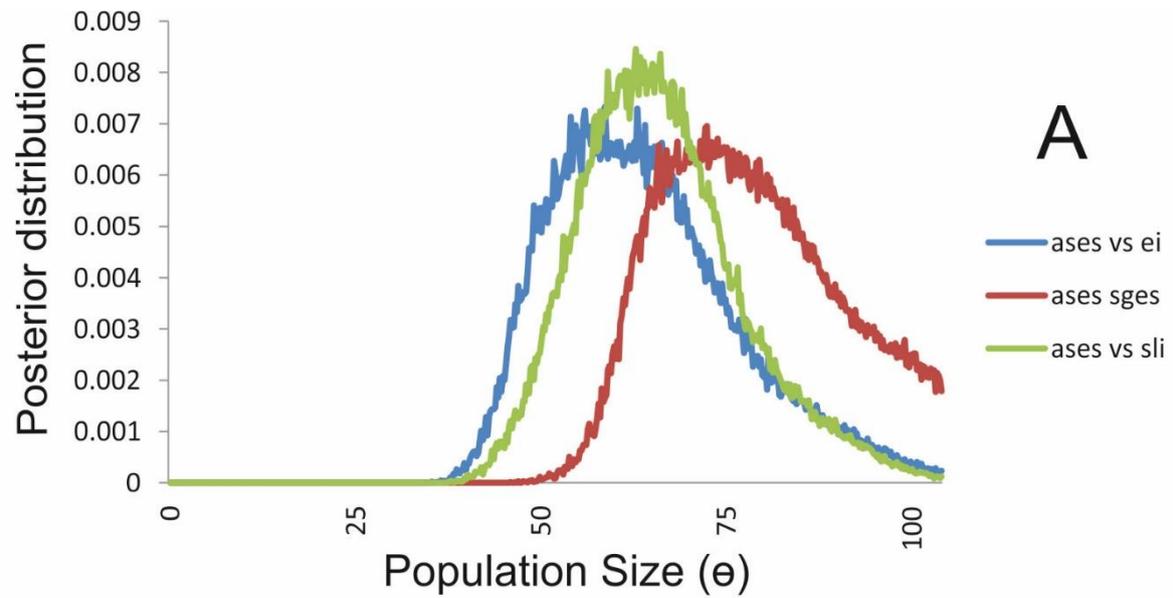


Figure 3.11. Posterior distribution of model parameter estimates for theta (θ). This value was used to calculate the effective population size. (A) Island populations against Argentinean population. (B) Comparison between islands.

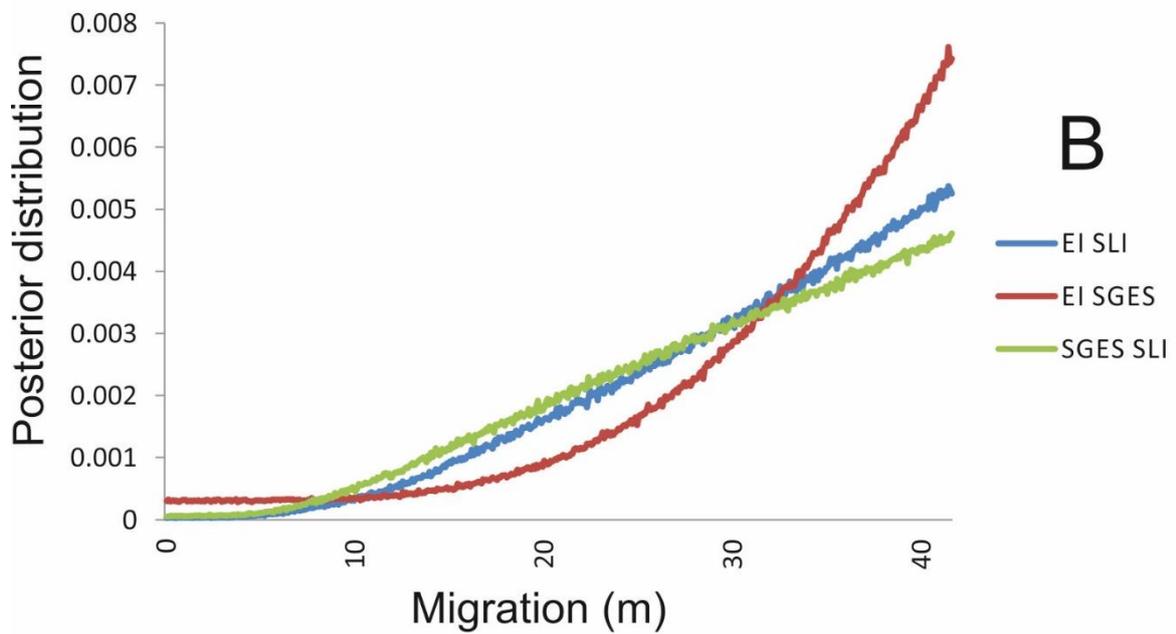
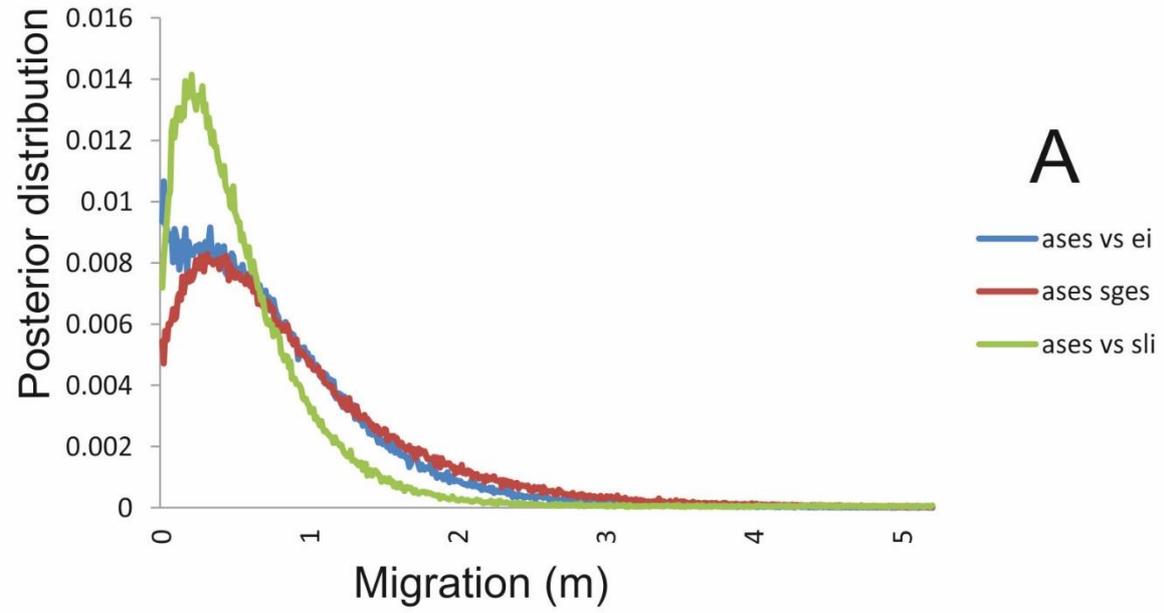


Figure 3.12. Posterior distribution of model parameter estimates for m , migration. (A) Island populations against Argentinean population. (B) Comparison between islands.

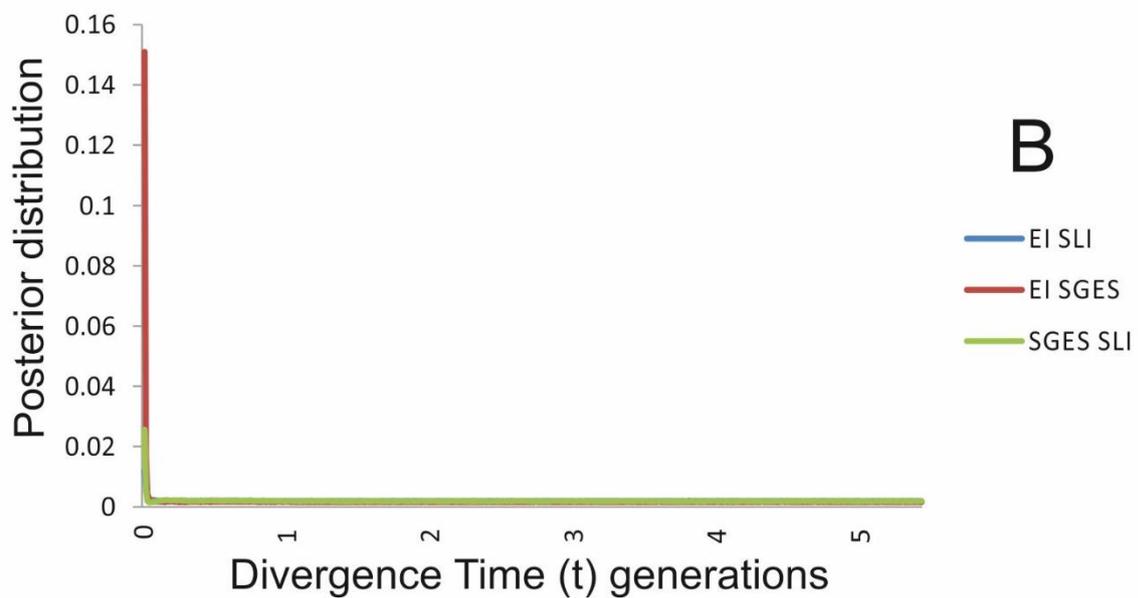
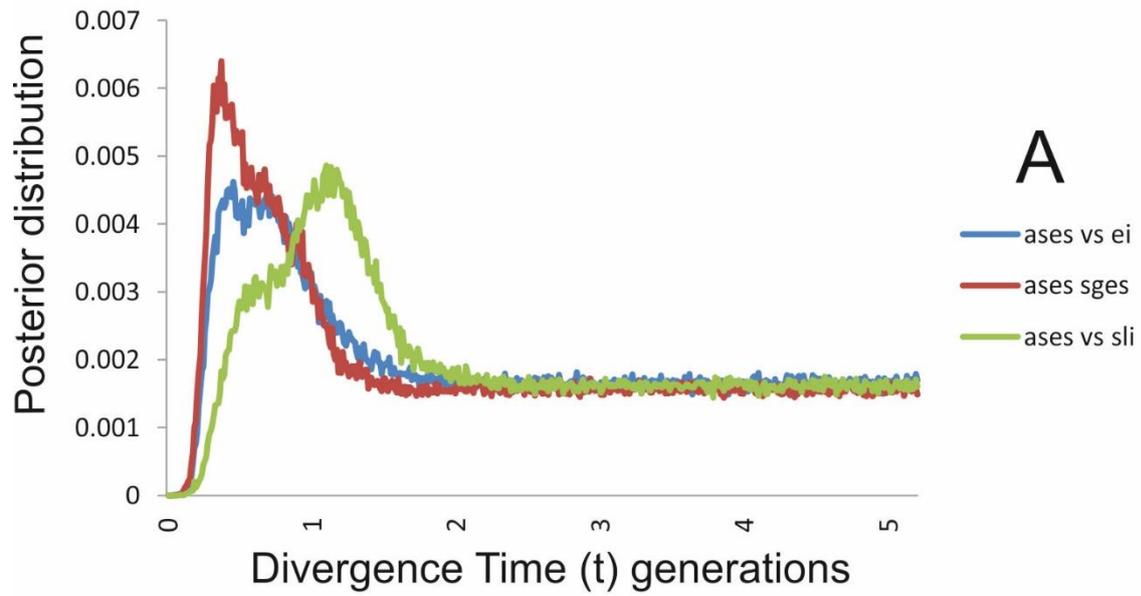


Figure 3.13. Posterior distribution of model parameter estimates for t , divergence time. (A) Island populations against Argentinean population. (B) Comparison between islands

3.5 Discussions

This study focused on sequencing the whole mitogenomes of Southern Elephant Seals from several populations, where genetic diversity and structure has been tested in previous studies using a small part of the mitochondrial genome (200- 400 bp control region). Similarly to previous studies (Hoelzel *et al.* 1993; Slade *et al.* 1998; Hoelzel *et al.* 2001; Fabiani *et al.* 2003; de Bruyn *et al.* 2014; Corrigan *et al.* 2016), the Argentinean colony presented very low genetic diversity, and it does not share any haplotype with the other colonies. Nonetheless, in the present study, a higher number of haplotypes from the Argentinean colony than in previous studies was found, this as a direct consequence of the inclusion of much longer sequences (16100 bp).

In a median joining network, the ASES haplotypes were not only on a separate branch, but they were also well isolated from other lineages. However, the ASES haplotypes all showed elevated frequencies compared with the other colonies and several differed from the others by a single bp mutation. These results are similar to the genetic composition of ASES that Hoelzel *et al.* (2001) reported, proposed to have resulted from a single founder event for ASES followed by an expansion with no further significant female recruitment. The elevated differentiation between ASES and the other islands, the data from the genetic distance and divergence, and the absence of shared haplotypes are all consistent with this interpretation. At the same time, multiple interpretations are possible for a given level of divergence. For example, weak differentiation may imply on-going gene flow, or that populations have been separated only very recently.

All phylogenetic trees support the clear separation of a group of haplotypes that are unique to Argentina, also observed in the phylogenetic network. All other haplotypes were distributed on the trees with no evident pattern of segregation, though separated into a series

of different lineages. The closest group of haplotypes to those from Argentina is formed mainly by samples from SGES and EI, and in the networks, the closest node is from EI (Figures 3, 4 & 5). These results are unexpected based on geographic distance alone since SLI is closer to ASES than EI or SGES. However, Elephant Island has been under ice several times since the last 9000YBP (Etourneau *et al.* 2013) so it is possible that during the relevant time periods, lineages currently represented on EI were instead on islands further north, such as SLI and SGES. The history of accessibility on EI was also discussed in the context of the nuclear DNA results reported in Corrigan *et al.* (2016). It is also the case of studies that reported movement between Elephant Island and South Georgia, further contributing to the potential for admixture (Hunt 1973; Bornemann, *et al.* 2000).

No differentiation was found between South Georgia and Elephant Island according to the Φ_{ST} test, and in fact, SGES has the highest number of unique haplotypes, whereas in EI shares most haplotypes with other colonies. On the contrary, the F_{ST} values suggested very low genetic structure among all colonies, showing the lowest structure between SGES and EI. Given that these sequences are 16 kb long, the Φ_{st} analysis is more appropriate given that it considers nucleotide distance between mutations. The Φ_{st} reported in this study, represents the highest level of structure between mainland and island populations, compared with previous studies using HVRI and nuclear markers. This result highlights the relevance of including longer sequences in studies of genetic structure, providing higher resolution to elucidate the connectivity between different sub-populations.

While all earlier studies have identified strong genetic differentiation between SGES and ASES, low differentiation had also been found among the three oceanic colonies in earlier studies. For example, Corrigan *et al.* (2016) using 15 microsatellite DNA loci obtained very low F_{ST} values when comparing SGES with either SLI or EI ($F_{ST}=0.006$ and 0.007 respectively), though somewhat higher values when comparing SLI and EI ($F_{ST}=0.01$). Slade

et al. (1998) included both Heard and MacQuarie Islands in their study, and based on genetic similarity suggested that Heard Island and SGES may have shared a common origin in South Africa. Corrigan *et al.* (2016) also suggest that similarities between SGES and Marion Island (concerning genetic distance and similar demographic histories) may indicate shared ancestry, perhaps on a mainland colony.

The nucleotide diversity obtained in the present study for ASES and SGES was of 0.000908 ± 0.000470 and 0.0038 ± 0.001950 respectively for the entire mitogenome, which represent at least an order of magnitude lower than those reported by previous studies for the HVRI (Hoelzel *et al.* 1993; Slade *et al.* 1998; Hoelzel *et al.* 2001). However, proper comparisons with previous works about genetic diversity are not possible given that the present study is the first that include sequences of the entire mitochondrial genome of Southern Elephant Seals. On the contrary to the nucleotide diversity, the haplotype diversity (or genetic diversity) was very high given that few haplotypes were shared between individuals. In this matter, ASES was the colony with the lowest haplotype diversity and SGES the highest. The haplotype diversity value of SGES was equal to one, which means that all the haplotypes sampled in that locality were different, whereas ASES resulted on 0.9447 ± 0.0303 due to a higher frequency of repeated haplotypes.

The demographic analysis also showed an apparent tendency of a sort of isolation between ASES and the other islands. The demographic parameters were escalated by considering a generation time on years at the age of the first reproduction on this species. Most studies reported first breeding age around four years (Hindell 1991; Ling & Bryden 1992; Bester & Wilkinson 1994; McMahon *et al.* 2003), whereas a study by Slade *et al.* (1998) considered a generation time of 8 years in their calculations. Depending on which generation time is being used to do the calculations, different values are obtained about the migrants per generations (m), effective population size (N_e), and divergence time (t) using

the program MDIV (Table 5). For this reason, the generation times of four and eight years was considered as upper and lower limits, in order to obtain a wider range of possible values.

In the present study, the value of migrants between island colonies was significant enough to avoid structure between them, according to the one-migration-per-generation rule (Mills & Allendorf 1996). On the other hand, all comparisons between the continental colony and island colonies suggested a very low number of migrants per generation, specifically, the lowest number of migrant per generation was between ASES and SLI, having only 0.0001123 female migrants per generation, the lowest rate of migration for the whole study. This result is unexpected due to the close distance of ASES and SLI compared with the other islands, which would suggest easier movements between these islands. When comparing the migration between SLI, SGES, and EI, the parameters are very high, and it is not possible to reach a peak in the posterior distribution graph, which means a significant number of migrants per each generation.

The N_e calculated by MDIV for ASES combined with the other islands were the lowest for the whole study, ranging from 890.27 for ASES-SLI to 991.46 breeding females for ASES-SLI. In contrast, when calculated the N_e for the different combinations of the sub-Antarctic islands, they all presented higher N_e ranging from SGES-SLI with 1408.60 breeding females to EI-SGES with 1296.92. The general results suggest a small N_e in ASES given that all comparisons with that colony resulted in lower values; whereas confidence ranges overlap with those comparisons that exclude ASES.

When the divergence time between ASES and the other islands was estimated, SGES-ASES were the populations that diverged more recently (2702.68 YBP, CI=0-8883.76 YBP), followed by EI-ASES (4917.64 YBP, CI=75.6-14510.92 YBP), SLI-ASES (7549.44 YBP CI= 1243.32-18712.2 YBP). The average divergence times between ASES and the other colonies indicated that island and mainland populations separated during the Holocene,

though the upper confidence limits for the separation of ASES and EI or SLI fall outside the Holocene (14510.92 and 18712.2 YBP). However, this is only considering the dispersion of females, while the dispersion of males could be different given that females tend to have high fidelity to the breeding site (Fabiani *et al.* 2006).

The work of Hoelzel *et al.* (1993), as updated in Corrigan *et al.* (2016) suggests an ancient event around 7,000 YBP that separated the populations of SGES and ASES, based on a model whereby a single control region sequence was left after the founder event, and later mutated twice. Corrigan *et al.* (2016) based on microsatellite DNA data, estimated divergence times (based on the isolation with migration model; Hey 2010) between ASES and SGES ranged from 383-8,519 YBP, very similar to the obtained results of this study for the same locations. The estimates based on MDIV for the divergence time between the Sub-Antarctic Islands were close to zero.

The vast differences in the results of effective population size, migrants per generation, and divergence time between studies might have different sources like the mutation rate used to do the calculations or the length of the sequences. For instance, Slade *et al.* (1998) used a mutation rate of $7.5E^{-8}$ S/S/yr (HPDI $2.9E^{-8}$ - $1.21E^{-7}$ S/S/yr) based on an external calibration by fossil records, and the sequence length of the fragments was 299 bp. This calibration affects the calculations of the demographic parameters, therefore obtaining higher values in comparison of the presented results. In the case of Corrigan *et al.* (2016) they used a mutation rate of $9.8E^{-7}$ S/S/yr (HPDI $1.67E^{-9}$ - $2.06E^{-6}$ S/S/yr) calculated for the CRI and calibrated using ancient DNA to have internal points of calibrations specific for the species (de Bruyn *et al.* 2009).

For the present study, a mutation rate of $4.9379E^{-7}$ S/S/yr (95 HPDI $2.63 E^{-7}$ to $7.12 E^{-7}$) was used for the entire mitogenome and was calculated using ancient DNA to calibrate internal nodes. The first demographic parameter to be estimated is N_e which uses the

mutation rate and the sequence length, thus depending on these factors N_e can vary. Previous studies used slower mutation rates and sequences 50 times shorter than the sequences used in the present study, which could cause this tendency in to show a smaller N_e . Even when values about the divergence times, N_e , and migration were not similar between studies, they all agree on the isolation and population structure between ASES and the other islands (Hoelzel *et al.* 1993; Slade *et al.* 1998; Hoelzel *et al.* 2001; Corrigan *et al.* 2016).

The demographic trend of each population to increase or decrease through time was calculated using Fu's F_s , Tajima's D , mismatch distribution and Bayesian Skyline Plots (Table 3.6). The P-values related to Fu's F_s , and Tajima's D shows that their results are not significant for any of the colonies, while the closest values to be meaningful is for SGES colony which suggest sudden expansion for both neutrality tests (Table 3.1). These results were consistent with the results of Corrigan *et al.* (2016), suggesting an expansion for SGES, while the other colonies remained as stable populations.

In order to investigate further about any possible expansion, a mismatch distribution analysis was performed for all the studied populations using a dataset of sequences of the entire mitochondrial genome, and other dataset with sequences of the HVRI (Figure S11, S12, S13, S14, S15, S16, S17, and S18). However, no signal of expansion was detected for either of the datasets given that the P-values associated with Harpending's Raggedness index were not significant. Finally, the BSP indicated that changes in N_e are not so drastic or evident for any of the four colonies, which indicate stable populations the last 4000 YBP.

BSP calculates an estimated trend in N_e over time under the assumption of panmixia, whereas MDIV provides a long-term average N_e of two different geographic populations, making a comparison between these approaches rather difficult. However, is useful to note the consistent indication that N_e is relatively low in ASES. The lowest N_e calculated by the Bayesian method belongs to ASES, followed by SLI, and finally SGES and EI with very

similar values (Table 6). The effective population size is typically a fraction of the census population size (at a ratio $N: N_e$, 10:1), though that relationship may vary (Frankham *et al.* 2014).

When comparing these results with the census of this species on these colonies, some significant differences can be noted. For instance, this study calculated a N_e of 20,000 breeding females for SGES and EI, whereas Boyd *et al.* (1996) reported 113,444 breeding females for SGES and Hunt (1973) reported 6,000 individuals in EI. Moreover, a reported N_e of 8,000 breeding females for SLI is presented, while Galimberti *et al.* (2001) reported only 1,827 individuals. Finally, Campagna & Lewis (1992) reported around 9,000 breeding females for the locality of ASES, whereas the presented study reports a N_e of 750 breeding females (Table 6).

Given that ASES presented a very low N_e compared with other island colonies, has the lowest nucleotide diversity, and present unique haplotypes not shared with other colonies, it is critical to preserve this population even if is not threatened in the short term. Usually, the majority of recorded extinctions and a substantial proportion of currently endangered species are on oceanic islands (Frankham *et al.* 2004). However, the data reported in the present study indicates that in this case, the system is working inversely, given that the mainland population is more vulnerable than the oceanic populations, though mainland is behaving like an "island" because it is isolated from the main stock.

Table 3.9. Effective population size for females as showed by the BSP in the present.

	Ne	HPDI
ASES	750	80-5000
SGES	20000	5000-100000
EI	20000	5000-100000
SLI	8000	2000-40000

Given that the BSP work under the assumption of panmixia, if these populations are not completely isolated from each other, results should be projecting the Ne of all those sub-populations. When considering the Φ_{ST} values, no structure is present between SGES and EI, which could explain why the values between these islands through time were the same. The studies that reported the abundance in the population of EI were considering this island as a separate colony from SGES, but according to the present study there is enough genetic connectivity between these islands to be regarded as part of the same population. On the other hand, an apparent isolation pattern was found in ASES which is reflected in the small Ne projected by the BSP. For these reasons, if all the island colonies are considered as one single population and ASES is completely isolated from them, then the Ne calculated by the BSP for the continental population would be possible.

The data on this study about population dynamics can be considered as a comparison with a study that investigated a now lost colony on the Victoria Land Coast in the Ross Sea, Antarctica. Hall *et al.* (2006) used ancient DNA samples to study the disappearance of this colony and the level of connection with some extant colonies, finding that Macquarie Island was the likely source population. The study also concluded that the colony was established around 8000 YBP during a warming period and open access at Victoria Land, and

disappeared around 1000 YBP when the sea-ice returned, and proposed that this colony was stable between 6000-4000 YBP. In this study, the BSP analyses suggest relatively stable populations for the four colonies studied, though there was a suggestion of a slight decline in N_e at ASES in the last 100 YBP (though highly overlapping the confidence limit range; Figure 7).

The BSP for MacQuarie Island obtained by de Bruyn *et al.* (2009) showed a similar pattern of stability and female N_e to the one found in this study for EI, SGES and SLI. The key comparison, however, may be that these estimates for the timing of the founding of the ASES colony are consistent with the timing of transitions elsewhere (all within the last ~8,000 years). On this timeframe, the Antarctic mainland population at Victoria Land was founded and according to Corrigan *et al.* (2016), differentiation occurred among oceanic island colonies, considering that this could have been associated with shifting patterns of sea-ice extent and breeding habitat availability after the end of last glacial period.

3.6 Conclusion

Previous studies about the genetic connectivity and demographic history of Southern Elephant Seals inhabiting the South Atlantic Ocean have used a small region of the mitochondrial genome (HVRI, around 300 bp) or microsatellite DNA loci. This study is the first that incorporates full mitochondrial genome datasets. Additionally, A mutation rate was used in this study for the entire mitochondrial genome that was internally calibrated a much higher rate than used in previous analyses. Even so, the divergence time estimates between ASES and the oceanic colonies were comparable to those determined using nuclear DNA markers (c.f. Corrigan *et al.* 2016).

The timing coincides with the major climatic changes during the Holocene, which could suggest a rearrangement in the connectivity of these colonies, migration to more suitable areas, or even momentary abandonment and posterior re-colonization of some inaccessible areas. It is known that EI has been under ice several times during the Holocene which could cause directional migration between EI and more northern islands. The results of this research suggest that EI, SLI, and SGES should be considered part of the same genetic stock, as there were no significant F_{ST} values among them, whereas the ASES population was genetically separated from the rest of the islands, as seen in earlier studies.

Probably one of the most unexpected results is the fact that ASES and SLI have the highest F_{ST} values, the lowest level of migration per generation, and the oldest divergence time regardless of the close geographic distance. The phylogenetic trees were consistent, indicating greater branch length distances between ASES and SLI than for the other population comparisons with ASES.

The mismatch distribution, Tajima's D , and Fu's F_s were not significant suggesting relatively stable populations for all the colonies, except for SGES which suggested

demographic expansion by the Tajima's D and Fu's F_s , as reported in earlier analyses. ASES is the population with the lowest N_e , though not very different to what is reported in previous genetic studies using HVRI and nuclear markers. It does not share any haplotype with other island colonies, which could be due to the disposition of matriline among oceanic islands may have been different at the time when ASES was founded.

According to the MDIV results, the separation between ASES and SGES colonies could take place during the Holocene, though the separation between the mainland and the other two island populations could happen before earlier than this. Furthermore, ASES has the lowest N_e of all the sampled colonies, highlighting the importance of preserve this unique mainland colony.

Generally, island colonies as observed for other species, represent very isolated populations compared with mainland colonies, with lower genetic and nucleotide diversity and its contribution to the overall genetic pool of the species is unique. This applies to the Argentinean population, being completely isolated from the other oceanic populations. The low genetic variability reduces the chances of the population to adapt to different scenarios of environmental change, have a better response to threats such as predators, or create resistance to disease. For these reasons, the conservation of the mainland colony is crucial, even if the existence of the whole species is not immediately threatened, but due to the possible loss of a unique gene pool and the reduction of the evolutionary potential of this species.

CHAPTER 4. GENERAL CONCLUSION

Studies in molecular ecology on wildlife benefit greatly from NGS technology, given that it allows the sequencing of entire genomes and only requires a small amount of tissue. Consequently, demographic studies are more feasible with evasive species (using non-invasive samples such as scats), or from aDNA to provide reliable information to compare with modern populations (Ekblom & Galindo 2011). In this study, these methodologies were used to generate novel information about Phocids in the Southern Ocean and provide higher resolution on their population dynamics compared with previous studies. The use of datasets of mitochondrial genomes has the potential to generate enough informative sites to answer evolutionary questions with a higher resolution than other traditional markers. When comparing the results of the demographic analysis obtained in the present study with the climatic alterations of the Holocene, changes in population size and genetic structure occur during the periods of major climatic changes. In other words, the results of this study suggest that both Southern Elephant Seal and Leopard Seals responded dynamically to climatic changes during the Holocene. Given that these species have different ecological niches and ranges of distribution, they react differently towards climatic events.

The Southern Elephant Seals form colonies preferentially on beach and tussock areas on sub-Antarctic islands, and rarely on mainland sites including the Antarctic continent (Ling & Bryden 1992). In contrast, Leopard Seals are solitary and prefer the sea ice on the Antarctic continent, sub-Antarctic islands and occasionally sub-tropical areas (King 1983; Bester & Roux 1986). The primary results suggest that the population size of Leopard Seals have increased during the Holocene, whereas the Southern Elephant Seals have remained stable, though the separation between the continental colony and the South Georgia Colony occurred during the same period. Regarding the demographic history of the Leopard Seal, these results propose a rapid expansion between 7,500 to 2,500 YBP, when two major

climatic changes took place, which might provide the conditions for this species to expand. During this period the effective population size increased from 8,000 to 60,000 females and then leveled off. Additionally, the increases in N_e at 7,500-5,500 YBP and 3,500-2,500 YBP, overlaps with the longest periods of glacial advances (Mayewski *et al.* 2004).

A possible explanation for the sudden increase in Leopard seals population is that they breed and spend the winter months in the sea ice (Southwell *et al.* 2003), in consequence, the cold periods promote longer presence of ice in the Southern Ocean which might facilitate an increase in the population size. Also, the abundance of Antarctic krill is dependent on the extension of the sea-ice (Atkinson *et al.* 2004; Meredith & King 2005; Parmesan 2006), and it is known that Leopard Seal spend winter in the Ross Sea waters searching for resources like krill, as well as other mesopredators that feed on krill (Ainley 2010). Moreover, given that the Leopard Seal feeds on some key species highly dependent on Antarctic temperatures like Krill, increments on N_e are associated with cold periods and the increasing of the ice shelf, which is important for the nursing stage of Krill. Finally, these changes in the extent of sea ice might affect the abundance of other ice-breeding Seals, and thus affect several trophic levels in the Antarctic ecosystem through an impact in alpha- and mesopredators.

The use of aDNA to calibrate molecular clocks is giving us a new perspective about previous estimations using fossil records, which points out to have much slower mutation rates due the lack of more recent calibration points. There is a tendency of faster mutation rates generated by molecular clocks that have been calibrated by using aDNA from different time periods (Fu *et al.* 2014; Barnes *et al.* 2007; Subramanian *et al.* 2009). For the Leopard Seal, the use of aDNA allowed the calculation of a mutation rate for the entire mitogenome, providing a more precise estimate than previously available. The rate obtained was higher than traditionally derived from phylogenetic estimates for Antarctic Seals (Wilson *et al.*

1974; Slade *et al.* 1998; Lambert *et al.* 2002), evolving approximately 14 times faster. However, this rate is consistent with rates in previous analyses using ancient DNA of other vertebrates (Ho *et al.* 2007; de Bruyn *et al.* 2009). A much smaller error interval was observed in the estimation of the mutation rate, in comparison to those with external calibration nodes. These internal calibrations represent more accurate estimates when using this rate to calculate effective population sizes and divergence times, which can be crucial when trying to assess the conservation of a species.

In the case of Southern Elephant Seals, no significant differences were observed between islands, having similar N_e and remaining almost without change through time. This is opposite to an initial hypothesis suggesting that different conditions among islands would be reflected in different demographic trends, genetic diversity, and genetic connectivity. A possible explanation for this could be that colonies inhabiting islands tend to migrate depending on the resource availability and climatic conditions, allowing the interchange of haplotypes between islands.

However, the Argentinean colony presented marked differences in N_e , genetic diversity and genetic structure compared with the other sub-Antarctic islands. The causes for this genetic isolation between continental and Island colonies are not very clear. However, they might be related to the quality of the habitat given that high availability of resources, lack of competitors, and preferences for sandy beaches have been reported in this area (Campagna *et al.* 1993). According to the results of the present study, the separation between ASES and SGES colonies took place during the Holocene, though the separation between the mainland and the other two island populations may have been earlier (though confidence limits on these estimates are broad and overlapping). The average divergence times occurred during significant climatic changes in the Holocene, which could suggest a rearrangement in

the connectivity of these colonies, migration to more suitable areas, or even temporary abandonment and later re-colonization of some inaccessible areas.

Studies like this can estimate effective population size and assess which populations are more threatened than others. Even though the results show that the Leopard Seal population is not threatened (~60,000 current N_e according to the BSP), an improved understanding of the environmental context of population dynamics in this species allows better predictions of the potential effects of contemporary rapid warming in the Antarctic ecosystem. Additionally, even when human activities do not compete directly with Leopard Seals for food resources, fisheries may impact directly on the mesopredators that the Leopard Seals depredate, therefore, the human impact should not be discarded in future scenarios of demographic change.

The four Antarctic phagophilic Pinniped species are to some extent separated geographically occupying different ecological niches (Davies 1958), whereas, the Leopard Seal shares similar traits with the other three phagophilic seals, and so, these data on the Leopard Seal have the potential to provide useful transferable inference. Although the four species of ice-breeding seals have not been as exploited as the Elephant Seals, important competitor species have been hunted extensively (e.g. large whales). For these reasons, the role of the Leopard Seal becomes more important with the reduction in the abundance of most large whales, the primary consumers of Antarctic krill in the Southern Ocean.

Similarly, the abundance of Southern Elephant Seals in a region plays a significant role in the local dynamics of food resources due to their high energy demands, mainly on squid and fish (Bornemann *et al.* 2000). By studying the genetic structure of Southern Elephant Seals, it was possible to assess the current genetic status of the South Atlantic Ocean populations and the degree of susceptibility of each colony based on its genetic diversity. When little genetic diversity is detected in a species or population, several

problems can arise as a consequence such as loss of evolutionary potential, susceptibility to diseases, mutational meltdown, and more (Amos & Balmford 2001). Therefore, the Argentinean colony has the lowest levels of genetic and nucleotide diversity, which places this population at a disadvantage to survive compared with the other colonies. The low diversity measurements indicate that ASES has the most moderate N_e of all the sampled colonies, highlighting the importance of preserve this single mainland colony. ASES has remained as a stable population since 600 YBP according to the BSP, followed by a sudden decline 200 YBP which is around the time of the commercial sealing, although, this estimate has wide confidence intervals. For these reasons, the conservation of the mainland colony is important, even if the survival of the species is not immediately threatened.

Very low values of effective population sizes can be the result of highly polygynous systems and short generation times, for instance, in those species where one male have harems with large numbers of females (Nunney 1993). Therefore, the degree of polygyny in the populations of Southern Elephant Seals might explain the differences between census and the N_e obtained in the present study by Bayesian methods. In the case of the SES, the population size of SGES and ASES in 1990 was estimated at around 400,000 individuals (Laws 1994), whereas in this study the N_e for SGES and ASES is 20,000 and 700 breeding females respectively. The low N_e of ASES might be the result of a recent founder event of a small group of females with no further migration and the presence of extreme polygyny. The previous hypothesis is supported by the MDIV results which suggest that divergence times between ASES and SGES is 675 generations, the most recent divergence among the islands and mainland colonies. Moreover, females tend to have high fidelity to the breeding site (Fabiani et al. 2006), which might have prevented the migration of haplotypes between SGES and ASES.

On the other hand, many aspects of the ecology and biology of Leopard Seals remain unknown given that they have broad distribution, low densities, and the inaccessibility to the pack ice (Southwell et al. 2003; Walker et al. 1998; Forcada and Robinson 2006). The solitary behavior of Leopard Seals (King 1983; Bester & Roux 1986) also hinders the investigation about site fidelity for some breeding areas or if they compete extensively for females. Contrary to the case of the Southern Elephant Seals, the Leopard Seals analyzed in this study shared very few haplotypes and did not show significant differences between modern and ancient samples. Also, the effective population size calculated by Bayesian methods was much higher than expected, exceeding the calculations of previous surveys (Erickson & Hanson 1990; Southwell et al. 2012). If this is true, it could mean that previous surveys have been greatly underestimated, highlighting the importance of studies where surveys and genomic approaches are integrated. Moreover, the high N_e and lack of genetic structure in Leopard Seals compared with Southern Elephant Seals could be the result of very opposite breeding systems like strong polygyny and female philopatry.

By calibrating a mutation rate specific for the mitochondrial genome of the Leopard Seal, it was possible to determine that the increase in N_e happened during cold periods that prompted the growth of food resources in the Antarctic. However, the Southern Elephant Seal showed stable populations in all colonies, though strong genetic structure was found between the continental colony and all sub-Antarctic islands, with ASES showing significantly smaller N_e . Also, the haplotype diversity and the divergence times indicate that the founding event that generated the mainland population happened during the Holocene and could be shaped by a different set of a few non-representative matrilineal haplotypes from sub-Antarctic islands, followed by no gene flow and subsequent removal of the haplotypes on the islands that founded the continental colony. Finally, these results suggest that climatic events during the Holocene impacted differently to each species; the Leopard Seal suffered changes in the population

size, whereas in the case of the Southern Elephant Seals, these conditions might have influenced the founding of the Argentinean colony. Past climatic changes have impacted the biology of many species around the world; thus the present study is helping to understand better how species react to environmental changes to predict future responses of species, which could be a critical aid to long-term conservation and management of sensitive habitats such as the Antarctic ecosystem.

SUPPLEMENTARY MATERIALS

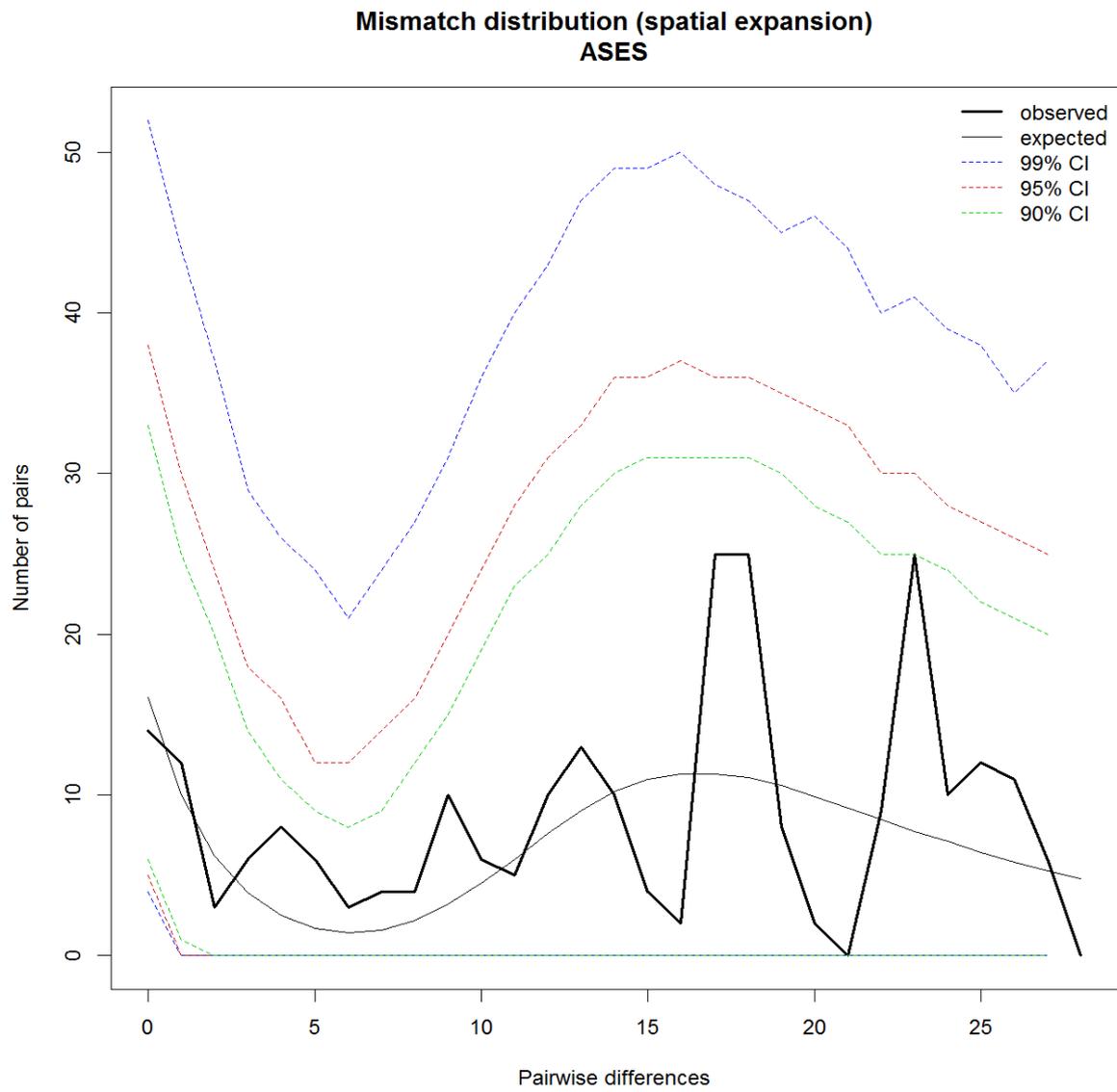


Figure S1. Mismatch distribution to investigate spatial expansion in ASES.

Mismatch distribution (spatial expansion)
EI

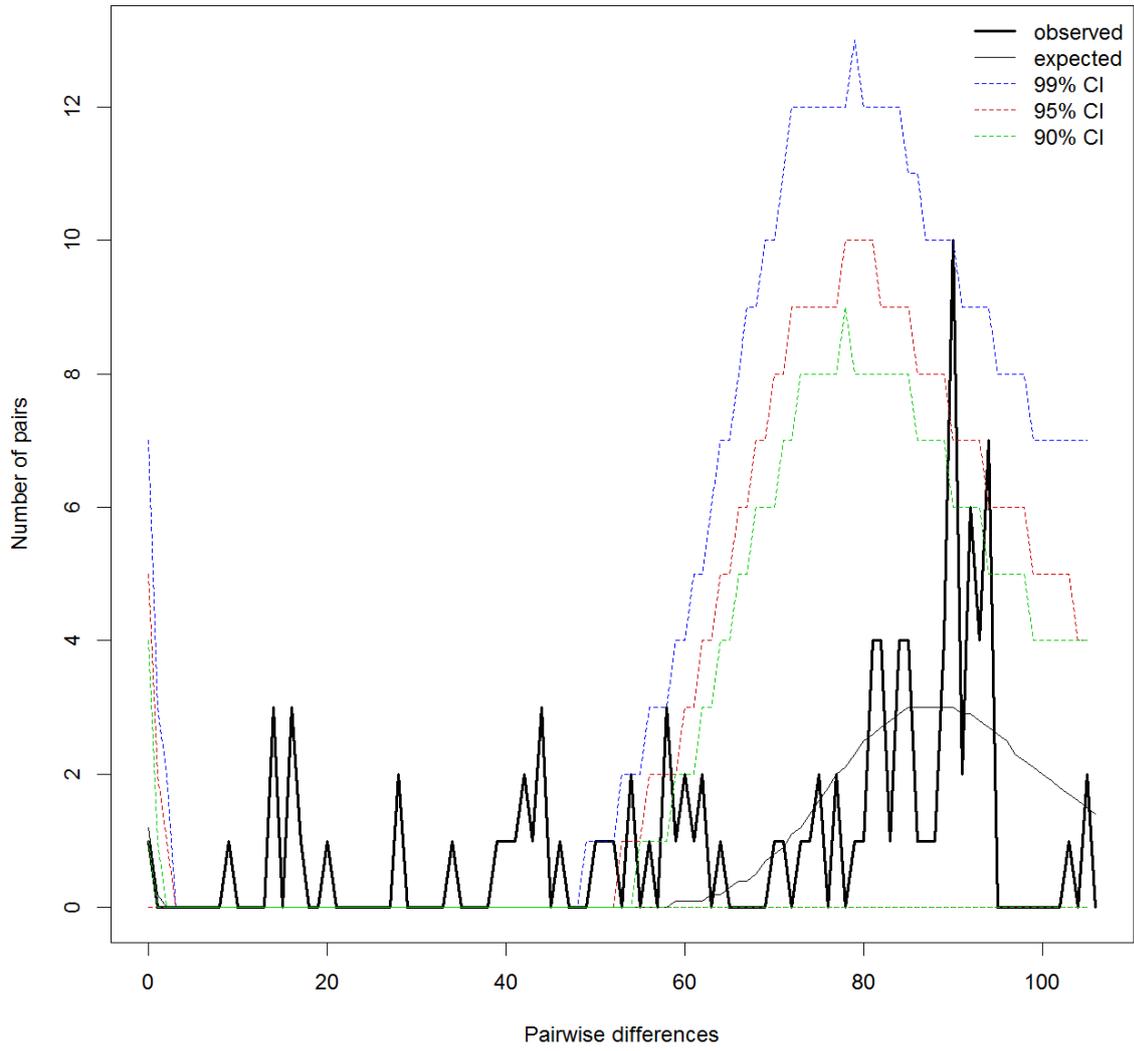


Figure S2. Mismatch distribution to investigate spatial expansion in EI.

Mismatch distribution (spatial expansion)
SGES

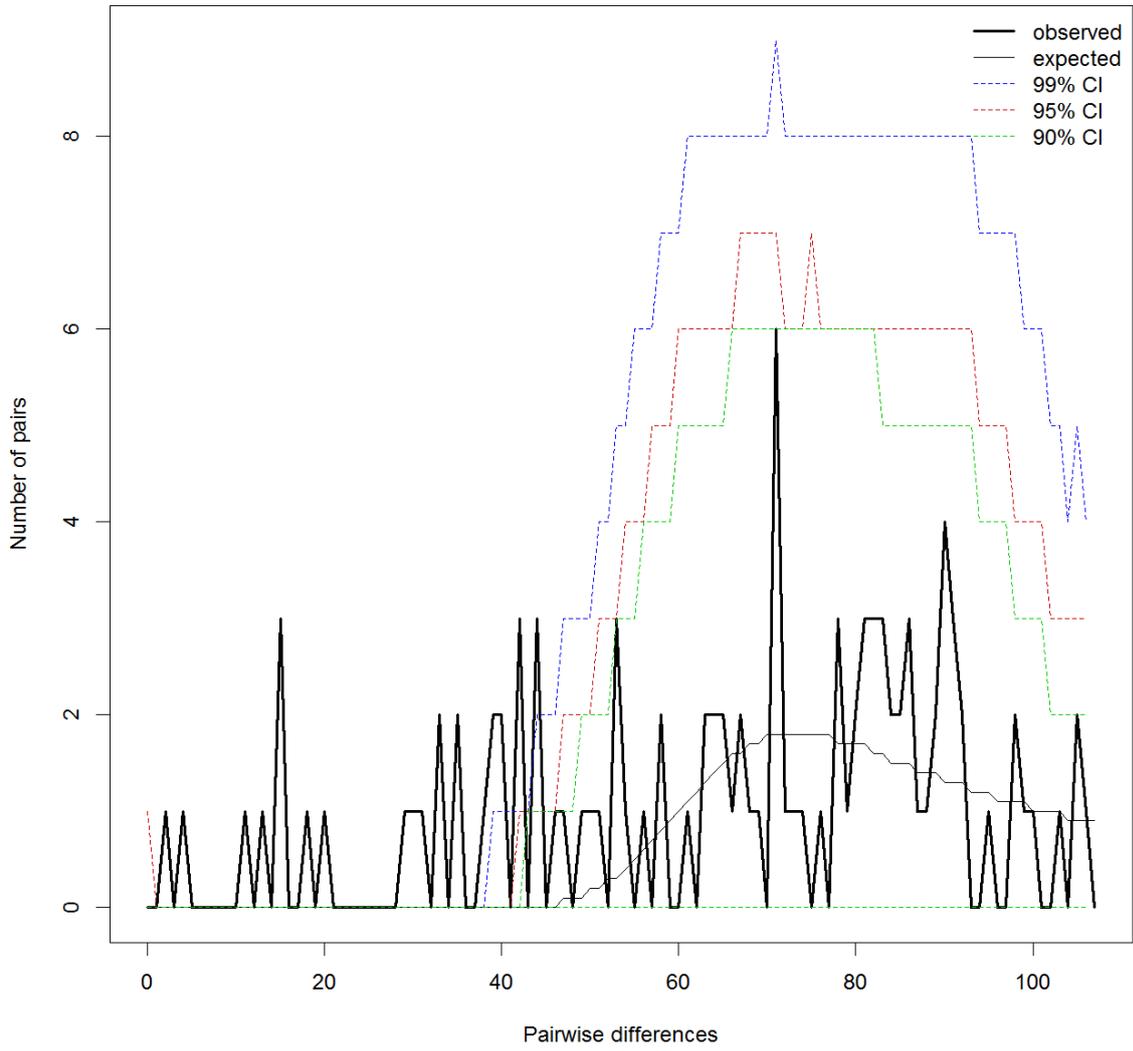


Figure S3. Mismatch distribution to investigate spatial expansion in SGESES.

Mismatch distribution (spatial expansion)
SLI

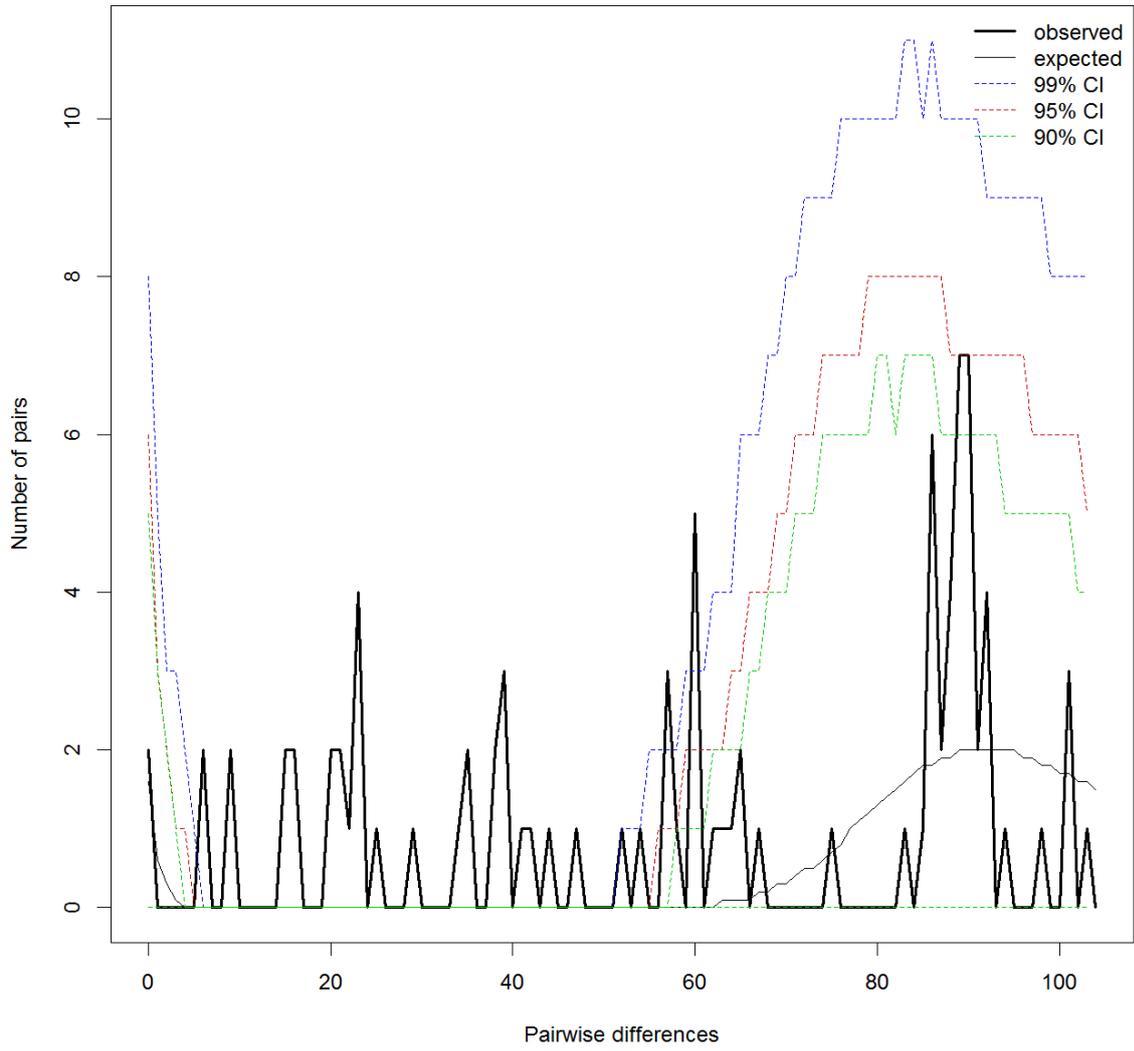


Figure S4. Mismatch distribution to investigate spatial expansion in SLI.

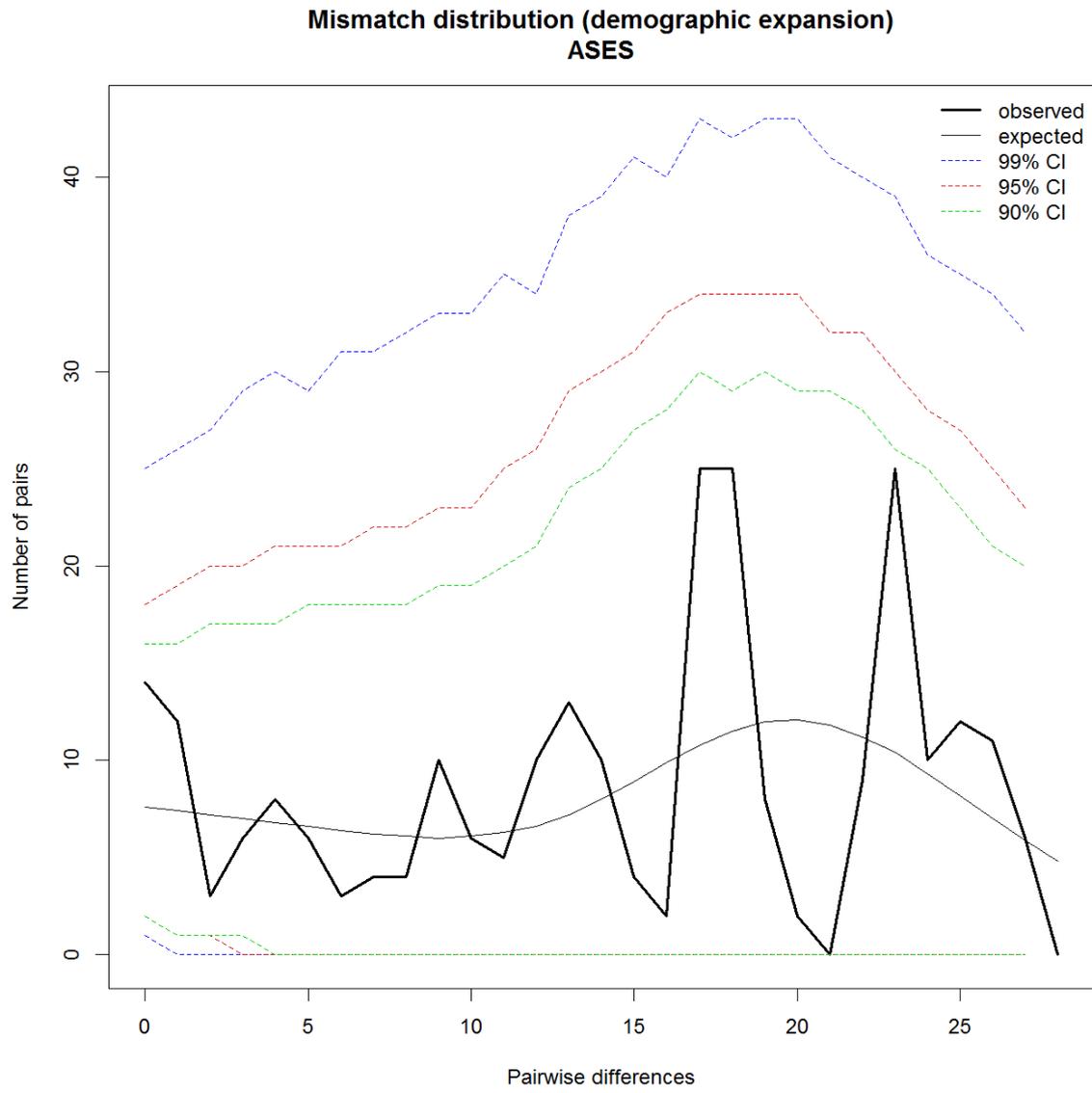


Figure S5. Mismatch distribution to investigate demographic expansion in ASES.

Mismatch distribution (demographic expansion)
EI

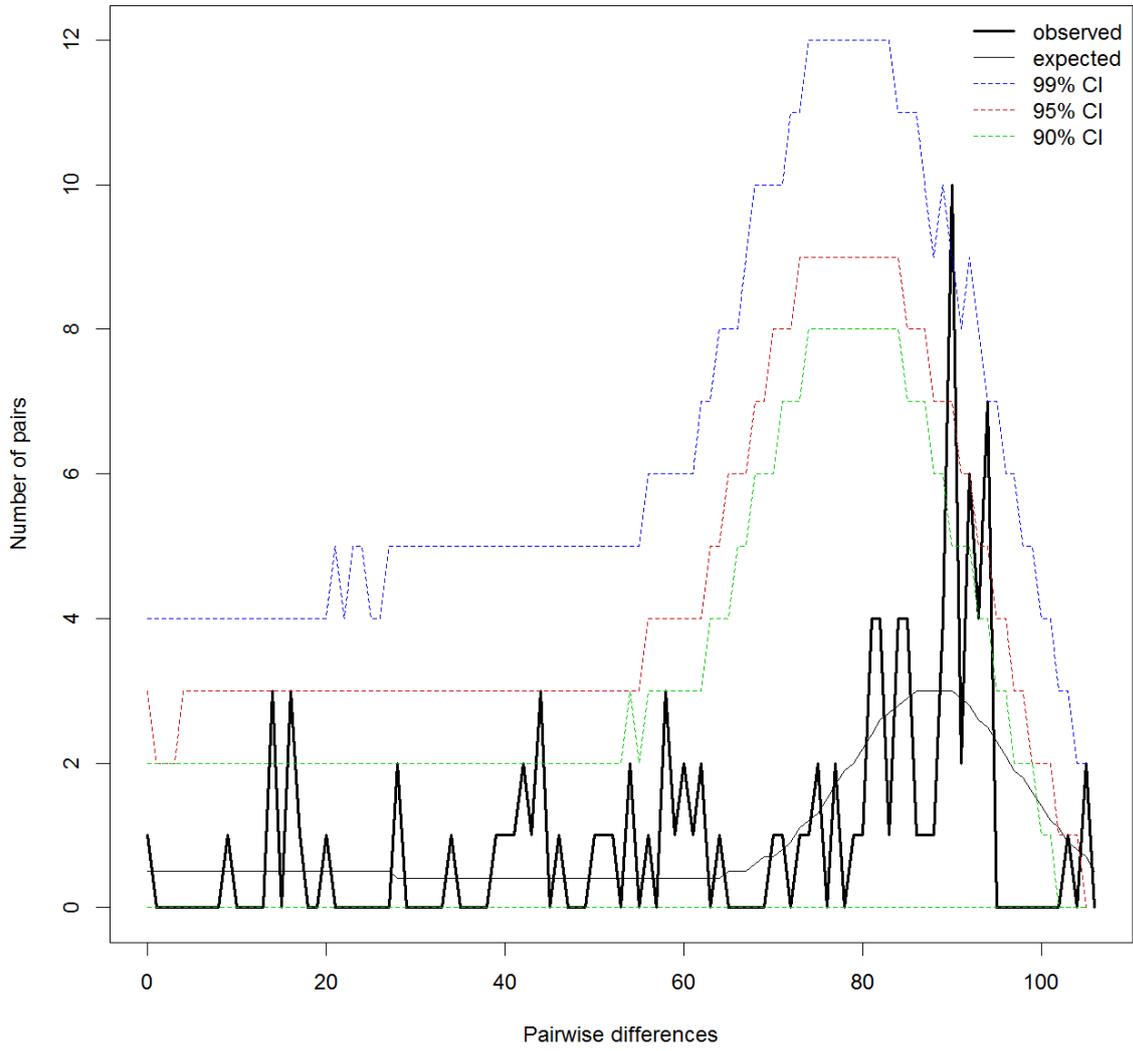


Figure S6. Mismatch distribution to investigate demographic expansion in EI.

Mismatch distribution (demographic expansion)
SGES

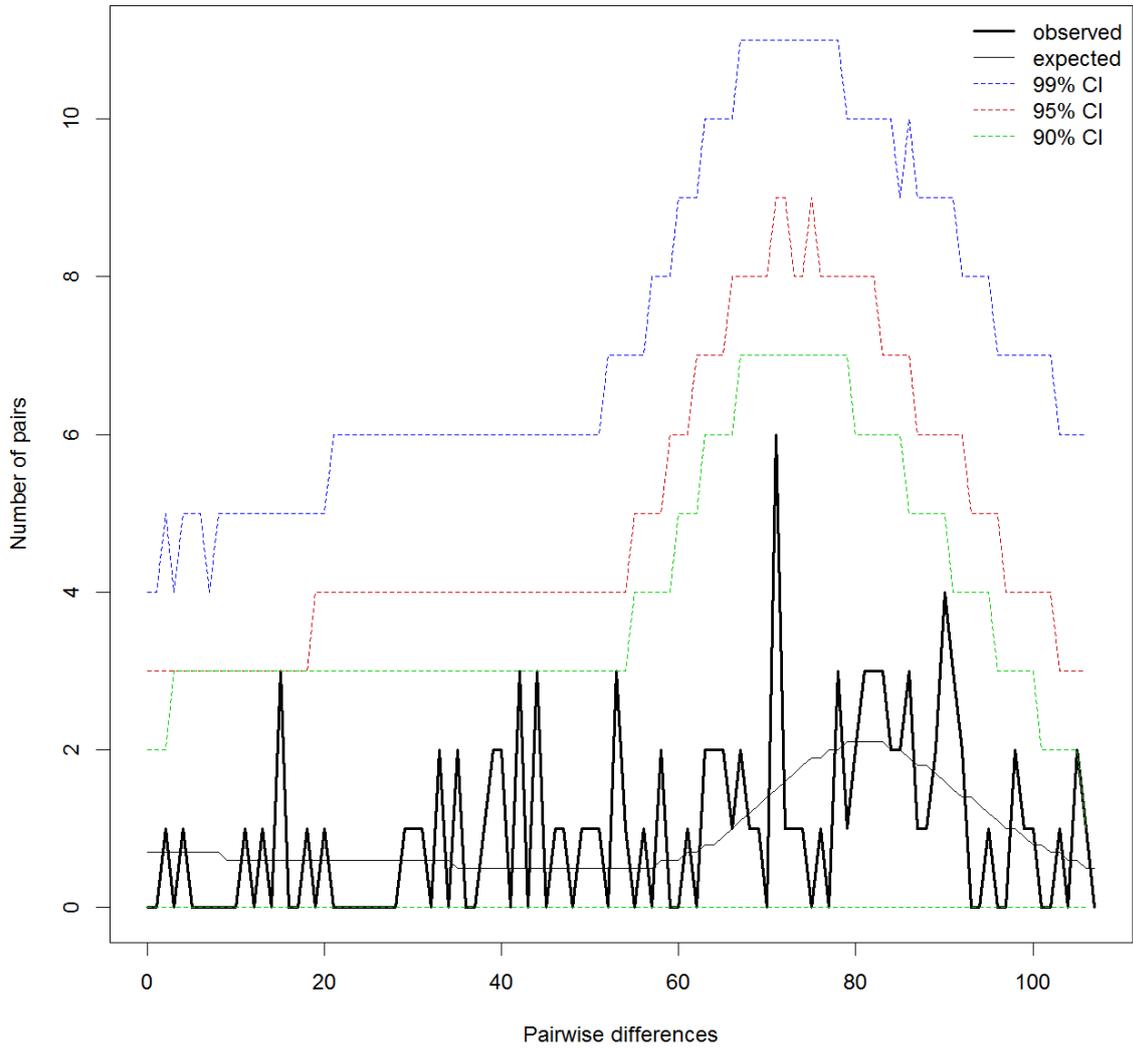


Figure S7. Mismatch distribution to investigate demographic expansion in SGESES.

Mismatch distribution (demographic expansion)
SLI

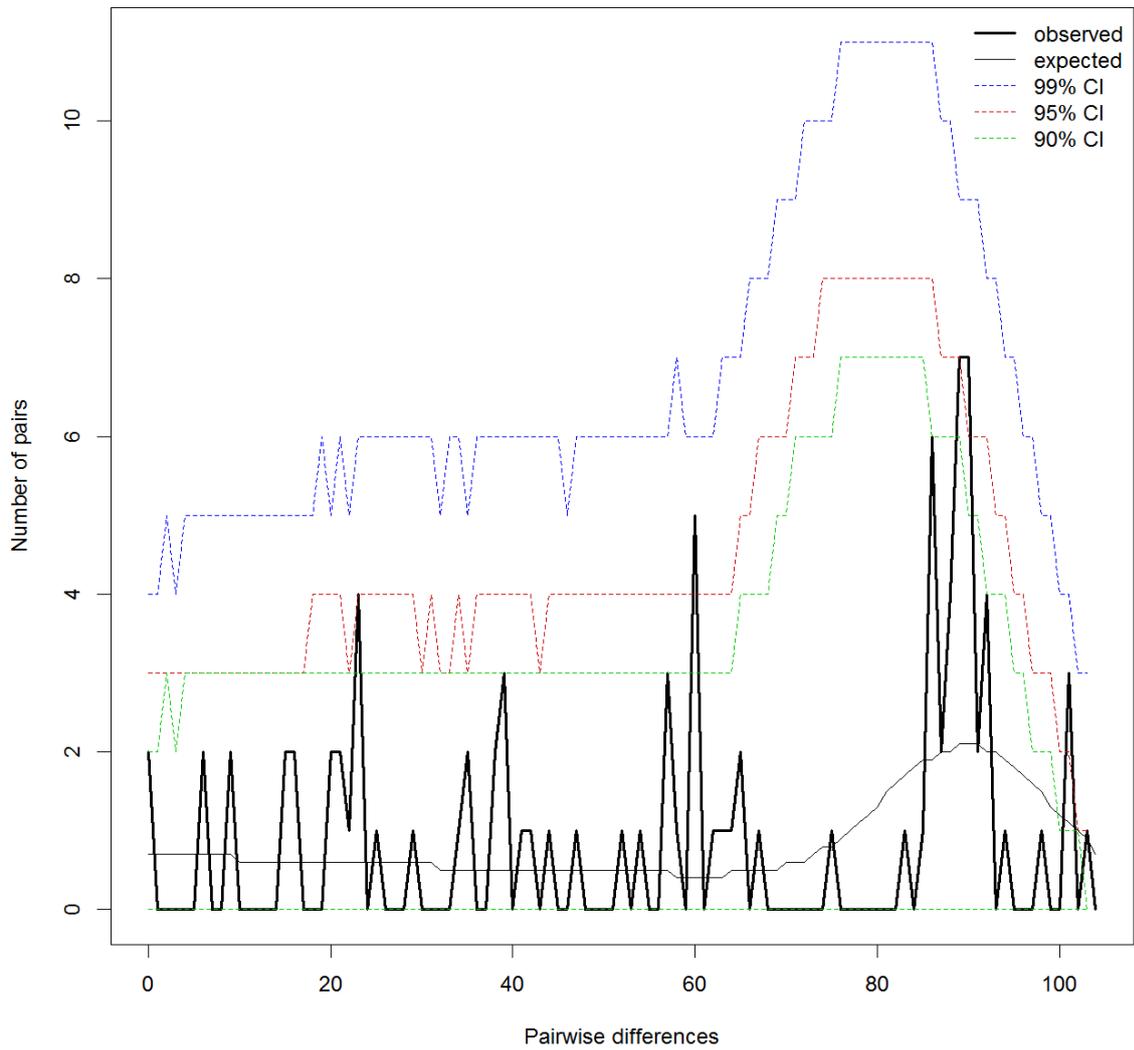


Figure S8. Mismatch distribution to investigate demographic expansion in SLI.

Molecular distances within and between populations

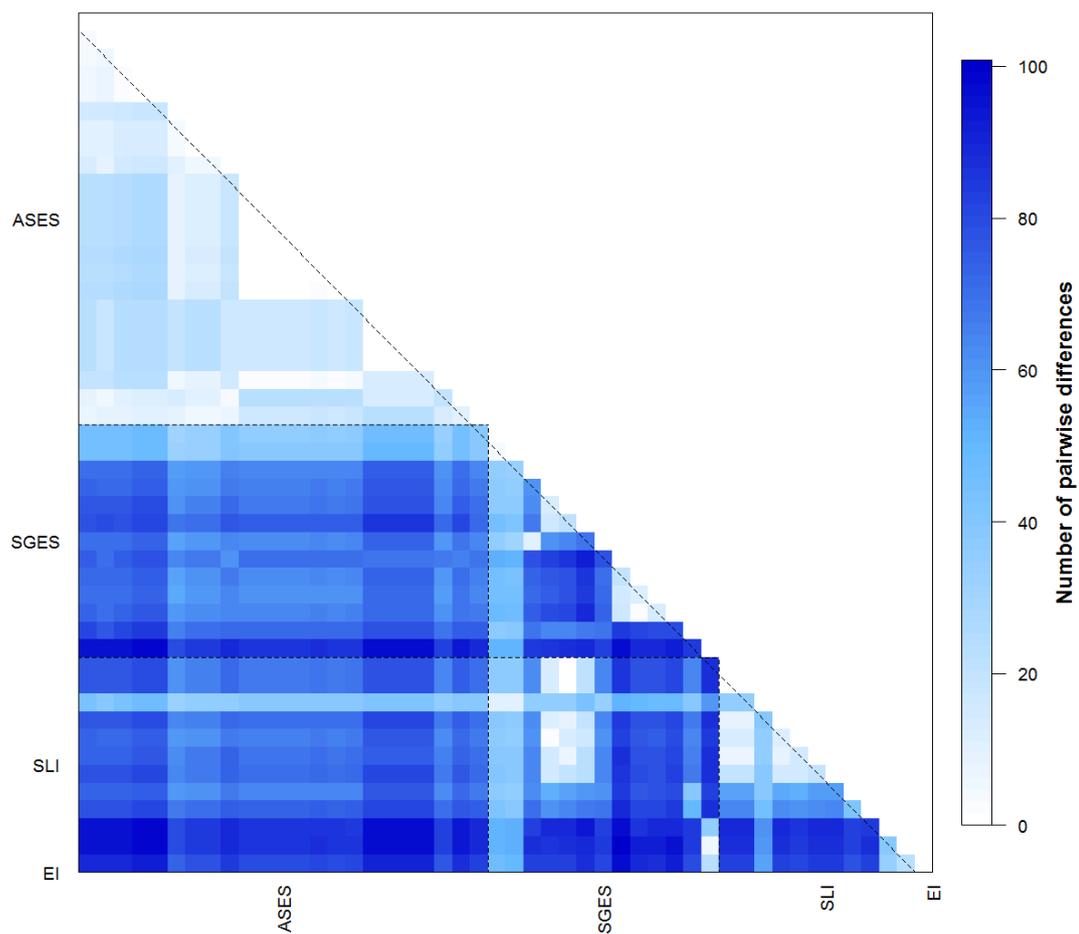


Figure S9. Molecular distances for the entire dataset.

Mismatch distribution (demographic expansion)
ASES

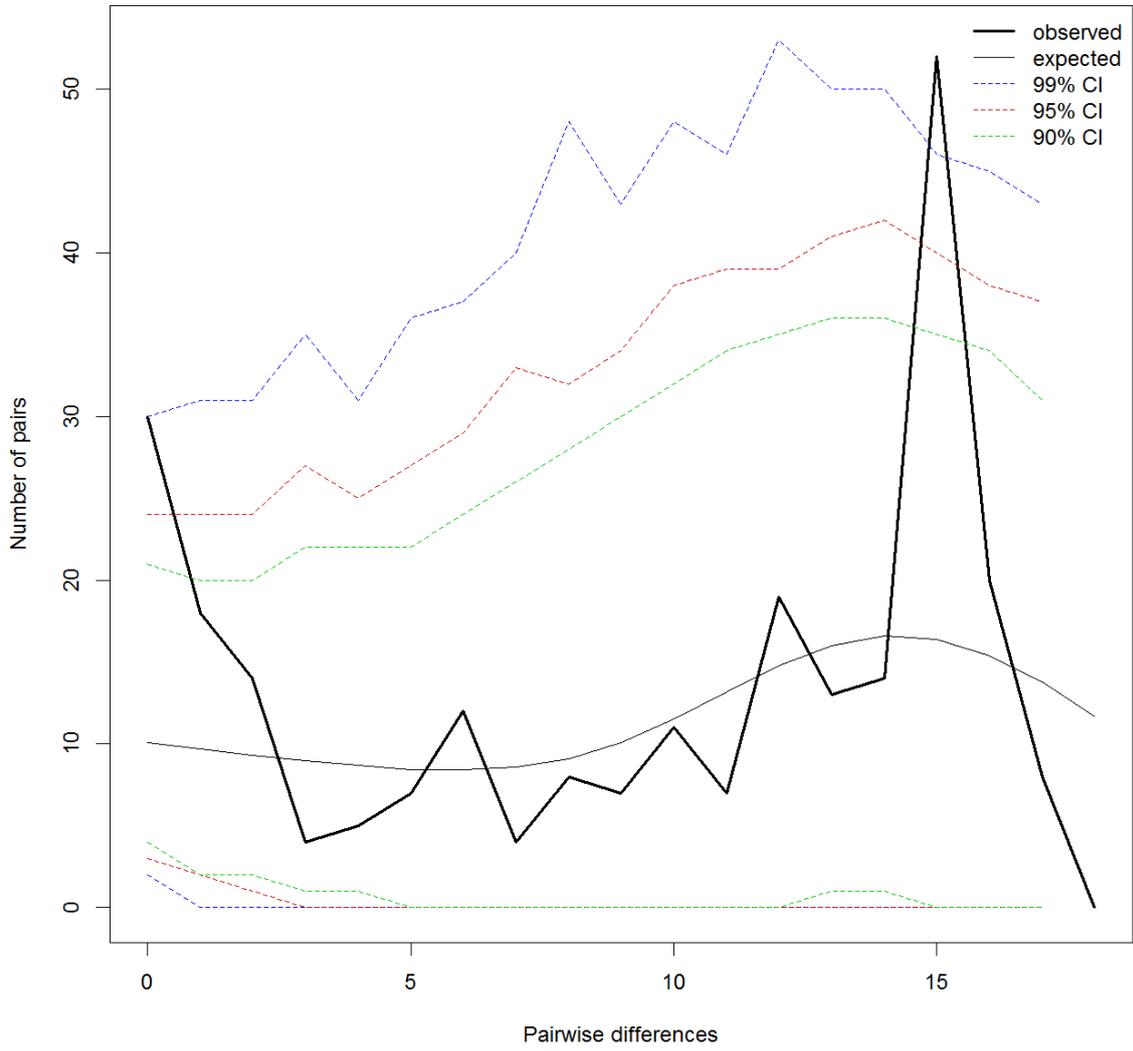


Figure S10. Mismatch distribution to investigate demographic expansion in ASES using HVRI.

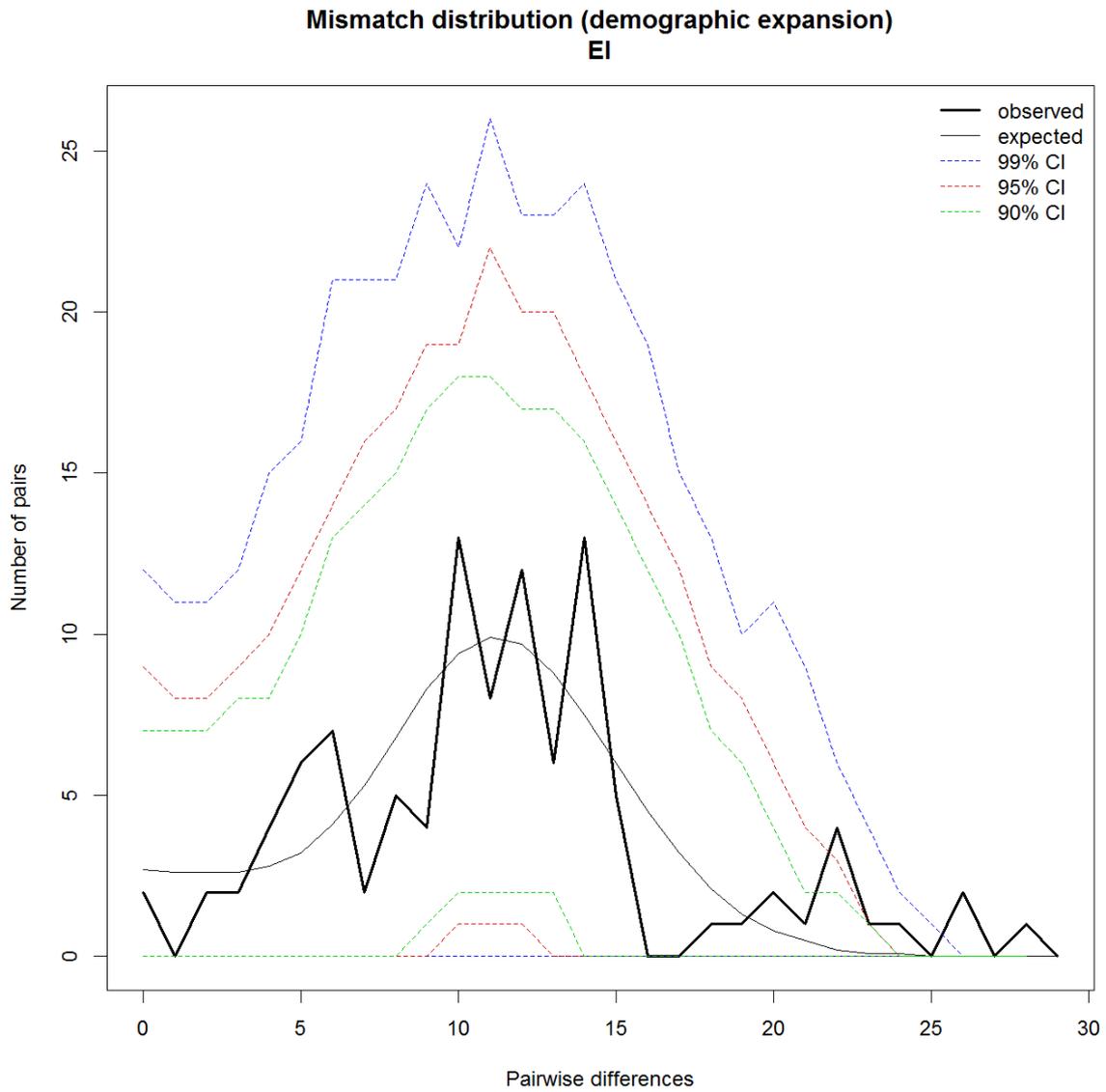


Figure S11. Mismatch distribution to investigate demographic expansion in EI using HVRI.

Mismatch distribution (demographic expansion)
SGES

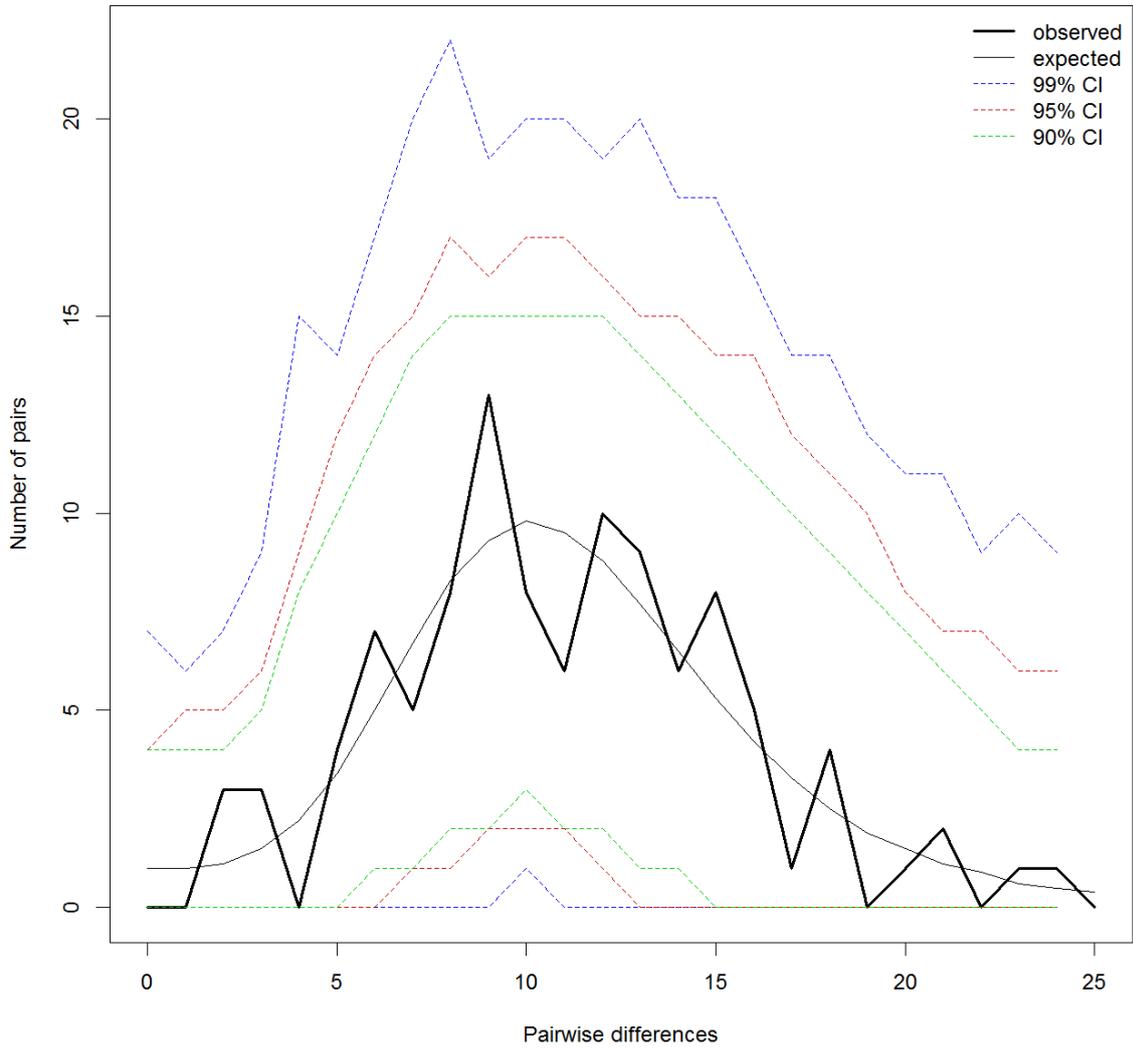


Figure S12. Mismatch distribution to investigate demographic expansion in SGES using HVRI.

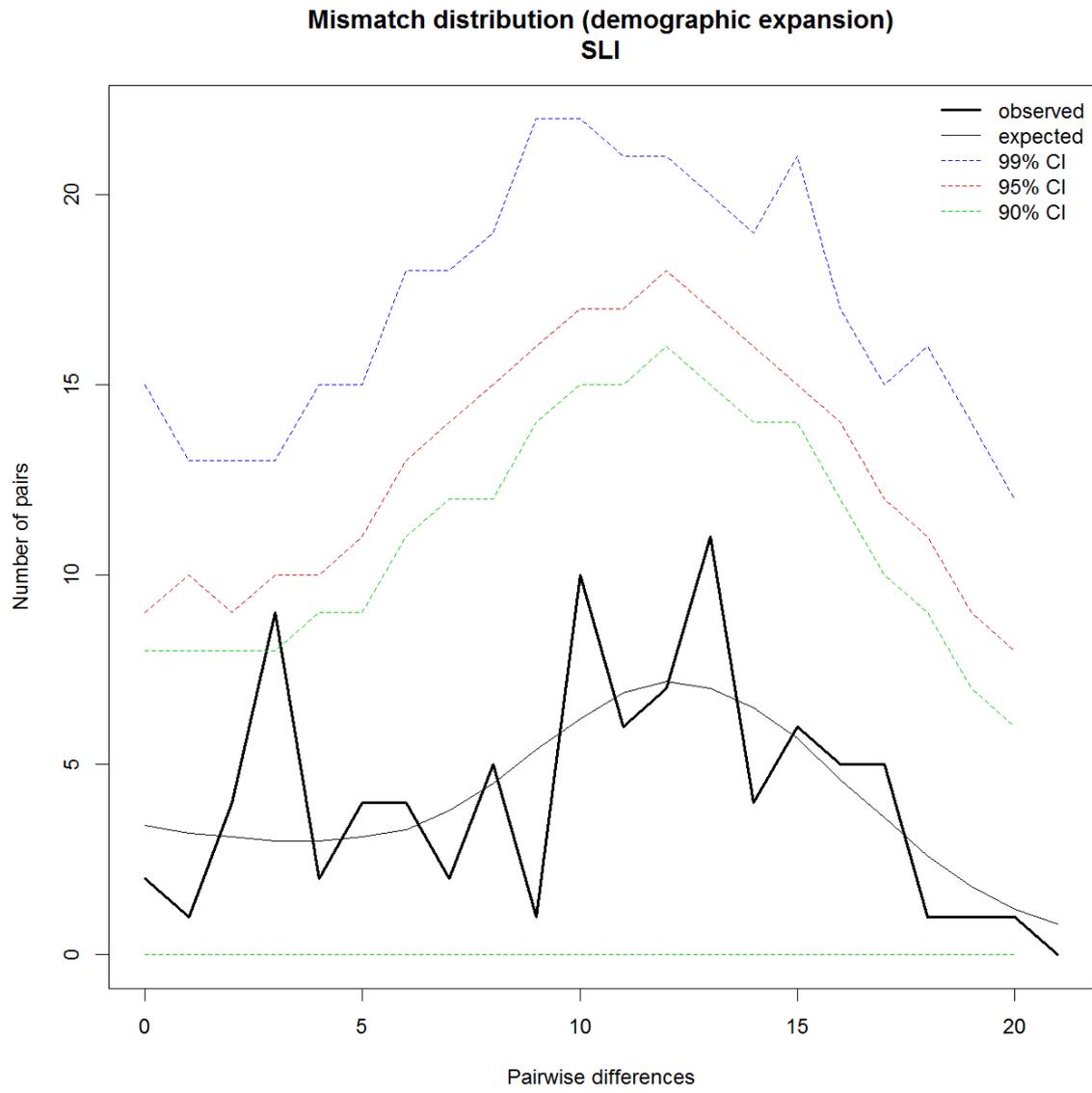


Figure S13. Mismatch distribution to investigate demographic expansion in SLI using HVRI.

Mismatch distribution (spatial expansion)
ASES

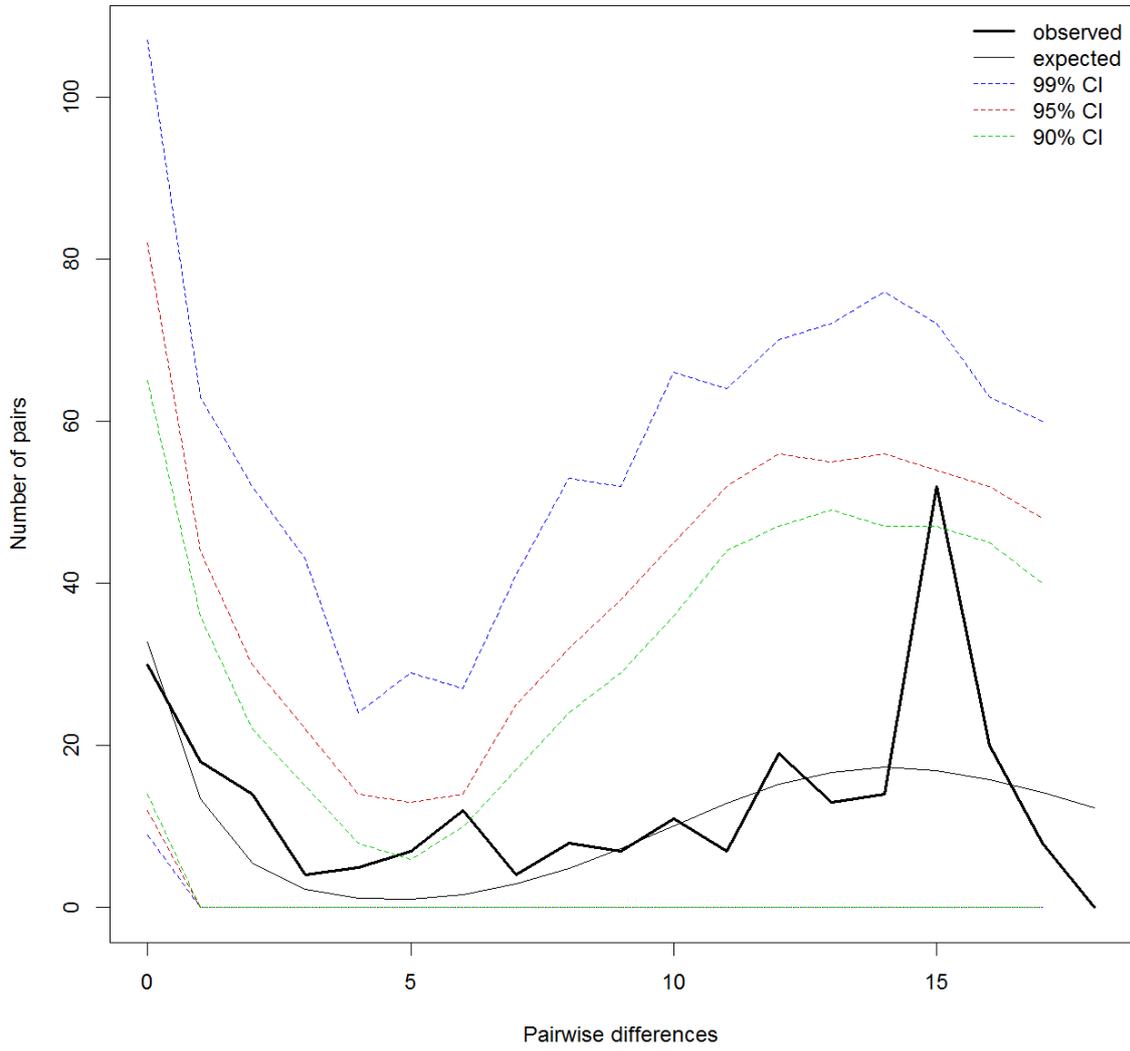


Figure S14. Mismatch distribution to investigate spatial expansion in ASES using HVRI.

Mismatch distribution (spatial expansion)
EI

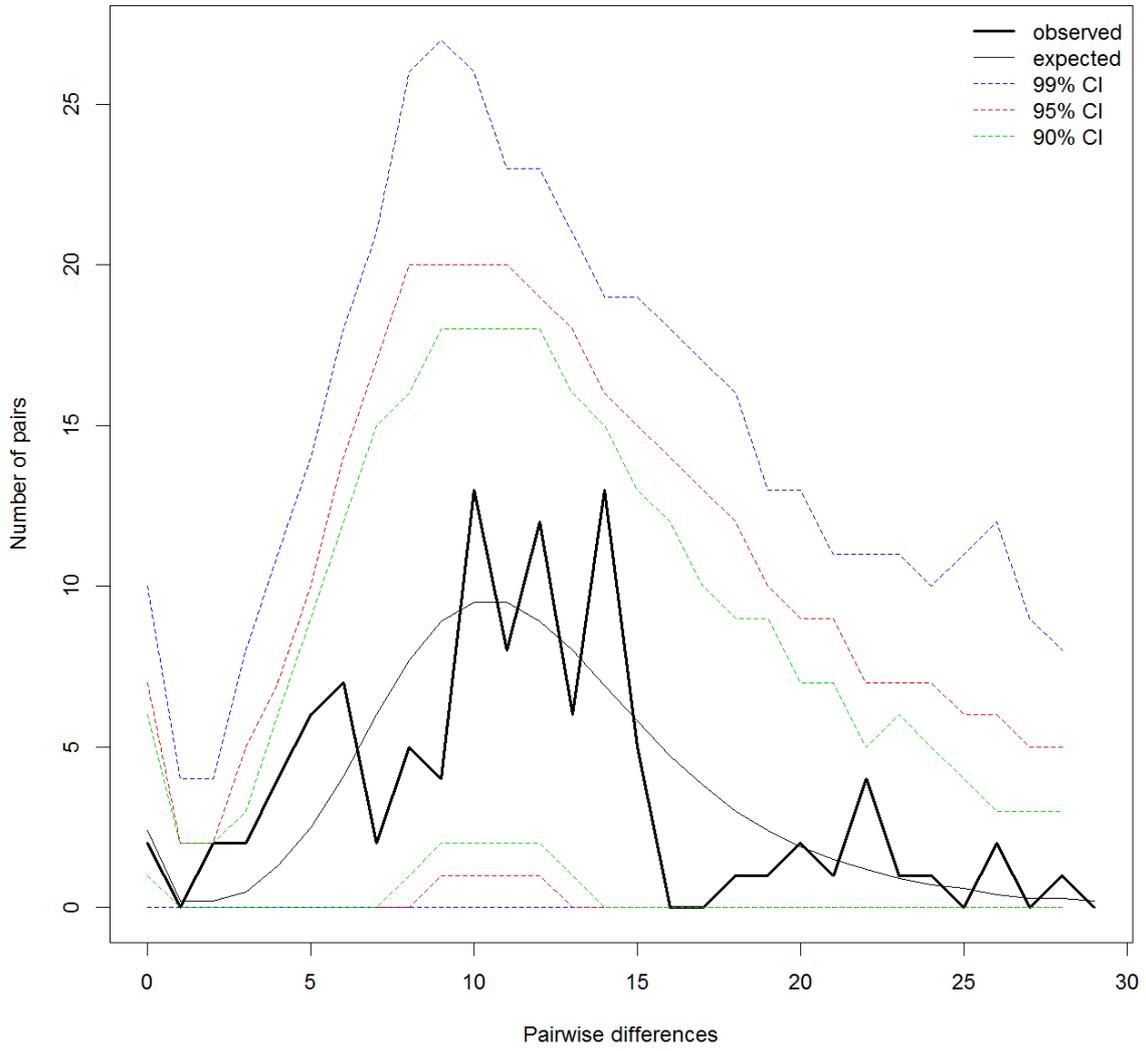


Figure S15. Mismatch distribution to investigate spatial expansion in EI using HVRI.

Mismatch distribution (spatial expansion)
SGES

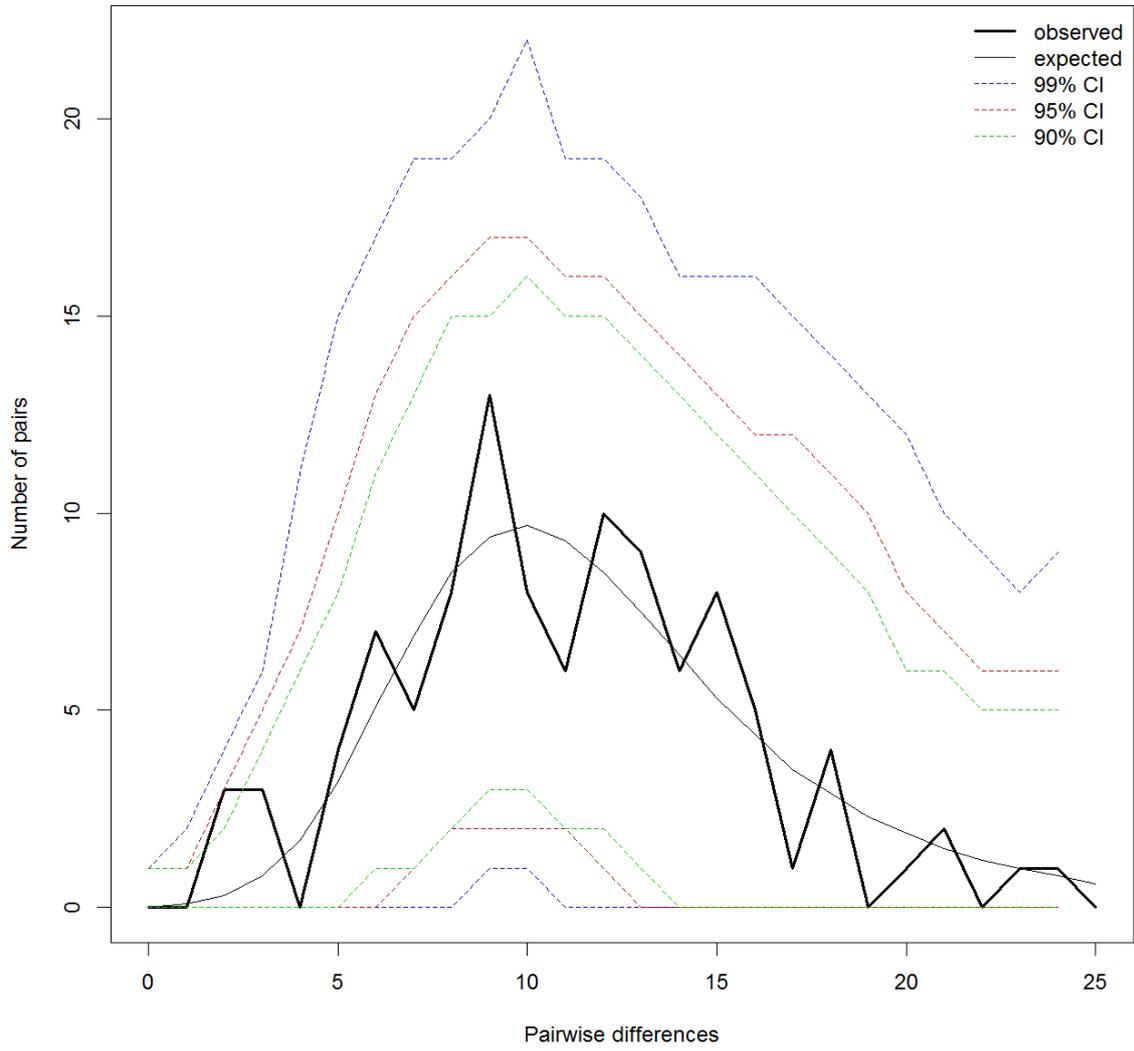


Figure S16. Mismatch distribution to investigate spatial expansion in SGESES using HVRI.

Mismatch distribution (spatial expansion)
SLI

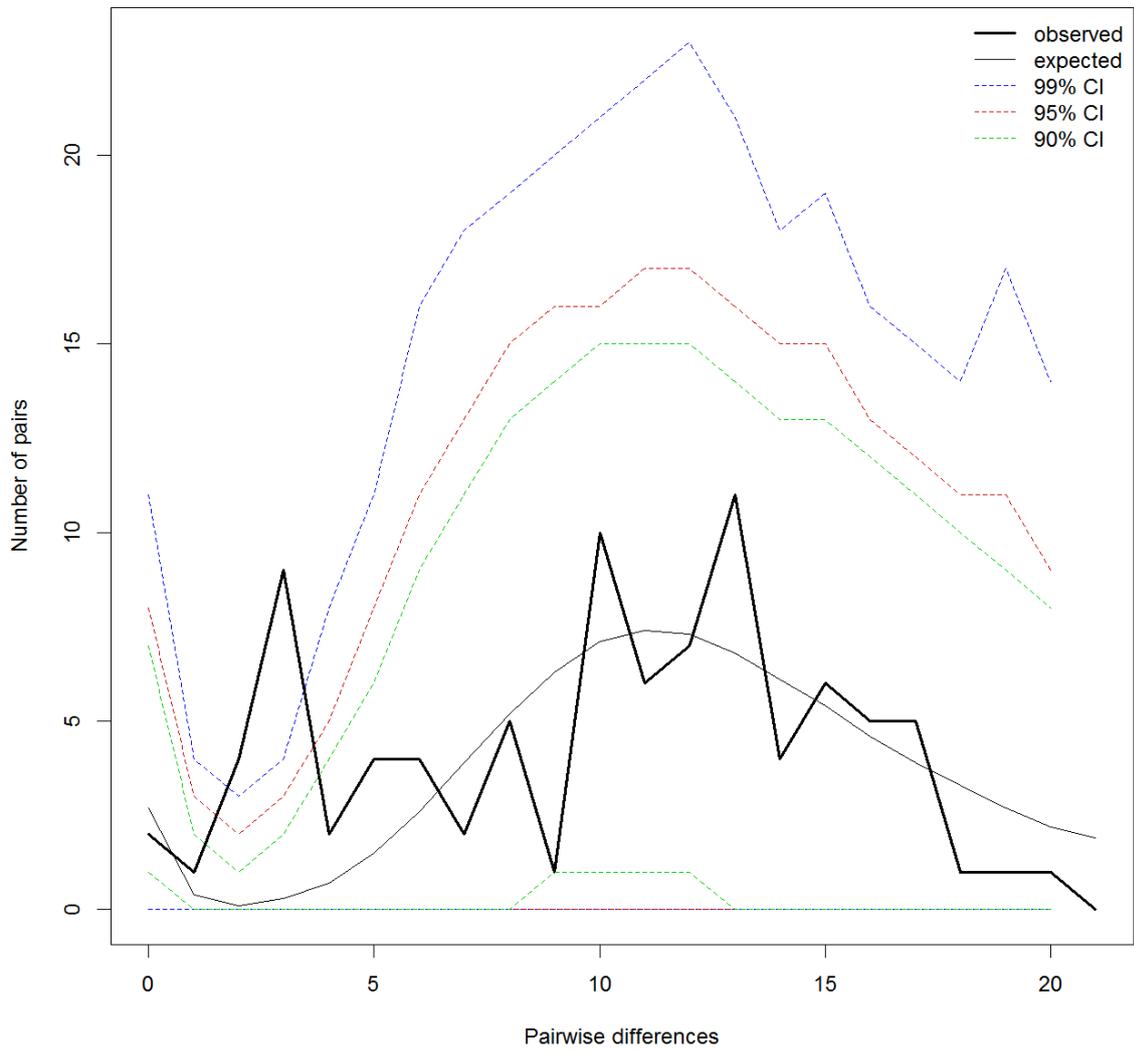


Figure S17. Mismatch distribution to investigate spatial expansion in SLI using HVRI.

Table S1. Sampling locations and number of samples from each location

Sampling location	Coordinates	N	Modern/Ancient
Kolich Point	77°21'00.0"S 163°34'00.0"E		2 Ancient
Explorer's Cove	77°34'00.0"S 163°35'00.0"E		2 Ancient
Salmon Valley	77°03'30.2"S 163°29'49.8"E		1 Ancient
Spike Cape	77°18'23.1"S 163°33'43.2"E		1 Ancient
Lake. Fryxell	77°37'00.0"S 163°11'00.0"E		3 Ancient
Howard Glacier	77°41'00.0"S 163°05'00.0"E		3 Ancient
Canada Glacier	77°37'00.0"S 162°59'00.0"E		2 Ancient
Goldman Glacier	77°42'00.0"S 162°51'00.0"E		2 Ancient
Moa Gl.	77°43'00.0"S 162°47'00.0"E		1 Ancient
Valley west of Mummy Pond	77°40'00.0"S 162°39'00.0"E		1 Ancient
South of Ferrar Glacier	77°49'00.0"S 162°42'00.0"E		6 Ancient
Northwest of Koettlitz Glacier	78°15'00.0"S 164°15'00.0"E		1 Ancient
Siniff Bay	74°40'00.0"S 135°50'00.0"W		7 Modern
Bird Island	54°00'00.1"S 38°01'09.1"W		5 Modern
Total			37

Table S2. Sampling locations and population size of each colony

Sampling location	Coordinates	N samples	Population size	Study
Peninsula Valdes	-42.311513, - 63.604553	24	19,000	Campagna & Lewis (1992)
South Georgia	-54.317790, - 36.245744	15	113,444	Boyd <i>et al.</i> (1996)
Elephant Island	-61.033275, - 55.649405	15	6,000	Hunt (1973)
Seal Lion Island	-52.421946, - 59.081319	15	1,827	Galimberti & Sanvito (2000)
Total		69	121271	

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