Reconstructing Northern Fur Seal Population Diversity through Ancient and Modern DNA Data

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Abstract

Archaeological and historic evidence suggests that northern fur seal (*Callorhinus ursinus*) has undergone several population and distribution changes (including commercial sealing) potentially resulting in a loss of genetic diversity and population structure. This study analyzes 36 unpublished mtDNA sequences from archaeological sites 1900-150 BP along the Pacific Northwest Coast from Moss *et al.* (2006) as well as published data (primarily Pinsky *et al.* [2010]) to investigate this species' genetic diversity and population genetics in the past.

The D-loop data shows high nucleotide and haplotype diversity, with continuity of two separate subdivisions (haplogroups) through time. Nucleotide mismatch analysis suggests population expansion in both ancient and modern data. AMOVA analysis (F_{ST} and Φ_{ST}) reveals some 'structure' detectable between several archaeological sites. While the data reviewed here did not reveal dramatic patterning, the AMOVA analysis does identify several significant F_{ST} values, indicating some level of ancient population 'structure', which deserves future study.

Keywords: Northern fur seal; ancient DNA; genetic diversity; archaeology

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List of Acronyms

aDNA	Ancient deoxyribonucleic acid (DNA)
AMOVA	Analysis of molecular variance
F_{ST}	Fixation index (Wrights F-statistics)
MJ	Median-joining
ML	Maximum likelihood
mtDNA	Mitochondrial DNA
NJ	Neighbour-joining
nuDNA	Nuclear DNA
SNP	Single nucleotide polymorphism

Chapter 1.

Introduction

Northern fur seal (*Callorhinus ursinus*) populations appear to have undergone dramatic population declines and reduction in ranges over the past few thousand years, with particularly dramatic declines documented over the past two hundred years. Archaeological evidence suggests that northern fur seals may have once been far more widely distributed, with rookeries potentially located along the Pacific Coast of North America from Southern California to Alaska (Crockford et al. 2002; Dickerson *et al.* 2010; Etnier 2002; Moss *et al.* 2006; Newsome *et al.* 2007; Pinsky *et al.* 2010). By historic times, however, only two colonies appear to have persisted in the Pacific Northwest: the South Farallon Islands colony (Central California) and the Pribilof Island colony (Alaska) (Newsome *et al.* 2007; Pyle *et al.* 2001; Sydeman and Allen 1999). Commercial harvesting from the 1700's to the 1900's resulted in further dramatic declines in northern fur seal populations, including the extirpation of northern fur seal from the South Farallon Islands, and a near decimation of the Pribilof Island population to less than a tenth of its population size (Burton *et al.* 2001; Sydeman and Allen 1999; Trites 1992). Ancient DNA (aDNA) research can help enable an understanding of the genetic diversity of populations, such as those of the northern fur seal, by providing ecological baselines for species dramatically affected by commercial harvesting (Chan *et al*. 2006; Herrmann and Hummel 1994; Speller *et al.* 2012).

There are several issues associated with using archaeological and historic evidence for determining northern fur seal population size and distribution. Archaeological evidence of past rookeries, often based on fragmented osteological remains continues to be debated as the evidence comes from human occupation sites and not from northern fur seal colonies themselves. While the evidence for local northern fur seal rookeries is compelling, there may still be alternate hypotheses to consider and

eliminate, including whether these remains come from high-latitude-breeding northern fur seals that were hunted while foraging far offshore, or whether they were perhaps hunted on land during non-breeding season 'haul outs' (Burton *et al.* 2001; Pinsky *et al.* 2010). The debate regarding presence of local rookeries also includes whether or not these remains came from northern fur seals breeding on nearby offshore islands or whether there is evidence of northern fur seal rookeries on the mainland.

For instance, Burton *et al.* (2001) posit the former existence of rookeries on the mainland due to northern fur seal remains being found at archaeological sites dating back up to 8,500 years. These archaeological sites were located some distance from offshore islands, such as the Moss Landing site on the coast of central California. Historic records are also problematic, as many of these records were generated before it was known that there are substantial differences between *Callorhinus ursinus* and *Arctocephalus townsendi* (Etnier 2002; Starks 1922). As a result, many of the harvest records may not reflect *C. ursinus* and thus may be unreliable (Pyle *et al.* 2001).

Other approaches to the study of northern fur seal populations in the past have used genetic evidence. Some studies have used modern northern fur seal genetic data to extrapolate past patterns (Dickerson *et al.* 2010); however, these may pose challenges as more recent events may erase signals of past ones (through mechanisms like replacement or dilution) (Leonard *et al.* 2002, Willerslev and Cooper 2005). The use of ancient DNA (aDNA) can mitigate some of the issues associated with the use of indirect (modern DNA) evidence, as it allows for direct observation of past genetic data, allowing for an improved resolution of genetic diversity and changes over time (De Bruyn *et al.* 2011). Ancient DNA is of particular use to the study of marine mammals, whose broad distribution and long-generation times can provide challenges for the study of demographic changes using only modern samples (Foote *et al.* 2012). There have been several studies conducted using aDNA from archaeologically recovered northern fur seal (Moss *et al.* 2006; Newsome *et al.* 2007; Pinsky *et al.* 2010; Winters *et al.* 2011); however, these studies are all limited by relatively small sample sizes and the range of specimen dates at each site. Genetic research into ancient northern fur seal populations can yield valuable information for both species conservation and an understanding of past human behaviour.

Gaining knowledge about how northern fur seal range and population size has changed over the years, and the effect of these changes on genetic diversity, is crucial for informing conservation practices (Allendorf *et al.* 2012). Temporal dynamics of population structure, including changes to population boundaries through time, gene flow between populations, and the impacts of dispersal/gene flow on the resiliency of a species to disturbance are all factors relevant to conservation (Newsome *et al.* 2007; Pinsky *et al.* 2010). This is especially important for northern fur seals given that the Pribilof Island population is currently in decline[1] due to unknown causes (Dickerson *et al.* 2010; Towell *et al.* 2006; Trites 1992). Of additional use to conservation biology is that evidence of local rookeries would indicate behavioural plasticity through differences in breeding and migrational behaviour between past and present northern fur seals. Both archaeological and genetic investigation can aid in developing a better understanding of population dynamics over a long time scale through both direct observation (archaeology, ancient DNA) and predictive models (i.e. Bayesian skyline plots).

Archaeological evidence in support of local rookeries is based on the identification of juvenile northern fur seals by morphological, morphometric, and isotopic data that appear to be below weaning (and, therefore, below migration) age. The existence of local rookeries along the British Columbian, Washington, Oregon, and Californian coasts would indicate year-round occupation in the middle latitudes. This would indicate a difference in migration behaviour between past and present populations as the predominant modern pattern is of migration to rookeries located on high-latitude offshore islands. Knowing more about the past population size and distributions of northern fur seals is also important for reconstructing human behaviour in the past. Archaeological evidence found in human occupation sites suggesting the presence of local rookeries along the coast of North America is of particular importance as rookeries may have represented an abundant, easily accessible resource, in contrast to the more difficult and time consuming process of hunting northern fur seals from boats while paddling offshore where seals would exhibit comparatively low densities (Burton *et al.* 2001; Crockford *et al*. 2002; Etnier 2002).

[1] This modern decline began in the mid-1990's following the cessation of experimental harvests and a brief period of population stability

1.1. Research Questions

The debate about the existence and nature of local rookeries for past northern fur seal populations has raised questions about their genetic diversity and population genetic structure dynamics. If northern fur seals had breeding colonies along the coast of North America, given the behavioural observation of high philopatry, it seems likely that these separate colonies may have been reproductively isolated, leading to distinct subpopulations (Dickerson *et al.* 2010; Etnier 2004; Moss *et al.* 2006). The possible existence of local northern fur seals breeding colonies generates the potential for the existence of subpopulations which would allow for the accumulation of genetic differences, leading to the hypothesis of 'population genetic structure' or accumulated genetic differences that may be detectable using statistical means between geographically separate samples (Dickerson *et al.* 2010; Moss *et al.* 2006). The term 'population structure' broadly refers to the mechanism by which genes are passed to subsequent populations (Templeton 2006). The term 'structure', or 'genetic structure', though broad in potential usage, is used to refer to barriers or limitations to gene flow (Dickerson *et al.* 2010; Speller *et al.* 2012; Templeton 2006). That is, limitations to gene flow lead to the existence of 'population genetic structure' which is the genetic reflection of the existence of local subpopulations (Dickerson *et al.* 2010; Templeton 2006). For example, if some event divides a population and poses a barrier to gene flow, genetic differences will accumulate between the populations. These genetic differences will eventually allow for the identification of 'population genetic structure', or simply as 'structure' in the jargon commonly used by population geneticists (Pinsky *et al.* 2010; Schrey *et al.* 2011; Speller *et al.* 2012). This genetic structure is a reflection of the social structure whereby gene flow is limited. Genetic evidence of local differences would support the hypothesis of local rookeries (Lyman 1995; Moss *et al.* 2006). In contrast, a lack of evidence of genetic difference neither supports nor refutes this hypothesis, as it may be that the split is too recent to show genetic differences or that a more sensitive marker may be needed (Beebee and Rowe 2008; Holsinger 2012; Moss *et al.* 2006; Templeton 2006). Despite these expectations for differences in genetic diversity and structure, studies on ancient northern fur seal genetics have not yet shown the expected strong signal of structure, though they may provide some weak evidence (Moss *et al.* 2006; Pinsky *et al*. 2010).

This thesis aims to reconstruct the past genetic diversity of northern fur seals using sequences obtained from archaeological samples to investigate the dynamics of population genetic structure, or the changes in population structure through time. This study seeks to lend support to the broader question of the existence of local breeding colonies, given the abundance of archaeological evidence. The main question is whether there is genetic evidence to support the idea of local rookeries. As discussed above, the possible existence of local rookeries leads to the hypothesis that ancient populations would exhibit genetic evidence of subpopulations. The null hypothesis to investigate these questions is that there is no discernable difference in genetic diversity between past and present populations, nor is there evidence of genetic population structure/population subdivision in past northern fur seal populations.

This thesis seeks to use original and unpublished mitochondrial DNA sequence data from a project by Moss *et al.* (2006) to further investigate the question of northern fur seal genetic diversity and population genetics dynamics. The objectives of this thesis are: 1) to retrieve and analyze mitochondrial DNA (mtDNA) sequence data from previously sequenced archaeological remains from three sites (described in Moss *et al.* 2006) to search for evidence of regional patterning, 2) to integrate this dataset with previously published sequence data (primarily that of Pinsky *et al.* [2010]) to observe how the expanded data from these three sites correlate to any broad geographic pattern, and 3) to explore and use multiple methods of data analysis to enhance our ability to detect population structure/evidence of subpopulations.

1.2. Data Sources and Methodology

Moss *et al.* (2006) undertook preliminary analysis of osteologically identified northern fur seal remains from three archaeological sites located in Alaska, southern British Columbia, and Oregon. A small proportion of the remains that were morphologically identified as belonging to northern fur seals were used for ancient DNA analysis. The team conducted DNA extraction, amplification, and sequencing in the Ancient DNA Laboratory at Simon Fraser University in Burnaby, BC (Moss *et al.* 2006). The resultant phylogenetic trees for *cytochrome b* and the D-loop/control region were

used to make inferences regarding population structure/population subdivision by searching for patterns of haplotype distribution between the three regions.

This primary source dataset used from Moss *et al*. (2006) was also compared with published sequence data derived from GenBank (Benson *et al.* 2008). The data obtained through the GenBank database included both contemporary and aDNA sequences from a number of studies on northern fur seals and Otariids. For the analysis of *cytochrome b* sequences, this data included sequences from Pinsky *et al.* (2010), Winters *et al.* (2011), and Wynen *et al.* (2001). For the analysis of D-loop data, the sequences came from studies by Pinsky *et al.* (2010) and Wynen *et al.* (2001). While a third large data set was available (Dickerson *et al.* 2010), it did not represent the results of random sampling and thus could not be used in these analyses. The *cytochrome b* and D-loop (also known as the hyper-variable displacement loop or control region) are mtDNA regions used as markers for species identification (Dawnay *et al.* 2007). However, they have a substantial difference as *cytochrome b* codes for a protein involved in the electron transport chain, while the D-loop is a non-coding region (Alberts *et al.* 2002). As a non-coding region, the D-loop is less conserved and is under 'neutral selection' (free from selection pressures). That is, because it is not expressed, it is expected to have greater variation than *cytochrome b*, providing the potential for finer resolution when looking at questions of the past, particularly among closely related species through aDNA analysis (Beebee and Rowe 2008) (see Appendix A for more discussion on use and challenges of aDNA). In order to distinguish between populations, species, or higher orders, a marker is required that is variable enough to distinguish the desired group from the others, but not so variable that the variations obscure patterns at the desired level of analysis (Beebee and Rowe 2008; Brown and Brown 2011). This analysis found the D-loop region to be more suitable than *cytochrome b* for population genetics-level analysis.

The unpublished sequence data used in this thesis is of particular use to the study of northern fur seal phylogeography and population genetics because these archaeological sites fill a geographic 'gap' present in the published data. These unpublished sequences include data from archaeological sites in southeast of Alaska (Cape Addington) and southern British Columbia (Ts'ishaa), locations not present in the

data obtained from previously published GenBank sequence data (see Figure 4.1 for archaeological site locations).

Together, these sources of genetic sequence information provide a foundation for exploring genetic diversity and population structure dynamics among northern fur seals in both past and present. The approach used in this thesis includes generating phylogenetic trees, using analytical software that employs algorithms based on common calculations to assess polymorphism and diversity measures (such as haplotype and nucleotide diversity), analyzing the pairwise diversity, using network analysis to examine the relationships between haplotypes, and using statistical software to assess how the genetic variance is partitioned.

1.3. Thesis Outline

Following this introductory chapter, this thesis is comprised of six additional chapters. Chapter 2 provides a review of the literature on northern fur seals. It begins with a brief summary of the taxonomic status and phylogeny of the species to define what northern fur seals are and how they are related to other pinnipeds (further detail available in Appendix C). One key issue is the classification of northern fur seals and whether it represents a unique clade separate from (and basal to) other Otariids. Chapter 2 also discusses evidence for changes in breeding and migration patterns through time using modern behavioural observations and archaeological evidence, as well as population size estimates of presumed population declines and expansions that may have impacted the genetic diversity of northern fur seals. The final section of Chapter 2 discusses previous studies investigating the genetic diversity and population structure dynamics of northern fur seals in the past and present.

Chapter 3 discusses key concepts behind population genetics, including the methods used in constructing phylogenetic trees and networks, basic evolutionary principles, and the methods applied specifically to the study of population genetics. The chapter provides an overview of those concepts essential to understanding and interpreting data on genetic diversity and population structure dynamics.

Chapter 4 describes the materials and methods used in this study, including methods employed by researchers who obtained the sequence data that informs this thesis, and the methods employed in this thesis work to expand the analysis. It then describes information relevant to the GenBank data to support the analysis used in this thesis. This is followed by a detailed discussion of the sampling strategy and sample processing methodology used by Moss *et al.* (2006). This is followed by a discussion of the various ways the contextual archaeological data can be sorted and grouped to generate more robust, meaningful datasets. The remainder of this chapter describes the approach and methodology employed in this thesis. A number of methods were employed to view, edit, and align sequence data; to generate phylogenetic trees; and to investigate population genetics using measures of genetic diversity, nucleotide mismatch distributions, network analyses, and AMOVA analyses of population variance.

Chapter 5 presents the results from phylogeny and species identity tests, measures of the diversity values (i.e. haplotype diversity, nucleotide diversity), and nucleotide mismatch, phylogenetic networks, and statistical AMOVA analysis (which describes the partitioning of variance within a given population). These results came from a variety of programs including MEGA 6 (Tamura *et al*. 2013), DnaSP 5(Rozas *et al*. 2003; Rozas 2009), NETWORK 4.6 (http:// www.fluxus-engineering.com; Bandelt *et al*. 1999), PopART (http://popart.otago.ac.nz; Bandelt *et al*. 1999), Arlequin 3.5 (Excoffier and Lischer 2010), and GenAlEx6.5 (Peakall and Smouse 2012).

Chapter 6 interprets the results within the framework of the above research questions and objectives, namely whether the results presented support evidence of differences in genetic diversity between the past and present, and whether there is a difference in the population genetic structure/evidence of subpopulations that may indicate the potential presence of local rookeries. The majority of analyses generated results similar to those in the literature, with little evidence of population subdivision in ancient northern fur seals and little difference between past and present. The exception was in the AMOVA analyses which show evidence of structure leading to the tentative rejection of the null hypothesis.

Chapter 7 discusses how the findings relate to the research question. The tentative rejection of the null hypothesis provides some genetic evidence in support of population subdivision in ancient northern fur seals. The thesis concludes with a bibliography and appendices. The Appendix A is an essay on ancient DNA including its potential applications and challenges, as well as some basic information on the structure and degradation of DNA. As this thesis was conducted with an ancient DNA laboratory, this information was included here. Appendix D includes sample calculations to illustrate how the equations for haplotype and nucleotide diversity values work.

1.4. Findings

This research showed results that are fairly consistent with the work undertaken in other studies on the population structure of northern fur seals. Despite archaeological evidence suggesting population structure/population subdivision may be expected, the results show little evidence of such subdivision, though there are a handful of statistically significant differences. This thesis appears to be the first to show some statistical support for 'population structure' in ancient northern fur seal, though much of this statistical support comes when using the less stringent of the two P-values employed. The research has also identified a number of issues with respect to the population structure of the northern fur seal population.

Despite poor resolution of pinniped taxonomy (See Appendix C for further discussion: Figures C.6a-c, Figures C.7 a-c) when using D-loop sequence data phylogenetic trees of both *cytochrome b* (Figure C.3, Figure C.4) and D-loop sequence data (Figure C.5, Figures C.6 a-c, Figures C.7a-c) were able to successfully identify northern fur seal remains to species. Appendix C illustrates some of the problems with determining pinniped taxonomy as D-loop trees were found to vary with outgroup species/sequence and method of tree-building). Despite this, all of the trees generated in Chapter 4 and Appendix C were able to successfully identify northern fur seal sequences with both cytochrome b and D-loop sequence data.

Callorhinus sequence data was used to investigate questions of population diversity and genetic structure. This study found that for D-loop data, there was a high

degree of diversity (Figure 5.2, Tables 5.4 and 5.5) in both past and present populations when compared to other studies. Unfortunately, due to alignment gaps (Table 5.1), the analysis of *cytochrome b* sequence data (Table 5.3) was extremely poor (including a modern sample size of only five sequences). As expected due to the differences between coding and non-coding regions, *cytochrome b* diversity was substantially lower than that of the D-loop data. For *cytochrome b*, the haplotype diversity appeared to be 'moderate' compared to other coding-region diversity values (see Discussion, Section 6.3.2). Nucleotide diversity is expected to be much smaller than the haplotype diversity (Goodall-Copestake *et al.* 2012). For *cytochrome b* data, the nucleotide diversity values appeared quite high compared to diversity ranges for a similar mitochondrial coding gene for a number of species. This relatively high *cytochrome b* diversity is interesting due to the low haplotype diversity observed (for more detail, refer to Discussion, section 6.3.2). In contrast, the D-loop data analysis revealed both a high proportion of unique haplotypes and high haplotype diversity when compared to D-loop haplotype diversity of other mammalian species (Lau *et al.* 1998; Naderi *et al.* 2007; Weiss *et al.* 2000). The nucleotide D-loop diversity was similarly high compared to nucleotide diversities of D-loop sequences from other species (Chaves *et al.* 2011; Dickerson *et al.* 2010; Melchior *et al.* 2010). Also of note is that the values of D-loop nucleotide diversities were fairly similar to those reported by Pinsky *et al.* (2010); this is also not surprising given that this data set makes up the majority of sequences used in this analysis.

Nucleotide mismatch analysis can be used to infer population demography, wherein a single smooth curve represents a recent population expansion, while a ragged line suggests a stable population (Holsinger 2008; Ingman and Gyllensten 2003; Matisoo-Smith and Horsburgh 2012). The analyses presented here revealed a smooth curve for the modern northern fur seal populations and a slightly more ragged curve for the ancient populations (Figures 5.12 a-b). Both of these indicate recent population expansion, though the ancient population appears a bit more stable but still affected by a recent expansion (Holsinger 2008; Ingman and Gyllensten 2003; Matisoo-Smith and Horsburgh 2012; Milá *et al.* 2000; Rogers and Harpending 1992).

The network analysis (Figures 5.13-5.16) revealed that the ancient D-loop haplotypes seemed to form two larger clusters that may indicate a deeper split between

these groups of haplotypes. Of the two groups, the one with a greater number of branches has a large number of reticulations (or 'cycles'). These may be the result of homoplasious mutations, where a number of the same mutations occurred in different branches (as mtDNA cannot undergo recombination). The deep split between groups may potentially indicate some evidence of structure/subdivision in these past northern fur seals. The modern network analysis of D-loop haplotypes also appears to be divided into two main groups, and there is clear evidence of a number of star-like phylogenies, indicating recent rapid population expansion (Edwards *et al.* 2004). The deep split between groups is preserved from the past to the present. It also appears that a handful of ancient haplotypes are not represented in the modern sample; this may represent extinct haplotypes or may be an artefact of DNA damage (Figure 5.11).

Finally, the AMOVA analyses of how the genetic variance was partitioned revealed that the majority of ancient variance was within populations rather than among populations (5% among populations, 95% within populations) (Figures 5.12 and 5.18, Tables 5.6 and 5.10). This 5% among-population variance was found to be non-significant at P≤0.005, but significant at P≤0.05. A high proportion of variation distributed within populations/subpopulations suggesting high dispersal/high gene flow between these groups. That is, most of the variation is widely distributed across the entire range, or conversely, that each subpopulation contains most of the variation found amongst all individuals across the range (Falk *et al.* 2001). The ancient northern fur seal data, therefore, appears to represent a population close to being panmictic (i.e. freely interbreeding/intermixing with no subdivision), though there is some statistical evidence for population subdivision (see Figure 3.1 for illustration of hypothetical population subdivision). At the 0.005 cutoff, the only 'population genetic structure' or population subdivision seen were a few pairwise comparisons on an archaeological site-by-site basis (Table 5.7, Table 5.11). While modern analyses have previously reported some site-by-site differences (Dickerson *et al.* 2010), such differences were not found in the ancient analysis by Pinsky *et al*. (2010). This further supports the finding of little 'population structure' (Falk *et al.* 2001; Gibbs 2008). When the P-value cutoff is increased to P≤0.05, the overall 5% among population diversity F_{ST} then becomes significant and the number of pairwise comparisons that are significant rises to a total of seven. While this is just an exploratory result, such evidence of structure was not found

in other studies on population genetics using northern fur seal aDNA. The modern data shows no evidence of population subdivision overall at either significance level, and only a single pairwise comparison (for both F_{ST} and Φ_{ST}) is significant at P≤0.05 (Figure 5.20, Tables 5.8, 5.9, 5.12, and 5.13).

To summarize, the data presented in this thesis was analyzed and found some evidence of geographic patterning. There was no specific clustering of haplotypes among geographic locations. First, the unpublished northern fur seal sequence dataset did not show evidence of patterning amongst the three sites. Second, when this data set was placed in broader geographic context with other sites, there was still no evidence of geographic patterning with regards to either phylogenetic trees/networks or diversity calculations.

Despite the lack of geographic patterning, the AMOVA analyses suggest some level of population structure in the ancient dataset. Under the more conservative P-value cutoff of 0.005, there is no overall ancient structure and only a few archaeological pairwise population comparisons show statistical differences. When the P-value is raised to the 0.05 cutoff there are a total of seven significant ancient population pairwise comparisons, with variance in the range of 12-24%, and the low ancient 5% F_{ST} (among population variance) became significant. However, the overall lack of strong support for structure (low overall F_{ST} values, little geographic patterning of haplotypes in phylogenetic trees etc.) does not refute the hypothesis of local rookeries, given the lack of structure/population differentiation in modern northern fur seal genetics.

While modern populations have been observed to have high philopatry, modern genetic data does not indicate strong population differentiation, though this may be a result of recent recolonization (Dickerson *et al.* 2010; Pinsky *et al.* 2010; Pyle *et al.* 2001). This observation of modern diversity may impact our assessment of ancient diversity, as it is highly probable that if there were local rookeries in the past, ancient northern fur seal genetic diversity may also have shown little evidence of this. None of the analyses found overall significance at either P-value for the modern northern fur seal data, and those two significant pairwise population comparisons, which became significant at P≤0.05, were only a 1% among population variance.

The findings presented here are consistent with those of other studies (Dickerson *et al.* 2010; Newsome *et al.* 2007; Pinsky *et al.* 2010); however, this thesis appears to be the first to describe evidence of significant population differentiation (F_{ST}) values for ancient northern fur seal. Despite archaeological observation of northern fur seal remains of pups of weaning age, adult males, and females and the suggestion of potential year-round northern fur seal occupation at sites, past studies on genetic data have not been able to confirm the existence of local subpopulations or existence of barriers to gene flow. While most of the analyses conducted in this thesis showed little difference between past and present diversity, the AMOVA analysis does indicate some possible level of ancient population structure. This AMOVA data, therefore, tentatively refutes the null hypothesis of no difference between past and present populations, given the difference in diversity or population genetic structure between the ancient and modern northern fur seal sequences. If this is confirmed with further analysis, this would support expectations for the existence of mid-latitude subpopulations under the working hypothesis of the existence of local rookeries.

In conclusion, these data tentatively reject the null hypothesis of 'no genetic difference', as the observation of some significant partitioning of variance in ancient northern fur seals appears to support 'genetic structure' or population subdivision in ancient northern fur seals. However, much of this support is only present at the $P \leq 0.05$. For both past and present northern fur seal populations, the high genetic diversity and lack of strong population genetic structure of northern fur seals may reflect the lack of discriminative power of mitochondrial DNA markers of this length, or may simply be a species trait perhaps related to the high mobility and dispersal potential of this species.

Chapter 2.

Literature Review on Northern Fur Seal

2.1. Introduction

The northern fur seal (*Callorhinus ursinus*) is particularly abundant at many archaeological sites along the northwest coast of North America (Braje and Rick 2011; Calvert 1980; Crockford and Frederick 2011; Burton *et al*. 2001; Gifford-Gonzalez *et al.* 2004; Gifford-Gonzalez 2011; Huelsbeck 1994; Lyman 1995; Moss *et al.* 2006; Newsome *et al*. 2007; Wigen and Stucki 1988). Given this abundance, it seems probable that northern fur seals played an important role as prey for human populations in this area. The modern population of northern fur seals is currently in decline with no clear cause (Dickerson *et al.* 2010; Towell *et al.* 2006; Trites 1992). A study of the genetic diversity of northern fur seals is thus important for understanding past human behaviour and for using the past to inform the present in issues related to conservation. For instance, the presence of locally breeding northern fur seal populations would be expected to be a far more energetically efficient resource for humans to exploit. Northern fur seals today only come ashore during non-breeding periods when sick or injured (Gifford-Gonzalez *et al.* 2004). In general, while many groups had proficient offshore hunting capabilities, given that northern fur seals while foraging off-shore are at low-density, it would be more efficient to hunt them at breeding sites where they cluster in high densities on land (Burton *et al.* 2001; Hildebrandt and Jones 1992; Lyman 1995; Smith and Smith 2001). Though today the majority of northern fur seals migrate to high-latitude offshore islands to breed, archaeological harvest profiles and isotope data indicate the presence of young fur seals all along the Pacific Coast of North America from southern California to Alaska (Braje and Rick 2011; Burton *et al.* 2001; Crockford *et al.* 2002; Etnier 2002; Lyman 1995; Newsome *et al.* 2007; Pyle *et al*. 2001). This has led to the hypothesized existence of local rookeries along the coast of North America, with

northern fur seals occupying middle latitudes year-round rather than migrating north as is the pattern seen today. Given these hypothesized local rookeries, population genetics predicts that these rookeries may have accumulated genetic differences between rookeries across their range due to reproductive isolation, under the assumption of northern fur seal philopatry to natal rookeries and the geographic separation between sites. This leads to the hypothesis that one might expect to find genetic signatures of subpopulations resulting from these barriers to gene flow. To investigate this question, the null hypothesis of no genetic difference between geographically separated archaeological northern fur seal was tested for using the previously unpublished sequence data obtained by Moss *et al.* (2006) in this thesis by applying methods of investigating population genetics. This goal of this thesis is to look for changes in the patterns of genetic diversity and population genetic structure/population subdivision of northern fur seal between the past and present.

To determine whether there have been any changes in the genetic diversity and population genetic structure/population subdivision of northern fur seals, an understanding of past and present northern fur seal distribution, population, and breeding and migrational patterns is necessary. To begin, it is important to know what 'northern fur seal' refers to and to ensure the sequence data obtained and used in this thesis is of northern fur seal origin. This requires an understanding of the taxonomy of northern fur seals. Since the hypothesis of local rookeries predicts different migration behaviours in the past than can be seen in the general pattern today, this chapter then discusses the patterns of distribution and breeding/migrational behaviour in the present and the evidence for these patterns in the past. Archaeological and historic evidence indicates a much greater population size and range than found today. In addition to the dramatic declines by commercial harvesting, there is additional evidence that northern fur seal populations may have experienced a number of fluctuations in population size and changes in regional distribution/species range (Betts *et al.* 2011; Burton *et al.* 2001; Crockford and Frederick 2007; Crockford and Frederick 2011; Dickerson *et al.* 2010; Newsome *et al.* 2007).

These population fluctuations are important in establishing patterns of genetic diversity and population genetics as bottlenecks and population radiations can have

great effect, so an understanding of past population dynamics is of great use for both generating and interpreting data (Allendorf *et al.* 2012; Leonard *et al.* 2007; Russell 2006; Templeton 2006; Willerslev and Cooper 2005). First, population size is important in interpreting population genetics data as it is an important contributor to patterns of genetic structure, along with social structure/social organization and patterns of dispersal/movement (Chaves *et al*. 2011). For northern fur seals, these social characteristics would include the highly polygynous mating system and philopatry exhibited by both sexes observed in modern fur seal studies[2] (Dickerson *et al*. 2010). Comparisons between past and present genetic variability may be explainable by population history. For example, population declines followed by expansions would be expected to result in a bottleneck effect, reducing genetic variation and may also diminish fitness through reduction in heterozygosity (Dickerson *et al.* 2010; Weber *et al*. 2004). Studies on other otariid species have reported loss of genetic diversity, with greater variation in ancient compared to modern populations (Weber *et al.* 2004). The degree of change in genetic diversity may be correlated with the severity of the population bottleneck (Chan *et al*. 2006), and observed/recorded population demographic sizes may be compared with simulated values to estimate 'posterior probabilities' (Alter *et al*. 2012). These can be used in Bayesian approaches (see Sections 3.3.1 and 7.8 for more discussion) such as the approximate Bayesian computation (ABC) framework - which is one method that can be employed to estimate demographic parameters from genetic data and which allows for incorporation of ancient DNA data from different time points (Alter *et al.* 2012; Chan *et al.* 2006; Chaves *et al.* 2011). After discussing the observed and predicted changes in population size in northern fur seal, the chapter concludes with an examination of the evidence from past studies focused on northern fur seal population diversity and changes in population genetic structure to examine whether northern fur seal in the past belonged to unique subpopulations, and whether there is genetic evidence to support the existence of local rookeries.

[2] High philopatry is expected to increase genetic differentiation, while dispersal would lessen it (Figure 3.1).

2.2. Taxonomic Classification

Northern fur seals belong to the superfamily Pinnipedia. This taxonomic group represents a number of marine mammals, including fur seal and sea lions, or 'eared seals' (Otariidae family), true seals (Phocidae family), and walruses (Odobenidae family) (Benson *et al.* 2008; Moss *et al.* 2006). The Otariidae family is split into two subfamilies: Arctocephalinae (or fur seals) and Otariinae (sea lions). The Arctocephalinae sub-family is divided into two genera: *Arctocephalus* and *Callorhinus*, the genus to which northern fur seals are often classified (see Figure C.1 in appendices). The other sub-family in Otariidae is the Otariinae, or the sea lion sub-family, which includes the genera *Phocarctos*, *Neophoca*, *Zalophus*, *Eumetopias*, and *Otaria* (Arnason *et al.* 2006; Baird and Hanson 1997). There is much debate regarding whether this taxonomy represents the actual phylogenetic relationships between species, as the Otariidae taxonomy is primarily based on morphology, and thus may not reflect a true evolutionary history. This debate permeates through the different taxonomic levels from species level to the sub-family division of fur seals and sea lions, and sometimes even to the monophyletic Pinnipedia grouping species level (Arnason *et al.* 2006; Bininda-Emonds *et al.* 1999; Moss *et al.* 2006; Wynen *et al.* 2001) (See Appendix C for more information).

A phylogenetic analysis and species identification was the first analysis done on the data so as to ensure that only northern fur seals were used for the population genetics analysis (for more detail, see Appendix C). In understanding the genetics of northern fur seal, once the species is clearly defined, a finer scale analysis of northern fur seal genetics such as population genetics can be undertaken, such as is done in this thesis where sample identity was confirmed prior to further study at the population scale. Essential to the investigation of northern fur seal population genetics is an understanding of how the species distribution/population size has changed over time. Ancient DNA can be beneficial in investigating past populations by providing information on past population genetics and estimates on population size. The changing population size and geographic distribution, as well northern fur seal behavioural observations in the present and projections for the past, will be discussed in the following sections given the crucial role these have on population genetic structure/population subdivision and genetic diversity of a species.

2.3. Distribution, Breeding, Migration, and Foraging Behaviour

This section summarizes what is known about northern fur seal distribution, breeding and migration patterns along the Pacific Coast of North America. Some information on ancient northern fur seal breeding and migration patterns can be inferred from archaeological assemblages, which by definition involve human activity. Information from these remains can be derived from archaeofaunal analysis to assess age/sex profiles and through the use of stable isotope data and ancient DNA. Stable isotopes can be used to infer data on foraging patterns of various species which can provide information about life histories of ancient northern fur seals (Burton *et al.* 2001, 2002; Moss *et al.* 2006). It begins by presenting information about modern distribution and breeding, migration, and foraging behaviour. It then discusses the archaeological evidence for these patterns in the past, drawing from archaeological studies ranging from California to Alaska, dating from 8,500 BP (Burton *et al.* 2001; Moss *et al.* 2006; Newsome *et al.* 2007). This includes the use of archaeofaunal analysis for age estimates to show the presence of pre-weaning age seal pups, stable isotope data on ancient foraging patterns to show continuity in offshore foraging, isotope data to indicate lack of migration/year round occupation at middle latitudes, and isotope data on tropic levels to indicate nursing pups to strengthen the archaeofaunal age estimates. The isotope data can be used to identify whether northern fur seals may have maintained similar behavioural patterns in the past as seen today. Whether or not the past behaviour is the same as that observed today may affect their population dynamics and distribution. The isotope data suggests that more southerly northern fur seals in the past remained in middle latitudes all-year round (non-migratory) including nursing pups and that their feeding behaviour remained pelagic, suggesting that remains found were likely caught on land (haul-outs, strandings, or local rookeries). Together this evidence of local rookeries and separate 'populations' of northern fur seal indicates some level of population isolation between northern fur seals at different latitudes and, therefore, increase the expectations of population genetic structure in past populations.

Today, all northern fur seals breed on offshore islands, predominantly in high-latitude areas (Burton *et al.* 2001; Newsome *et al.* 2007). The primary rookeries for

northern fur seals are on the Pribilof Islands (primarily St. Paul and St. George) in Alaska (Dickerson *et al.* 2010; Newsome *et al.* 2007). Approximately 65% of the world's population of northern fur seals breed here (Newsome *et al.* 2007). Other breeding colonies can be found on Bogoslof Island (Alaska), off the coast of San Miguel (California), and on Robben Island (a.k.a. Tyuleniy Island, Russia) (Dickerson *et al.* 2010). The majority of female northern fur seals migrate north to the Pribilof Islands between June and July where they give birth to pups within a day or two of arrival, on average (Gentry 1998; Newsome *et al.* 2007). They typically mate and return to foraging trips less than a week after the birth of the pups (Gentry 1998). Younger females tend to arrive and mate later, often with younger males. Northern fur seals experience an embryonic diapause, where implantation of fertilized egg occurs following the end of nursing the current season's young (Gentry 1998). Weaning occurs in early November, just prior to the migration of females and their young to the south for winter (Gentry 1998; Newsome *et al.* 2007). This four-month lactation period in modern northern fur seals is considered to be relatively short among the otariids. It is therefore unlikely that past populations weaned at an earlier age than modern populations (Burton *et al.* 2001; Moss *et al.* 2006; Newsome *et al.* 2006; Newsome *et al.* 2007). Unlike the females, male northern fur seals do not migrate, with the Pribilof males remaining in the Gulf of Alaska over the duration of the winter. Both male and female northern fur seals are pelagic; that is, they forage far offshore (Burton *et al.* 2001; Newsome *et al.* 2007). Northern fur seals may travel long distances on foraging trips, with travel distances often encompassing several breeding colonies (Dickerson *et al.* 2010). Modern studies indicate a highly polygynous mating system with high philopatry (return to natal breeding site) exhibited by both sexes. The expectation for high philopatry is typically genetic differentiation between breeding colonies, though this is not clearly seen in genetic studies (see section on Population Structure for further discussion) (Dickerson *et al.* 2010). Archaeological abundance and site distribution is the first indicator of the widespread presence of northern fur seal in areas they are not seen in today.

The archaeological study by Burton *et al*. (2001) examined the remains of northern fur seals from Californian sites dating from 8,500-100 BP, with a handful of carbon dated elements ranging from 2,920 to 2,470 BP (±50-60 years). The archaeofaunal assemblages suggest that northern fur seals were the predominant

pinniped along the Californian coastline, with *Callorhinus* remains comprising up to half of the pinniped remains (Burton *et al.* 2001). Moss *et al.* (2006) examined remains from three archaeological sites: the Cape Addington (Alaska [Moss 2004]) site dating from cal AD 50–1680; the Ts'ishaa (Barkley Sound, British Columbia [McMillan and St. Claire 2005]) site consisting of a terrace component dating from 3370-1000 BC (31 samples from this area) and a village site dating from cal AD 70-1700 (with the majority of northern fur seal remains recovered from the village site); and the Netarts Sandspit (Oregon [Losey 2002]) site which had samples dating from cal AD 1300–1800. The northern fur seal remains were fairly abundant, particularly at the Ts'ishaa site where they comprised the majority of the pinniped remains recovered (Moss *et al.* 2006)[3]. A third study on archaeological remains used samples from numerous sites from Alaska to California, dating between 8,150-8,390 cal BP to around 20-340 cal BP (Newsome *et al.* 2007). More specifically, these sites were Shemya, Chaluka, and Rolling Bay (Alaska); McNaughton Island, Hesquiat, and Ts'ishaa (British Columbia); Ozette (Washington); Seal Rock and Umqua/Eden (Oregon); and Duncan's Point, Point Año Nuevo, Moss Landing, Santa Cruz, and Point Mugu (California) (Newsome *et al.* 2007). An abundance of northern fur seal remains were found at these archaeological sites.

Archaeometric analysis of the assemblages often indicate that some of the recovered remains belong to young seal pups which could suggest year-round occupation if the pups were of pre-weaning age (Braje and Rick 2011; Burton et al. 2001; Crockford *et al.* 2002; Etnier 2002; Moss *et al.* 2006; Newsome *et al.* 2007). The Moss Landing and the Mendocino Coast sites revealed the remains of small or young seal pups, estimated to be three months old or younger (Burton *et al.* 2002). Given that modern northern fur seal pups do not leave their rookeries and continue to nurse until they are four months old, this finding suggests the presence of local rookeries along the coast of California (Burton *et al.* 2001). Further evidence of young seal pups comes from the northwest Pacific Coast region. The age at death of these remains was estimated using comparative collections (Moss *et al.* 2006). The northern fur seal assemblage at Cape Addington, Rockshelter contained 20 bones/bone fragments identified as northern

[3] This correlates with the findings from another Vancouver Island site (Hesquiat Harbour), where some layers also found northern fur seal to be the most abundant mammal (Calvert 1980).

fur seal, with half of the elements coming from seal pups. Assemblage composition at the Ts'ishaa site included both male and female northern fur seal remains as well as those of young seal pups (under four months old), suggesting again year-round occupation (Moss *et al.* 2006). At the Netarts Sandspit site, 31 elements were identified as northern fur seal, seven of which were from seal pups. Further remains of young northern fur seals were documented by Newsome *et al.* (2007), providing potential evidence of local rookeries. These pups were estimated to be around four months old at death, and thus most likely were of pre-weaning age. These young pups make up a substantial proportion of the remains found at these sites and therefore indicate the likely presence of local breeding colonies, or rookeries, along the coast of California, the Pacific Northwest, and the Eastern Aleutians (Newsome *et al.* 2007). Isotopic analysis of archaeometric age-estimated bones and a bone growth series suggests that these ancient pups grew slower[4] than their modern counterparts and maintained the nursing signal longer (elevated $\delta^{15}N$, discussed shortly in the sub-section on isotope data) (Newsome *et al.* 2007).

In addition, young northern fur seals, ranging from 5-12 month old individuals, are represented in the archaeological assemblages from the Eastern Aleutians and the Olympic Peninsula. This range of age classes indicates the seals were present in the area at a variety of time points in their yearly pup growth cycle rather than at only a single point in this cycle, indicating that northern fur seal occupied the site year-round rather than simply 'passing through' it as part of their yearly migratory cycle. Post-weaning age remains may be the result of haul-outs or strandings.

Archaeometric analysis and isotopic data from these assemblages are generally consistent with the findings from Burton *et al.* (2001) and Moss *et al.* (2006) (Newsome *et al.* 2007). Given the lack of islands/offshore rocks near some of these sites, it seems likely that there may have been at least one mainland beach rookery (such as the Moss Landing site where some seal pup remains were found), something which has not been observed in modern northern fur seals (Burton *et al.* 2001). In addition to the Moss

[4] Regarding northern fur seal growth rates, there is much support among historic-age specimens indicating that growth rates fluctuated, and that such fluctuations may have been correlated with a variety of factors (such as population size, prey availability, etc) (Etnier 2004).

Landing site, two other sites have also been proposed as candidates for mainland rookeries: Duncan's Point Cave (Sonoma, California) and Point Mugu (southern California) (Burton *et al.* 2001). The likely presence of mainland rookeries also indicates a greater northern fur seal distribution and abundance than is seen today; that is, the archaeological abundance over such a large range of sites suggests either a large number of rookeries or a very large migrating population to be hunted during haul-outs and strandings. Whether this abundance of northern fur seals at archaeological sites is due to the presence of local rookeries, or perhaps is the result of different foraging strategies in the past, needs to be considered (Burton *et al.* 2002).

Stable isotope data from all three studies indicate that northern fur seals appear to have maintained a similar strategy of far offshore feeding (Burton *et al.* 2001; Moss *et al.* 2006; Newsome *et al.* 2007). Moss *et al.* (2006) analyzed bone collagen in samples from Cape Addington and Netarts for stable isotope composition ($\delta^{13}C$ or $\delta^{15}N$) and compared the results with those of harbour seals (who feed in the littoral zone, as opposed to the pelagic foraging behaviour of northern fur seals) and found data suggesting that archaeological northern fur seals were dissimilar to harbour seals (i.e. far offshore foragers), which is expected given modern behaviours. Burton *et al.* (2001) analyzed modern and archaeological northern fur seal remains from Californian and found a similar pattern represented in carbon and nitrogen isotope ratios, suggesting that ancient northern fur seals followed the same strategy of offshore feeding as is seen today. Given the low density of northern fur seals while foraging offshore, the technology required to travel and hunt far offshore species, and the lack of remains of other offshore species (fish), it is likely that the northern fur seal remains found in these assemblages were hunted on land (Burton *et al.* 2001; Moss *et al.* 2006; Newsome *et al.* 2007). This evidence that ancient northern fur seals were pelagic foragers provides support for the hypothesis that the abundance of northern fur seals in archaeological assemblages is not the result of an alternate foraging strategy that may have made them more vulnerable to hunting in the past, and thus supports the current hypothesis of breeding colonies throughout their range (Newsome *et al.* 2007).

Stable isotope data also provides evidence that the northern fur seal remains found at these sites remained at mid-latitudes year-round and were therefore non-

migratory. The $\delta^{13}C$ and $\delta^{15}N$ stable isotope values vary according to latitude and are higher in temperate regions (Burton *et al.* 2001, 2002). Burton *et al.* (2001) indicated that the ancient female northern fur seal remains that were analyzed fell into three distinct groups: a Californian grouping, a North Pacific/Gulf of Alaska/Eastern Aleutian grouping, and a Western Aleutian grouping. This finding lends support to the notion that archaeological northern fur seals were year-round residents, even among more northern colonies. This isotope foraging pattern data indicated two geographically distinct archaeological northern fur seal groupings: a northern set (Oregon, Washington, British Columbia, and Alaska), and a Californian set (see chapters in Braje and Rick 2011). Burton *et al.*'s (2001) stable isotope data comparing middle Holocene samples from various sites indicated that those samples from higher latitude sites (North California/Mendocino) had lower $\delta^{13}C$ and $\delta^{15}N$ values than those from sites further south (Central California). This variation between sites suggests local differences which conform to the expected pattern of changes with latitude. In comparing $\delta^{13}C$ stable isotope values, Burton *et al.* (2001) saw no difference between the modern and ancient Californian populations. However, both Californian groups showed significant differences from modern Alaskan (male) and migratory (female) northern fur seals. This finding supports the notion that these ancient northern fur seals were non-migratory with offshore feeding, much like the San Miguel population today. The $\delta^{15}N$ values for both ancient and modern Californian northern fur seals were significantly higher than those of modern Alaskan northern fur seals, which again supported latitudinal differences between populations. The stable nitrogen values are believed to be caused by the vertical mixing of new nitrogen in the oceans, and the similarity of the ancient sample values to those of non-migratory modern Californian (San Miguel Island) samples suggests that these ancient northern fur seals did not migrate (Burton *et al.* 2001). Burton *et al.* (2001, 2002) thus conclude that ancient Californian northern fur seals were not seasonal migrants and that there is evidence that the pattern of breeding and migration was different in the past for at least some northern fur seal populations. Newsome *et al.* (2007) likewise found no evidence of migration for past populations, not even among the colonies in the higher latitudes, through the finding of distinct isotopic signatures for a Californian grouping, an eastern Aleutian/Gulf of Alaskan/Pacific Northwest grouping, and a western Aleutian grouping.

Isotope data (of an age-estimated series) as well as modern observations of longer lactation periods for lower latitude populations both suggest that the remains of young northern fur seals were pre-weaning pups, which supports the argument for local rookeries. Nursing is typically reflected in pups that have $\delta^{15}N$ values that indicate higher trophic levels than their mothers, with the lactation signal persisting longer in ancient specimens (ages based on skeletal age classes) than in modern (Pribilof) northern fur seal in the same age class (Burton *et al.* 2001; Moss *et al.* 2006; Newsome *et al.* 2007; for modern see Newsome *et al*. 2006). The isotope data in these studies suggest prolonged periods of nursing. Both the remains of young archaeological pups and those from modern Pribilof Islands populations showed similar signals due to nursing between the ages of 2-6 months (Newsome *et al.* 2007). However, among older archaeological northern fur seal pups, this nursing signal persisted much longer (present in 9-12 month old pups from the Umnak Island site and 12-15 month old pups from the Ozette site) (Newsome *et al.* 2007). The isotopic data also suggest that the Cape Addington site contained juvenile fur seals (less than one year old) due to $\delta^{15}N$ enrichment (indicative of higher tropic level as a result of nursing) (Burton *et al.* 2001). The most likely explanation for this is that these ancient pups were weaned at a much older age than *Callorhinus* pups today (Moss *et al.* 2006). It has been shown that migrating high-latitude pinnipeds nurse for shorter periods than those at temperate latitudes (Burton *et al.* 2001), and that the breeding/nursing season of northern fur seals is constrained by the southward migration (Gentry 1998). It is, therefore, conceivable that past populations, particularly non-migratory populations, may have weaned at a later age. Many Otariidae often wean between ten and fourteen months of age in order to provide some protection against minor climate and resource fluctuation events (Lyman 1991; Newsome *et al.* 2007). A longer weaning period may have been especially beneficial for ancient northern fur seals, particularly given that such fluctuation events have been shown to dramatically affect northern fur seal and other pinniped populations (Lyman 1991; Newsome *et al.* 2007). Modern observations have also supported the notion that the lactation period of northern fur seals would likely have been longer in lower latitude areas in the past, since even the recent migrants to San Miguel Island in California have a longer lactation period than their high-latitude breeding counterparts (Burton *et al.* 2001). A longer breeding season would lend further support that the remains of young northern fur seals

found at these sites did indeed represent pre-weaning age pups, and that there were local rookeries present.

Collectively, the archaeological research presented above all seems to support the presence of local rookeries. The distribution of northern fur seals appears to be much greater in the past than it is today, with evidence of breeding colonies all along the Pacific coastline from California to the Aleutian Islands. As northern fur seal rookeries would represent a high-density food resource, the abundance of northern fur seal remains in archaeological contexts has been used to support the argument for presence of haul-outs / rookeries (Burton *et al*. 2001; Moss *et al.* 2006). Data from both past and present northern fur seals indicate a continuity of offshore feeding during non-breeding season; this archaeological abundance is likely the result of local rookeries being present rather than from different foraging patterns in the past (Burton *et al.* 2001; Moss *et al.* 2006; Newsome *et al.* 2007). There is archaeological evidence of rookeries from archaeometric and isotopic analysis of faunal remains, indicating juvenile northern fur seals that were likely of pre-weaning age, since migration occurs after weaning (Burton *et al.* 2001, 2002; Moss *et al.* 2006; Newsome *et al.* 2007). While the majority of northern fur seals today have a migratory breeding pattern, with females and weaned juveniles migrating south in the fall (while males remain at high-latitudes), archaeological assemblages and isotopic dating of these remains suggest a different pattern, with northern fur seals remaining locally in areas along the Pacific Coast year-round, including locations as far south as California (Burton *et al.* 2001; Dickerson *et al.* 2010; Moss *et al.* 2006; Trites 1992). This potential evidence for the presence of local rookeries leads to the question of whether there is genetic evidence to support local differentiation of subpopulations that would be expected from geographically separated rookeries. It is thus essential background for the hypothesis tested in this thesis.

2.4. Population Dynamics

Researchers have theorized that northern fur seal populations appear to have experienced a number of events that have led to dramatic population expansions and declines over time. This is extremely important to the focus of this thesis; dramatic changes in population size may have distinct effects on population diversity and genetic

structure (Hewitt 1996). Various lines of evidence, including genetic data from both modern and archaeological samples, historic records, and modern studies suggest a number of population expansions and declines (Dickerson *et al.* 2010). More specifically, this includes the use of archaeological remains to obtain ancient DNA, the extrapolation of modern sequence data to infer past populations using models (such as Bayesian skyline analysis), written records on sealing harvests, and modern population estimates using ecological sampling counts. Unfortunately many of these datasets are hampered by problems including small sample size and DNA degradation for archaeological remains (see Appendix A on Challenges of Ancient DNA); inadequate historic records which occasionally do not distinguish taxa (i.e. northern fur seal and the Guadalupe fur seal are often not differentiated); and the challenges associated with modern ecological sampling for a marine mammals with a large range and the extrapolation of modern data into the past (Foote *et al.* 2012; Moss *et al.* 2006; Newsome *et al.* 2007; Starks 1922). Some of the potential causes for these population changes include climatic events, pre-contact human hunting, and commercial sealing. The major population expansion and decline events are estimated to have occurred around 11,000 BP (expansion), 2,000-800 BP (some localized declines), and in recent history (decline, some recovery, and current decline) (Burton *et al.* 2001; Dickerson *et al.* 2010; Moss *et al.* 2006; Newsome *et al.* 2007). This section will review the estimated northern fur seal population size over time, as well as discuss the potential factors in these decline and expansion events.

A population expansion around 11,000 BP has been extrapolated from genetic data (Dickerson *et al.* 2010). Following the Wisconsin glaciation after around 10,000 BP, there was an increase in available habitat, with presumed population increases (Burton *et al.* 2001). Archaeological sites in California dating between 8,500 to 100 BP often contain high numbers of northern fur seal remains, high enough to suggest that northern fur seals may have been the most predominant pinniped along the Californian coastline (Burton *et al.* 2001).

Various lines of evidence suggest that sometime between 2,000-800 years BP, particularly in certain areas such as in northern and central California, the population appears to begin declining. Several factors have been posited to explain this decline. The likely population decline around a thousand years ago has been largely attributed to

predation by human hunters (Burton *et al.* 2001; Dickerson *et al.* 2010; Newsome *et al.* 2007). Climate change has also been posited as another possible explanation for declining populations, though Burton *et al.* (2001) suggest that the presence of northern fur seal rookeries in middle latitudes (San Miguel) indicates that if climate change were a cause, it must have been a transient change. Since there is scientific evidence of the occurrence of some climatic events (Burton *et al.* 2001), it seems plausible that these events may have indirectly affected northern fur seal populations through impacts on predators or niche-competitors. As terrestrial predators, in particular grizzly bears, are thought to limit northern fur seal rookeries on mainland beaches (Burton *et al.* 2001), terrestrial ecosystem dynamic changes may have had an impact on the mainland beach rookeries (such as the Moss Landing site). Given the current distribution of other pinnipeds (such as California sea lions, Stellar sea lions, and northern elephant seals, which all appear to have been more abundant in archaeological record than today), it is also conceivable that niche-competition may have played a role in the chain of events that lead to northern fur seal rarity in these regions (Burton *et al.* 2001). Newsome *et al.*'s (2007) study on archaeological remains (using archaeometry and isotopic data) pushes the decline of the northern fur seal population in central and northern California to the more recent date of around 800 years ago, while populations in southern California, the Pacific Northwest, and the eastern Aleutian Islands persisted until around 200 years ago when commercial hunting by Europeans seems to have been a contributing factor. Newsome *et al.* (2007) note a general pattern of marine mammal decline along the eastern edge of the Pacific Ocean and suggest a number of possible causes, including European commercial harvests, increased predation by prehistoric humans, or climatic events directly affecting marine productivity. One major climatic event that occurred towards the end of this period was the Medieval Climatic Anomaly/ Medieval Warm Period which occurred around 900-1,350 AD. The warming of Pacific waters may have increased mortality[5], while droughts on land may have increased harvesting pressures by indigenous populations (Gifford-Gonzalez 2011; Graham *et al.* 2010; Newsome *et al.* 2007).

[5] Sea warming has been shown to have effects on northern fur seal populations. For instance, El-Nino Southern Oscillation events have been correlated with decline in pup production resulting from decreased prey availability (Gifford-Gonzalez 2011; Newsome *et al*. 2007).

One of the major factors that has contributed to a dramatic decline of northern fur seal populations has been commercial harvesting. This species has been hunted commercially for hundreds of years into the 20th century. While assemblages from archaeological sites ranging from Southern California to the Aleutian Islands suggest there were abundant northern fur seals throughout this range by the time of early European explorers, only two colonies in the north-eastern Pacific appear to have existed: the Pribilof Island and the Farallon Island colonies (Newsome *et al.* 2007; Pyle *et al.* 2001; Sydeman and Allen 1999). There is evidence that the fur trade decimated rookeries along California and the Pacific Northwest region (Burton *et al.* 2001; Starks 1922).[6] Historical records indicate the presence of a northern fur seal colony on the South Farallon Islands (Central California) in the late 1700's, with a population estimated around 50,000 (Burton *et al.* 2001; Newsome *et al.* 2007; Pyle *et al.* 2001; Sydeman and Allen 1999). This population was decimated by commercial harvesting and has only recently been recolonized (Sydeman and Allen 1999). It is estimated that around 1867, the population of northern fur seals in the Pribilof Islands was around 3 million; however, by 1910, commercial harvesting (particularly on land) reduced the population to around 200-300,000 (Trites 1992). These dramatic declines led to the implementation of hunting restrictions. By the 1940's, restrictions were imposed to limit land-based hunting of sub-adult males, resulting in a degree of population recovery to around 1.5 million individuals (Trites 1992). While there is evidence of recolonization of the South Farallon islands, there is little data on the northern fur seals there (Burton *et al.* 2001; Newsome *et al.* 2007; Pyle *et al.* 2001; Sydeman and Allen 1999). San Miguel Island was recolonized in the 1960's (Gifford-Gonzalez 2011). The rookeries on San Miguel (southern California) as well on Bogoslof Island (eastern Aleutians, Alaska) are also believed to have been colonized by migrants from Pribilof Islands (Burton *et al.* 2001; Newsome *et al.* 2007).

Between 1956 and 1968, experimental harvesting was done on northern fur seals, leading to a population decline which extended through to the 1980's (Dickerson

[6] Starks (1922) notes that many of the reports from this time did not distinguish between northern fur seal and the Guadalupe fur seal. He also notes the presence of fur seals on the Farallon islands but believed these to be Guadalupe fur seal because they were caught at a time which did not fit with the typical migratory pattern of northern fur seals and thus could not be residents year-round.

et al. 2010; Trites 1992). The Pribilof Islands population has not recovered since this decline (Towell *et al.* 2006). While the population was relatively stable into the mid 1990's, there has been a precipitous decline in pup production in recent years (Towell *et al.* 2006). Currently, the population of northern fur seal is estimated to be around 1.2-1.4 million individuals (Dickerson *et al.* 2010; Ream 2002; Trites 1992). This current decline thus makes a study of northern fur seal population dynamics, both in the present and throughout the past, especially important.

Failure of the northern fur seal population to recover in the Pribilof Islands and the current population decline has been attributed to a number of possible factors including high juvenile mortality and the impact of intensified fishing. Intensification of commercial fisheries has been thought to potentially impact northern fur seal populations indirectly by affecting fish populations that may be preyed upon by northern fur seal, or directly by increasing mortality rates of northern fur seals due to abandoned fishing gear (Trites 1992). Evidence against this latter hypothesis of mortality increase due to fishing gear is that other pinnipeds (such as other northern fur seal populations and Antarctic fur seals) who have been observed to have similar entanglement frequencies on haul-out sites have not suffered the same decline (Trites 1992). Further, other pinniped species (California sea lions, northern elephant seals, harbour seals) that may be expected to be affected by entanglements in fishing gear are increasing in population size. However, intensified fishing may still have a significant impact by affecting prey species. While there appears to be sufficient, if not abundant, food in the spring for seal pups off the coast of BC and Alaska, the data on fish abundance off of the Aleutian archipelago is lacking; a decline in fish stocks there could potentially affect young seals during the fall migration (Towell *et al.* 2006; Trites 1992). In addition, the population at Bogoslof Island in the Aleutians has seen some level of increase in pup production (though this increase is less than the declines seen on the Pribilofs and thus not a matter of northern fur seals emigrating from the Pribilofs) (Towell *et al.* 2006). The hypothesis of commercial fishing effects on fish stocks affecting northern fur seal populations may be supported by population declines of Stellar sea lions and harbour seals in the same area; however, far more data on fish abundance is required to make such inferences (Trites 1992). One part of the argument in support of possible decreased prey availability and its effects on northern fur seal populations is centred around measures of nutrition (based on dental

and size data) and how well these nutritional indicators would reflect nutrition during this fall migration period to investigate if nutrition is affecting pup mortality (Trites 1992) . A more recent hypothesis is that northern fur seal decline may be due to commercial whaling/sealing/fishing which affects alternative orca prey, causing them to rely more heavily on northern fur seal (Springer *et al.* 2003).

Northern fur seals have undergone dramatic changes in both population size and distribution. Where once they were the most numerous pinniped in archaeological harvest profiles, they have experienced declines in population and have disappeared from northern and central California. These expansions and declines in population size can be anticipated to strongly affect northern fur seal population genetics studies.

2.5. Population Genetic Structure Dynamics of Northern Fur Seal

The previous sections of this chapter discussed several of the factors that may affect population genetics studies of northern fur seals. Northern fur seal populations have experienced a number of dramatic decline and expansion events over the past few thousand years. They also appear to have experienced changes to their migration and breeding patterns, with past evidence indicating they remained in temperate latitudes year-round. These changes in population size and migration/recolonization events may strongly affect genetic structure in studies of northern fur seal (Dickerson *et al.* 2010). As will be discussed in more depth in Chapter 3, in the field of population genetics, 'population structure' refers to the means by which genes are passed onto the next generation (Templeton 2006), though 'structure' is effectively used to refer to barriers or limitations to gene flow and the existence/formation of local subpopulations that can be seen through the genetic variance (de Thoisy *et al.* 2006; Dickerson *et al.* 2010; Holsinger and Weir 2009; Pritchard *et al.* 2000; Schrey *et al.* 2011). The study of population genetics and changes over time may also be complicated by past expansion and decline/bottleneck events. Founder effects or population bottlenecks are often investigated when looking at changes in population genetics through time as more recent events can also obscure the genetic signals of previous events or cause misleading interpretations on genetic diversity. In northern fur seal, such events may

include the glacial retreat of the Wisconsin glaciation around 10,000 BP and range expansion, the commercial harvesting of northern fur seal, and the cessation of this harvesting. All of these events may result in the homogenization of populations, something which has been seen in other Otariidae species (where recolonization can result in anywhere from 'moderate' differentiation to a completely homogenous/panmictic population) (Dickerson *et al.* 2010). Given the geographic separation of *C. ursinus* breeding colonies and the high philopatry (fidelity to breeding sites) exhibited by both sexes, some evidence of population genetic structure might be expected; however, there is little genetic evidence to indicate this. DNA data has been used to investigate population genetics for northern fur seal. It has been applied to both mitochondrial sequences, such as the more conserved *cytochrome b* and the hypervariable D-loop/control region sequences), as well as microsatellite data which is based on nuclear DNA. This section will investigate the population genetics level work done on northern fur seals, on both archaeological and modern samples, to date. Thus far, there is little evidence to support population structure/population subdivision in both past and present northern fur seals despite expectations for it (Dickerson *et al*. 2010; Gifford-Gonzalez 2011; Newsome *et al*. 2007; Moss *et al*. 2006; Pinsky *et al*. 2010; Ream 2002).

There are a number of analytical approaches to the study of population structure. Moss *et al.* (2006), in their study investigating whether the northern fur seal remains from three archaeological sites indicated local breeding sites or whether they belonged to a migrating population, endeavoured to confirm species ID. They conducted genetic analysis on 11 Cape Addington, 10 Ts'ishaa, and 16 Netarts samples, sequencing the extracted aDNA and generating neighbour-joining trees (Kimura 2 parameter model) using reference sequences from a number of pinniped species. The resultant trees were used to examine haplotype diversity for both D-loop and *cytochrome b* sequences, and to identify geographic patterns (or lack thereof) by looking for location-associated groupings. Of the 37 pinniped bone samples, 35 yielded positive DNA results for *cytochrome b* and 36 for D-loop amplifications, indicating good DNA preservation. Of these, 29 clustered with *C. ursinus* in neighbour-joining trees. The mitochondrial *cytochrome b* sequences were found to be more useful for species ID as they were a bit more conservative than the hypervariable D-loop control region sequences (Moss *et al.* 2006; Yang *et al.* 1998). For the D-loop sequences, species identification could still be

determined, though there was significant variation amongst sequences. For the 29 samples that were species-identified (clustered with) northern fur seal, 23 haplotypes were found. This variability of haplotypes makes the D-loop a possible target to investigate phylogeographic and population-level differences (Moss *et al.* 2006).

Moss *et al.* (2006) suggest that the high variability of haplotypes found for the ancient remains indicates that past northern fur seal genetic diversity was much greater than it is in the present, though more modern haplotypes would need to be considered to further investigate this question. The genetic variation among modern D-loop haplotypes were within the range of variation seen in ancient populations and there was no clear clustering of haplotypes by site, suggesting a lack of population structure; that is, no evidence that this population is subdivided into subpopulations by barriers to gene flow (Beebee and Rowe 2008; Moss *et al.* 2006). The genetic evidence thus does not appear to support the hypothesis that there were local breeding sites (as evidenced from the presence of juvenile remains, age estimated by morphological analysis, and isotopic analysis of trophic level). However, Moss *et al.* (2006) argue that a lack of support is not the same as lack of evidence against local breeding sites; they suggest that certain conditions such as possible high gene-flow between hypothetical 'residential' populations and migratory populations, or a lack of time between separation of migratory and residential populations, may not yield sufficient genetic variation at this level of analysis[7] to be able to identify population structure/subdivision.

Newsome *et al.* (2007) conducted a similar study on archaeological northern fur seal remains. The authors performed a genetic analysis on nineteen of their samples from Chaluka (Alaska), Ozette (Washington), and San Miguel Island (California). They amplified the same section of the control region as Moss *et al.* (2006). Samples were sequenced in both directions, aligned, and used to build both neighbour-joining and maximum likelihood trees. Bootstrapping for these trees supported the monophyletic grouping of northern fur seals. The genetic data showed that samples were genetically diverse (haplotype diversity of 0.8). Despite this genetic diversity, the archaeological

[7] Wynen *et al.* (2001, cited in Moss *et al.* 2006) suggests that D-loop is not sufficiently informative to look at population genetic structure.

samples from temperate regions grouped among modern sequences from high-latitude breeding populations (Newsome *et al.* 2007). This suggests that these temperate groups did not form unique genetic groupings (lack of structure) (Gifford-Gonzalez 2011; Newsome *et al*. 2007).The lack of structure suggests that migration between groups may have been sufficient to prevent accumulation of genetic differentiation (Gifford-Gonzalez 2011; Newsome *et al*. 2007.

The field of population genetics includes numerous other analytical approaches that can be applied to the study of northern fur seals. Dickerson *et al.*'s (2010) study of modern northern fur seals utilized a two-pronged approach using both microsatellite and mtDNA analyses. The microsatellite approach found that there was a good deal of genetic variation in modern northern fur seals, with between 12 and 22 alleles at each locus analyzed (sample size n=728 NFS). The microsatellite analysis used GENEPOP v. 3.1 to calculate the observed and expected heterozygosity, to check whether populations were in Hardy-Weinberg equilibrium (no deviations were found, so null alleles were not an issue), to examine genotypic distribution and population differentiation (using F-statistics), and to test for isolation-by-distance (Dickerson *et al.* 2010). The study showed high observed heterozygosity (with all but one locus showing 83% heterozygosity), no significant population differentiation (F-statistic values), and no evidence of isolation by distance between populations. Additionally, STRUCTURE was used to estimate the number of populations represented by the sample and FSTAT and GENEPOP were used to calculate allelic richness and to estimate the F-statistic variable F_{ST}. STRUCTURE's admixture model suggested that all samples could be grouped into a single population. Dickerson *et al.* (2010) suggest that the lack of evidence for population differentiation/structure may be a result of too few loci being used. They then cite a similar study on Stellar sea lions where an increase from 6 to 13 microsatellite markers was enough to show population structure.

Dickerson *et al.* (2010) also examined a 381 bp mtDNA control region (D-loop) fragment to look at structure/population subdivision in modern northern fur seal populations. As the study by Moss *et al.* (2006) indicates, the D-loop region is more likely to yield relevant data on population genetics than the less variable *cytochrome b* region. After the sequence data was aligned, Arlequin was used to determine the

number of variable sites, identify haplotypes, do genetic diversity calculations, and undertake AMOVA analysis for population structure to search for evidence of population subdivision (Dickerson *et al.* 2010). The majority of genetic variations were limited to singlets: of 332 different haplotypes found, 227 of them were only found in a single individual. Despite this high haplotype diversity, the nucleotide diversity was considered to be 'moderate', showing 87 variable sites, with 106 nucleotide substitutions and one indel (insertion-deletion variation). The authors suggest that the high haplotype diversity and moderate nucleotide diversity indicates that the haplotypes are closely related, suggesting recent population expansion. The AMOVA analysis, which looks for population structure among numerous levels of groupings (using F_{ST} and the related statistic, Φ_{ST}), found little population differentiation. This lack of population differentiation occurred when genetic data was compared between clusters of islands as well as on the larger scale of eastern versus western Pacific. The only differences seen were at an island to island scale (Dickerson *et al.* 2010). The PhiST data (which included both haplotype frequencies and genetic distance) showed far more island-island pairwise comparisons with significant differences than the F_{ST} statistic (which is only based on haplotype frequencies). These pairwise differences suggest some possible population structure, particularly between the breeding colony islands in the United States and the Bering Island/Robben Island (also known as Tyuleniy Island) colonies, though the test for isolation by distance (using GENEPOP with the PhiST statistics) did not yield significant results.

An earlier study, by Ream based on his dissertation work also involved modern DNA sampling and population assessment using eight microsatellite markers to determine population genetics variables. The study found a high degree of genetic variation and high heterozygosity, with no clear evidence of population subdivision. There were some significant differences in allele frequencies between populations for a handful of comparisons. However, neither F_{ST} nor R_{ST} estimates were significant overall, nor for population pairs (Ream 2002). Further, there was no evidence of isolation by distance as evidenced by no significant correlation between geographic distance and genetic differentiation ($F_{ST}/(1-F_{ST})$ or the R_{ST} equivalent) (Ream 2002).

To examine the recent population history of northern fur seals, Dickerson *et al.* (2010) used Arlequin (Excoffier and Lischer 2010) to generate a minimum-spanning network (see methods employed in this thesis, Section 4.5). This network showed three maternal lineages; however, these lineages did not have a geographic correlation, with haplotypes of each lineage mixed amongst the islands. The network pattern was 'star-like,' which is an indicator of a fairly recent population expansion. The authors suggest that this may be due to a recolonization of northern fur seals. Evidence for sudden population expansion was suggested by the unimodal nucleotide mismatch frequency distribution (determined using DnaSP 5, Rozas *et al.* 2003). Finally, past population sizes were estimated using a Bayesian skyline plot created in BEAST. This plot indicates a population increase around 11,000 BP following the last glacial retreat, with a decrease starting around 2,000 BP. Like Burton *et al.*'s (2001) isotopic study, Dickerson *et al.* (2010) hypothesize that this population decrease may be the result of hunting by coastal human (First Nations) populations after 2,000 BP. The Bayesian skyline plot also suggests a very recent increase in population size, likely the result of recovery from commercial harvests.

The only study to employ similar population genetics approaches on ancient northern fur seal was Pinsky *et al.* (2010). This study used 40 ancient remains and compared these with modern samples of northern fur seal. This study used mitochondrial DNA D-loop and *cytochrome b* sequence data. Statistics such as haplotype diversity, nucleotide diversity, AMOVA and pairwise F_{ST} analysis was conducted in Arlequin. Additionally, Bayesian methods were used (Approximate Bayesian Computation framework) to estimate population densities and dispersal rates. The AMOVA analysis found no significant partitioning of variance overall; nor any significant pairwise differences when different regional groupings were considered. Thus, the only study to look at the partitioning of variance in ancient northern fur seals supports a condition close to panmixia rather than structure reflecting population subdivision.

The research to date provides little evidence to support population structure/evidence of subpopulations in northern fur seal populations. Despite both modern and ancient DNA data indicating a high level of genetic diversity in both the past

and contemporary northern furs seal populations, there is little evidence of population genetic structure for either. Thus far, the greatest support for structure comes from the control region analysis by Dickerson *et al.* (2001) which showed low level differentiation in some island level comparisons for modern northern fur seal. The lack of population structure (in either ancient or modern populations) might be considered surprising given the high philopatry of both male and female northern fur seals to return to natal islands for breeding and the geographic separation of northern fur seal populations/breeding sites. This lack of evidence may be the result of an insufficient number of ancient sequences being included in the analysis, or the result of the short fragment length analyzed. Alternative explanations for this may include high gene-flow between hypothetical residential populations and migratory populations (Gifford-Gonzalez 2011; Holsinger and Weir 2009; Matthee *et al.* 2005; McMillan and Bermingham 1996; Morjan and Rieseberg 2004). It is also possible that there has not been enough time for population separation to show at the level of genetic analysis used. This general lack of population structure/population subdivision could also be explained by the processes of modern gene flow and/or high migration rates (including expansion after the retreat of glaciation, post-harvest expansion, and modern migration) (Dickerson *et al.* 2010; Newsome *et al*. 2007; Pinsky *et al.* 2010). Dickerson *et al.* (2010) additionally suggest that male-based genetic dispersal could contribute to this pattern. These processes of gene flow could be sufficient to homogenize the population and generate the overall lack of population structure observed. Additional data and expanded analyses, especially on archaeological remains, may help to elucidate any population structure, particularly the inclusion of additional microsatellite markers, longer mitochondrial DNA fragments, and more northern fur seal sequences. The fairly high levels of haplotype diversity and moderate nucleotide diversity suggest fairly recent population expansion with a number of closely related lineages (Dickerson *et al.* 2010; Ream 2002), while the archaeological data (though weak due to low sample size) suggests higher diversity in past populations compared to present ones (Moss *et al.* 2006).

2.6. Summary

Though *Callorhinus ursinus* is taxonomically grouped with other fur seals in the sub-family Arctocephalinae, it may form a completely separate taxonomic group, perhaps basal to the sea lion/fur seal split 6 million years ago which was followed by an evolutionary rapid radiation. Following the end of the Wisconsin glaciation, northern fur seals appear to have had a rapid population expansion, colonizing the new territory. They appear to have become the dominant pinniped in most faunal assemblages along the Northwest Coast of North America from southern California to the Aleutian Islands. Archaeofaunal and isotopic analysis suggest that many of these fur seals may have been non-migratory, a pattern that starkly contrasts with the dominant pattern of northern fur seals today. The predicted presence of rookeries and presumed year-round occupation in more temperate latitudes in the past, and the high philopatry of northern fur seals would potentially allow for some level of population structure among ancient populations. However, there is little evidence of population structure/genetic population subdivision today. Given the devastating population bottlenecks and the complete extirpation of some northern fur seal colonies, particularly those due to commercial sealing, this lack of structure/subdivision is not surprising as high migration as a function of recolonization tends to make populations genetically more similar (Wynen *et al.* 2000). In addition, the recolonization of San Miguel Island may also have been too recent to allow for structure at this level of analysis. Thus, in order to gain insight into the genetics of northern fur seal in the past, the remains of archaeological northern fur seal need to be considered.

Only a few studies thus far have been conducted specifically looking at the population genetics of ancient northern fur seal using aDNA (Newsome *et al.* 2007; Pinsky *et al.* 2010).[8] These studies seem to indicate a high level of genetic variation in northern fur seals. Of course there are challenges associated with ancient, historic and modern sample sets that make further study of this question important. There is little

[8] Of these two studies, only Pinsky *et al.* 2010 assessed the partitioning of diversity. Newsome *et al.* (2007) was limited to assessment of the statistical value of Hd, or 'haplotype diversity' (see section 4.5.2) and clustering within phylogenetic trees. This thesis seeks to apply population genetics methods to the unpublished sequence data from Moss *et al.* (2006).

evidence of population structure/population subdivision and it may be that methods with greater analytical power need to be considered, such as the inclusion of more samples/sequences, other methods of analysis, and the selection of targets that may show more variation. This study will attempt to use an additional number of analytical methods and the inclusion of additional data sequences to look at the population genetics of northern fur seal over time.

Chapter 3.

Analysis of Genetic Data: Phylogenetics and Population Genetics

3.1. Introduction

DNA research can allow insight into phylogenetics; the genetic relationships between populations, species, and genes; population genetics; and phylogeography, the study of the geographical distribution of these through time and the mechanisms by which this distribution occurs. Phylogenetics is most concerned with the relationships of species to one another, while population genetics is more concerned with genetic variation within species. There are many methods available to look for these patterns in DNA including phylogenetic trees or networks, cluster analyses (such as principal component analysis), genetic distance statistics or population statistics (e.g. F_{ST} or F_{IT}), and simulation analyses (e.g. models of population continuity) (Kaestle and Horsburgh 2002). By examining ancient DNA, it is possible to look at the direct evidence of the relationships of past species and populations (Pääbo *et al.* 1989).

This section will begin by discussing some fundamental evolutionary concepts and will then examine some tree-building/phylogenetic and population genetics methods. The sub-section on tree-building methods will provide an overview of several of the common methods and models used (such as neighbour-joining, maximum likelihood, maximum parsimony, and Bayesian approaches) that can be sorted under a few broad approaches to classification. Tree-building methods can be classified as cladistic vs. phenetic; distance vs. character-state, and clustering vs. search methods. The sub-section on population genetics will summarize the basic approaches in this field (which is typically based on diploid genetic data), and then will focus more specifically on

approaches that can be used for mitochondrial DNA, which is the subject of study in this thesis.

3.2. Evolutionary Concepts

This thesis relies heavily on evolutionary concepts for both phylogenetics used for species identification and population genetics to look at the diversity of northern fur seal populations. When discussing phylogenetics, population genetics, or phylogeography, it is essential to have a basic understanding of these evolutionary concepts. Evolution is, at its core, 'descent with modification' (Hall and Hallgrimsson 2008). Current views on evolutionary theory are based on 'modern synthesis' which was developed in order to reconcile two schools of thought: Darwinian selection and Mendelian genetics (Weber 2011). Modern synthesis was designed to help explain both macroevolutionary (the large species changes seen in the fossil record) and microevolutionary (changes at the population level) patterns (Delisle 2009; Hall and Hallgrimsson 2008; Weber 2011). Modern synthesis envisions natural selection as changes in gene frequencies in populations that can arise from natural selection or genetic drift. The changes in gene frequencies can be caused by two main mechanisms leading to species differentiation (speciation) – by natural selection or by random chance ('genetic drift') – when reproductive barriers are in place (Reece *et al.* 2013). These reproductive barriers limit gene flow and allow for the accumulation of genetic differences, such as changes in allele frequencies and the appearance and dissemination of new mutations between populations.

The occurrence of mutations is a random process (Russell 2006; Templeton 2006). Specifically, a 'gene' refers to a segment of DNA that 'codes' for a product, such as a polypeptide chain or a sequence of RNA (Hall and Hallgrimsson 2008; Russell 2006). Mutations can result in alternative forms of a single gene. These different forms of a gene residing at the same locus, and thus affecting the same feature, are called 'alleles' (Hall and Hallgrimsson 2008; Russell 2006). The existence of multiple alleles in a group of individuals is a genetic variability known as 'allelic polymorphism' (Templeton 2006). It is these alleles, and the changes in their frequencies due to natural selection or genetic drift, that are the basis for modern synthesis.

The theory of natural selection relies on a few key concepts. These concepts are: 1) that DNA has the ability to replicate, and 2) that mutations can occur. Because of the existence of mutations, different phenotypes will exist and natural selection posits that these different phenotypes can result in differences in reproductive fitness. The differences in reproductive fitness can then affect the transmission of genes from one generation to the next (Templeton 2006). Natural selection cannot act directly on the genotype. The interaction of genes with the environment creates the phenotypic variation on which selection can act (Reece *et al.* 2013; Templeton 2006). There are several constraints on natural selection, including that it can only act on existing variation, and by historical structures or limitations of other elements required for survival and reproduction (Reece *et al.* 2013).

Genetic drift occurs when allelic frequencies are altered or fluctuate by random chance (Holsinger and Weir 2009; Reece *et al.* 2013). Allele frequencies can undergo fluctuations over time which can lead to the loss of some alleles and the fixation of others due to the chance events inherent in survival and reproduction (Reece *et al.* 2013). Genetic drift has a much stronger effect in small populations, such as under bottleneck and founder effect conditions (Beebee and Rowe 2008). Examining changes in the relative frequencies of these alleles is one of the common ways that population genetics is studied.

However, not all mutations occur in the coding regions of the DNA, and some methods of studying genetic relationships do not distinguish between gene and non-coding regions. For instance, one of the commonly studied regions of mitochondrial DNA is the D-loop which, because it is non-coding, is not affected by natural selection and thus is able to accumulate mutations faster. The inclusion of non-coding areas of the genome in the study of phylogeny and population genetics leads to the use of more generic terms. The term ‘polymorphism’ refers to the “presence of two or more genetic or phenotypic variants in a population” (Hall and Hallgrimsson 2008, p. 608). This can be anything from a point mutation (i.e. a single-nucleotide polymorphism or SNP), a duplication or deletion of a repetitive element (such as microsatellites or minisatellites), or a mutation that results in a change to an enzyme cleavage site (such as restriction

fragment length polymorphism or RFLP) (Allendorf *et al.* 2012). The specific type of polymorphism being used depends on the method of analyzing genetic diversity.

The study of genetic differentiation/speciation uses the various types of genetic data (i.e. SNP, microsatellite, RFLP) by analyzing them with phylogenetic trees/networks and using population genetics methods. The following sub-sections discuss some of the methods used to investigate phylogenies and generate phylogenetic trees, and to examine population genetics/phylogeography.

3.3. Networks and Trees

Phylogenetic networks and trees are both able to show the genetic relationships between sequences. Both work on the same principles; however, networks are able to show 'cycles' or alternative potential phylogenies (Bandelt *et al.* 1999; Huson *et al.* 2010; Kaestle and Horsburgh 2002). This different presentation can allow for different factors to be assessed. Trees are more often used for phylogenies, or the relationship between species (Arnason *et al.* 2006; Sorenson *et al.* 1999), while networks are frequently used to show intra-species relationships between haplotypes (Bailey *et al.* 1996; Jansen *et al.* 2002; McMillan and Bermingham 1996). Trees typically have a bifurcating pattern that mimics speciation, while networks allow for clear visualization of other types of relationships, such as starlike radiations and multiple possible evolutionary relationships that are common with intra-population analyses. For instance, Bollongino *et al.* (2006) provided a series of phylogenetic networks which very clearly showed the change in haplotypes in cattle over time. Despite these common uses, both trees and networks may be used for both inter- and intra-specific relationships (Bruford *et al.* 2003; Kistler and Shapiro 2011; Larson *et al.* 2007).

3.3.1. Tree-building

Ancient DNA can allow for the examination of phylogenies of extinct animals and species, and using trees and networks are essential to this process. There are a number of approaches that can be utilized to determine how organisms are related to one another, with the most common methods resulting in phylogenetic trees. A phylogenetic

tree is a representation of an 'evolutionary hypothesis'; it is created with the premise that the tree will reflect evolutionary relatedness (Higgs and Attwood 2005; Kaestle and Horsburgh 2002). There are a number of ways that phylogenetic trees can differ. They can differ by general approach, the type of algorithm used, and the model being employed (including the different underlying assumptions of each model). There are also many different models for tree-building and data analysis that affect how the data is analyzed, such as various formulas that can be used to compare the relatedness of members or the use of different substitution models which can account for differences in the rates at which various transitions and transversions occur (Kaestle and Horsburgh 2000). This sub-section examines typical methods for categorizing tree-building methods, and then examines the features of the most typical approaches, including their merits and limitations, and concludes with some examples of these approaches.

Categorizing Tree-building Methods

There are three main approaches to categorizing tree-building methods: phenetic versus cladistics, distance versus character-state, and clustering versus search trees. The first is based on basic methods of determining relationships: phenetics or cladistics. For phylogenetics, however, the division of phenetics versus cladistics is not always the most useful categorization because phylogenetic methods are not always reflective of the phonetic and cladistics division. Distance versus character-state based methods are often considered to be a better classification for phylogenetic approaches (Kaestle and Horsburgh 2002; Opperdoes 1997). The third main categorization is whether the trees are built by clustering methods or by search methods.

Phenetic and cladistics methods are the two basic approaches to determining relationships. Phenetic methods group based on similarity, typically using morphological characteristics (Hall and Hallgrimsson 2008; Higgs and Attwood 2005), while cladistics (which are more commonly used for genetic data) use shared derived characters, or 'synapomorphies', to infer common ancestry (Hall and Hallgrimsson 2008; Reece *et al.* 2013). When the characters considered are genetic, the study of these relationships is called phylogenetics. In phylogenetics, cladistics are the more common approach; however, some phylogenetic analyses do apply some phenetic approaches, with neighbour-joining trees as the classic example (Hall and Hallgrimsson 2008; Higgs and

Attwood 2005). The terms phylogenetic tree and cladogram are often used interchangeably, though the latter term may be used to suggest a hypothesis, while phylogenies are often used to imply a 'true evolutionary history' (Beebee and Rowe 2008). In a phylogeny, the branch lengths are often scaled to represent the degree of character change. For the purposes of this thesis, the term 'phylogenetic tree' will conform to the common definition of an evolutionary hypothesis and will cover all branching diagrams generated from genetic data showing inferred relationships (including trees generated by cladistics and phenetic methods). However, as mentioned previously, not all approaches to tree-building are best characterized in this way either, particularly methods often employed for phylogenetics, so other categorizations are also useful (Holder and Lewis 2003; Kaestle and Horsburgh 2002; Opperdoes 1997).

Another broad categorization that is often applied to phylogenetic tree-building methods is whether the approach is character-state or distance-based. Character-state based trees rely on specific sequence data, while distance-based methods compress this data into pairwise distances, where some data is lost but the processing time is also reduced (Kaestle and Horsburgh 2002). The phenetic Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and neighbour-joining methods are both distance-based, while the cladistics method of maximum parsimony is considered a character-based method (Higgs and Attwood 2005; Opperdoes 1997). The maximum likelihood method uses the entire sequence data and thus is free from some of the limitations affecting the other two approaches (Opperdoes 1997).

Finally, the third categorization separates those trees that are singularly constructed from the data by clustering algorithms versus those search approaches where a number of possible trees are compared for the best fit. Clustering methods generate a single tree, while search methods involve the construction of multiple trees which are then searched for the best fit with the data according to various optimality criteria (as in trees built on parsimony or likelihood methods) (Kaestle and Horsburgh 2000). The methods that search the 'tree space' for the optimal tree can be divided into those using an optimality criterion (parsimony and likelihood are the most common) and those that take a Bayesian approach (Holder and Lewis 2003). Distance methods of tree-building include neighbour-joining (NJ) and UPGMA. These two methods are based

on calculations of the pair-wise differences in the multiple alignments and generate a single tree using clustering algorithms (Higgs and Attwood 2005; Holder and Lewis 2003). For those using an optimality criterion, there are a number of different criteria that may be used, including maximum likelihood, maximum parsimony, and minimum evolution (Hall and Hallgrimsson 2008; Holder and Lewis 2003). The selected optimality criterion is used to 'score' possible trees to search for the 'correct' one (Kaestle and Horsburgh 2002). For instance, when the optimality criterion is parsimony, the parsimonious tree (i.e. the one that is based on the fewest evolutionary events) will be selected. Related to this are Bayesian approaches where tree space is searched for the correct tree using posterior probabilities (Holder and Lewis 2003).

Common Approaches

Some of the most common approaches to tree-building are neighbour-joining, maximum parsimony, maximum likelihood, and Bayesian statistics methods. Each method processes the available data differently. This section will describe the general features of each of these common methods. The neighbour-joining method is a distance-based method which groups species by similarity of evolutionary distances (Hall and Hallgrimsson 2008; Holder and Lewis 2003). It tends to be a relatively fast method of analysis and is thus often used as a starting point for more in-depth analyses (Hall and Hallgrimsson 2008; Holder and Lewis 2003). Because the sequences are grouped based on similarity, this is a phenetic method of analysis (Leht and Jaaska 2002). This method can apply different models that weigh various factors differently. For instance, the Jukes-Cantor model operates under the assumption that mutation frequencies of all four types of bases are equal, while the Kimura 2 parameter model takes into account the differences between transitions and transversions (Hall and Hallgrimsson 2008). The default model for neighbour-joining on MEGA6 program is the maximum composite likelihood model (Tamura and Nei 1993). This model assumes that substitution patterns are equal among lineages as well as equal substitution rates between sites (http://www.megasoftware.net). It includes consideration of differences in the mutation rates of purines and pyrimidines; it is thus useful under situations of high mutation rates and non-coding areas where the Kimura-2-parameter model may underestimate the ratio of transitions to transversions (http://www.megasoftware.net). For the purposes of the analyses to be conducted in this thesis, either the Jukes-Cantor or the maximum

composite likelihood method should be acceptable models to use. This is especially true for the D-loop region, where high mutation rates are expected.

The maximum parsimony (MP) method is a cladistic, character state-based method that is based upon the informative differences (shared derived characters, or synapomorphies) between the different sequences (Hall and Hallgrimsson 2008; Higgs and Attwood 2005; Leht and Jaaska 2002; Opperdoes 1997). The optimality criterion for this method is parsimony, which assumes that the correct tree is the one with the minimum number of mutations producing the sequences observed (Hall and Hallgrimsson 2008). Thus, for maximum parsimony this optimal tree is the one with the fewest mutations (Reece *et al.* 2013).

The maximum likelihood (ML) method compares multiple pathways and works under the optimality criterion of choosing the tree which is most likely, given a specific set of parameters (Hall and Hallgrimsson 2008; Holder and Lewis 2003). This method is a statistical phenetic method (Opperdoes 1997). That is, it compares the probability distribution and maximizes the most likely one, taking into account parameters such as the base frequencies and the transition/transversion ratio (Hall and Hallgrimsson 2008). Likelihood methods choose the hypothesis/tree with the highest probability of having produced the DNA data, given certain probability rules and how DNA changes over time. Thus, for maximum likelihood trees, the tree with the highest probability of generating the observed sequences is chosen, and calculations of this probability may take many different factors into account, such as the various substitution models which can account for differences in the rates at which various transitions and transversions occur (Holder and Lewis 2003; Kaestle and Horsburgh 2002; Reece *et al.* 2013).

To test the reliability of the trees generated by clustering algorithms or those using an optimality criterion, a method of using randomized subsets of data called 'bootstrapping' can be performed[9] (Higgs and Attwood 2005). The bootstrap values, as given on the trees, can provide some indication of which branches appear to be more

[9] Minimum of at least 500 bootstrap replicates is standard.

strongly supported. Larger bootstrap values indicate that lineages group together a larger percentage of the time.

A new method of tree-building is also coming into use; this method is based on Bayesian statistics and prior probabilities using other known information. Bayesian phylogenetic trees are designed to maximize the posterior probability, which is dependent upon the likelihood calculations and prior probability, both of which are based upon other knowledge or previous experiments (Holder and Lewis 2003). This method is one which may be employed in future studies on this thesis material.

Choosing a Tree-building Method

There is much debate on which method of tree-building should be employed (maximum likelihood, parsimony and Bayesian approaches are the most popular), though there seems to be no solid rule for identifying the 'best method' (Kolaczkowski and Thornton 2004; Sanderson and Kim 2000). Different models can lead to different results and careful consideration must be given to the model used as this is critical to how results are interpreted. There are a number of considerations that may affect how useful a particular tree-building method may be.

When examining phylogenetic data, a number of parameters can affect the results. One of the most important parameters is the choice of analytical model as different models may indicate different levels of relatedness between the organisms considered. One example of different analytical models leading to different results is that of the human Neanderthal divergence[10] where various studies obtained different results. The importance of choosing which model to use was also noted by Weaver and Roseman (2005) who suggested that the result of a previous study considering Neanderthal mtDNA within the range of modern human variation was an artefact of the bootstrapping process. Because of the numerous analytical models which may be

[10] Gutiérrez *et al.* (2002) recommends a change in several of the parameters, such as the model of substitution and accounting for varying substitution rates, in order to generate a more accurate model of human and Neanderthal divergence. Varying parameters may help to avoid long-branch attraction, which occurs where two lineages appear to be more similar to one another than to more related groups due to a large number of polymorphic changes.

selected, even when the exact same genetic data is considered, there may be variations in the supported conclusions between studies.

Using maximum parsimony tends to be less accurate than the neighbour-joining and maximum likelihood methods when there is fast evolution and unequal evolutionary rates. Parsimony methods often do not work well and may even generate incorrect trees when there is substantial homoplasy (similarity resulting from convergence rather than shared ancestry), which more commonly occurs in rapidly evolving characters (Graur and Li 2000). Different mutation rates between branches or considerable variation in the branch lengths also tend to skew the analyses for parsimony methods (Holder and Lewis 2003; Kolaczkowski and Thornton 2004; Kuhner and Felsenstein 1994). Similarly, when there are long genetic distances between the taxa under consideration, 'long branch attraction' can make distantly related groups seem more similar (Graur and Li 2000). This long-branch attraction happens when groups accumulate a large number of polymorphic changes between the two lineages, which can result in the two branches beginning to appear more similar to one another than they are to more related groups. This was suggested as a possibility by Gutiérrez *et al.* (2000) who commented that some models of human and Neanderthal divergence could be improved by changing the model of substitution to mitigate this problem.

Neighbour-joining methods run the risk of losing information as the sequences are compressed into genetic distances. For instance, conditions such as having multiple mutations at the same site can result in data loss when character-state data is compressed into genetic distances between sequences (Holder and Lewis 2003). The loss of data can obscure relationships, a factor to consider when using this method (Holder and Lewis 2003). In addition, it has been noted that accurate estimates of pairwise distances can be difficult to obtain when sequences are fairly divergent (Holder and Lewis 2003). Overall maximum likelihood methods tend to perform better than other tree-building methods; however, this may not always be the case for very short distances (where neighbour-joining may be better) (Kuhner and Felsenstein 1994). One major problem with maximum likelihood methods is that there may be difficulty in determining if the method has run long enough to find the best tree since it searches at random for better pairings (Holder and Lewis 2003). In addition, bootstrap values are not

definitive proof that a phylogeny is good since it is possible to have a high bootstrap value for an incorrect node if the model of evolution is poor (Higgs and Attwood 2005).

Employing multiple phylogenetic tree-building and network-building methods can provide a way of overcoming some of the limitations of using single approaches by validating the findings through cross-comparison. There have been several studies which have utilized multiple methods. In three studies on cave bears, Hofreiter *et al.* (2002) used maximum likelihood, neighbour-joining, and Bayesian approaches; Orlando *et al.* (2002) used both distance and parsimony methods, while Loreille, Orlando *et al.* (2001) used neighbour-joining trees with Kimura-2-parameter models. All of these findings supported a monophyletic cave bear phylogeny with the polar bear clades nested within the brown bear lineages. In examining the phylogenetic relationship of moas, both Baker *et al.* (2005) and Huynen *et al.* (2003) used various methods such as neighbour-joining, maximum parsimony, maximum likelihood, and Bayesian approaches. Van Der Kuyl *et al.* (1995) used a neighbour-joining tree to show the relationships of primate groups, while Bunce *et al.* (2005) used both maximum likelihood and Bayesian approaches to assess how the extinct Haast's eagle was related to modern eagles (also using multiple genes).

This thesis will utilize a number of tree-building methods. To begin, neighbour-joining trees will be used because this method is relatively fast and thus a good starting point for analysis. Though data can often be lost using this method, it has been shown to be useful when genetic distances are short (Holder and Lewis 2003; Kuhner and Felsenstein 1994). This then seems a good method to use for the more recent divergence of the pinniped lineage and for intra-species differences. This method was used in conjunction with the maximum composite likelihood model of substitution since this model is useful with fast mutation rates, such as one would expect from the D-loop region. Next, since maximum likelihood trees generally tend to perform better than other tree-building methods, this method will be used as well. This method of tree-building was used with the Jukes-Cantor substitution model, the Tamura and Nei model, and the Kimura-2-parameter model as these are some of the more commonly used substitution models. As mentioned above, there are differences between true phylogenies of species and trees generated from one or a few genes. An important point to remember is that

gene divergence is not the same as species divergence. Events such as hybridization polymorphism in the ancestral state (which can become fixed differently in different lineages) and homoplasy can all contribute to differences in the phylogenies (Arnason *et al.* 1996; Patterson *et al.* 2006). Thus, while gene divergence usually precedes species divergence, certain factors can obscure these relationships. Because of this, it is also useful to consider more than one gene, as different genes may have different rates of nucleotide substitution and may result in different trees (Graur and Li 2000; Reece *et al.* 2013).

This thesis utilized trees and networks for a variety of purposes. Trees were used for both species sample identity confirmation (Section 5.2), phylogeny testing (Appendix C), and were also employed to specifically look at the relationship between haplotypes (Section 5.3).

3.3.2. Population Genetics and Phylogeography

Population genetics is the study of genetic variation within a species and its amount, distribution, and change over space and time (Templeton 2006). The term 'population' is a fluid one that can be used for everything from a single group of locally breeding individuals, to encompassing a number of groups over the landscape that occasionally have gene flow between them. Local population (or deme) is used to refer to a group of individuals in geographic proximity who comprise a breeding population (Templeton 2006). The term 'local' needs to be treated carefully since different sampling locations do not necessarily indicate different populations. For instance, if the 'similarities (such as in a pairwise analysis) indicate no statistical difference, this may suggest that all samples are from a single, large, interbreeding population (Wasser *et al.* 2004). The 'gene pool' is used to refer to all the genetic information shared by this population. It can also be used to refer to all the potential gametes and thus the potential genetic information that can be passed on to the next generation. Quantitatively, evolution/microevolution examines changes in the frequencies of genes or gene combinations in the population of study (Templeton 2006).

Given the definition of population above, 'population structure' then refers to the means by which genes are passed onto the next generation, including factors such as genetic exchange between populations, population size, and whether the population shows random mating (Templeton 2006). The main goal of analyses on population structure is to look for population substructure or population subdivision (Miller *et al.* 2011; Pritchard *et al.* 2000). Thus, despite this broad definition, effectively the term 'structure' is used by many in the field of population genetics to refer to barriers or limitations to gene flow; this includes the resulting formation of local subpopulations (Speller *et al.* 2012; Wynen *et al.* 2000). That is, a population showing evidence of structure, therefore, shows evidence of population subdivision.

For this thesis, the premise that northern fur seals exhibit high philopatry, returning to natal islands to breed, and the archaeological evidence of breeding colonies along the coast lead to the expectation that breeding between potential rookeries may be limited, thereby allowing for accumulation of genetic change. Since mtDNA is haploid, it has a smaller effective population size than nuclear markers and can show genetic drift more rapidly, though selection may make this pattern less visible (Beebee and Rowe 2008). This leads to the hypothesis that ancient northern fur seal populations would exhibit genetic evidence of subpopulations/show evidence of some level of structure that indicates inter-breeding between these separate colonies is limited. The null hypothesis tested in this thesis is that there is no evidence of structure/population subdivision.

Underlying Assumptions and Deviations from Hard-Weinberg Equilibrium

Most common approaches to population genetics use the Hardy-Weinberg model. This model examines heterozygosity in a population and therefore requires diploid data (such as nuDNA). It uses a null hypothesis which assumes conditions of no evolution to test if evolutionary forces are at work (Reece *et al.* 2013). It requires the fundamental assumptions that an 'infinite' (or extremely large) population is operating under a system of random mating, that segregation of parental alleles is random, that there is no population structure nor gene flow in or out of the population, and that there are no mutations or natural selection (Reece *et al.* 2013; Templeton 2006). The null hypothesis works as non-random mating is expected to affect the frequencies of homozygotes/heterozygotes, but not the overall allele frequencies (Reece *et al.* 2013).

Deviations from Hardy-Weinberg equilibrium indicate that some kind of evolutionary mechanism is at work such as natural selection, genetic drift, or gene flow. Natural selection will result in the favouring of some alleles over others; genetic drift is stronger in smaller populations and can lead to the fixation of harmful alleles; and gene flow tends to reduce the genetic differences between populations (Reece *et al.* 2013). One commonly used method, F-statistics, applies this model.

Wright's F-Statistics

Wright's F-statistics, including F_{ST}, can be used to describe how the variance is partitioned within and between subpopulations. Whether or not the distribution of this variance is significant can also be tested by looking at random distributions of the samples (Beebee and Rowe 2008; Excoffier *et al.* 1992). One such method of assessing the distribution of variance is an analysis of molecular variance (AMOVA). To examine phylogeography, F_{ST} and equivalent values can be combined with data such as geographic distance and latitude to examine how the genetic variation is distributed among and between the populations studied.

Wright's F-statistics are measures originally designed to assess the neutral, biallelic loci under the island model (Rousset 1997; University of Auckland 2005; Whitlock 2011). In structured populations with limited gene flow, heterozygosity is expected to be lost at a greater rate than in panmictic populations (this is known as the Wahlund effect). The most commonly used F-statistic, F_{ST}, is the proportional loss in heterozygosity from this population structure (Kane 2011; Templeton 2006; Whitlock 2011). F_{ST} is the 'fixation index', described by the equation $F_{ST}= (H_T-H_S)/H_T$, where H_T is the expected heterozygosity in the total population and H_S is the expected heterozygosity within the subpopulation (assuming random mating) (University of Auckland 2005). F_{ST} is a standardized measure of the variance among these local subpopulations. It can also be described as $F_{ST}=Var[p]/(\bar{p} (1-\bar{p}))$, where Var[p] is the variance among local population allele frequency and $\bar{p}$ is the mean allele frequency.

This F-statistic value F_{ST} is related to the other two F-statistic values F_{IS} (the inbreeding coefficient) and F_{IT} (the overall fixation index) through the relationship (1-

F_{IT})=(1-F_{IS})(1-F_{ST}) (University of Auckland 2005). There is also a relationship between F_{ST} and number of migrants, since migration reduces F_{ST}. Together, F_{ST} and N_M (number of migrants) can be used to characterize gene flow (Rousset 1997).

Genetic drift will be expected to increase F_{ST} by increasing the differences between populations, while migration will reduce F_{ST} and the uniqueness of populations (Whitlock 2011). While mutation rates generally do not have a strong impact on F_{ST} values (Rousset 1997), comparisons of F_{ST} values across the genome can be used to identify high mutation rates/selection (Whitlock 2011). While mutation might be expected to increase the genetic difference between populations, it can actually lower the F_{ST} values through both homoplasy and by increasing the number of heterozygotes, both within and among the populations (Whitlock 2011). Since mutation rates are not equal across the genome, F_{ST} values that differ across different genetic regions can indicate high mutation rates/selection (Whitlock 2011).

F-statistics allow the comparison of among versus within population diversity, as well as population diversity between specific populations (using pairwise population diversity F-statistics). With the first, results are interpreted by comparing the amount of diversity with assigned sub-groups (or 'subpopulations') to that of the entire regional/'population' sample (Peakall and Smouse 2012). If the 'among population' diversity is low (with high 'within population' variance), it would indicate that most of the variation observed is distributed across the range (Falk *et al.* 2001). That is, a high within but low among population diversity would represent high dispersal/high gene flow between populations. This would indicate a population close to panmixis with no population structure. Since the F_{ST} values are represented in a decimal form, an F_{ST} of 0 would therefore indicate complete panmixis (that is, 0% of the diversity is among populations) with no subdivision, while an F_{ST} of 1 (100% of the diversity is among populations) would indicate that all of the subpopulations are completely separate with no shared genetic diversity/no gene flow, and therefore all of the variation observed is attributable to this population structure (Figure 3.1).

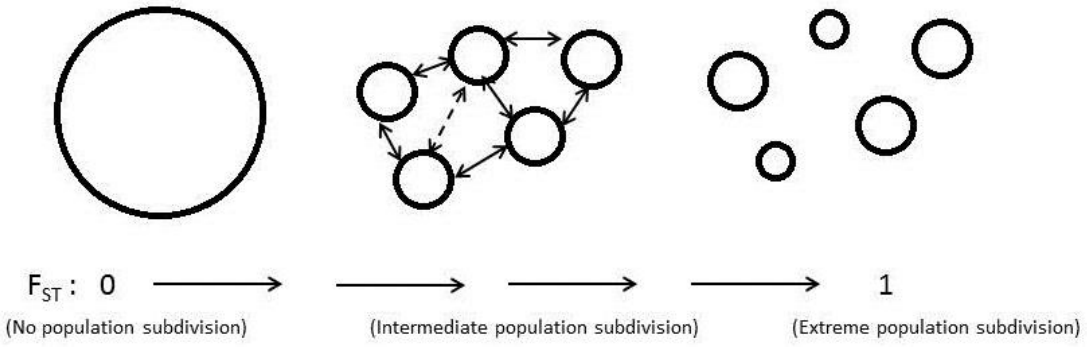


Figure 3.1. Illustration of the range of population subdivision.
Population subdivision ranges from a panmictic population with no population subdivision (F_{ST} of 0, with no 'among population' variance) to a population with an F_{ST} of 1 (100% 'among population' variance), representing a population with extreme subdivision where all differences can be explained by this population subdivision.

F_{ST} is designed to examine 'structure' in diploid populations and is based on measures of heterozygote frequency relative to that expected under conditions of Hardy-Weinberg (Holsinger and Weir 2009). Unfortunately, due to the mechanism of inheritance for mitochondrial genes, where only the maternal contribution is passed on and where all individuals are haploid, other methods of analyses are needed for interpretation of mtDNA data, such as D-loop haplotypes. For these calculations, analogous measures may be employed such as Φ-statistics (i.e. PhiPT or Φ_{ST}) (Excoffier *et al.* 1992; Peakall and Smouse 2012). The AMOVA analysis, for example, was specifically designed for haplotype frequencies (Michalakis and Excoffier 1996).

F_{ST} was originally designed for a single biallelic neutral locus; as a result, other versions of this measure may be used for other situations (University of Auckland 2005). For such calculations involving haploid data, F_{ST} can be considered as a measure of haplotype diversity, as opposed to heterozygosity (University of Auckland 2005). This is because both haplotype diversity and heterozygosity are calculated using the equation $1-\Sigma p_i^2$ where p is the haplotype frequency (for haplotype diversity) or the frequency of each allele (for heterozygosity with more than 2 alleles) (University of Auckland 2005). Similar measures of F_{ST} can utilize nucleotide diversity (Nei 1987; Nei and Tajima 1981) to generate F_{ST} equivalents using these values (University of Auckland 2005). These

values include γ_{ST}, and Φ_{ST}, among others. γ_{ST} refers to the proportion of interpopulational gene diversity using the equation $\gamma_{ST}=\delta_{ST}/\pi_T$, where π_T is the total nucleotide diversity (equivalent to H_T) and δ_{ST} (equivalent to 'D_{ST}') is the gene diversity between populations (Nei 1982). Φ-statistics are an analogue of F-statistics often used with AMOVA analyses (Excoffier *et al.* 1992). For nucleotide diversity and Φ_{ST}, the equation $(\pi_T-\pi_S)/\pi_T=\pi_B/\pi_T$ can be used, where π_T is the nucleotide diversity for the entire population, π_S is the average nucleotide diversity within subpopulations, and π_B is the average nucleotide diversity between subpopulations (University of Auckland 2005). The difference between F_{ST} and Φ_{ST} results in different implications for calculating and interpreting data: F_{ST} assumes equal distances between alleles, while Φ_{ST} includes the fact that alleles have different distances from one another, and that as a result of the differences F_{ST} is more likely to first show population differentiation following population subdivision (University of Auckland 2005). Other similar values may be used under other situations. For instance, one version of F_{ST} calculations is an equivalent value which is designed to be used under conditions of multiple alleles (sometimes specifically designated as G_{ST}). This G_{ST} value uses both the heterozygosity within populations and the heterozygosity of all populations as if they were under Hardy-Weinberg frequencies (Whitlock 2011). Values of diversity commonly used include F_{ST}, ΦST, γST and G_{ST} (described above) as well as D, and R_{ST}; each of which takes into account different factors.

3.4. Summary

This chapter discussed methods involved in generating phylogenetic trees and methods employed in the study of population genetics. This discussion is important as phylogenetic trees will be used in this thesis to confirm species identity. As mentioned in the section on taxonomic classification, the otariids are a group with an unresolved phylogeny, as different studies have found different relationships between species and have suggested that the modern taxonomy based on morphology may not be reflective of genetic relationships. An understanding of population genetics and phylogeography is essential to understanding whether there is evidence of structure/subdivision in the population.

Chapter 4.

Materials and Methods

4.1. Introduction

This thesis is based on unpublished sequence data for northern fur seal remains from three archaeological sites that were obtained and sequenced by Moss *et al.* (2006). Comparing this data to previously published ancient and modern sequence data, this thesis seeks to further investigate the genetic diversity and evidence for population genetic structure dynamics in northern fur seals. This extends the previous analysis of sequence data obtained by Moss *et al.* (2006) by using additional analytical methods to calculate values of genetic diversity, as well as a larger set of sequences for comparison with additional data from GenBank (Benson *et al.* 2008). This chapter provides a summary of the methods used by these researchers to prepare and extract the data, as well as the methods used in this thesis to contribute to the discussion of northern fur seal phylogeny and population structure. The first section will provide a review of the relevant archaeological and methodological information for the two sources of genetic data. This will include an overview of the site and sample data available, and a summary of the DNA extraction, amplification and sequencing methods employed by Moss *et al.* (2006). The second section will then discuss the methods employed in this thesis to analyze this genetic data. This will begin with a discussion on potential ways to group the small sample sets into meaningful sets given the problems that can arise from small sample sizes. This will be followed by a description of the methods employed for sequence analysis to investigate the phylogeny and population genetics.

4.2. Source Data

The data for this thesis is the previously unpublished DNA sequences from archaeological northern fur seal remains that were sequenced by Moss *et al.* (2006). This chromatogram data are derived from previously sequenced archaeological remains from three sites that had been previously analyzed in the Ancient DNA Lab at Simon Fraser University (SFU), Burnaby, BC (Moss *et al.* 2006). This data represented two mtDNA regions: a portion of the coding *cytochrome b* gene and a portion of the non-coding D-loop region (Table 4.1). This data was compared with previously published modern and ancient northern fur seal sequences from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (Benson *et al.* 2008).

The archaeological remains used to generate the chromatogram data (used as primary data in thesis) came from three sites excavated by Moss *et al.* (2006) (see Table 4.1). The first site, Cape Addington, Rockshelter, Alaska (49-CRG-188), is located on Noyes Island in the Prince of Wales Archipelago (dated to cal AD 50-1680). The second site is the Ts'ishaa site (DfSi-16) on Barkley Sound, Vancouver Island, British Columbia (which dates to 5320 years [cal BP], with the village sites dating between AD 80-1700). At this site, 250 bones were identified as northern fur seal. Of these, 31 bones were recovered from the older region of the site, while the majority of the bones were found in the more recent village occupation (Moss *et al.* 2006). Archaeological evidence from this site also suggests an increase in the relative frequency of northern fur seal remains (Frederick and Crockford 2005; Moss *et al.* 2006). The third site analyzed by Moss *et al.* (2006) was the Netarts, Sandspit site (35-TI-1) in Oregon, which dates to cal AD 1300-1800. At this site, all of the sequenced samples came from village site house layers and house fill. Site locations are shown in Figure 4.1. To confirm species identity, these sequences were analyzed in this thesis (protocols described in the following sub-section, Initial Data Processing, describing the sequence alignment and generation of phylogenetic trees). Species identifications obtained from these sequences are shown in Table 4.1 (see Chapter 5 for results).

Table 4.1. List of chromatogram sequence data analyzed in this thesis and the results of species identification.

Sequences were generated from archaeological material by Moss *et al.* (2006) to provide the chromatogram data that was further analyzed in this study. Species identity was assessed using phylogenetic trees generated with *cytochrome b* and D-loop sequences; specific identifications obtained agreed with the identities determined by Moss *et al.* (2006).

Site Location	Species Identity (*CytB*)	Species Identity (D-loop)
Netarts, OR 35-Tl-1 (AD 1300-1800)		
NF1	*C. ursinus*	*C. ursinus*
NF2	*C. ursinus*	*C. ursinus*
NF3	*C. ursinus*	*C. ursinus*
NF4	*C. ursinus*	*C. ursinus*
NF5	*C. ursinus*	*C. ursinus*
NF6	*C. ursinus*	*C. ursinus*
NF7	*C. ursinus*	*C. ursinus*
NF8	*C. ursinus*	*C. ursinus*
NF9	*C. ursinus*	*C. ursinus*
NF10	*C. ursinus*	*C. ursinus*
NF11	*A. philippii/ townsendi*	*A. townsendi*
NF12	*C. ursinus*	*C. ursinus*
NF13	*E. jubatus*	*E. jubatus*
NF14	*C. ursinus*	*C. ursinus*
NF15	*C. ursinus*	*C. ursinus*
NF16	*C. ursinus*	*C. ursinus*
Ts'ishaa, BC DfSi-16 (AD 80-1700)		
NFS17	*C. ursinus*	*C. ursinus*
NFS18	*C. ursinus*	*C. ursinus*
NFS19	Unknown, basal to *Arctocephalus* and *Eumetopias jubatus*	*A. philippii*
NFS20	*C. ursinus*	*C. ursinus*
NFS21	*C. ursinus*	*C. ursinus*
NFS22	*C. ursinus*	*C. ursinus*
NFS23	*C. ursinus*	*C. ursinus*
NFS24	*C. ursinus*	*C. ursinus*
NFS25	*C. ursinus*	*C. ursinus*
NFS26	*C. ursinus*	*C. ursinus*
Cape Addington, AK 49-CRG-188 (AD50-1680)		
NF28	*E. jubatus*	*E. jubatus*
NF29	N/A	*E. jubatus*
NF30	*C. ursinus*	*C. ursinus*
NF31	*E. jubatus*	*E. jubatus*
NF32	*C. ursinus*	*C. ursinus*
NF33	*C. ursinus*	*C. ursinus*
NF34	*C. ursinus*	*C. ursinus*
NF35	*C. ursinus*	*C. ursinus*
NF36	*E. jubatus*	*E. jubatus*
NF37	*C. ursinus*	*C. ursinus*

To examine how this data set fit with broader geographic patterns, modern and ancient sequence data from both the *cytochrome b* gene and for the D-loop/control region was obtained from GenBank. For the analysis of *cytochrome b*, these additional sequences consisted of population sets from Pinsky *et al.* (2010), Winters *et al.* (2011), and Wynen *et al.* (2001).[11] Though the population set from Wynen *et al.* (2001) only includes five *C. ursinus* sequences, it also includes sequences representing eleven other otariid species. These other species were used to generate phylogenetic trees for phylogeny and species identity tests. The five sequences from Winters *et al.* (2011) were ancient sequences from Unalaska, Alaska (AK). The *cytochrome b* data set from Pinsky *et al.* (2010) represents 28 ancient sequences, though no identifiers of sample location were given.

For D-loop sequences, the data available included sequences from Dickerson *et al.* (2010), Pinsky *et al.* (2010), and Wynen *et al.* (2001). The primary source of ancient sequences from GenBank used in this thesis was Pinsky *et al.* (2010). The samples were dated (either directly or indirectly by C14 dating) to 2,450 years or less. These remains came from San Miguel Island (California), Umpqua (Oregon), Seal Rock (Oregon), Chaluka (Alaska), Ozette (Washington), and one sequence from Duncan's Point (California) (see Figure 4.1) (Pinsky *et al.* 2010). For the modern D-loop data, the primary source was also Pinsky *et al.* (2010), with 363 modern sequences from a number of sites (Bogoslof Island, Saint George Island, and St. Paul Island, Alaska; and San Miguel Island, California). Wynen *et al.* (2001) had five sequences from St. Paul Island (Alaska) that were also used.

[11] The Winters *et al.* (2011) article is presumed to be the Runnells *et al.* (2011) Genbank entry, despite neither first author name appearing in the other entry. All other authors remain the same, the article titles are identical, and the species and number of sequences match.

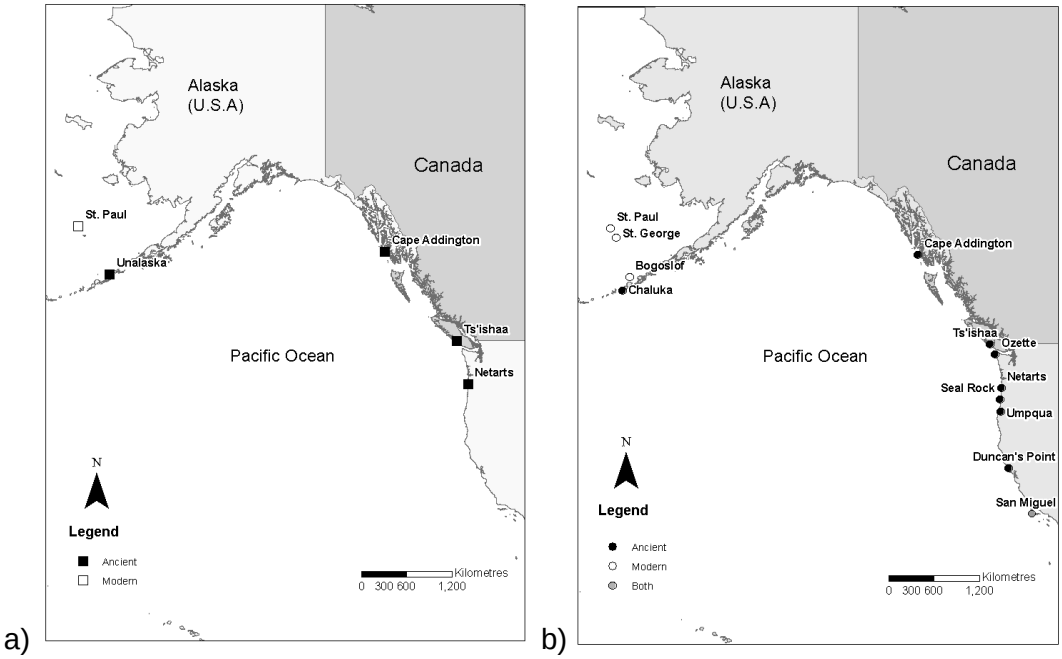


Figure 4.1. Site locations for sequence data for northern fur seal (*C. ursinus*) samples. a) *cytochrome b* b) D-loop.
Square boxes indicate sites where *cytochrome b* data was obtained, while circles indicate sites where D-loop sequence data was obtained. White markers indicate source locations for modern sequence data collection; black markers indicate archaeological sites where *C. ursinus* remains were used for DNA sequence analysis, and grey markers indicate locations where both ancient and modern sequences were obtained (Pinsky *et al.* 2010). *Cytochrome b* data derived from Moss *et al.* (2006), Winters *et al.* (2011), and Wynen *et al.* (2001). D-loop data was obtained primarily from Moss *et al.* (2006) and Pinsky *et al.* (2010) with a few sequences from Wynen *et al*. (2001). The unpublished sequence data analyzed in this thesis comes from Cape Addington, Ts'ishaa and Netarts. None of these sites were represented in the published data from GenBank (Maps courtesy of Colin Halseth).

Unfortunately, one large potential source of northern fur seal D-loop sequences from GenBank had to be excluded due to the format of the dataset. Dickerson *et al.* (2010) had 332 haplotypes[12] with data from a number of source locations (Bering Island, Bogoslof Island, Lovushki Island, Medney Island, Robben Island, San Miguel Island, St. George Island, and St. Paul Island). However, there was no information embedded in the sequence data regarding which location each haplotype came from or the frequency

[12] This number became only 273-274 haplotypes when cut to the length of 160 bp to match sequence data from Moss *et al.* (2006).

of each haplotype. That is, these 332 haplotypes actually came from a total of 619 sequences that had been analyzed, but there was no information regarding how many times each specific haplotype was represented in the dataset. Thus, because the dataset was simply a list of haplotypes, it was not useful in a population study as it was not representative of random sampling.[13]

4.3. DNA Extraction, PCR and Sequencing in Moss *et al.* (2006)

Ancient DNA analysis includes sample preparation, DNA extraction, amplification and sequencing of samples described in Table 4.1. The ancient northern fur seal remains (37 bone samples[14]) were analyzed in the Ancient DNA Laboratory at SFU, following the established protocols (Moss *et al.* 2006); for more details, also see Appendix B (Speller *et al.* 2005; Yang *et al.* 1998; Yang *et al.* 2004; Yang *et al.* 2005).

To minimize cross-contamination, DNA lab work was carried out in a dedicated ancient DNA laboratory following strict contamination control protocols. This included UV irradiation and bleaching of surfaces and equipment, positive air pressure and HEPA air filters in the pre-PCR DNA laboratory, the use of protective clothing, and physical separation of pre- and post-PCR labs in different buildings (Tuross 1994; Yang 2008).

4.4. Determination of Analytical Groupings

Given the extremely small data sets for the ancient sequence data, analyses are likely to be skewed. As population genetics analysis of DNA is strongly affected by sample sizes and sequence length, increasing both the number of nucleotides analyzed and the number of sequences can increase the opportunity for genetic differences (genetic polymorphisms) to be observed (Allendorf *et al.* 2012; Nei 1987). Due to the

[13] Since each sequence represented a different haplotype, rather than a randomly sampled individual, there was no data on how often each haplotype was represented/the frequencies of each haplotype.

[14] Of these, only 36 samples successfully amplified.

limited sample sizes for the ancient sequence sets, it may be useful to consider grouping some of the data together into larger sub-sets. As discussed above, different sample sites do not necessarily reflect different populations. However, as the evidence of local breeding sites is based on the remains of animals found at archaeological sites, the precise location of these proposed breeding sites is difficult to determine (Burton *et al.* 2001; Pinsky *et al.* 2010). In addition, with extinct populations, one cannot directly observe the movements of individuals for differences in migration, foraging, philopatry, and other movements that may affect gene flow/genetic exchange. This makes defining past populations challenging. This section will discuss the merits and detriments of various potential ways of grouping the data (particularly focused on geographic distance) to increase sample size and acquire more meaningful information for population genetics analysis. The DNA data analysis software package DnaSP v.5 (Rozas 2009) was utilized to make comparisons between potential groupings and determine whether any potential patterns emerged from the data.

Given the distances that northern fur seals may travel during breeding seasons for foraging trips and during their migration, the geographic distance from one site to another may be one useful way of grouping the smaller sample sets of data into larger, more robust, sets. Northern fur seals can travel up to 5,000 km during migration, and telemetry studies have shown that during the breeding season, female northern fur seals often travel up to 200 km to forage, while juvenile males travel up to 400 km from their rookery to forage (Dickerson *et al.* 2010; Gentry 1998). Many of the archaeological sites where northern fur seals have been found are within these typical travel distances from one another (Figure 4.2), so potential gene flow between rookeries with overlapping foraging distances would not be surprising. Both migrating and foraging distances are often long enough to cover multiple breeding areas. Though the tendency to return to natal breeding grounds, or 'philopatry', among northern fur seals is fairly high, there is still room for gene flow. Philopatry is lower among juvenile males and increases with age (Gentry 1998). There is measurable evidence that northern fur seals have immigrated to breeding sites, and that they have made 'brief visits' to other sites (Gentry 1998). Given the distances that northern fur seals may travel during the breeding season, geographic proximity of sites may be a useful way of grouping the small data sets together. As the majority of population genetics approaches focus on the D-loop data, which is expected

to be more informative for intra-population comparisons since it is non-coding, the following paragraphs will discuss how the various analytical approaches were applied to group the D-loop sites in Figure 4.2.

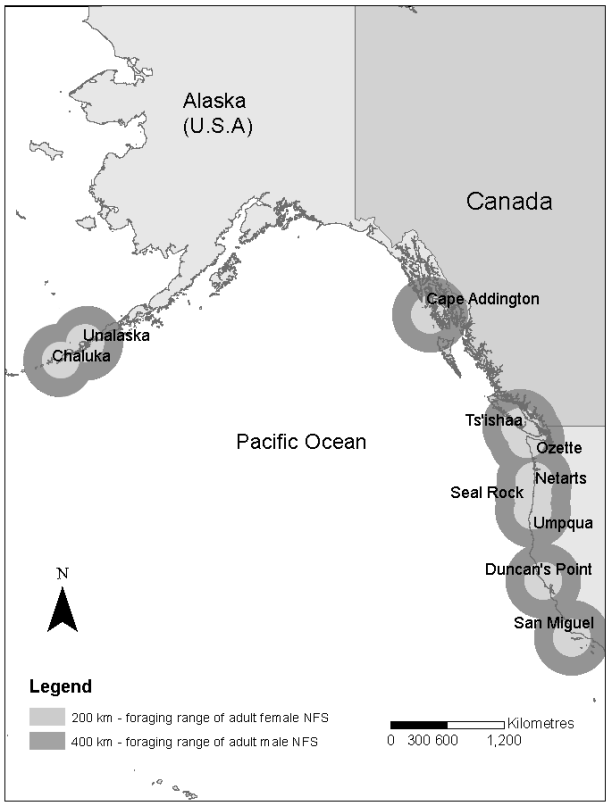


Figure 4.2. Geographic proximity of archaeological sites from which northern fur seal (*C. ursinus*) sequence data (*cytochrome b* and D-loop) was obtained.
D-loop data obtained from all archaeological sites except Unalaska, *cytochrome b* data only obtained from Unalaska, Cape Addington, Ts'ishaa, and Netarts. It seems likely that gene flow or hunting (and therefore sampling) of northern fur seals (*C. ursinus*)from nearby areas could have easily occurred during breeding season between these rookeries due to travel of northern fur seals during foraging trips (map courtesy of Colin Halseth).

The British Columbia and Washington sites are located fairly close together, with a distance from Ts'ishaa to Ozette just under 100 km. More importantly, both sites border the Strait of Juan de Fuca/Salish Sea. Since northern fur seals are aquatic, the sharing of a water body would likely be more important than direct geographic distance. However, *C. ursinus* tend to be pelagic foragers rather than near-shore foragers, so this body of water may not have been as highly utilized as might be expected. Nevertheless,

the BC/Washington grouping may be expected to be a fairly valid grouping given both the short distance and the shared resources/body of water. Additionally, southern BC and Washington are both part of a highly productive region due to the transition between the Alaskan and Californian systems (i.e. coastal runoff differences) as well as the end of the coastal upwelling domain which spans from Baja California to the northern end of Vancouver Island (Beamish *et al*. 2005; Ware and Thomson 2005).

In Oregon, the distance from Netarts to Seal Rock is around 100 km, while the distance from Seal Rock to Umpqua is around 90 km. Again, both of these distances are easily within the range of female foraging bouts. Though the distance from Netarts to Umpqua is a bit longer, around 200 km, the presence of northern fur seals at an intermediary site could allow for a potentially high degree of gene flow between all three sites.

Another possible option for grouping datasets could be grouping all of the BC, Washington, and Oregon samples together. Though they all belong to the same marine ecozone, the Pacific-Marine ecozone (which also includes Cape Addington) (Wiken *et al.* 1996), they are all fairly spread out, though there may be some cline in genetic variation over these distances. Even though the distance between Ts'ishaa and Umpqua is approximately 575 km - a little over the distance regularly travelled by males and females (Dickerson *et al*. 2010; Gentry 1998), there are a number of sites between these two locations. These sites may potentially represent a cline with gene flow across the region. The downside of this latter comparison option is that grouping these sites together could potentially obscure genetic structure over this region. A minor consideration is that not all of these sites are part of the same terrestrial ecoregion (US Environmental Protection Agency 2015). While aquatic conditions and prey species distributions most likely have greater impact on northern fur seal distribution, terrestrial ecoregion may have some impact on breeding sites through the possible terrestrial predators in the region. Additionally, it may be possible that ecological land conditions may factor into northern fur seal haul-outs and rookeries in other ways[15]. The Ts'ishaa,

[15] One might speculate that other land mammals may be a competitor for space, depending on the particular environment. Additionally, it seems conceivable that depending on ecological conditions/vegetation may affect the quality of the beach as a rookery site.

Ozette, Netarts, Seal Rock, and Umpqua sites are all located in a “Marine West Coast Forest,” the Ts’ishaa site is located in the subtype “Coastal Western Hemlock-Sitka Spruce Forests” terrestrial ecozone, while both the Netarts, Seal Rock, and Umpqua sites are located in the subtype “Coast Range” ecozone (US Environmental Protection Agency 2015). Most of these sites are coastal, however, the Umpqua site is an estuarine site located 4 km inland from the ocean (personal communication with Dr. Madonna Moss, University of Oregon; Hildebrandt and Jones 1992; Lyman 1991). Thus, a grouping of all five sites from BC, Washington, and Oregon would include two ecozone subtypes and span over 600 km, making this option a less likely grouping.

In Alaska, the Aleutian Islands and Pribilof Islands are all fairly close together, with St. George and St. Paul located 370-300 km from Bogoslof Island in the Aleutians. Among the Aleutian Islands, Bogoslof is located only 122 km from Chaluka. These northern islands may together form a relevant grouping based on proximity. Another possible grouping may be to include all samples from Alaska together as they are all high-latitude sites; all these sites are currently located above 52°N. However, given the distance between the Aleutian/Pribilof Islands and Cape Addington, this option may not be the most useful grouping.

As the disappearance of northern fur seal remains from archaeological sites in northern, central[16], and southern California occurred at various points over the mid to late Holocene (Gifford-Gonzalez *et al.* 2004; Newsome *et al*. 2007; Whitaker and Hildebrant 2011), it may be best to consider Duncan’s Point and San Miguel Island as two separate groups, as Duncan’s Point is located in northern California while San Miguel Island is located in southern California. The drawback to analyzing the two sites separately is that this leaves quite small sample sizes. It might therefore also be useful to group the two sites together, as both share a similar environment including both belonging to the larger marine ”Coastal Upwelling Domain”, shared with other sites along the coast north to Vancouver Island (Beamish *et al.* 2005). They are both part of the same ecoregion subtype (US Environmental Protection Agency 2015). In addition, the distance between the two sites is about 500 km, a distance that is somewhat further than

[16] Central California starts just south of San Francisco Bay.

a male's average foraging trip yet may still allow for some gene flow/shared sampling between the two regions. As mtDNA is passed from females to offspring, it is conceivable that some of the remains sampled may be visiting/immigrant males from neighbouring sites or that females may have immigrated to another breeding ground, as northern fur seal immigration has been observed in living populations (Gentry 1998). The sequence data from California may potentially be most useful when each site is treated separately as other studies on northern fur seals have broken the analysis of ancient northern fur seal in California into separate south, central and northern California regions (Newsome *et al.* 2007). However it may also be worthwhile to attempt to group these sites together as they fall within the Mediterranean California ecozone. Based on the groupings by other studies though, this latter option may be expected to provide weaker comparisons than those that divide these regions of California.

Grouping with the use of marine ecoregions/ecozones is not a very effective grouping for northern fur seal either. First, the entire range of ancient sites presented here belongs to the Temperate Northern Pacific ecoregion (Spalding *et al.* 2007). Second, this range refers to only the coastal regions extending to 370 kilometres offshore (Spalding *et al.* 2007), while northern fur seals are pelagic foragers. The separation by oceanographic regions may not be of great use in separating these northern fur seal sites as it would only really result in two groupings of terrestrial sites. The North Pacific can be divided into three bio-physical regions 1) the Coastal Upwelling Domain which covers the entire coastline from Baja California to the northern limit of Vancouver Island, 2) the Coastal Downwelling Domain from Haida Gwaii then following the along the coast of Alaska north then west along the Aleutian islands, and 3) the Central Subarctic Domain, which is the open ocean (primarily the Alaskan Gyre which is bounded by the Subarctic and Alaskan Currents, and the Alaska Stream) (Beamish *et al.* 2005). When this is applied to the sites in this study, it would result in only two divisions: those in Alaska (corresponding to terrestrial sites along the Coastal Downwelling Domain), and the remainder of sites (corresponding to those in British Columbia, Washington, Oregon, and California which border the Coastal Upwelling Domain). This division of sites may be too broad to show regional differences. However, such a broad division may be useful as isotope data on archaeological northern fur seal foraging can be divided into a northern and a southern set, thought the divisions differ in

that this isotope data grouped the Oregon, Washington, and British Columbian northern fur seal with the northern set, rather than with the Californian set (Braje and Rick 2011; Newsome *et al*. 2007).

One final consideration on grouping data from different locations is that source data for ancient sequences comes from archaeological sites. Potential human transportation of northern fur seal remains should also be acknowledged. Given that northern fur seals congregate at breeding season (representing a dense, predictable resource), it is possible that such a potential resource may have resulted in longer than normal human travel. One might conceive of seasonal habitation to take advantage of the yearly breeding season. Most hunter-gatherers typically move between 5-10 km during residential relocation, though these relocations can occasionally reach 60-70 km per trip, cumulating in some travel distances that in some cases may reach up to 490 km/year (Panter-Brick *et al.* 2001, Ames 2002). Short-term hunting trips may further extend this range of human travel, with a typical foraging radius of 4-6 km (Ames 2002). Hunting travel by boat may have greatly increased this range of human transport of northern fur seal remains, with a foraging radius expanded to 30km (Ames 2002). For an example of human seasonal movement within the geographic regions under examination there is evidence that some winter villages in northern California were located over 20km inland from summer coastal sites (Lightfoot 1992). Many groups on the northwest coast would make 2-3 moves per year, generally around distances of 10- 50km (see Ames 2002). This illustrates that human transport of remains cannot be discounted. These distances support the use of some geographic grouping (as the remains at nearby archaeological sites may have come from the same breeding site), though the distance of typical human travel (foraging trips, residential relocation) is typically far shorter than that travelled by northern fur seals on their foraging trips.

The population genetics analyses undertaken in this thesis attempt to discern whether any of these potential ways of grouping the data may yield meaningful patterns, whether the sites should all be treated independently if they represent unique populations, or whether there is no structure and all samples represent individuals from a large panmictic population. Geographic proximity may be one method of grouping the data into more robust sample sets, particularly for the BC and Washington sites, the

cluster of Pribilof/Aleutian Islands in Alaska, and the three sites in Oregon. These various groupings were used to increase the sample sizes of the population genetics analyses by DnaSP v. 5 to make potentially more robust data sets (Rozas 2009).

4.5. Research Methodology

4.5.1. Initial Data Processing

To this point, the materials and methods chapter has reviewed the methods used by Moss *et al.* (2006) to obtain the sequence data that serves as the primary data source for this thesis and the GenBank datasets available to supplement this sequence data, as well as discussed possible ways of grouping the sample data. The following sub-sections describe the methods employed in this thesis to expand upon the analysis of this data set beyond that done by Moss *et al.* (2006). This sub-section describes the analysis of phylogeny and sample identification, which were essential in authenticating those sequences belonging to northern fur seal for analysis by the methods described below on assessing population structure.

Sequence Alignment

The analysis for this thesis begins with the raw chromatogram sequencing data from the successful amplifications obtained by Moss *et al.* (2006). This data was visualized using ChromasPro (Technelysium Pty Ltd) to view the traces, edit the sequences, and assemble 'contigs' or contiguous DNA sequences (formed from a set of overlapping DNA sequences to represent a consensus sequence) for each sample with more than one trace (sequenced in both directions or repeats). Sample 29 only had successful amplification of the D-loop sequences, so identification of this sample was based solely on the phylogenetic tree from this data set.

The additional pinniped sequences described in the source data section were imported from GenBank (Benson *et al.* 2008). Sequences were aligned in MEGA 6 with a Clustal W sequence alignment program (Tamura *et al.* 2013). Once all sequences were aligned, they were trimmed to the same length with primer sequences removed. For *cytochrome b*, this length was 139 bp while for D-loop, this was 160 bp.

Phylogeny Testing

To confirm species identity of archaeological samples with those determined by Moss *et al.* (2006), neighbour-joining trees (using the Maximum Composite Likelihood method) were generated using MEGA 6 (Tamura *et al.* 2013). The trees were then rooted using an outgroup belonging either to the true seals (family Phocidae) or walrus (family Odobenidae, only 1 extant species). The resulting trees for *cytochrome b* are shown in the Appendix C. For D-loop, further analysis of northern fur seal phylogeny involved the additional generation of maximum-likelihood trees using different outgroups. To simplify the analysis, northern fur seal sequences from Dickerson *et al.* (2010) and Pinsky *et al.* (2010) were omitted due to the large number of northern fur seal sequences and lack of sequences from other species. This large northern fur seal dataset would only slow computation time and unnecessarily complicate the tree by overwhelming it with northern fur seal sequences. Rather, the multiple pinniped species data set of Wynen *et al.* (2001) was used. With its more simplified data set, these resultant trees were used both to test the phylogeny/taxonomy of northern fur seals (as discussed in the literature review and Appendix C, how northern fur seals are related to other pinniped species is an area of contention), and to confirm the species identity of the archaeological sequences from (Moss *et al.* 2006). Table 4.1 shows the confirmed species identity of these samples.

4.5.2. Population Genetics Analysis

This section describes those methods employed that further delve into the genetic diversity of northern fur seals and patterns of change in the population genetic structure. The first step in the analysis was the removal of non-northern fur seal sequences. This was done using the phylogenetic trees to confirm the species identity of the remains (described in section 4.5.1). Next, several analyses were conducted to investigate the population genetic structure and diversity changes between the past and present northern fur seal sample sets. These analyses included assessing the diversity in haplotypes and in nucleotide variation, testing for evidence of geographic patterning with these values, nucleotide mismatch analysis for population history, network analysis

of haplotype relatedness, and an AMOVA analysis (F_{ST} and Φ_{ST}) to assess how the molecular variance is partitioned.

Haplotype and Nucleotide Diversity Calculations

Once the data set was limited to only include northern fur seals, haplotype diversity was assessed in two ways. First, the number of branches represented in phylogenetic trees (generated with MEGA 6 [Tamura *et al.* 2013]) were manually tabulated to give the number of haplotypes and assess broad geographic patterns. The use of phylogenetic trees is a crude starting point to assess whether there is any dramatic patterning of haplotypes, such as a cluster of haplotypes unique to a geographic region. Second, the software analysis program DnaSP (Rozas *et al.* 2003) was used to calculate a few measures of the diversity of haplotypes, particularly the number of unique haplotypes and the calculation value 'Hd' ('haplotype diversity', as defined below).

Haplotype diversity[17] is a calculation of the relative proportions of each haplotype. The program DnaSP employs the approach of Nei's (1987) Equation 8.4, or a modification of Equation 8.4 which replaces *2n* with *n* according to the manual. Nei's Equation 8.4 is actually used for diploid populations, and replacing 2n with n yields Nei's Equation 8.5. The reason for this is because 'h' and the terms haplotype diversity/gene diversity[18] are used interchangeably, with the latter applying to both haploid and diploid organisms (Nei 1987). As the DNA used in this analysis is haploid mtDNA (and is designated as such in the DnaSP data format box), Equation 8.5 would be the equation expected to be employed for analysis. To confirm the equations for haplotype diversity (and nucleotide diversity) used under the chosen settings were the desired equations, a small sample data set of artificially generated sequences was used as a test to compare the values obtained by DnaSP and those by manual calculation using the desired equation from Nei (1987) (see Appendix D). The selected setting generated the same values to the haplotype diversity values using Nei's Equation 8.5, which is the same

[17] 'Nucleon diversity' in Nei and Taijima (1981).

[18] There is a related measure, termed "gene identity", equivalent to 1-*h* that may also be useful under some circumstances (Nei 1987)

equation as Nei and Tajima's (1981) Equation 8.7 (shown below in its two common forms):

$$\hat{h} = \frac{n\left(1 - \Sigma_{i=1}^{l} x_i^2\right)}{n-1} \quad \text{or} \quad H = \frac{N}{N-1}\left(1 - \Sigma_i x_i^2\right)$$

where H/h(hat)/Hd = estimated heterozygosity, n (or N) represents the number of sequences, *l* equals the number ofhaplotypes[19], and χ_i^2 represents the frequency of (each) haplotype (Nei and Tajima 1981). Low haplotype diversity values indicate the dominance of one or a few haplotypes, while high haplotype diversity values indicate an abundance of haplotypes. The lowest possible value would be 0 (indicating only 1 haplotype is present), while the greatest value is 1, indicating that every haplotype is unique (see Appendix D for how calculations work).

Nucleotide diversity is calculated in DnaSP using Equation 10.5 (Nei 1987):

$$\hat{\pi} = \frac{n}{n-1}\Sigma_{ij} x_i x_j \pi_{ij}$$

where π is the nucleotide diversity (sometimes shown as a $\hat{\pi}$ symbol to represent that the value is an approximation of nucleotide diversity), π_{ij} is the proportion of the nucleotides between the *i*th and *j*th sequences, X_i and X_j represent the population frequencies of the *i*th and *j*th sequences, and n is the number of sequences. Since genetic variability declines are a function of the effective population size (with variation lost at a rate of $1/N_f$ for haploid genes), with larger populations experiencing slower declines, a reduction in π would be expected to be the result of population decline (Herrmann and Hummel 1994).

To ensure that the program DnaSP was using these formulas and that the correct analytical variables were selected, the previously created artificial dataset for equation testing was again used to check the results generated by DnaSP with manual calculations using the formulas above (see Appendix D for sample calculations). Once

[19] Nei and Tajima (1981) used the term 'nucleomorphs'

both haplotype and nucleotide diversity calculations used by the program were verified to be the desired formulae for these calculations, these same program settings were then used to analyze the northern fur seal dataset. DnaSP was used to obtain calculations of nucleotide diversity, haplotype diversity, number of haplotypes, number of polymorphic sites and mutations, and number of sites analyzed for *cytochrome b* and D-loop sequences that had been sorted into various groupings.

Geographic and Temporal Patterning

Since one of the goals of this thesis was to assess population genetic structure differences in the past and present, and whether there was any difference in how diversity varied with geography, the values of diversity determined by the DnaSP analysis above were graphed. First, the various potential groupings were compared by graphing the haplotype diversity against the number of sequences to see if there were any particular differences between past and present diversity and whether these differences were simply due to low sample size.

Regarding geographic patterning, there are a few possible expected patterns of genetic diversity that one might expect to find in northern fur seals based on potential models. The 'latitudinal model' predicts decreasing diversity with increasing latitude (Schrey *et al.* 2011). This model is based on climate oscillations and the prediction that following the retreat of glaciation, there was a poleward species range expansion (Schrey *et al.* 2011). This model may fit with northern fur seal, as Bayesian skyline analyses have indicated a possible population expansion of northern fur seals around 11,000 years ago following the last glacial retreat (Crockford and Frederick 2007; Dickerson *et al.* 2010; Gifford-Gonzalez 2011). The second model that may have merit is the 'centre-marginal model' which predicts that the centre of a species distribution will have the highest diversity while peripheral sites will have lower diversity (Schrey *et al.* 2011). This is based on the idea that centre of species distribution will represent the most optimal habitat, with regions further out representing peripheral or marginal habitat. The centre of this range may also be expected to have greater gene flow. This model may have some merit if ancient northern fur seals indeed had breeding colonies all along the coast of North America. In this case, it could be that in the past, the centre of this range may have been the optimal habitat, but as northern fur seals were extirpated from

their ranges, only the peripheral colonies in the far north survived. To test these models, the values of genetic diversity (haplotype diversity and nucleotide diversity) were graphed against latitude to search for patterns of genetic diversity that may have changed over geographic distance, and which may represent a change in the genetic diversity and population genetic structure from the past to the present.

Nucleotide Mismatch Analysis

D-loop/control region nucleotide diversity and population dynamics were further assessed using nucleotide mismatch distribution showing the number of pairwise differences between sequences. Pairwise differences were calculated using DnaSP 5 (Rozas 2009), which also generated the distribution graph (Figure 5.7).

Network Analysis

To assess how the D-loop haplotypes related to one another, NETWORK 4.6 (http:// www.fluxus-engineering.com) and PopART 1.7 (http://popart.otago.ac.nz.) were used to generate median-joining networks. A network is a means of showing alternative potential phylogenies, where a reticulation indicates alternative possible phylogenetic pathways. Median-joining networks are a common method employed in the study of mtDNA for various species (Cai *et al.* 2009; Huson *et al.* 2010; Jansen *et al.* 2002; Speller *et al.* 2010; Zhao *et al.* 2011; see discussion on network building in Chapter 3, sub-section on tree building above).

The median-joining method is particularly useful for intraspecific data with no recombination, but is capable of handling sites with multiple mutations (Bandelt *et al.* 1999; Huson *et al.* 2010). Given that there are a number of sites in which multiple mutations are present (i.e. non-binary data),[20] the median-joining algorithm was used. In addition, the NETWORK manual (http:// www.fluxus-engineering.com) recommends the

[20] While single nucleotide polymorphism (SNP) data typically represents bi-allelic data, there were more mutations than polymorphic sites, indicating that a few sites had more than one mutation. This was also relevant for data analysis/methodology. See also section 3.3.1 on tree-building for methods impacted by use of polymorphic versus biallelic data and section 5.3.1 on D-loop haplotype diversity by manual tabulation.

median-joining methods for general, 'first choice' use. A study of a number of network and tree-building methods found that under conditions of no recombination, most methods generated the correct topologies (though minimum-spanning methods performed worse), so the network generating methods employed here should be expected to generate representative results (Woolley *et al.* 2008). Networks were generated for both ancient and modern data, as well as a combined network with both data sets to attempt to identify persistence of haplotypes. The combined networks were generated using both NETWORK (http://www.fluxus-engineering.com) and PopART (Population Analysis with Reticulate Trees) (http://popart.otago.ac.nz). While networks have some use in showing the relationship between haplotypes, statistical methods (such as F-statistics) are a very common approach used to better describe the diversity.

F-Statistics (F_{ST}) Analysis

F-statistics are extremely useful to the study of population genetics as these statistics can describe the partitioning of variance within and among populations. The hierarchical analysis of molecular variance (AMOVA) framework allows for F-statistics estimations (Michalakis and Excoffier 1996). F-statistic analysis was conducted using two programs, the excel add-in statistical program GenAlEx 6.5 (Peakall and Smouse 2012) and the integrated software package Arlequin 3.5 (Excoffier and Lischer 2010) to assess the partitioning of genetic diversity in northern fur seal ancient and modern populations. Both programs were used to cross-check the analyses, to visualize the partitioning of variance in graphical format (GenAlEx), and provide a more robust analysis of the variance (Arlequin) (personal communication with Dr. Brent Murray, UNBC). In MEGA6 (Tamura *et al.* 2013), aligned sequence data was exported in excel format. The bases were manually re-coded numerically as per the user guide (A=1, C=2, G=3, T=4) (Peakall and Smouse 2012). Non-polymorphic sites were removed and proper designators (including site assignment) were added to the workbook. The GenAlEx 6.5 program was used to test the data, then perform an AMOVA analysis (with 999 permutations) on the D-loop mtDNA data (haploid) (Peakall and Smouse 2006). For Arlequin, the sequence data was sorted into discrete population groups in DnaSP 5 (Rozas 2009) and exported as an Arlequin file format. AMOVA analysis was run in Arlequin using 1000 permutations. Results were compared with two levels of significance. The first was $P \leq 0.005$, which was chosen to be a highly stringent P-value

from past experience. Second, given that the modern study by Dickerson *et al.* (2010) used a P-value cutoff of 0.05, this P-value was also used to make results more comparable. Further, the P≤0.05 was the original standard for significance as designated by Fisher in 1925 (Rice *et al.* 2008).

4.6. Summary

This thesis provides a detailed analysis of the original sequences from a project by Moss *et al.* (2006), with a more specific focus on assessing genetic diversity patterns and population genetic structure dynamics, supplementing this data with previously published sequence data. This chapter provided a review of the archaeological site data and sample processing methods used by Moss *et al.* (2006) to obtain the data that was used in this thesis, and reviewed the pertinent information on the sequences obtained from GenBank. This chapter also outlined the methods employed in this thesis for analyzing sequence data. The following chapter will review the results obtained from these analyses.

Chapter 5.

Results

5.1. Introduction

This chapter presents the results of the data analysis to attempt to identify whether the northern fur seal sequences show evidence of differences in population genetic structure between ancient and contemporary populations. First, a phylogenetic analysis was conducted to generate a northern fur seal phylogeny to re-confirm the species identity of the Moss *et al.* (2006) sequences so that more in-depth population genetics analysis could be conducted. This was done for both *cytochrome b* and D-loop sequence data. The data from samples that were genetically confirmed to be northern fur seal were then examined for evidence of population structure/population subdivision using a variety of methods. These included determining haplotype and nucleotide diversity, generating median-joining networks and performing AMOVA analyses. Haplotype diversity was determined through manual examination of phylogenetic trees and tabulation using the DnaSP 5 (Rozas *et al.* 2003) software. Nucleotide diversity was also determined using DnaSP 5. The DnaSP 5 analyses were conducted on a variety of different sample combinations following the logic presented in Chapter 4.4, "Determination of Analytical Groups." Next, to obtain a better view of how the diversity was distributed, median-joining networks were generated and AMOVA analyses performed. First, NETWORK 4.6 and PopART 1.7 were used to generate median-joining networks to attempt to view the relationships between the various haplotypes (Bandelt *et al.* 1999; fluxus-engineering.com; http://popart.otago.ac.nz). Finally, AMOVA analysis was performed using Arlequin 3.5 and GenAlEx 6.5.

5.2. Phylogeny and Confirmation of Species Identification

Neighbour-joining trees generated using MEGA6 were used to re-confirm the identities of the archaeological specimens. Even though this is a step that had been previously undertaken by Moss *et al.* (2006), given the debate on northern fur seal taxonomy (as outlined in Appendix C), it is essential to verify that northern fur seal sequences can be reliably grouped by species when different taxa are considered in the taxonomy. This is particularly important for investigating population genetics.

Most samples were able to be identified at the *cytochrome b* gene level, and all were identified to species with the D-loop region (though the *cytochrome b* data was sufficient to distinguish *Callorhinus ursinus* from non-*Callorhinus ursinus* samples in all cases) (Table 4.1, Appendix C). Species identity of northern fur seals was consistent between all trees generated (Table 4.1, Appendix C). Species identity generally correlated with the findings of Moss *et al.* (2006), with a minor improvement (i.e. identification to species of *Arctocephalus* sample NF19 (from Ts'ishaa) which Moss *et al.* (2006) had only previously identified to the genus level) and the observation of how easily impacted the D-loop phylogeny was by small changes, such as outgroup species, outgroup sample (differences based on individual sequences), and on tree-building parameters (see Appendix C for full discussion).

5.3. Genetic Diversity of Northern Fur Seals

Having confirmed the species identity of the archaeological samples, it was then possible to investigate only northern fur seal sequences to gain further insight into the population genetics and phylogeography of this species. To search for evidence of population structure/evidence of population subdivision, measures of haplotype and nucleotide diversity were assessed. First, the number of haplotypes was tabulated and the distribution of these haplotypes was examined using phylogenetic trees of only northern fur seal sequences to show how these haplotypes related to one another. Next, haplotype diversity, nucleotide diversity, and other measures were assessed using DnaSP 5. Third, DnaSP 5 was used to generate a nucleotide mismatch distribution graph. Finally a network analysis was performed to examine the relationships between

phylogenetic sequences. The following sections will present the results obtained from these analyses.

5.3.1. Haplotype Tabulation and Distribution

The first stage was to investigate haplotype diversity by tabulating the number of haplotypes and attempt to identify geographic patterning among the related haplotypes. The removal of non-northern fur seal sequences expanded the range of analysis by removing several alignment gaps, thereby increasing the number of sites analyzed. This was done using both visual identification of the number of branches on phylogenetic trees, manual inspection of sequence alignments in MEGA6 (Tamura *et al.* 2013), and tabulation using the program DnaSP 5 (Rozas *et al.* 2003).

Cytochrome b

The phylogenetic tree of *cytochrome b* sequences was examined for number of haplotypes and any evidence of geographic patterning. The *cytochrome b* NJ tree reveals three haplotypes present in the *C. ursinus* data set analyzed, with all but one sequence belonging to one of the two main haplotypes (Figure 5.1). This unique haplotype is distinguished by a single nucleotide difference, a G→A transition at site 41 (see Table 5.1). Though the NJ tree revealed only three haplotypes, the DnaSP 5 analysis of the northern fur seal *cytochrome b* data calculates between two and four haplotypes (Table 5.3). The manual tabulation of mutations based on sequence alignments found a total of thirteen mutations (Table 5.1). Further investigation of the NFS *cytochrome b* alignment was conducted.

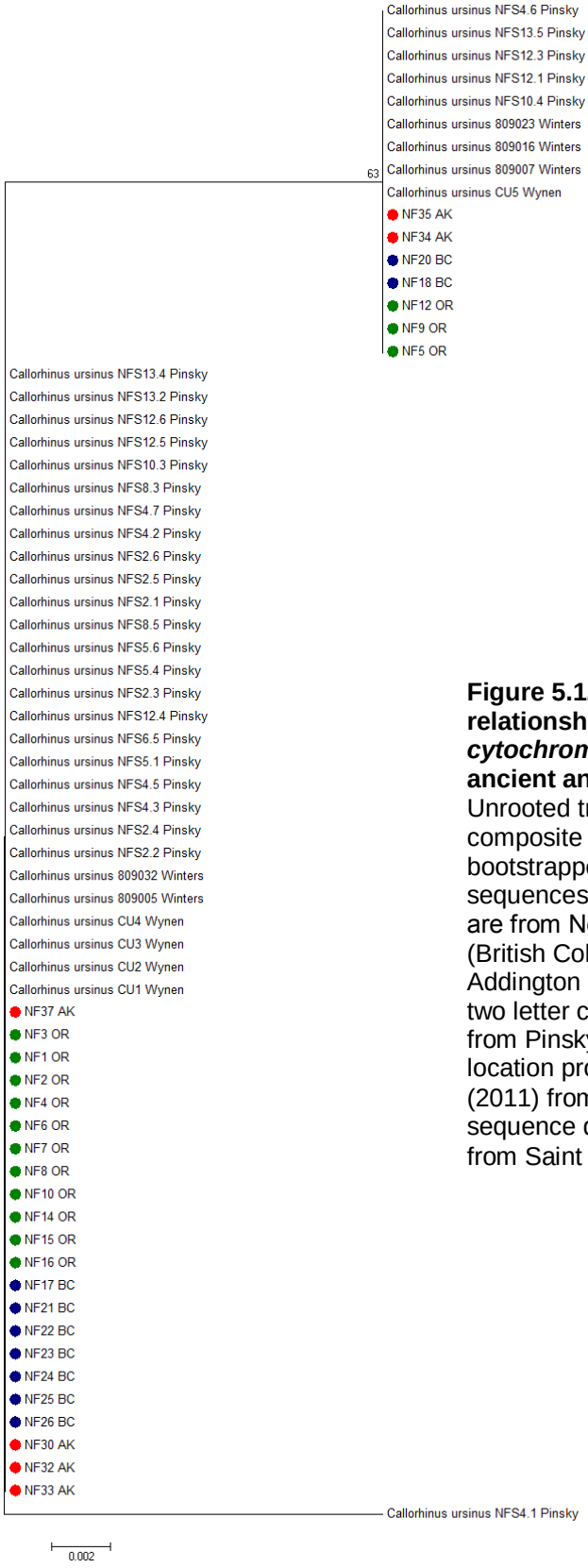


Figure 5.1. NJ tree showing the relationships between the *C. ursinus cytochrome b* sequences for both ancient and modern sequence data. Unrooted tree generated using maximum composite likelihood model and bootstrapped with 1000 replicates. Ancient sequences obtained by Moss *et al.* (2006) are from Netarts (Oregon), green, Ts'ishaa (British Columbia), blue, and Cape Addington (Alaska), red, designated by two letter code. Ancient sequences are from Pinsky *et al.* (2010) (no geographic location provided), and Winters *et al.* (2011) from Unalaska (Alaska). Modern sequence data from Wynen *et al.* (2001) is from Saint Paul (Alaska).

The initial assessment of *cytochrome b* sequence relatedness (based on the NJ tree-building method) revealed only three *cytochrome b* haplotypes. The first haplotype contained only a single sequence belonging to one of the ancient sequences from Pinsky *et al.* (2010). The second haplotype contained a mix of sequences including a modern NFS sample (CU5), three of the five ancient Unalaska sequences (Winters *et al.* 2011), and five of the ancient sequences from Pinsky *et al.* (2010), as well as sequences from all three of the sites sampled by Moss *et al.* (2006). The third haplotype included the remainder of the sequences, and therefore included sequences from all of these data sets. Because the second and third haplotypes contained sequences from all of the different sampling locations, there seems to be no evidence of geographic patterning from this tree (see section 6.3.1).

Table 5.1. *Cytochrome b* sequence mutations from manual identification of the MEGA 6 (Tamura *et al.* 2013) sequence alignment
Alignment revealing a fairly high number of G→A and C→T substitutions indicative of cytosine deamination.

Base position	Sequence	Mutation
10	NF10	G→A
21	NFS 4.3	G→A
25	NF24	G→A
41	4.1 (NFS4.1)	G→A
121	NF6	A→C
122	NF6	T→C
130	NF16	C→T
130	NF2	C→T
133	NF12	G→A
133	CU2	G→A
133	CU5	G→A
133	10.4 (NFS10.4)	G→A
139	CU 5	T→C

The *cytochrome b* sequence alignment reveals substantial alignment gaps. These alignment gaps resulted in a number of base positions being eliminated from the analysis. The impact of these alignment gaps can clearly be seen in a comparison of the number of bases used in the comparison (Table 5.3). The DnaSP 5 analysis indicates

that the Alaskan data set (modern and ancient, n=16) has four haplotypes and three polymorphic sites. When these sequences were compared base by base (manual comparison of the sequence alignment generated with the program MEGA6), one polymorphism found at position 10 was excluded from analysis due to alignment gaps at the start of the DNA sequences. Ten sequences belonged to the same type, while four had a single mutation at position 79, one sequence had a single mutation at position 133, and one sequence had multiple mutations at position 10, 79, 133, and 139. For the entire data set (all NFS *cytochrome b* sequences), polymorphisms were found at positions 10, 21, 41, 79, 121, 130, 133, and 139; of these positions 10, 21, 121, 130, 133, and 139 were excluded from analysis by DnaSP 5 when the entire data set was considered as a result of the alignment gaps (Table 5.3).

The results of this analysis revealed that the number of alignment gaps makes this data set a poor indicator of population structure/subdivision and haplotype relationships. There is no evidence of geographic patterning discernable from the *cytochrome b* phylogenetic tree (Figure 5.1). As *cytochrome b* is a coding gene with constraints on mutations, it may not be as useful for population genetics as a non-coding marker (such as the D-loop/ control region).

D-loop Haplotype Diversity

The D-loop NJ phylogeny to investigate the relationships between haplotypes does not reveal strong patterns of geographic patterning (Figure 5.2). Samples from Alaska, British Columbia (BC), Washington, Oregon, and California are jumbled throughout the tree. This indicates there is little evidence of geographic patterning (Figure 5.2). This suggests that haplotypes are for the most part distributed all around the geographic range. There is no evidence discernable from this tree that would exclusively group a geographic subset.

A manual examination of the D-loop sequence alignment revealed that there were far more mutations than in the *cytochrome b* data and that a handful of these sites had multiple mutations (Table 5.2). Typically, single-nucleotide polymorphism (SNP) markers are bi-allelic, so the presence of multiple mutations at these sites is one factor that may need to be taken into consideration when choosing further analytical methods

as some require binary data (see also Discussion sections 6.2, 6.3 and Materials and Methods sections 4.5.1 and 4.5.2).

Tabulation of the number of D-loop haplotypes using DnaSP 5 revealed a greater proportion of the haplotypes were unique among the ancient set. For the ancient samples, a total of 57 different haplotypes (out of 66 sequences) were identified, while for the modern data, 187 haplotypes were observed (out of 367 sequences) (see Tables 5.3 and 5.4). This indicates a total of 244 haplotypes for both ancient and modern northern fur seals, not including the excluded haplotypes from the Dickerson *et al.* (2010) study. There is a major difference in the proportion of unique haplotypes between the past and present. In the ancient D-loop data set, 86% of the haplotypes are unique (57 different haplotypes out of 66 sequences). Among the modern data, the DnaSP 5 analysis shows only 51% of the haplotypes are unique (187/367). In comparison, the data set from Dickerson *et al.* (2010) (which was excluded in the above calculation) shows 54% of their haplotypes were unique using their longer (original, GenBank) sequence length. These two separate modern calculations of haplotype uniqueness are consistent with one another. When the sequence data from Dickerson *et al.* (2010) is cut to the length of 160 bp, the number of haplotypes drops to between 273 and 274 (out of 619 sequences; this represents only 44% of the haplotypes that are unique at this level of analysis).

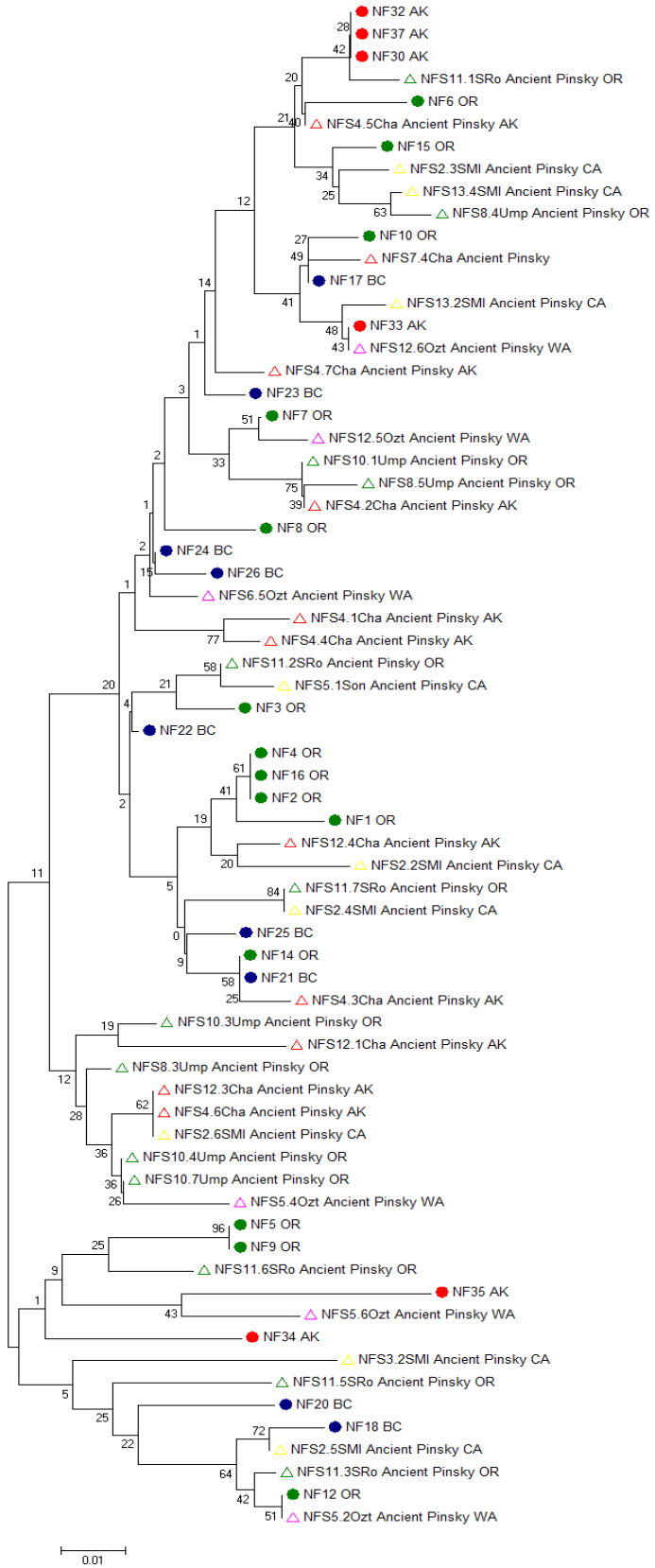
Table 5.2. List of sites and mutations in MEGA6 (Tamura *et al.* 2013) D-loop alignment.

Mutations tabulated manually to investigate the polymorphism and potential DNA damage resulting from cytosine deamination.

Site	Mutation
3	A→G
4	G→A, G→C, G→T
7	G→A
31	T→C
36	G→A
37	T→C
38	A→G
39	A→G
42	G→A
45	G→A
46	A→G
48	C→T
55	A→G
57	C→T
59	G→A
64	A→G
65	A→G
67	C→T
68	T→C
69	T→C
72	T→C
75	C→T
80	A→G
82	C→T
84	A→G
85	G→A; G→T
86	A→G
87	C→T
88	A→T; A→C
90	T→C
91	G→A
92	T→C

Site	Mutation
93	T→C
94	A→T
95	A→G
98	C→T
100	T→C
101	C→T
103	G→A)
104	A→G
105	A→G
109	A→G
113	C→T
116	C→T; C→G
120	C→T
121	C→T
125	T→C
128	G→A
129	T→C; T→A
130	A→G
131	G→A; G→T; G→C
134	C→G
137	A→G
138	A→G
145	G→A
157	*End of analysis

Figure 5.2. NJ tree of ancient D-loop/control region sequences showing the relationships between the *C. ursinus* sequences from different geographic locations.
Unrooted tree generated using maximum composite likelihood model and bootstrapped with 1000 replicates. All sites are listed with the two letter provincial/ state code and colour coded for assessment of geographic patterns. Solid circled indicate ancient sequences obtained by Moss *et al.* (2006) are from Netarts (Oregon), Ts'ishaa (British Columbia), and Cape Addington (Alaska). Ancient sequences from Pinsky *et al.* (2010) are designated using traingles with the designation Ancient Pinsky, with the preceeding letters referring to the site location of San Miguel Island (SMI), Umpqua (Ump), Seal Rock (SRo), Chaluka (Cha), Ozette (Ozt), and Duncan's Point/ Sonoma (Son). For both data sets, Alaskan sites are designated in red indicators, British Columbian sites in blue, Washington sites in purple, Oregon in green, and Californian sites in yellow.



5.3.2. Haplotype and Nucleotide Diversity Analysis

DnaSP 5 (Rozas *et al.* 2003) was used to investigate polymorphism and diversity for both *cytochrome b* and D-loop sequence data. A number of variables were considered, such as the number of sites used in the analysis (important for *cytochrome b* where alignment gaps resulted in sites being skipped), number of haplotypes (as discussed in the previous sub-section), haplotype and nucleotide diversity (using the equations specified in Chapter 4.5.2), and the number of polymorphic sites/number of mutations. Due to a variety of analytical options available through the program, it was first tested with a sample dataset. This sample data set was cross-checked by manual calculation to ensure comprehension of how the resulting values were produced (see Appendix D for manual calculations). This was done as the manual often refers to more than one equation used for the measurements. Once the methodology had been verified as the one outlined by the equations presented in Chapter 4, analysis of the *cytochrome b* and D-loop data sequence data was performed.

Cytochrome b

The ancient sample set was far more robust than the modern sample set of *cytochrome b* sequences. For this gene, there were only five sequences in the modern analysis, all of which came from St. Paul, AK (Wynen *et al.* 2001). Given this small sample, ancient and modern sequences can all be seen in Table 5.3. For the analysis of the five modern sequences, no sites were excluded, three haplotypes were identified (haplotype diversity (Hd) of 0.7), and nucleotide diversity (π) was calculated to be 0.01295 (Table 5.9). The *cytochrome b* sequences were grouped in different combinations for DnaSP 5 analysis and the results showed lower haplotype and nucleotide diversity in the ancient compared to the extremely small (n=5) modern set (Table 5.3).

Table 5.3. DnaSP 5 analysis of *cytochrome b* DNA polymorphism examining the haplotype diversity and nucleotide diversity of *C. ursinus* sequences for both modern and ancient sequence data.
Length of sequence analyzed was 139 base pairs.

Comparison Groups	#sites used (/139)	#haplotypes	Haplotype diversity (Hd)	Nucleotide diversity (Pi)	Number of polymorphic sites
All Northern Fur Seal (n=67)	79	3	0.393	0.00505	2
Ancient Netarts, Oregon (n=14)	112	3	0.385	0.00451	2
Ancient Ts'ishaa, BC (N=9)	107	2	0.389	0.00727	2
Ancient Cape Addington, Alaska (n=6)	124	2	0.533	0.00430	1
Modern St. Paul Alaska (Wynen) (n=5)	139	3	0.700	0.01295	4
Ancient Unalaska, Alaska (Winters) (n=5)	139	2	0.600	0.00432	1
Ancient North America from Pinsky (n=28)	85	3	0.362	0.00442	2
All ancient (n=62)	79	3	0.397	0.00513	2
All modern (n=5)	139	3	0.700	0.01295	4
All ancient Alaska (n=11)	124	2	0.545	0.00440	1
All Alaska (modern and ancient) (n=16)	124	4	0.617	0.00692	3
Ancient BC and Oregon (n=23)	80	2	0.356	0.00445	1

The DnaSP 5 analysis found that the haplotype diversity values for *cytochrome b* ranged from 0.356 to 0.700. Given the amount of data that was excluded from the analysis due to alignment gaps, these values are not very informative. For instance, some analyses only included 57% of the sites (79 of the 139 sites). Since the D-loop is a non-coding region and has a greater number of mutations, the data from the D-loop set was expected to be more informative (this is also why D-loop is the preferred region to compare intra-specific variation (see Chapter 3.1, 6.2, 6.3.2, and Appendix A for more discussion).

D-loop

Given the non-coding nature with a high amount of polymorphism and the greater number of sequences in the D-loop data, it was expected to be a better candidate for

analyzing measures of population genetics. Analysis of the D-loop data involved both haplotype diversity and nucleotide diversity calculations (as well as other measures) from DnaSP 5 (Rozas *et al.* 2003), as well as an analysis of the nucleotide mismatch distribution, network analyses and AMOVA analysis. The results of these analyses are shown below and in the following sections (sections 5.3.3, 5.3.4, and 5.3.5).

The D-loop data was first analyzed using DnaSP 5 to generate relevant values of haplotype and nucleotide diversity. Trimming the number of ancient sequences analyzed from 68 to 66 and cutting all the sequences to a length of 157 bp resulted in all sites being analyzed by the DnaSP 5 program. A number of potential groupings (as suggested in Chapter 3) were analyzed. The results are shown in Table 5.4 (ancient) and Table 5.5 (modern).

Table 5.4. DnaSP 5 analysis of ancient DNA polymorphism examining the haplotype diversity and nucleotide diversity of *C. ursinus* D-loop/control region sequence data.
All comparisons across all of the 157 bp.

Comparison groups	#haplotype	# of polymorphic sites	#mutations	Haplotype diversity (Hd)	Nucleotide diversity (Pi)
All sequences (All ancient) (n=66)	57	40	42	0.995	0.04724
Netarts OR (n=12)	9	20	20	0.939	0.04227
Ts'ishaa BC (n=9)	9	16	16	1.000	0.03680
Cape Addington AK, (n=6)	4	14	14	0.800	0.04374
Umpqua OR (n=7)	6	14	14	0.952	0.03700
Seal Rock OR (n=6)	6	17	17	1.000	0.04926
Chaluka AK (n=11)	11	23	23	1.000	0.04331
Ozette WA (n=6)	6	21	22	1.000	0.05817
San Miguel (n=8)	8	20	21	1.000	0.05778
Duncan's Point (n=1)	/			/	/
Ancient Alaska (n=17)	15	29	29	0.978	0.04646
Ancient N. Alaska (see Chaluka)	(11)	(23)	(23)	(1.000)	(0. 04331)
Ancient BC Washington (n=15)	15	24	25	1.000	0.04440
Ancient Oregon (n=25)	21	26	26	0.983	0.04671
Ancient California (n=9)	9	22	23	1.000	0.05697

Table 5.5. DnaSP 5 analysis of modern DNA polymorphism examining the haplotype diversity and nucleotide diversity of *C. ursinus* D-loop/control region sequence data.
Comparisons across all 157 bp.

Comparison groups	#haplotype	# of polymorphic sites	#mutations	Haplotype diversity (Hd)	Nucleotide diversity (Pi)
All modern (n=367)	187	55	63	0.989	0.04782
St. Paul, AK (n=94)	60	39	42	0.986	0.04583
Bogoslof, AK (n=95)	65	42	44	0.988	0.04854
St. George, AK (n=92)	69	40	43	0.990	0.04802
SanMiguel, CA (n=86)	64	40	45	0.990	0.04868
All Modern Alaska (n=281)	148	52	56	0.988	0.04748

To visually represent the data and search for overarching patterns, the D-loop data presented in Table 5.5 was graphed against the number of sequences to attempt to control for the dramatically different sample sizes. A test rarefaction curve (not shown) revealed that neither set appeared to reach the plateau that would indicate that the majority of haplotypes in each population have been sampled. Additionally, the ancient and modern samples appear to follow the same curve which may suggest a potential continuity in pattern for diversity of haplotypes in the population, though this analysis is too course to gain any strong insight into patterns between the ancient and modern populations (Smith and Smith 2001). To specifically look at the patterns between the past and present and assess the impact of the dramatic difference in sample size between past and present, the values of haplotype diversity (Hd) were graphed against the number of sequences analyzed (Figure 5.3). This graph revealed that haplotype diversity was high, with the majority of values between about 0.950 and 1.000 for both ancient and modern populations. Overall, the pattern for haplotype diversity is not very different between the past and present, though there are two points below this range – the Netarts set at Hd= 0.939 and the Cape Addington set (which was a clear outlier) with Hd around 0.800 (Table.5.4). It was possible that this point may have been an outlier because of stochastic differences/statistical variance from the small sample size, though the Cape Addington grouping contained a total of six sequences. That this outlier could be due to low sample size does seem plausible, though the other two sites containing only six samples (Ozette and Seal Rock) both have a haplotype diversity of 1.

Through an examination of the nucleotide diversity (π) for D-loop sequences, the past and present samples appear to follow a similar linear pattern when graphed against the number of sequences (Figure 5.4). The possible outliers appear to be the ancient Ozette, WA set (with a π = 0.05817) and San Miguel (with π= 0.05778) having greater than average nucleotide diversity, and the Ts'ishaa BC /Umpqua, OR combined set showing values lower than π =0.04000 (4%). This larger range of nucleotide diversity in ancient samples may be the result of random chance sampling (stochastic variance) which could easily result from very low sample size. One clear trend visible from this data, despite the poor sample sets, is that most samples have a nucleotide diversity of around the range of π = 0.04000-0.05000.

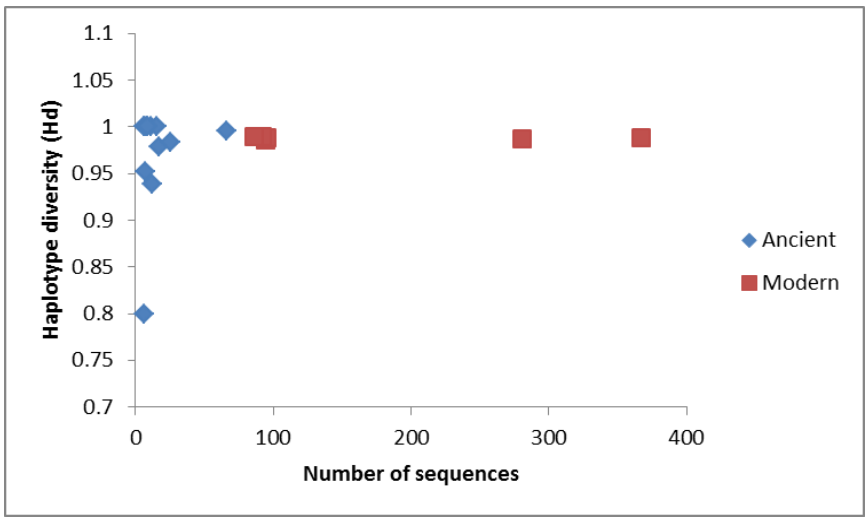


Figure 5.3. Graph of D-loop/control region haplotype diversity versus number of sequences analyzed for *C. ursinus*
Graph can be used for comparison of patterns in modern and ancient diversity.

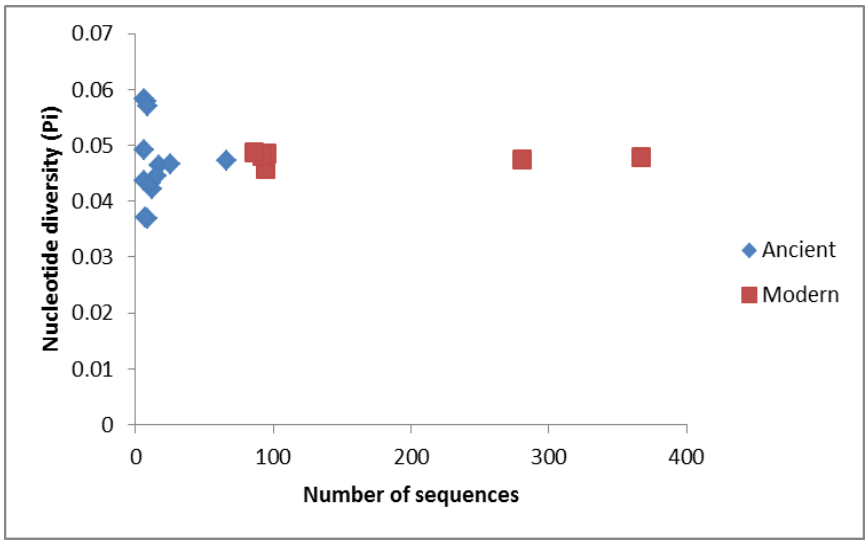


Figure 5.4. Graph of D-loop/control region nucleotide diversity versus the number of sequences analyzed.
Graph can be used for comparison of patterns in modern and ancient diversity in *C. ursinus*.

To search for geographic patterning, the same data points as shown above (Table 5.4, Table 5.5) were graphed against latitude (Figures 5.5 and 5.6). This was done using the non-combined data set to use more specific data points (rather than

ranges of latitude values). There is no clear pattern visible for either the haplotype diversity (Figure 5.5) or the nucleotide diversity (Figure 5.6). The strongest pattern that can be identified with respect to haplotype diversity (Figure 5.5) may be the similar Hd values for all modern northern fur seals, despite geographic separation in these contemporary populations. For the ancient northern fur seal, this graph suggests a possible pattern of either a consistent haplotype diversity value or slight decline in diversity with increasing latitude.

Regarding nucleotide diversity, there is also little evidence of patterning. No clear trend lines were observable showing change with latitude. The modern samples show no difference in nucleotide diversity across latitude, with consistently high values for all four sites, while the ancient data forms a nebulous cloud centered around similar values with no clear patterning visible (Figure 5.6). That is, neither the ancient nor the modern show any trend of increasing or decreasing nucleotide diversity (π) with latitude.

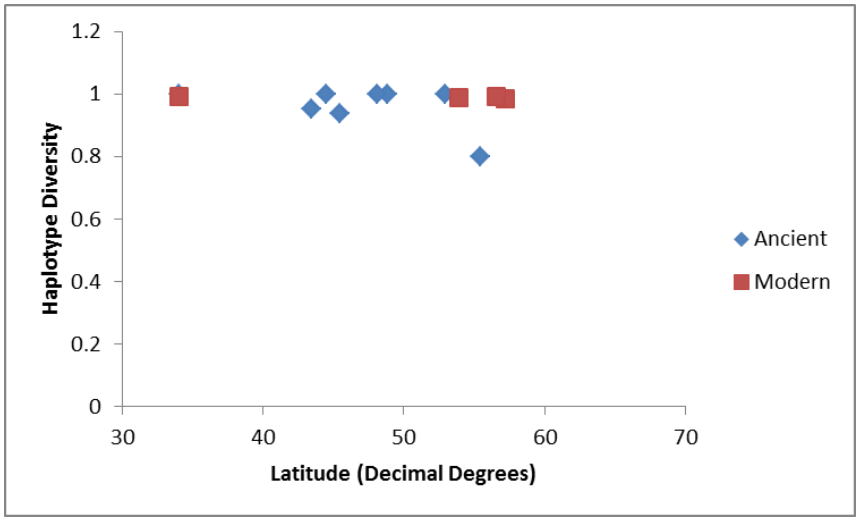


Figure 5.5. Graph of haplotype diversity determined by DnaSP 5 of northern fur seal *(C. ursinus)* D-loop sequences for modern and ancient samples against latitude to assess geographic patterning.

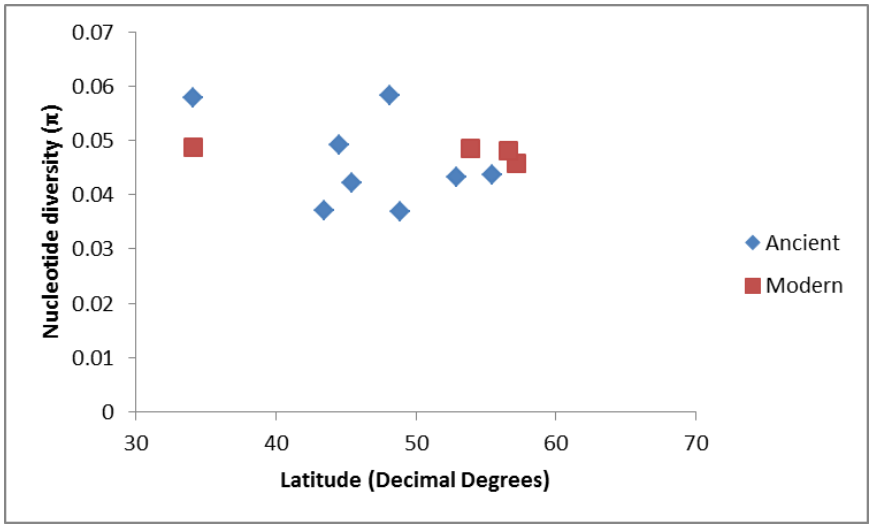


Figure 5.6. Graph of nucleotide diversity (π) determined by DnaSP 5 of northern fur seal (*C. ursinus*) D-loop sequences for modern and ancient samples against latitude to assess geographic patterning.

Nucleotide mismatch distributions are often used to examine population dynamics. The shape of a mismatch distribution is affected by past population changes (Rozas *et al.* 2003). Mismatch distribution graphs involve graphing the number of pairwise differences between the nucleotides on the x-axis and the frequency with which each number of pairwise differences occurs on the y-axis. For this thesis, the mismatch distributions were also generated using DnaSP 5 (Okello *et al.* 2005; Rozas 2009).

5.3.3. Nucleotide Mismatch Distribution

Nucleotide mismatch distributions are compared by the "smoothness" of the curve. Smoother curves generally correlate with recent population expansions, while more 'ragged' graphs typically indicate a long-term, stable population (Holsinger 2008; Ingman and Gyllensten 2003; Matisoo-Smith and Horsburgh 2012). The mismatch distribution for the aDNA set appears to generally form a single wave or peak, though this peak is not very smooth (Figure 5.7a). This ancient graph thus appears to have some properties of both ragged and smooth graphs as the overall shape is a single large peak rather than a ragged series; however, this large peak is not particularly smooth and appears to contain potentially another peak. In contrast, the modern appears to have a single smooth curve (Figure 5.7b). The increased raggedness of the aDNA set can be

seen by comparing the raggedness statistic r, which is 50% larger in the ancient (0.0066) set compared to the modern (0.0044).

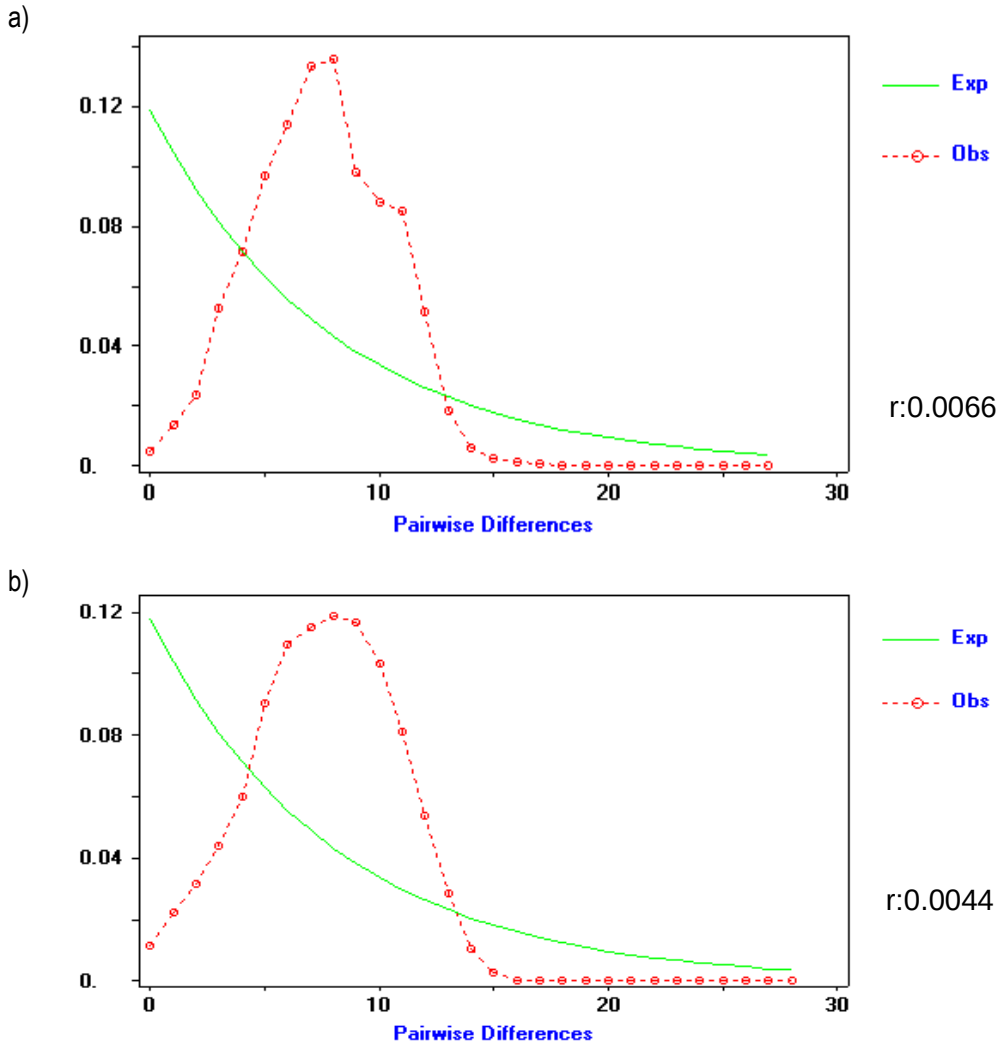


Figure 5.7. Nucleotide mismatch distribution graphing the frequency against the number of pairwise differences for ancient and modern DNA D-loop fragment.
Observed frequencies of pairwise differences graphed with a red line, compared to the expected curve (green) for 157bp regions a) Ancient distribution: Raggedness statistic (r) of 0.0066, graph generated using DnaSP for 66 sequences. b) Modern distribution: Raggedness statistic (r) of 0.0044, graph generated using DnaSP for 368 sequences.

5.3.4. Network Analysis

The program NETWORK was used to generate median-joining networks (Bandelt *et al.* 1999) separately for the ancient and modern D-loop sequence data to attempt to identify genetic patterns, while NETWORK and PopART were used to generate combined ancient and modern graphs to identify whether there is a continuity of haplotypes. As discussed in Chapter 3, networks have many similarities to trees; however, they allow for more than simple bifurcating patterns and can even form 'cycles' or alternative potential phylogenies. A number of these 'cycles' can be seen in the networks generated using both ancient and modern D-loop sequence data (Figures 5.8, 5.9) (Bandelt *et al.* 1999; Huson *et al.* 2010). The nodes represent sequences with specific mutations; in this case, each refers to a specific haplotype. The size of the nodes corresponds to the number of sequences with that haplotype. Typically such networks include branch lengths with markers to represent the number of character changes between these nodes. However, given the densely packed information in Figures 5.13 and 5.14, these have been omitted for clarity. Where the reticulations form 'cycles', the mutations may have occurred at multiple times, such as parallel mutations or homoplasies (see Chapter 4 Materials and Methods and Chapter 3: Phylogenetics and Population Genetics for more discussion) (Bandelt *et al.* 1999; Edwards *et al.* 2004; Huson *et al.* 2010).

The network analysis revealed that both the ancient and modern networks appear to be split into two deep branches (which may be considered 'clades' or 'haplogroups'); there is only a single connection between them (Figures 5.8 and 5.9) (http:// www.fluxus-engineering.com). Each of these branches contained a large number of haplotypes that formed a fair number of cycles. With the larger data set of the modern DNA, the network was obviously far more densely packed. As a result, evidence of star-like phylogenies begins to emerge (Figure 5.11).

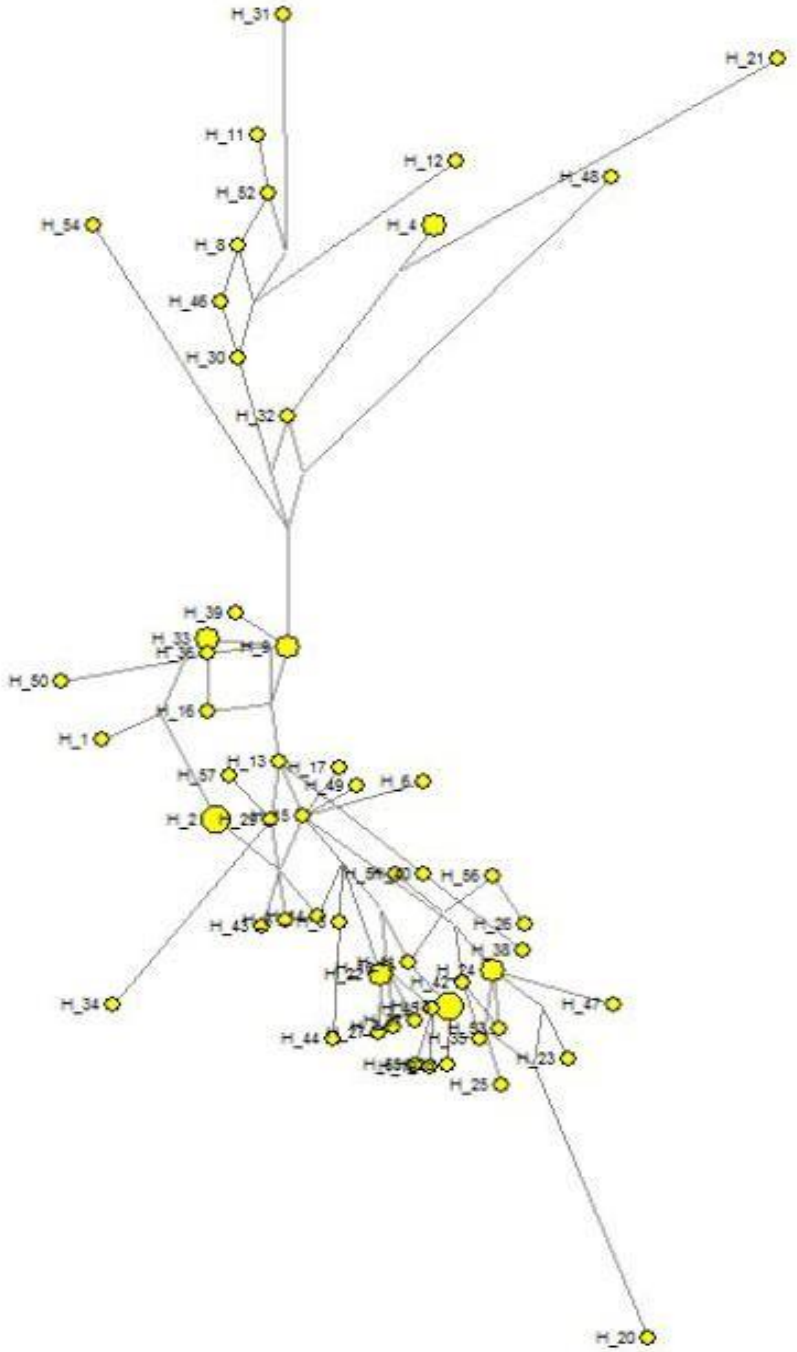


Figure 5.8. Median-joining network of ancient northern fur seal (*C. ursinus*) 157 bp D-loop sequences generated by NETWORK to illustrate the relationship between haplotypes
Node size correlates to number of haplotypes.

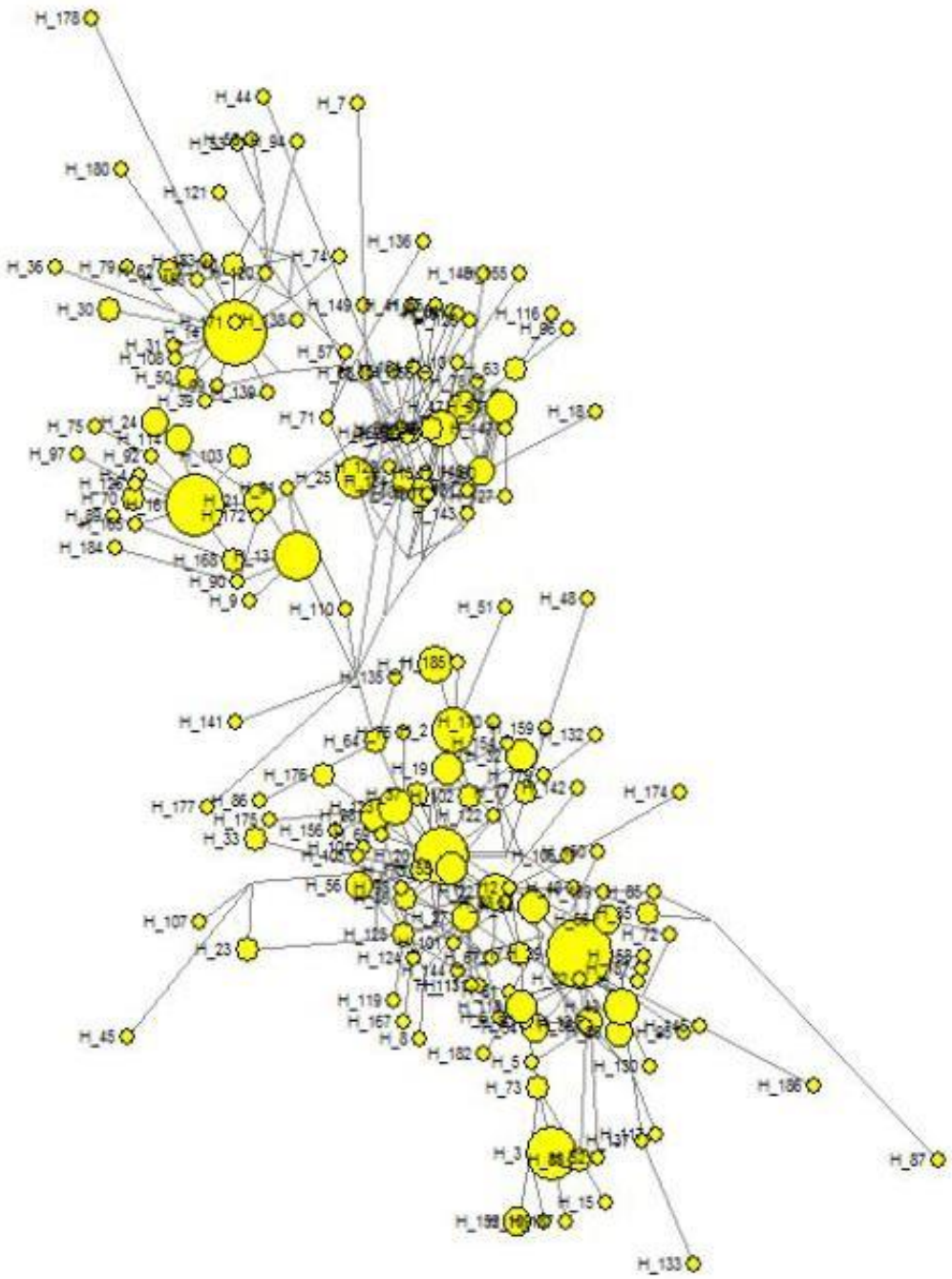


Figure 5.9. Median-joining network of modern northern fur seal (*C. ursinus*) 157 bp D-loop sequences generated by NETWORK to illustrate the relationship between haplotypes.
Node size correlates to number of haplotypes.

Given the persistence of two clades separated by only a single branch in both the modern and ancient data sets, it appears that there may be a persistence of haplotypes between modern and ancient northern fur seal. To investigate this, the two data sets were combined and MJ networks were generated to identify the relationship between the ancient and modern haplotypes. PopART was used to generate a clear network with non-overlapping nodes (Figure 5.10), while NETWORK was used to generate a colour-coded network to illustrate the location and relative proportion of modern and ancient haplotypes (Figure 5.11).

The network shown in Figure 5.10 clearly shows the single branch connection between clades which exists when both ancient and modern sequence data are combined. Therefore, this branch relationship is the same in the past as it is in contemporary northern fur seal populations. There also appears to be three large haplotype clusters in the top half of the figure.

The persistence of a number of ancient haplotypes into contemporary times can be seen through the number of nodes containing both ancient and modern sequences (Figure 5.11). This median-joining network shows a number of isolated ancient haplotypes that are not present in the modern sample set, with these haplotypes connected to extant haplotypes often by only a single branch rather than being intermediary/ancestral haplotypes. Further analysis of ancient and modern northern fur seal population genetic data particularly focused on the distribution of molecular variance (AMOVA).

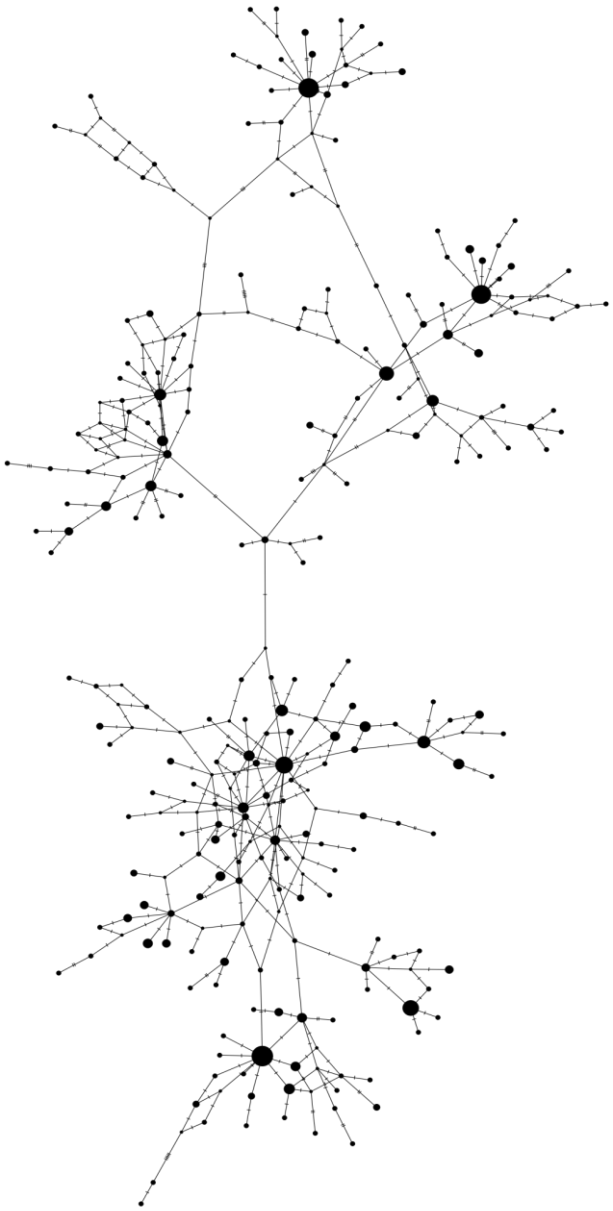


Figure 5.10. Median-joining network generated by PopART to illustrate the relationship between haplotypes for the combined ancient and modern 157 bp D-loop sequence data.
Node size correlates to number of haplotypes.

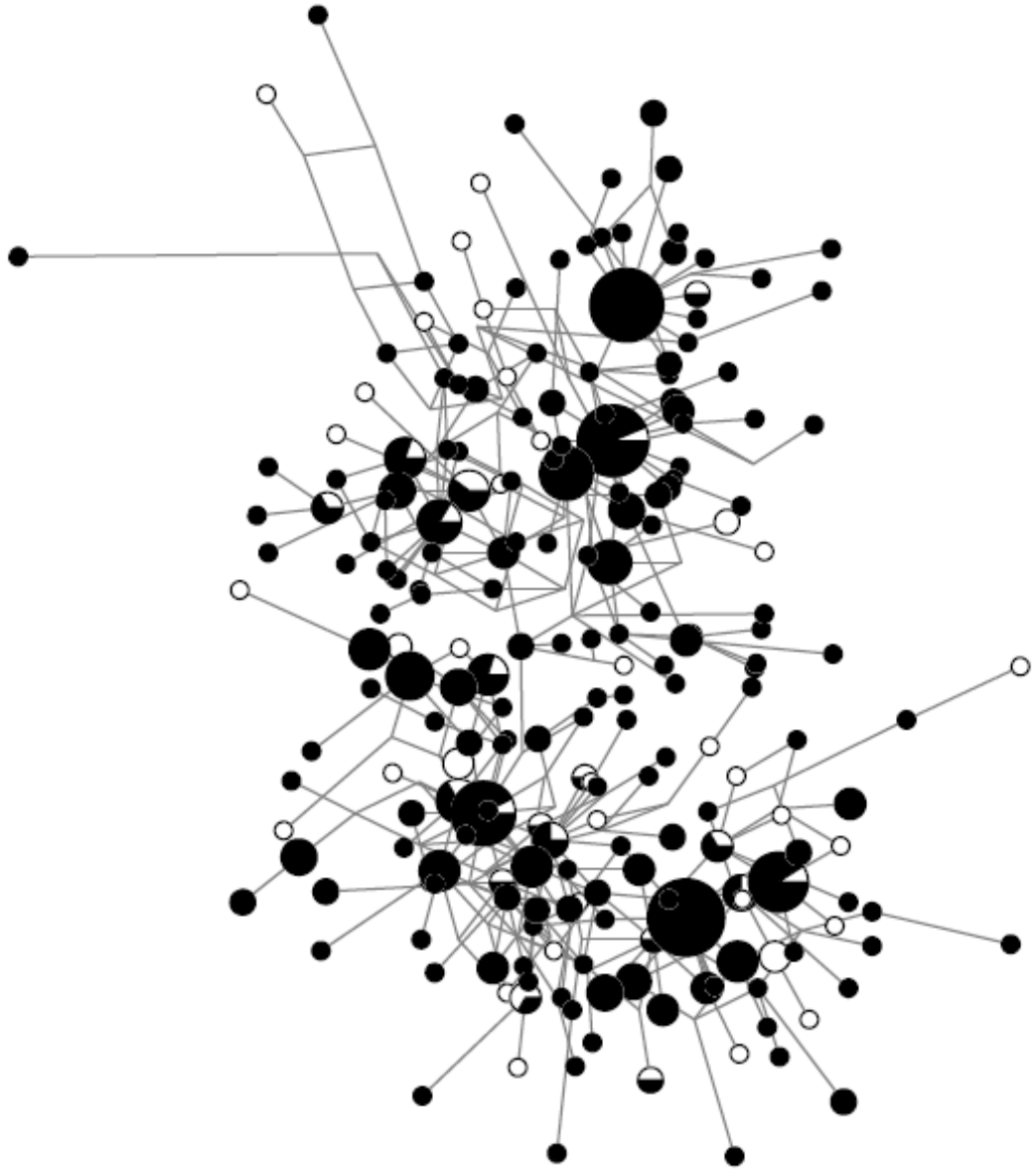


Figure 5.11. Median-joining network generated by NETWORK to illustrate the relationship between modern and ancient 157 bp D-loop sequence data.
Ancient sequences are shown as white in the pie-chart nodes, while black is used to represent modern sequence. Node size correlates with number of haplotypes.

5.3.5. AMOVA Analysis of F-Statistics

To assess whether there are differences between 'populations' at the different archaeological sites and modern rookeries, and whether these vary between modern and ancient data sets, AMOVA analyses were performed using GenAlEx 6.5 (Peakall and Smouse 2012) and Arlequin 3.5 (Excoffier and Lischer 2010). An AMOVA analysis is particularly useful in assessing potential differences in population genetic structure as it describes the way the observed variance is partitioned among subpopulations. Overall partitioning of the variance as well as population differentiation for 'population' (rookery or archaeological site) pairs were both calculated. Results were assessed at two different significance levels (P-value cutoffs of 0.005 and 0.05).

Ancient D-Loop AMOVA Analysis by GenAlEx

For the ancient data set, the frequency distribution of the observed F_{ST} equivalent Φ_{PT}/("PhiPT") versus the expected F_{ST}/(PhiPT) shows that the expected (red bar) value is located on the upper range of the distribution generated with random data (black bars) (Figure 5.12). The AMOVA results of the ancient mtDNA haplotype data indicate that 5% of the total variation is found among populations, while the remaining 95% of the variance is found within populations (Figure 5.12, Table 5.6). The F_{ST} (PhiPT) value (P-value is 0.024) is not significant at the 0.005 cutoff, though it is at the 0.05 cutoff (Table 5.6). However, despite this significance at the 0.05 cutoff, as mentioned above, this value is still within the distribution of random PhiPT values (Figure 5.12). The number of migrants was estimated to be 9.192.

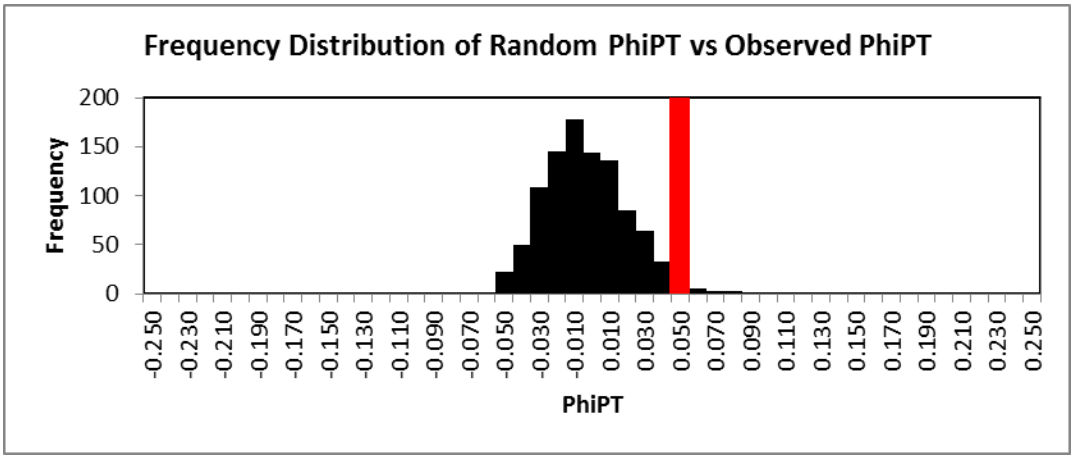


Figure 5.12. Frequency distribution of ancient *C. ursinus* mitochondrial DNA D-loop haplotype data.
Graph showing the observed PhiPT(Φ_{ST}) compared to the predicted PhiPT(Φ_{ST}) value if the data is sorted randomly from *Callorhinus ursinus* 'populations' from the separate archaeological sites. The observed data is located within the upper distribution of the expected data with a PhiPT(Φ_{ST}) of 5% (0.050) (red) using 999 permutations (P value is 0.024). This variance is only significant at the 0.05 P-value cut-off.

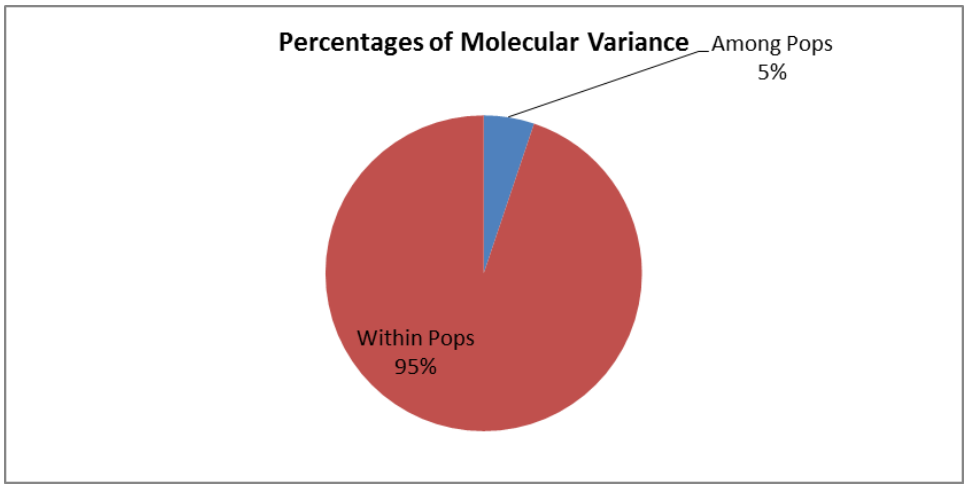


Figure 5.13. AMOVA graph of relative percentage of molecular variance (PhiPT or Φ_{ST}) for ancient mitochondrial DNA D-loop haplotype data.
Graph shows the within and among population variance of northern fur seal (*C. ursinus*) recovered from archaeological sites along the Pacific Coast of North America. Among population variance was significant at P≤0.05, but not at P≤0.005.

Table 5.6. Summary of AMOVA table analyzing the variance (PhiPT or ΦPT) in ancient mtDNA haplotypes within and among populations of *C. ursinus* from archaeological sites

Archaeological sites span the Pacific Coast of North America. Table shows the degrees of freedom (Df), sum of squares (SS), estimated variance (Est. Var), percent variance (%), PhiPT and the P-value (P(rand>=data)). This variance is only significant at the 0.05 P-value cutoff.

Source	Df	SS	MS	Est. Var.	%
Among Pops	7	35.677	5.097	0.193	5%
Within Pops	57	202.123	3.546	3.546	95%
Total	64	237.800		3.739	100%

Stat	Value	P(rand >= data)
PhiPT	0.052*	0.024

When the archaeological sites were compared to one another in pairwise population comparisons, the majority of archaeological sites showed no difference between populations. The pairwise PhiPT(Φ_{ST}) for archaeological site by site comparison showed no highly-significant values for all comparisons, except between Netarts/Cape Addington and Netarts /Umpqua (Table 5.7). These are the only two comparisons which yielded P-values less than the 0.005 cutoff and thus the differences were highly significant. For these significantly different populations, the number of migrants between Netarts and Cape Addington was estimated to be 1.603 migrants/generation, and the number of migrants between Netarts and Umpqua was estimated to be 2.238 per generation (estimates assume equal population size, which is not always the case). When the P-value cutoff was increased to 0.05, a handful of other archaeological site comparisons became significant: Ts'ishaa/Cape Addington, Ts'ishaa/ Umpqua, Cape Addington/Seal Rock, Cape Addington/Chaluka, and Umpqua/Seal Rock.

Table 5.7. PhiPT(Φ_{ST}) values for the pairwise comparisons (Pairwise Population PhiPT Values) between the *C. ursinus* mtDNA D-loop sequences for the eight archaeological sites.

Archaeological sites located along the Pacific Coast of North America, and only includes sites with multiple *Callorhinus ursinus* remains. The P-values (based on 999 permutations) are shown in brackets. Comparisons with significant P-values are shown in bold with ** indicating those significant at the 0.005 cutoff and * indicating those significant at the 0.05 P-value cutoff.

NETARTS	TSISHAA	CAPEADDINGTON	UMPQUA	SEALROCK	CHALUKA	OZETTE	SANMIGUEL	
0								**NETARTS**
0.000 (0.408)	0							**TSISHAA**
0.238 (0.004)**	**0.211 (0.008)***	0						**CAPEADDINGTON**
0.183 (0.003)**	**0.161 (0.019)***	0.130 (0.058)	0					**UMPQUA**
0.035 (0.230)	0.000 (0.367)	**0.204 (0.046)***	**0.187 (0.018)***	0				**SEALROCK**
0.021 (0.217)	0.000 (0.437)	**0.125 (0.021)***	0.051 (0.155)	0.047 (0.156)	0			**CHALUKA**
0.047 (0.163)	0.000 (0.409)	0.014 (0.359)	0.000 (0.349)	0.000 (0.428)	0.000 (0.441)	0		**OZETTE**
0.027 (0.210)	0.000 (0.453)	0.015 (0.327)	0.031 (0.236)	0.000 (0.390)	0.000 (0.426)	0.000 (0.482)	0	**SANMIGUEL**

Modern D-Loop AMOVA Analysis by GenAlEx

The AMOVA analysis of the modern northern fur seal D-loop sequence data revealed even less patterning. The very slight among population variance observed is non-significant as the P-value is 0.229, which is greater than 0.005, or the 0.05 P-value cutoffs (Table 5.8). The frequency distribution of the observed F_{ST} equivalent (PhiPT) versus the expected PhiPT shows that the expected (red bar) value is located in the middle of the range of the distribution generated with random data (black bars) (Figure 5.14). The AMOVA results of the modern mtDNA haplotype data indicate that a negligible amount of the total variation (0.2%, automatically rounded to 0% by the software package for Figure 5.15) is found among populations, while the remaining 99.8% of the variance (automatically rounded to 100% by the software package for Figure 5.20) is found within populations (Figure 5.15, Table 5.8). The estimated number of migrants was 259.416.

When the modern rookery islands were compared to one another in pairwise population comparisons, no islands showed a pattern of difference between populations at the 0.005 P-value cutoff (Table 5.9). The pairwise PhiPT(Φ_{ST}) for rookery site by site comparison did yield one significant difference when the P-value cutoff was raised to 0.05. This was a 1% difference between the San Miguel and Bogoslof islands (Table 5.9).

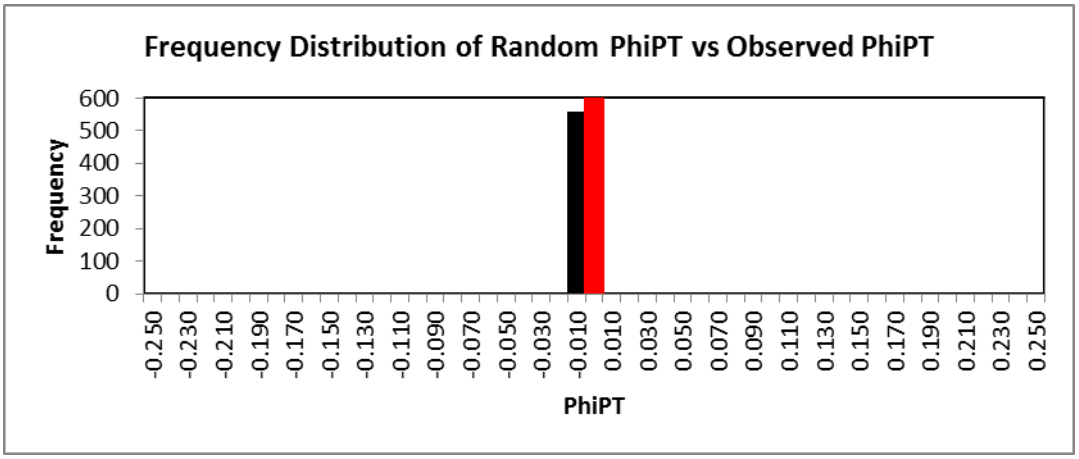


Figure 5.14. Frequency distribution of modern mitochondrial DNA D-loop haplotype data showing the observed PhiPT(Φ_{ST}) compared to the predicted PhiPT(Φ_{ST}).
Predicted PhiPT value based on if the data sorted randomly from *Callorhinus ursinus* island rookery populations. The observed data is located within the upper distribution of the expected data with a PhiPT(Φ_{ST}) of 0% (0.002) (red) using 999 permutations (P value is non-significant at 0.229).

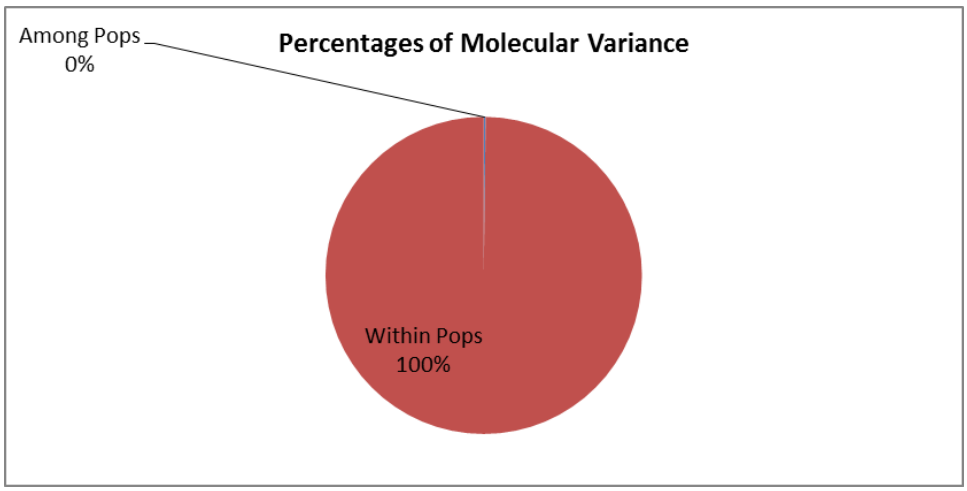


Figure 5.15. AMOVA graph of modern mitochondrial DNA D-loop haplotype data showing the molecular variance (PhiPT or Φ_{ST}) within and among populations of northern fur seal (*C. ursinus*).
Source data from modern rookery sites in Alaska and California. No significant variance at either P-value cutoff.

Table 5.8. Summary of AMOVA table analyzing the variance in modern mtDNA haplotypes within and among populations of *C. ursinus.*
Populations of *C. ursinus* sampled from rookery sites in Alaska and California. Table showing the degrees of freedom (Df), sum of squares (SS), estimate variance (Est. Var), percent variance (%), PhiPT, and the P-value (P(rand>=data)). The Φ_{ST} was not significant.

Source	Df	SS	MS	Est. Var.	%
Among Pops	3	13.232	4.411	0.007	0%
Within Pops	363	1360.602	3.748	3.748	100%
Total	366	1373.834		3.755	100%

Stat	Value	P(rand >= data)
PhiPT	0.002	0.229

Table 5.9. PhiPT(Φ_{ST}) values for the pairwise comparisons (Pairwise Population PhiPT Values) between the modern mtDNA D-loop sequences.
Sequence data obtained from four rookery island sites along the Pacific coast of North America with contemporary *Callorhinus ursinus* sequence data. The P-values (based on 999 permutations) are shown in brackets. No island comparisons were significant at the 0.005 P-value cutoff, though one site comparison was significant at the 0.05 (*) cutoff as highlighted in bold.

STPAUL	SANMIGUEL	BOGOSLOF	STGEORGE	
0.000				**STPAUL**
0.003 (0.218)	0.000			**SANMIGUEL**
0.005 (0.146)	**0.010 (0.050)***	0.000		**BOGOSLOF**
0.000 (0.396)	0.000 (0.394)	0.000 (0.414)	0.000	**STGEORGE**

AMOVA by GenAlEx 6.5 Summary

There is little evidence of the existence of separate populations or the partitioning of genetic variance among either the ancient or the modern data. While the ancient data shows some slight partitioning of variance as a whole (5% variance which was only significant at the 0.05 cutoff), more differences can be seen at the level of site by site comparisons. The only significant comparisons at the 0.005 cutoff came from the ancient island-by-island comparisons when Netarts was compared to either Umpqua or Cape Addington (Table 5.7). The number of significant comparisons increased when the P-value cutoff was raised to 0.05 (2 comparisons at the 0.005 P-value cutoff and 7 at the

0.05 cutoff for the site by site comparisons). In comparison to the slight partitioning of variance seen in the ancient dataset, the modern data is not significantly different from randomized data and there is no evidence of population genetic structure. As Arlequin is thought to yield better results (personal communication with Dr. Brent Murray, UNBC), the AMOVA analysis above was repeated using this program (Arlequin 3.5).

Ancient D-Loop AMOVA Analysis by Arlequin

An AMOVA analysis of the ancient northern fur seal D-loop sequence data was conducted using Arlequin 3.5 with 1000 permutations (using a minimum spanning network approach). This analysis yielded similar results to those generated using GenAlEx 6.5. The estimate of population variance determined for this ancient D-loop data set was 4.94%, and was non-significant at the 0.005 cutoff (Table 5.10). Again, only when the P-value cutoff was raised to 0.05 did this ~5% variance become significant.

At the level of pairwise comparisons, a few significant differences were found. At the 0.005 P-value cutoff, two pairwise comparisons between archaeological sites were found to be significantly different from one another: Cape Addington/Netarts and Cape Addington/Ts'ishaa (Table 5.11). These results are slightly different from those calculated with the GenAlEx program, as only one of these had been previously identified as significantly different at this 0.005 cutoff (the Cape Addington/Netarts comparison, Table 5.7). The Netarts/Umpqua comparison (which for GenAlEx was significantly different) is not significant here at the 0.005 cutoff, though the P-value of 0.00901 is only slightly greater than the 0.005/ 0.5% cutoff and is significant when the P-value cutoff is raised to 0.05) (Tables 5.7 and 5.11). The significant difference between Cape Addington and Ts'ishaa (Table 5.11) at the 0.005 cutoff is a new observation that was not found in the GenAlEx 6.5 AMOVA (though the P-value indicated a value close to significant at this cutoff level) (Table 5.7). Overall, these values are similar to those obtained by GenAlEx 6.5, though Arlequin 3.5 does yield some slight variation among this ancient D-loop 'population' data. When the P-value is raised to 0.05, the significant

Table 5.10. AMOVA results analyzing the variance in ancient mtDNA haplotypes within and among populations of *C. ursinus.*

Source data from archaeological sites along the Pacific Coast of North America. Table showing the degrees of freedom (Df), sum of squares (SS), variance (Est. Var), percent variance (%), F_{ST}, and P-value (significance tests based on 1023 permutations). Variance is only significant at the 0.05 P-value cutoff, and not at the 0.005 cutoff.

Source of Variation	Df	SS	Variance	% Variance
Among populations	8	38.938	0.18416	4.94
Within populations	57	202.123	3.54602	95.06
Total	65	241.061	3.73017	100.00%
Fixation Index:	FST= 0.04937			
P-Value	0.01564*±0.00368			

Table 5.11. F_{ST} values for the AMOVA pairwise comparisons (Pairwise Population F_{ST} Values) between the mtDNA D-loop sequences for the eight archaeological sites.

Archaeological sites located along the Pacific Coast of North America. Site data only including those sites with multiple *Callorhinus ursinus* remains. The P-values (based on 110 permutations) are shown in brackets. Comparisons with significant P-values are shown in bold with 2 stars (significant at the 0.005 cutoff) and 1 star (significant at the 0.05 cutoff).

NETARTS	TSISHAA	CAPEADDINGTON	UMPQUA	SEALROCK	CHALUKA	OZETTE	SANMIGUEL	
0.000								**NETARTS**
-0.01760 (0.60360)	0.000							**TSISHAA**
0.23772 (0)**	**0.21077 (0) ****	0.000						**CAPEADDIN.**
0.18264 (0.00901)*	**0.16104 (0.04505)***	0.12972 (0.09009)	0.000					**UMPQUA**
0.03493 (0.25225)	-0.01043 (0.48649)	**0.20364 (0.03604)***	**0.18719 (0.01802) ***	0.000				**SEALROCK**
0.02130 (0.25225)	-0.03064 (0.80180)	**0.12475 (0.00901)***	0.05068 (0.15315)	0.04650 (0.13514)	0.000			**CHALUKA**
0.04685 (0.14414)	-0.02740 (0.55856)	0.01370 (0.28829)	-0.00981 (0.42342)	-0.02915 (0.55856)	-0.00924 (0.54054)	0.00000		**OZETTE**
0.02695 (0.13514)	-0.00923 (0.51351)	0.01534 (0.28829)	0.03105 (0.18018)	-0.00681 (0.47748)	-0.01907 (0.69369)	-0.09216 (0.99099)	0	**SANMIGUEL**

F_{ST} comparisons presented here are the same as the significant Φ_{PT} values above: Netarts/Cape Addington, Netarts/Umpqua, Ts'ishaa/Cape Addington, Ts'ishaa/Umpqua, Cape Addington/Seal Rock, Cape Addington/Chaluka, and Umpqua/Seal Rock (Tables 5.7 and 5.11). While Arlequin 3.5 generates more precise data, when these significant values are rounded to the same number of digits as those of GenAlEx 6.5, the values become identical (Tables 5.11 and 5.7).

Modern D-Loop AMOVA Analysis by Arlequin

The AMOVA analysis of modern D-loop sequence data for northern fur seals performed by Arlequin 3.5 yielded similar results to those presented above for the AMOVA analysis conducted using GenAlEx 6.5. The total variance found using this Arlequin 3.5 AMOVA yielded a variance of 0.19% (Table 5.12) , which rounds to the same value as previously obtained by GenAlEx 6.5 (Table 5.8), again yielding a non-significant P-value for either cutoff.

As with the previous AMOVA analysis by GenAlEx 6.5, the AMOVA by Arlequin 3.5 did not find any significant island-by-island pairwise comparisons at the 0.005 cutoff (Table 5.13). However, when the cutoff was raised to 0.05, one comparison became significant: San Miguel/St. George (Table 5.13). This comparison is different from the significant PhiPT comparison by GenAlEx 6.5 at the same significant level, though both AMOVA analyses found a significant comparison at the 0.05 cutoff between San Miguel and an island in the Bering Sea (Tables 5.9 and 5.13).

Table 5.12. AMOVA results (F_{ST}) analyzing the variance in contemporary mtDNA haplotypes within and among populations of *C. ursinus.*
Source data from rookery sites along the Pacific Coast of North America. Table showing the degrees of freedom (Df), sum of squares (SS), variance (Est. Var), percent variance (%), F_{ST}, and P-value (significance tests based on 1023 permutations). Variance is not significant at either cutoff.

Source of Variation	Df	SS	Variance	% Variance
Among populations	3	13.232	0.00722	0.19
Within populations	363	1360.602	3.74821	99.81
Total	366	1373.834	3.75544	
Fixation Index:	FST : 0.00192			
P-Value	P-value = 0.21994+- 0.01097			

Table 5.13. AMOVA by Arlequin analysis of F_{ST} values for the pairwise comparisons (pairwise difference) between the mtDNA D-loop sequences for four contemporary *Callorhinus ursinus* rookery island sites.
Rookery sites located in Alaska and California. The P-values (based on 110 permutations) are shown in brackets. No island comparisons were significant at the 0.005 P-value cutoff. Significant comparison (*) at the 0.05 is highlighted in bold

STPAUL	SANMIGUEL	BOGOSLOF	STGEORGE	
0.000				**STPAUL**
0.00495 (0.10811)	0.000			**SANMIGUEL**
-0.00198 (0.51351)	-0.00153 (0.57658)	0.000		**BOGOSLOF**
0.00252 (0.19820)	**0.00979 (0.02703) ***	-0.00234 (0.67568)	0	**STGEORGE**

AMOVA by Arlequin Summary

The AMOVA analysis by Arlequin 3.5 resulted in little difference from the AMOVA conducted by GenAlEx 6.5. The modern data results of Arlequin 3.5 were almost identical to those previously determined, with no significant partitioning of the variance, nor any significant island by island differences at the 0.005 cutoff and only one island by island comparison significant at the 0.05 cutoff. For the ancient D-loop sequence data, the overall pattern of variance distribution was similar to that of GenAlEx 6.5, with almost 5% of the variance found among populations, though this value is again only significant

at the 0.05 cutoff (Table 5.10). The archaeological site-by-site pairwise comparisons found Cape Addington and Netarts to be significantly different from one another in both Arlequin (Table 5.11) and GenAlEx (Table 5.7) analyses at the 0.005 cutoff, but only Arlequin found Cape Addington and Ts'ishaa to be significantly different at this P-value cutoff. Additionally, Arlequin did not find any significant difference between Netarts and Umpqua (which was significantly different in the AMOVA by GenAlEx) at the 0.005 cutoff. When the P-value cutoff was raised to 0.05, the ancient D-loop population pairwise comparisons by Arlequin 3.5 yielded identical significant results to those of GenAlEx 6.5.

5.4. Summary

This chapter presented the data obtained by the analyses performed for this thesis. The phylogenetic trees proved a good way to identify sample sequences to species; however, their power in resolving northern fur seal phylogeny was weak as the two genes resulted in different trees and the tree topology varied for the D-loop sequences with the various outgroups and tree-building methods applied (see Appendix C for more detail). These findings (including low bootstrap support and a variety of trees) suggest that the majority of nodes are not supported in the D-loop phylogenetic trees. Regarding the genetic diversity, it becomes evident that the number of alignment gaps and lack of modern sequences makes the *cytochrome b* data set quite challenging as either the number of sites or the number of sequences had to be dramatically decreased in order to ensure that more polymorphic sites were analyzed. In addition, it only revealed a handful of haplotypes (Figure 5.1). In contrast, the D-loop data revealed an abundance of haplotypes in both ancient and modern samples with high haplotype diversity (values from 0.8 to 0.1 with a mean of .97 and a median value of 1 for all comparisons) (Tables 5.4, 5.5). The nucleotide diversity of the D-loop data for the ancient (range π = 0.037-0.058, median π =0.046, mean π = 0.047, all comparison groups) sequences gave a wider range of values though the average nucleotide diversity was fairly close to those obtained from the modern sequences (range π = 0.045-0.048, mean π =0.048, median π = 0.048) (Tables 5.4 and 5.5). The nucleotide mismatch analysis appears to show recent population expansion for both ancient and modern data

sets. The network analysis appears to cluster the lineages into two main branches that are maintained through time, with preservation of some ancient haplotypes into modern times (Figures 5.8-5.11). AMOVA analyses conducted by both GenAlEx 6.5 and Arlequin 3.5 found 0% of the variation was among populations for modern northern fur seal D-loop sequence data, but was around 5% (only significant at the 0.05 P-value cutoff) for the ancient data set. Thus, overall, all the AMOVA analyses suggest no partitioning of variance among modern populations and little to no partitioning of variance among ancient populations. The clearest evidence of difference are a few site-by-site comparisons for the ancient set (Cape Addington/Netarts, Netarts/Umpqua and Ts'ishaa/Cape Addington) at the 0.005 cutoff. Both GenAlEx 6.5 and Arlequin 3.5 yielded seven identical significant site by site comparisons at the 0.05 cutoff for ancient D-loop data.

In summary, the D-loop data generated high diversity with a large number of unique haplotypes with similar values between the ancient and modern data sets. When testing for geographic patterning, no strong correlations were found between diversity and latitude. Nucleotide mismatch distributions both resulted in a relatively smooth curve, with the ancient population appearing slightly more ragged. The networks revealed the same cluster division between the past and present data sets. The AMOVA analyses showed no modern division with a low ancient F_{ST}/Φ_{ST}. The following chapter will discuss how these results can be used to investigate the research questions and hypotheses proposed.

Chapter 6.

Discussion

6.1. Introduction

This section of the chapter interprets the results obtained from the analyses. It begins with a brief discussion of some considerations on northern fur seal behaviour regarding the appearance of northern fur seal on shore, followed by a discussion of the phylogeny and species identification results. It then examines the results of the analyses of northern fur seal sequence data to look for evidence of increased population structure/subpopulations in the past, where isolated breeding colonies distributed over substantial distance may have represented some type of barrier to gene flow. These analyses will include an assessment of haplotype and nucleotide diversity, pairwise nucleotide mismatch data, median-joining networks, and AMOVA analyses.

6.2. Sites: Offshore, Strandings, Haul-outs, and Rookeries

Before any discussion of the analyses can take place, it is important to emphasize that the remains recovered were obtained from human occupation sites. The source of these northern fur seal remains is uncertain. These remains may have come from northern fur seals hunted far offshore (with implications for human technology at these sites), from recovery of stranded northern fur seals or hunting at local haul-out sites, or from hunting at potential rookery sites (which would be expected to result in genetic population differentiation that is tested in this thesis).

Regarding the first option, given that far offshore hunting of northern fur seals is an energetically poor resource and there is little archaeological support for far offshore hunting, it seems unlikely this is the source of the northern fur seal remains. It seems

more likely that these northern fur seals were obtained while on land where they would be far more easily accessible (Burton *et al.* 2001; Moss *et al.* 2006; Newsome *et al.* 2007). Similarly, for the second option, the archaeological abundance of northern fur seals has been suggested to preclude the likelihood that these remains were the result of stranded individuals as this is not expected to be a common enough occurrence that it would account for the abundance of remains found (Newsome *et al.* 2007). While archaeological evidence supports active hunting of northern fur seals (Newsome *et al.* 2007), some studies argue that this possibility cannot be completely disregarded (Moss *et al.* 2006). It is therefore important to assess is whether these northern fur seal remains may have come from 'haul-out' locations where pinnipeds may come ashore preferentially, perhaps during migration periods. Today, there is a general trend of northern fur seals foraging far offshore, with most individuals found inland resulting from strandings (Moss *et al.* 2006). During their time at sea, northern fur seals are only reported hauling-out on shore while sick or injured (Gifford-Gonzalez *et al*. 2004). A study using satellite telemetry found that during migrational periods, northern fur seals were rarely found in shallower coastal waters (Ream *et al.* 2005). These modern migrational routes did not bring northern fur seals close to shore near either site of Ts'ishaa or Cape Addington (Moss *et al.* 2006; Ream *et al.* 2005). If northern fur seals in the past did not have local rookery sites and migrated north to breed, then it seems likely that their migrations in the past may have followed a similar pattern to that seen today, thus making it likely that northern fur seals would haul-out very infrequently at many of these sites. However, today occasional haul-out sites have been reported for northern fur seals in the Forrester Islands (South of Cape Addington, which is not located along the migratory routes reported by Ream *et al.* [2005]) and recently for Shell Island – Simpson Reef, Oregon (approximately 250 km south of Netarts) (Moss *et al.* 2006). Thus, despite the archaeological abundance and far-offshore foraging pattern of northern fur seals, the possibility of local haul-out sites rather than rookeries cannot be excluded entirely. This leaves the final, most probable option, that there were local rookeries within the vicinity of these archaeological sites. However, when interpreting these results, one needs to keep in mind the existence of other possibilities.

One further note regarding site descriptions is that much of the data on northern fur seal is recovered from archaeological sites less than 8,500 years old, with many of

the studies exclusively (Moss *et al.* 2006; Pinsky *et al.* 2010), or in large part (Burton *et al.* 2001; Newsome *et al.* 2007), using remains dating to within the last 2-2,500 years.

As sea levels have been relatively constant over the past 2000 years (Sivan *et al.* 2004), the coastal landscape with regard to rookery/haul out sites is not believed to be substantially different for the data used in this thesis. Further, even though a period of drought has been recorded over this time span, it is not believed to have substantially altered marine conditions at the time (Newsome *et al.* 2007). Compared to earlier time periods, the last 6-7,000 years has seen relative stability in ocean levels with changes of only a few meters (Balsillie and Donoghue 2004; Lambeck and Chappell 2001). Thus most studies referred to in the literature, and in this thesis, make the assumption that the geography over the relevant archaeological time frame was fairly close to that of today.

6.3. Phylogeny and Species ID

The phylogenetic and species identification methodology appears sound. The species identifications agree with those determined by Moss *et al.* (2006). Both D-loop and *cytochrome b* trees appear to sufficiently identify most sequences to genus, if not species, and both clearly identified northern fur seal sequences. One minor improvement was made over sample identification by Moss *et al.* (2006), courtesy of the different data set used for comparison from GenBank (Benson *et al.* 2008). This was the species identification of sample NF19 to the species *Arctocephalus philippii* (formerly only identified to genus with *A. philippii* and *A. townsendi* as possible matches), though the bootstrap values are still very low for all tree groupings.

The phylogenies generated here reveal two things. First, these trees are useful for confirming species identity, particularly of northern fur seal samples. Secondly, the high degree of variability between the trees generated here highlights the overall debate in the classification of pinnipeds (see Appendix C for full discussion). While this study only utilized maternally inherited mtDNA, the taxonomy of pinnipeds is further complicated by the use of other genes and genetic regions. Further differences would be expected when considering other genetic regions as a result of varying mutation rates in different genetic regions, or mitochondrial/nucleotide/Y-chromosome differences from

differences in male/female inheritance such as patterns of behaviour/movement (i.e. migration and reproductive strategies) (Lau *et al.* 1998).

6.4. Genetic Diversity of Northern Fur Seals

The following sub-sections will discuss the evidence for population structure/population subdivision in northern fur seals. This evidence is based on the various approaches to investigate haplotype and nucleotide diversity. It will begin by discussing the number of haplotypes identified and whether the phylogenetic trees revealed any geographic patterning. Next, it will discuss the haplotype and nucleotide diversity measures that were determined using DnaSP and MEGA 6. Finally, it will discuss the results of the nucleotide mismatch distribution, network analysis, and AMOVA analyses. Since these analyses are only to examine northern fur seal populations, the sequences morphologically misidentified as northern fur seal that were genetically identified as other species were removed from the analysis.

6.4.1. Phylogenies and Number of Haplotypes and Geographic Patterning

The neighbour-joining tree of *cytochrome b* haplotype data revealed little genetic difference, with only two main haplotypes (Figure 5.1). There was a single sequence that formed a third haplotype. This sequence is not the result of a new mutation, as the sequence in question belongs to an ancient northern fur seal specimen (Pinsky *et al.* 2010). Therefore, this sequence could represent an ancient haplotype or may perhaps have been the result of post-mortem DNA damage, which is common in ancient samples. An inspection of the sequence data for this haplotype revealed that it was identifiable by a G→A (at site 41). This type of transition is typical of DNA damage that results from the hydrolytic deamination of cytosines (Brotherton *et al.* 2007; Hofreiter, Jaenicke *et al.* 2001; Schuenemann *et al.* 2011). It may, therefore, be that this is not a true ancient haplotype; however, more data is needed to investigate this question, such as the use of repeat extractions or cloning, or the use of a method such as uracil-N-glycosylase to reveal if this was the result of cytosine deamination (Briggs *et al.* 2010; Hofreiter, Jaenicke *et al.* 2001). Though the neighbour-joining tree revealed only three

haplotypes, the DnaSP analysis of the northern fur seal *cytochrome b* data calculates between two and four haplotypes (Table 5.3). The manual tabulation of mutations based on sequence alignments found a total of thirteen mutations (Table 5.1). These differences highlighted the problem with this *cytochrome b* dataset – a number of sequences had substantial alignment gaps resulting in sites being omitted from analysis.

The neighbour-joining tree of D-loop sequences revealed a number of different haplotypes; many were unique with only a single sequence, though a handful of haplotypes had up to three sequences (Figure 5.2). There were fewer and smaller alignment end gaps so there was less of an issue with omitted comparisons. DnaSP identified 57 ancient haplotypes and 187 modern haplotypes. The ancient samples had a greater proportion of unique haplotypes compared to the modern samples. There are a few possible explanations for this. First, it may reflect DNA damage artificially inflating the haplotypes by conflating post-mortem DNA damage with true mutations. It may also be that there was greater genetic diversity in the past which was then lost through the dramatic population decline events. It could simply be a factor of a continuous relatively fast mutation rate and mutations accruing through lineages, combined with the way that archaeology often has difficulty narrowing in on a specific 'snapshot' of time by instead compressing a large window of time for the 'big picture'. That is, even if the proportion of unique haplotypes at any one time is the same as today, with the potential age range of samples, a number of mutational events may have occurred through the generations. Thus, when sampled in archaeological contexts, sequences are more likely to be diverse. Finally, it may be a factor of the increasing sample size which may result in a tapering off in haplotype richness (Chaves *et al.* 2011).

Neither the *cytochrome b* nor the D-loop tree showed much evidence for geographic patterning (Figures 5.1 and 5.2). Had any haplotypes consisted of only sequences from one location, or from a cluster of sites in close geographic proximity, this would have been clear evidence of geographic patterning/population structure. For instance, a study on water buffalo using a similarly sized D-loop fragment (158 bp) found clustering of some island haplotypes that were distinct from the mainland population (Lau *et al.* 1998). In this study, for the *cytochrome b* phylogeny, all but one of the sequences grouped with one of two main haplotypes. Each of these haplotypes

contained sequences from all of the different sampling locations. Because of this, there is no way to discern any evidence of geographic patterning from this tree. For the D-loop data, samples from all of the geographic regions were dispersed throughout the phylogenetic tree (Figure 5.2). There was no strong evidence of genetic patterning observable from this tree. This does not necessarily indicate an absence of population structure; there may be differences detectable in haplotype frequency/diversity or nucleotide diversity that may reveal some structure.

6.4.2. Diversity Values: DnaSP Analysis

When looking at diversity between populations within a species, the D-loop/hypervariable region is a favoured marker (Lau *et al.* 1998; Naderi *et al.* 2007; Weiss *et al.* 2000), while cross-species comparisons often use mitochondrial coding genes, such as *cytochrome c* oxidase subunit 1 (Goodall-Copestake *et al.* 2012). DnaSP was used to analyze a number of measures of genetic diversity to investigate population structure. These measures included haplotype and nucleotide diversity, calculated using the equations of Nei (1987) and Nei and Tajima (1981).

The impact of the analytical algorithms and how the treatment of alignment gaps can affect the analysis is evident in the results from the northern fur seal *cytochrome b* data set. As alignment gaps are excluded from the analyses, the presence of these due to poor sequence information may affect the number of sites, and therefore the number of haplotypes calculated (Table 5.3). For the *cytochrome b* data, when all 67 sequences were analyzed, of the eight polymorphic sites, six were excluded from analysis due to alignment gaps at the beginning and end of sequences. Typically sequences are trimmed to remove the alignment gaps that result from short sequences or when bidirectionally sequenced regions do not perfectly overlap unresolved bases with clear sequence reads. In this case, the 139 bp region[21] analyzed here was delimited by the sequences amplified by Moss *et al.* (2006) (excluding primers). Trimming all of these amplified sequences to the same length would have dramatically reduced the sequence

[21] This was the maximum length- end gaps further reduced this length for a number of comparisons

information, as can be seen in the reduction of eight polymorphic sites to two. The alternative would be to remove short sequences from the analysis. In the case of the *cytochrome b* data set, this would have resulted in far fewer sequences (reduced from 67 sequences to 34), essentially cutting the data set in half. Given the limited starting data set, in order to maximize the potential information obtainable from the sequence data, all sequences were left in for the DnaSP analysis to examine the nucleotide and haplotype diversity. The DnaSP analysis indicated that some of the comparisons included less than 60% of the sites. This dramatic decrease in polymorphism resulting from the exclusion of sites with alignment gaps clearly has a strong effect on the determination of haplotype diversity.

Haplotype Diversity

Haplotype diversity is a measure of the uniqueness of a haplotype in a population or the relative proportions of each haplotype (Naderi *et al.* 2007; Nei and Tajima 1981; University of Auckland 2005; Weiss *et al.* 2000). The lower the value, the more a single haplotype will dominate (McMillan and Bermingham 1996).

Cytochrome b

The DnaSP analysis found that the haplotype diversity of the *cytochrome b* analyses ranged from 0.356 to 0.700 for the various comparisons (Table 5.3). This represents moderate haplotype diversity (Deffontaine *et al.* 2005; Goodall-Copestake *et al.* 2012). More precisely, the haplotype diversity for all of the ancient set was calculated to be 0.397, though the Cape Addington-only and Unalaska-only comparisons both showed higher values in the range of ~0.5-0.6. The highest diversity values were from the modern northern fur seal set, with a haplotype diversity of 0.700 and a nucleotide diversity of 0.01295 (Table 5.3). While this is contrary to expectations of decreased diversity in the present due to population declines, these values are not unsurprising given the small number of sites analyzed for both past and present groupings, as well as the quality of these sequences. Given that the alignment gaps of the ancient *cytochrome b* dataset resulted in many positions (and therefore many polymorphisms) being excluded from comparison it is not surprising that when the modern set was examined alone that a higher haplotype and nucleotide diversity was seen, given that more

positions were being compared, though the small dataset may allow for stochastic differences.

Since haplotype diversity calculations are based on Equation 8.5 from Nei and Tajima (1981), $H = \frac{N}{N-1}(1 - \Sigma_i x_i^2)$ which includes only the number of sequences and the frequencies of each haplotype and does not account for sequence length, obviously using longer sequences would result in increased likelihood of new mutations. This would be expected to decrease the frequency of each haplotype (χ_i) for the same number of sequences, and this in turn would be expected to increase the haplotype diversity. This value makes it quite difficult to cross-compare between studies, as different genetic regions/genes and different amplicon lengths affect the haplotype diversity. These diversity values are moderate when compared to similar studies. For instance, a study on the *cytochrome b* gene in voles reported a subpopulation diversity of 0.758-0.786 as 'low' while values of 0.911-0.978 where reported as 'high diversity' for a sequence of 1011 bp (Deffontaine *et al.* 2005). The *cytochrome b* haplotype diversity values found in this study would of course be expected to be much lower, given the much shorter sequence length used in this study compared to that of Deffontaine *et al.* (2005). A second study of *the cytochrome b* gene in camel breeds reported an average Hd value of 0.833 (for 1140bp). A third study, this one on a different mitochondrial gene, *cytochrome c* oxidase I (*coxI*), compared diversity values across a range of species (Goodall-Copestake *et al.* 2012). This third study found that the median haplotype diversity value was 0.701 and the mean diversity was 0.636 for a sequence length of 456 bp (Goodall-Copestake *et al.* 2012). In comparison, the haplotype diversity values found in this study are around and below the median values found in Goodall-Copestake *et al.* (2012). This would suggest that the *cytochrome b* haplotype diversity values found in this study are around a low to moderate in range (with most ancient values on the lower end). However, when sequence length is taken into consideration, the haplotype diversity values reported here appear to be expected to fall into a more 'moderate' range of 'moderate diversity'.

D-loop

The DnaSP analysis revealed high haplotype diversity for the D-loop analysis for both modern (Table 5.5) and ancient (Table 5.4) northern fur seal. The various D-loop groups analyzed generated values between 0.800 and 1.000 (Table 5.4, Table 5.5). As expected, given that the D-loop is a non-coding region, the Hd values in this region were higher than those for the *cytochrome b* data. This increased diversity reflects the increase in unique haplotypes that can be seen on the trees (Dickerson *et al.* 2010; McMillan and Bermingham 1996; Nei and Tajima 1981; University of Auckland 2005). Values for the modern D-loop sets (0.986-0.99) were located in the upper range of the Hd for ancient sets, though likely due to the larger sample size, none of the modern groupings were composed of entirely unique haplotypes (which would be represented by a Hd=1) (Table 5.5).

The haplotype diversity was higher in the D-loop data compared to those values in the *cytochrome b* data. This increased haplotype diversity is expected since the D-loop, as a non-coding region, is more subject to mutations. As the D-loop accumulates mutations faster it can be better for revealing more recent diversity than coding regions (Nisan 2014). It may be that the difference in relative diversity between the *C. ursinus cytochrome b* and D-loop to those of other species may indicate a temporal pattern. This possible pattern of higher modern diversity with decreased diversity in the past, could potentially be a reflection of commercial sealing. However, it is important to consider that the *cytochrome b* data has a number of alignment gaps, and thus some of the values may be skewed. The potential for alignment gaps to skew the diversity is supported by the moderate to high nucleotide diversity in *cytochrome b* (see following section) as well as the number of polymorphic sites that were excluded from analysis as a result of these gaps (Table 5.1).

There was little difference in haplotype diversity between the modern and ancient D-loop datasets. All of the modern data fell within the range of the ancient set (Figure 5.5, Tables 5.4 and 5.5). The haplotype diversity of the entire ancient data (0.995) set was slightly higher than that of the entire modern data set (0.989). This may potentially indicate a slight loss of diversity over time (perhaps reflecting some impact of commercial sealing and the loss of haplotypes), or it may simply be that the D-loop

diversity in the past may be slightly inflated due to archaeological sampling of remains over larger timescales or may be an artefact of post-mortem DNA damage (see Appendix A for further discussion) (Weber *et al*. 2004).

Various site groupings (as outlined in Chapter 4.4) of both ancient and modern D-loop sets primarily yielded Hd values greater than or equal to 0.950 (Figure 5.3). The values found in this study were quite close to those obtained in Pinsky *et al.* (2010). This is not surprising given that the modern sample set was almost exclusively comprised of sequences from Pinsky *et al.* (2010)[22] due to the removal of haplotypes without frequency data (Dickerson *et al.* 2010). Further, the sequences generated by Pinsky *et al.* (2010) also comprised a large portion of the ancient dataset. Pinsky *et al.* (2010) reported a Hd of 0.989 (which rounds to the same value as found in this study) for their modern northern fur seal sequence set and 0.996 for their ancient (compared to 0.995 in this study). These D-loop Hd values would also be considered to be high when compared with other studies (Chaves *et al.* 2011; Dickerson *et al.* 2010; Lau *et al.* 1998; Naderi *et al.* 2007; Weiss *et al.* 2000).

As mentioned previously for *cytochrome b*, following Nei and Tajima's (1981) equation, Hd values are expected to correlate somewhat with sequence length, with longer sequences expected to show increased Hd values due to the increased likelihood of encountering new mutations and thus new haplotypes. The D-loop values presented here in this study would therefore be expected to show lower Hd values than those of comparative studies which used longer D-loop sequences. Instead, this is not the case. To compare with other studies on the same species, a study of modern northern fur seal that used a 375 bp sequence of the D-loop region reported high Hd values of 0.994; this is only slightly higher than the values reported in this study for the modern northern fur seal (range of 0.986-0.990) (Table 5.5), and is actually slightly lower than the Hd reported for the group containing all of the ancient northern fur seal sequences (Hd=0.995) (Table 5.4) (Dickerson *et al.* 2010).

[22] The only other source for the modern sample set was the five sequences from Wynen *et al.* (2001).

The D-loop Hd values in Tables 5.4 and 5.5 were also compared to those reported in other studies with different species and again found to be within a similar range, though the sequences in this thesis were of much shorter length. One study with a similar length (366 bp) fragment to that of Dickerson *et al.* (2010), on the mammalian species (*Brachyteles hypoxanthus*), also found a high Hd of 0.905 (Chaves *et al.* 2011). Further, all of the Hd values reported in this thesis for all ancient and modern northern fur seal sequences fall within the range of Hd values reported by Naderi *et al.* (2007) on their study on goats, though Naderi *et al.* (2007) used a much longer sequence length (558 bp, for a range of Hd= 0.8-1). Weiss *et al.*'s (2000) study on trout species (using a sequence length 310 bp) reported a generally high Hd at 0.85 (Weiss *et al.* 2000). Compared to these three studies, the values reported here are considered 'high' and roughly within a similar range to other studies reporting high diversity. However, once the shorter sequence length is taken into consideration, the Hd values found in this thesis are much higher; thus reclassification of the diversity found here to 'extremely high' for both ancient and modern sequence data is suggested.

Archaeological sites and potential archaeological site groupings were also compared to one another. Most of the data groupings (as outlined in Chapter 4.4) yielded fairly similar results to one another. However, there is one outlier clearly identifiable in Figure 5.3. This data point belongs to Cape Addington, with a D-loop Hd of 0.8. This anomalously lower Hd value may be due to low sample size, though data sets for other archaeological sites containing the same sample size showed much higher diversity. Since the samples are of archaeological origin, it is possible that a set of remains may have been sampled more than once due to the fragmentary nature of these finds. However, it is standard procedure to sample remains from separate excavation units, strata, and unique skeletal elements to optimize the likelihood that the remains are from different individuals. To further investigate this possibly, a closer look was taken at the haplotypes. Samples NF30, NF32 and NF37 were found to have the same haplotype and they came from Unit 7 strata 15-25 cm, Unit 7, 23-35 cm, and Unit 7, 25-37 cm respectively (raw data and excavation notes from Moss *et al.* [2006] not shown). Given the potential overlap in depositional context, it is possible that all of these remains may have come from one individual, unless skeletal element is accounted for. These remains represent a maxilla with canine, a left premaxilla, and a right maxilla. The (presumed)

complete ‘maxilla with canine’ and the ‘right maxilla’ must, therefore, come from separate individuals. However, it is possible that the ‘premaxilla’ could potentially have belonged to the same individual as either of the maxilla remains. It is, therefore, possible that some degree of this outlier may be due to resampling of the same individual; regardless, at least two separate individuals were found to have the same haplotype.

These two sets of remains with the same haplotype may represent randomly chosen individuals from the population or may be biased by the potential of sampling related individuals. For instance, related individuals may be present at the same location at the same time (such as a mother and offspring) at a rookery or haul-out, or maternally related individuals at the same rookery. This would not be unexpected given the typical northern fur seal pattern of high philopatry of returning to natal island to breed. Similar sampling concerns have been expressed by Pinsky *et al.* (2010), given that female northern fur seal fidelity to natal sites may extend to even particular sub-sections of a colony and human harvesting behaviour may conceivably be restricted/limited by ease of access.

Unfortunately, small sample sizes for archaeological remains in this study (which is in general a problem for ancient DNA) may also potentially over-inflate the estimates of ancient haplotype diversity. Future studies would potentially include far more samples from each archaeological site. Since many of the other studies on other species’ D-loop sequence data presented here are around 310-380 bp, it would be beneficial for any future analyses on these northern fur seal remains to attempt amplification of a longer region of the D-loop (Chaves *et al.* 2011; Dickerson *et al.* 2010; Naderi *et al.* 2007).

To summarize, the *cytochrome b* Hd values are within a moderate to low range when compared to other coding mitochondrial regions, with the higher values corresponding to the small modern dataset. In comparison, the D-loop haplotype diversity is extremely high in both modern and ancient datasets compared to other species. While the total ancient D-loop Hd was slightly higher than that of the total modern set, all of the modern groupings had Hd values within the upper ranges of the ancient dataset. This wider range of D-loop Hd values seen in the ancient dataset may be the result of sampling and low sample sizes. Further, the slightly higher D-loop Hd in

the ancient set may be due to population declines, or it may simply be the result of low sample sizes (with few to no repetitive haplotypes sampled), or the use of archaeological samples where DNA degradation and large time scales may artificially inflate haplotype diversity.

There is some general correlation between nucleotide and haplotype diversity, with the values of nucleotide diversity being smaller than those for haplotype diversity. For instance, Goodall-Copestake *et al.* (2012) found that the Bayesian Information Criterion indicated that for their gene of interested (*cox1*), a good model of approximation was that $\pi = (Hd)^2$. The authors rationalized that based on the diversity equations in Nei and Li (1979) and Nei (1987), where:

$$\hat{\pi} = \frac{n}{n-1} \Sigma_{i,j} x_i x_j \pi_{ij} \quad \text{and} \quad H = \frac{N}{N-1} (1 - \Sigma_i x_i^2)$$

, this relationship would be expected as π is based on "the pairwise comparison of nucleotide differences within distinct haplotypes and is dominated by the square term" (Goodall-Copestake *et al.* 2012, p. 55). The following sub-section will look at nucleotide diversity for the northern fur seal data presented here.

Nucleotide Diversity

Nucleotide diversity is the average of the nucleotide differences per site between randomly chosen sequences (Nei 1987; Nei and Tajima 1981). Unlike haplotype diversity, the measure of nucleotide diversity employed in this study (based on the equations in Chapter 4.5.2) does account for sequence length, as π is a measure of the average nucleotide differences *per site* (Nei 1987; Nei and Tajima 1981). This suggests that nucleotide diversity values could be directly compared one-to-one with other values for the same gene, regardless of sequence length. Of course, since this value is an average per site (Nei 1987; Nei and Tajima 1981), it would seem to follow that longer sequences should be less likely to have their diversity values dramatically affected by factors such as local mutational hotspots or regions under constraint which may skew the value one way or another.

Analysis of nucleotide diversity for *cytochrome b* ranged from 0.00432 to 0.01295 there was a huge range in diversity values (Table 5.3). The larger values were 2-3 times greater, however the only occurrence of values in this larger range were for the modern groupings. Among the ancient samples, the values were much more consistent (ranging from 0.00432 to 0.00720). In comparison, the median value reported for nucleotide diversity of the *cox1* gene by Goodall-Copestake *et al.* (2012) was π =0.00356. This would indicate that the nucleotide diversity found in this thesis for *cytochrome b* is moderate to high, despite the low-to-average haplotype diversity. This makes the modern diversity in the range of high to extremely high. This is an interesting finding since the ancient samples, which span a larger time frame and may therefore have been subjected to post-mortem DNA degradation resulting in sequence changes (such as through cytosine deamination), would be expected to have greater diversity. Since only selection (which is a possibility for *cytochrome b*) and population size are expected to change the frequency of an allele (Herrmann and Hummel 1994), one could conceive that perhaps the increase in nucleotide diversity in the modern populations may be due to recent population expansions and that with time, these additional haplotypes may be filtered out (i.e. by coalescence [Higgs and Attwood 2005]). However, one consideration is that this is a very small sample set (only 5 modern sequences) so it is difficult to draw conclusions.

The nucleotide diversity of the D-loop data for the ancient (range π = 0.03680-0.05817, median ~ π =0.046, mean ~ π = 0.047, all comparison groups) sequences gave a wider range of values than that of the modern sequences (range π = 0.04583-0.04868, mean ~ π =0.048, median ~ π = 0.048) (Table 5.4 and Table 5.5). As expected, given that the predominance of sequences used in this analysis are from Pinsky *et al.* (2010), the nucleotide diversity values reported in this study were extremely similar to those of Pinsky *et al.* (2010) who reported π =0.048 for both modern and ancient sequence data[23]. Pinsky *et al.* (2010) considered this to be high nucleotide diversity. In comparison to other studies on northern fur seal, such as Dickerson *et al.* (2010) who reported

[23] More precisely, the ancient nucleotide diversity was 0.0484, while the modern was 0.0477 (Pinsky *et al.* 2010).

'moderate nucleotide diversity' at π =0.024 (375 bp for modern northern fur seal D-loop sequences), these values do seem high as the average in this thesis is about twice those reported by Dickerson *et al.* (2010).

While cross-species comparisons of D-loop diversity would not be expected to be as useful as coding-region comparisons due to the increased range of variability, it is useful to understand how the diversity of northern fur seals compares to other species. However, different studies have reported varying values of π as being 'high', which makes it difficult to compare between different species. For instance, Lau *et al.*'s (1998) study on water buffalo using an almost identical length D-loop segment (158 bp) found this species had a range of nucleotide diversity for different groups from π = 0.0079 to 0.0749, with an overall diversity at 0.0526. They described this diversity as fairly high, which would suggest that the results reported in this study are perhaps a bit more representative of 'moderate' genetic diversity. In contrast, Melchior *et al.*'s (2010) study on human populations or Chaves *et al.*'s (2011) study on Muriqui suggest that northern fur seal nucleotide diversity values are quite high. Chaves *et al.* (2011) reported π =0.0135 as 'remarkably low' diversity, which would place the values reported here as moderate to high, while Melchior *et al.* (2010) referred to π = 0.0183 as 'fairly high', which would make the results in this study extremely high in comparison.

To summarize the nucleotide diversity values, the range found in this thesis would be considered moderate to high diversity (Chaves *et al.* 2011; Dickerson *et al.* 2010; Melchior *et al.* 2010; Pinsky *et al.* 2010). High nucleotide diversity may suggest a higher effective population size, population growth or high substitution/mutation rates (Berlin *et al.* 2008; Ingman and Gyllensten 2003).

Geographic Patterning of Diversity

The second objective of this thesis was to integrate the dataset utilized in this thesis work (unpublished sequence data drawn from the three sites in Moss *et al.*'s [2006] study) with previously published sequence data to observe whether there was any broad geographic patterning in the data. Given the distribution of sites along a north-south coastline, the haplotype and nucleotide diversity values reported in this study were

graphed against latitude. As outlined in the “Geographic and Temporal Patterning” subsection found in section 4.5.2, two models may be expected to describe the distribution of northern fur seal diversity. First, the ‘latitudinal model’ predicts decreasing diversity with increasing latitude based on the expectation of range expansion following glacial retreat, which may fit with some northern fur seal population size extrapolations in the past (Dickerson *et al.* 2010; Gifford-Gonzalez 2011; Schrey *et al.* 2011). The second option, the ‘centre-marginal model’, would similarly predict geographic patterning along a north-south axis for ancient samples given the north-south arrangement of archaeological sites along the coast. This model predicts the centre of a northern fur seal distribution to have the highest diversity, rather than a pattern of decreasing diversity with increasing latitude (Schrey *et al.* 2011). As can be seen from Figures 5.5 and 5.6, there is little evidence of geographic patterning visible. These figures show high haplotype and nucleotide diversity values across both ancient and modern sequence sets with no clear trend visible. This lack of geographic structure may potentially suggest a recent population expansion, though this is usually associated with low genetic diversity rather than the high diversity seen here (Milá *et al.* 2000; Okello *et al.* 2005). This pattern of high diversity (in both the past and present) with little population structure has been hypothesized to have been the result of a high dispersal rate of northern fur seals and the potential of the north to act as a refugium to ameliorate the impacts of loss of breeding range (De Bruyn *et al.* 2011; Foote *et al.* 2012; Newsome *et al.* 2007; Pinsky *et al.* 2010).

6.4.3. Mismatch Distribution

One technique often used to identify patterns in populations is the graphing of nucleotide mismatch distributions (Figure 5.7). This entails plotting the frequencies of pairwise distances; that is, the frequencies of pairs of individuals/sequences that differ across some number of sites, to generate a histogram. In such a graph, a period of population growth results in a ‘wave’ that travels left to right along the axis (from 0 to 1, 2, 3, 4 etc. sites) every 1/(2μ), with μ representing the standard symbol for mutation rate per generation (Rogers and Harpending 1992) since each new mutation is expected to increase the count of differences between sequences. Other types of events such as bottlenecks and population reductions also generate waves that travel through this

distribution curve over time (Rogers and Harpending 1992). Generally, the smoothness of the curve suggests a recent population expansion while a more 'ragged' graph indicates a long, stable population (Holsinger 2008; Ingman and Gyllensten 2003; Matisoo-Smith and Horsburgh 2012; Milá *et al.* 2000; Okello *et al.* 2005). Theoretically, a stable population in equilibrium should not result in any waves; however, computer simulations have shown instead a number of little peaks, or 'raggedness' (Okello *et al.* 2005; Rogers and Harpending 1992). The graphs typically show the number of site differences (Holsinger 2008; Okello *et al.* 2005; Rogers and Harpending 1992).

The nucleotide mismatch distribution of the ancient northern fur seal appear to form a single wave, though this wave is slightly more 'ragged' in appearance than is typical of a recent population expansion (Figure 5.7a). This may indicate a population expansion a little bit further back in time, with the recent expansion trending towards a stable population or it may be a factor of the limited sampling over an archaeological time scale, or may potentially be skewed by DNA degradation. In contrast, the modern samples clearly showed a recent population expansion. This evidence of population expansion is interesting because it contradicts observations of modern population decline. This is not a result of sampling older northern fur seals that may have been born prior to the decline, as the primary source of modern sequences was Pinsky *et al.* (2010) who collected samples from pups between 1993 and 1998. These pups were born during a relatively stable time period following the experimental harvesting decline (which lasted until the 1980's) and during the start of the current pup production decline (Dickerson *et al.* 2010; Towell *et al.* 2006; Trites 1992). It is possible that there is a delay between the population events and the nucleotide mismatch reflecting these events, or there may have been some greater population expansion/recovery during this 'stable period.'

This analysis can be expanded further in the future. Other programs such as Arlequin (Excoffier and Lischer 2010; Milá *et al.* 2000) can also be used to calculate nucleotide mismatch distributions to confirm these results. In addition to the raggedness statistic 'r' , more powerful statistical analyses such as 'Fu's F' can be used to more clearly define how 'ragged' or smooth the graph is and thus the type of population it represents (Rozas 2009; Rozas *et al.* 2003).

6.4.4. Network Analysis

A median-joining network analysis was undertaken to show the relationship between northern fur seal haplotypes. The network analysis revealed that both the ancient and modern networks appear to be split into two deep branches with only a single connection between them (Figures 5.8 and 5.9) and that this division is the same for both past and present (Figure 5.10). The ancient D-loop haplotypes seemed to form two larger clusters that may indicate a deeper split between these groups of haplotypes (Figure 5.8). Because the data being analyzed is haplotype data, these two clades may be considered 'haplogroups' (http://www.fluxus-engineering.com/). The deep split between groups may potentially indicate some evidence of structure/subdivision in these past northern fur seals. The modern network analysis of D-loop haplotypes also appears to be divided into two main groups (Figure 5.9), and there is clear evidence of a number of star-like phylogenies, indicating recent rapid population expansion (Edwards *et al.* 2004). As this deep split occurs in both ancient and modern sequence data (Figures 5.10 and 5.11), it may represent a preservation of some of the ancient haplotype diversity despite the recent population declines. Since there is undeniable historical and modern evidence for population declines, the star-like phylogenies may suggest population expansion/population recovery following these bottleneck events.

The larger of the two clusters in the network forms a large number of cycles. As mtDNA cannot recombine, this appears to indicate that a number of the same mutations occurred in different branches (homoplasy). Since mtDNA is unilinearly inherited, factors such as recombination and species hybridization may be expected to have no effect on the phylogeny (Allendorf *et al.* 2012; Arnason *et al.* 1996; Patterson *et al.* 2006). That is, all of the mitochondrial mutations should trace back to a single ancestral sequence (coalescence). The cycles seen in the networks are therefore unexpected and must represent significant homoplasy (Figures 5.8 through 5.11).

From the combined modern and ancient networks (Figures 5.10 and 5.11) it can be seen that the general pattern of two clades is preserved. Additionally, Figure 5.11 reveals a number of ancient haplotypes which are not represented in the modern sample. These may represent ancient haplotypes which have gone extinct, or they may be the result of DNA damage and not be true haplotypes. If these are true haplotypes,

this would indicate a high number of 'singleton' or allele/haplotype variants present only once in a sample. A high degree of singletons can be indicative of population expansions (Okello *et al.* 2005; Wynen *et al.* 2000; Zhao *et al.* 2006). Wynen *et al.* (2000) suggested this was one explanation for a similar phenomenon in a study of *Arctocephalus gazella* and *A. tropicalis*, though it may also have been a sampling artefact. A high proportion of singleton mtDNA haplotypes may also be a characteristic of ancient samples due to the effect of sampling lineages through time (i.e. as accumulated genetic changes over time could artificially inflate measures of diversity) (O'Rourke *et al.* 2000). Further investigation of whether these do represent true haplotypes could be accomplished by repeat extractions or cloning, or the use of a method such as uracil-N-glycosylase to test for damage.

6.4.5. F-statistics

The data hinted at potential population genetic structure/subdivision in the past, with limited evidence of structure in the present. The differences were determined using an AMOVA analysis to assess F-statistics (F_{ST} and Φ_{ST}). Neither PhiPT/ Φ_{ST} values derived from the AMOVA analysis by GenAlEx 6.5 (Tables 5.6, 5.7, 5.8, and 5.9) nor the F_{ST} values from Arlequin 3.5 (Tables 5.10, 5.11, 5.12, and 5.13) showed strong evidence of population genetic structure, though there were a few differences at the scale of archaeological site comparisons and 5% ancient variance at the 0.05 P-value cutoff. Both Arlequin 3.5 and GenAlEx6.5 results were identical for significant comparisons at the 0.05 cutoff, with both AMOVA analyses identifying seven comparisons with significant differences in F_{ST}/Φ_{ST}.

The AMOVAs by both GenAlEx 6.5 (Peakall and Smouse 2012) and Arlequin 3.5 (Excoffier *et al.* 2010) statistical software packages revealed slight differences in variance between modern and ancient DNA sets. Overall, at the 0.005 significance level, neither ancient nor modern datasets showed any statistical significance for among-population variance for the total range of northern fur seal distribution. At this P-value cutoff, while neither method found significant among-population variance for either the ancient or modern populations, the ancient did have greater (non-significant) variance. At the 0.05 P-value cutoff, the ancient ~5% among population variance does become

significant and thus is different from the modern population.[24] Overall, for the modern analyses, GenAlEx 6.5 found the variance to be 0.2% (P-value of 0.229) and Arlequin found the modern among-population variance to be calculated as 0.19% (P-value 0.21994), both of which are non-significant. Together, this suggests some minor population structure in the archaeological data, with some statistical support (at the 0.05 cutoff) and a complete lack of population structure overall in the present.

Regarding the ancient partitioning of variance, with only ~5% of the variance found among populations, the majority of the ancient D-loop variance was found within populations, and therefore most of the diversity is not unique to population sub-groupings (Falk *et al.* 2001; Whitlock 2011). The 5% variance among ancient populations may be a true reflection of past diversity or this may be an artefact of small sample sizes or the broad time-scale covered that may artificially inflate diversity. If this partitioning of the variance is valid and not an artefact, it could indicate that there was some slight population 'structure'/existence of subpopulations in the archaeological data (Falk *et al.* 2001; Peakall and Smouse 2012; University of Auckland 2005; Whitlock 2011). It appears, therefore, that overall there is some slight difference between archaeological and modern northern fur seal diversity (0.05 P-value cutoff). More differences became apparent when rookery/archaeological site by site pairwise comparisons were assessed.

The limited evidence of population genetic structure/subpopulations is further supported by the estimated number of migrants for both ancient and modern D-loop datasets. GenAlEx 6.5 estimated both ancient and modern to have greater than one migrant per generation. Less than one migrant per generation is the threshold under which population divergence resulting from drift is expected (Beebee and Rowe 2008). Values above this, such as those found in both the ancient and modern data sets in this analysis, suggest that population divergence would not be expected, which is consistent

[24] This 5% among population variance had a P-value of 0.016/0.024 (Arlequin/GenAlEx). Both P-values were greater than 0.005 or 0.5% cutoff, and therefore non-significant at the more stringent cutoff. Further, GenAlEx found the ancient 'observed PhiPT' value to be located within the frequency distribution range of random PhiPT values. This 5% variance is only significant at the 0.05 or 5% cutoff.

with the 0.005 P-value cutoff for the pairwise AMOVA analyses. However, the use of this threshold also assumes the populations are at equilibrium (Beebee and Rowe 2008).

Regarding the pairwise comparisons, the majority of sites showed no significant difference from one another, though there were a handful of site by site comparisons that did suggest some evidence of population genetic structure/existence of subpopulations (Beebee and Rowe 2008; Dickerson *et al.* 2010). For the ancient data, there were two significant comparisons for both F_{ST} and Φ_{ST} at the 0.005 cutoff (3 comparisons total) indicated site-by-site differences, which may hint at some local subpopulations/population genetic structure or this may simply be an artefact of the ancient DNA dataset (small sample sizes, archaeological sampling, DNA degradation). At the 0.05 cutoff, a total of seven comparisons for both F_{ST} and Φ_{ST} showed significant differences between archaeological sites. For the modern data, neither Arlequin 3.5's pairwise F_{ST} values nor GenAlEx 6.5's analogous PhiPT values showed evidence of significant differences between rookery sites at the 0.005 cutoff, and only a single comparison was significantly different at the 0.05 cutoff for each AMOVA.

The unpublished sequence data from Moss *et al.* (2006) was key to identifying archaeological site-by-site differences. For those comparisons showing population differentiation at the 0.005 cutoff, at least one of the two archaeological sites being compared was one of those analyzed by Moss *et al.* (2006). That is, strict comparison of the reported literature data did not find significant differences, and the only significant differences observed were site-by-site comparisons involving the new data presented here. When the cutoff was expanded to 0.05, six out of the seven significant comparisons involved at least one of the Moss *et al.* (2006) sites.

Both AMOVA analyses reported significant differences between the Netarts and Cape Addington ancient sites at the 0.005 cutoff (Tables 5.7 and 5.11). It seems likely that this may have some relation to the decreased haplotype diversity reported for Cape Addington (see Figure 5.3 and section 6.3.2), though Cape Addington is not significantly different from the other archaeological sites. As discussed, this decreased D-loop haplotype diversity may be related to sampling (re-sampling of remains or sampling of related individuals) which may have biased the sample set. It thus appears less diverse

than other archaeological sites or it could be a genuine reflection of a less-diverse subpopulation. More data is needed to confirm this hypothesis.

The PhiPT (Φ_{ST}) AMOVA analysis by GenAlEx 6.5 additionally reported significant differences between Netarts and Umpqua at the 0.005 cutoff (Table 5.7). This is particularly intriguing as both of these sites are in close geographic proximity (Figure 4.2). Given the geographic proximity of the three Oregon sites (Seal Rock, Netarts, Umpqua), it may be that remains from one or more of these sites may have come from the same rookery/location by direct human movement or trade with other coastal groups. This is the only site-by-site comparison which shows significant difference at P≤0.005 for either Φ_{ST} or F_{ST} between the DNA recovered by Moss *et al.* (2006) and the data of Pinsky *et al.* (2010)[25].

The pairwise F_{ST} analysis by Arlequin 3.5 also reported significant differences between Ts'ishaa and Cape Addington at the 0.005 cutoff (Table 5.11). This result may again be influenced by the low D-loop haplotype diversity of the Cape Addington data set; however, this result is not supported by the GenAlEx 6.5 AMOVA results, nor does the Cape Addington data set show statistical differences from other archaeological sample sets. As discussed in Section 6.1, modern migrational routes do not bring northern fur seals close to either Cape Addington or Ts'ishaa. If ancient populations were migratory, it follows that one might expect them to use similar migrational routes. If there was a rookery site at either (or both) of these locations, it may allow for some potential level of population isolation, with other seals bypassing these locations as they migrate to the Pribilofs, and thus some small degree of genetic separation.

For the ancient dataset at the 0.05 P-value cutoff, significant differences were found between Netarts/Cape Addington, Netarts/Umpqua, Ts'ishaa/Cape Addington, Ts'ishaa/Umpqua, Cape Addington/Seal Rock, Cape Addington/Chaluka, and Umpqua/Seal Rock (Tables 5.7 and 5.11). These comparisons are interesting because there does not appear to be any broad geographic patterning. For instance, there are significant differences between both Netarts/Umpqua and Umpqua/Seal Rock, yet all

[25] A total of three comparisons were significant at at P≤0.005 for either Φ_{ST} or F_{ST}: Netarts/Cape Addington (both Φ_{ST} and F_{ST}), Ts'ishaa/CapeAddington (F_{ST}), and Netarts/Umpqua (Φ_{ST})

three of these sites are in very close proximity to one another (see Figure 4.1). Because of this difference between sites in close geographic proximity, further AMOVA analysis of the larger regional groupings (as described in Section 4.4) was not conducted as it may have obscured these differences. Regarding the differences seen, it is interesting to note that while there are differences between sites in close geographic proximity (noted above), not all distantly separated sites had significant differences. For instance, while Chaluka/San Miguel and Cape Addington/San Miguel are the most geographically separated locations, there is no significant difference in these comparisons. Further, the ancient San Miguel sub-group was not found to be significantly different from any other archaeological site grouping and the Chaluka site was only found to be different from Cape Addington, and not any other distant archaeological sites.

It does seem that Cape Addington site is a bit unique, as it occurs in four of the seven significant ($P \leq 0.05$) archaeological site comparisons, more than any other site. This site is relatively geographically isolated compared to other sites represented in this study and more sampling between Cape Addington and the other locations would be beneficial in assessing patterns across this range. However, this isolation alone cannot explain the uniqueness of the site in the pairwise comparisons. As mentioned above, even more distantly separated sites failed to generate significant comparisons such as those between Chaluka and the other sites as well as the Cape Addington/San Miguel grouping, so another explanation might be required. As mentioned above and in section 6.1, the Cape Addington site is not particularly close to modern migrational routes (Moss *et al.* 2006; Ream *et al.* 2005). This may explain why this site appears a bit unique - perhaps in the past it may have been more isolated from migratory populations, or may have represented a local rookery with less genetic exchange with the other represented sites. This 'uniqueness' of the Cape Addington site is likely correlated with the low haplotype diversity at this site, which was previously discussed (for further debate on this issue see section 6.4.2).

Regarding the modern rookery site by site comparisons, no sites were significantly different at the 0.005 P-value cutoff for either AMOVA analysis. At the 0.05 cutoff, both GenAlEx 6.5 and Arlequin 3.5 identified a single significant comparison: each involved San Miguel Island. GenAlEx 6.5 found approximately 1% of the variance

(Φ_{ST}) was partitioned among rather than between San Miguel and Bogoslof, while Arlequin 3.5 reported approximately 1% F_{ST} variance among San Miguel and St. George. While the San Miguel population is non-migratory and would be expected to accumulate some genetic difference, the lack of strong differentiation may be expected, given that the recolonization of this island was a relatively recent event.

These results are generally consistent with those reported in other studies, but they also indicate slightly more partitioning of diversity than found in those other studies. This thesis suggests some possible overall population structure in the ancient set (only at the P≤0.05 cutoff), no overall modern partitioning of variance, and a handful of significant archaeological/rookery site by site comparisons. Most other studies on northern fur seal have found some slight evidence or no support for population structure. For instance, Pinsky *et al.* (2010) reported no significant F_{ST} value for either the ancient or the modern populations so statistically, both ancient and modern northern fur seals appeared to be a single population with no subdivision (which is more in support of panmixia rather than population genetic structure/subpopulations). Pinsky *et al.* (2010) also found no difference between ancient Californian and ancient Alaskan D-loop data (reporting negative F_{ST} values which are equivalent to zero) (Excoffier and Lischer 2010). In Dickerson *et al.*'s (2010) analysis of modern D-loop data (365 bp), while both F_{ST} and Φ_{ST} pairwise population comparisons yielded significant values, the only significant F_{ST} values were less than 1% of the variance, and Φ_{ST} less than 3%[26]. Dickerson found that Φ_{ST} values were more sensitive to population differentiation than F_{ST} values, with this analysis identifying a handful of differences between the various rookery island populations, primarily between the Russian and Eastern Pacific populations. Neither of the modern comparisons found to be significantly different in this thesis (F_{ST} or Φ_{ST}, P≤0.05) correlated with the significant pairwise differences identified by Dickerson *et al.*'s (2010) analysis of modern northern fur seals using the same significance level (P≤0.05, or 5% cutoff) (Tables 5.9 and 5.13). Since no modern Russian northern fur seal colonies were assessed in this analysis, whether or not there

[26] This is dramatically different compared to the ancient data presented here with significant pairwise population variance up to 24% in the ancient (both at P≤0.005 and P≤0.05). However, the modern data presented here reflects a similar range (around 1% variance for those comparisons which are significant at P≤0.05).

is structure at this geographic scale for ancient northern fur seal remains to be determined. Additionally, Dickerson *et al.*'s (2010) analysis of microsatellite data (7 loci) found no significant F_{ST} values and, therefore, no evidence of population structure.

The analysis presented here was conducted at two different significance levels. Overall, however, the general pattern of little to no structure/subpopulations among modern northern fur seal reported in the literature is consistent with the data reported here. The modern results presented in this thesis are generally consistent with other modern studies finding little to no population genetic structure and thus little evidence of subpopulations. While Pinsky *et al.* (2010) reported no significant F_{ST} values for ancient northern fur seal (either when assessed in total or when divided into geographic regions), this study has identified some evidence of structure, with overall partitioning of the variance among populations (for P-value cutoff of 0.05) as well as a small number of archaeological population site by site comparisons. The study by Pinsky *et al.* (2010) did not find any significant differences for the ancient data presented, which would suggest that this thesis is the first report of ancient partitioning of variance for northern fur seal. This study identified 5% overall among population variance (at the $P \leq 0.05$ cutoff). Additionally, a handful of archaeological population site by site comparisons were significant with F_{ST}/Φ_{ST} values of 13-24% among population variance identified (most at the $P \leq 0.05$ cutoff). This seems to clearly suggest at least some low level of variance partitioning/subpopulations in archaeological northern fur seal. Given the small sample sizes and possible impact of sampling over larger timescales and possible effects of DNA degradation, further study is still necessary.

Taken together, these findings suggest fairly high gene flow in both past and present northern fur seal populations, despite the behavioural observation of high philopatry (Pinsky *et al.* 2010). It appears there were few barriers to gene flow in northern fur seal populations, though there may have been some greater partitioning of diversity in the past, perhaps providing limited support to the hypothesis of local rookeries. F-statistic analyses have been supplemented in other studies with Bayesian analyses that have suggested northern fur seals may have retained a large refugia population despite commercial hunting, and that following this decline, had high dispersal rates which may have allowed the population to rebound (Pinsky *et al.* 2010).

One hypothesis regarding the existence of local rookeries, particularly those along the California coast where there are few off-shore islands (and, therefore, the possible existence of mainland rookeries), is that such rookeries may have only been temporary occurrences during years of *Callorhinus ursinus* abundance (Gifford-Gonzalez 2011). As well, there is a possibility that these 'rookeries' may not have been true rookeries, but instead may have acted as a training ground for sub-adult northern fur seals to come ashore during breeding season to gain experience with male-male competition while in the transition years between juvenile offshore foraging and breeding adulthood (Gifford-Gonzalez 2011). If either such behaviour is the case, the small-scale regional differentiation between Californian sites would be lessened, as remains obtained from these sites would show affinity with the source population. However, if such behaviours are only prevalent in lower latitudes, those southerly northern fur seals may have represented a larger meta-population which may still have had genetic differentiation from more northerly populations.

Dr. Iain McKechnie (personal communication) has also posited the potential for both migratory and local northern fur seal populations. A situation such as this may explain isolated genetically unique sampling sites, with some sites corresponding to local rookeries, which would be unique from other sites corresponding to the sampling of this migratory population (i.e. through haul-outs/strandings or pelagic hunting). Under such conditions, cross-sampling of individuals from both populations may confound possible patterns. However, as previously discussed, migrating northern fur seals rarely come ashore until they reach their rookery destination (Gifford-Gonzalez *et al.* 2004; Ream *et al.* 2005), so cross-sampling would not be expected to pose a substantial problem. The movement of northern fur seals between such populations would, however, lessen genetic differentiation between these posited groups of ancient northern fur seal through gene flow. Tandem analysis of ancient DNA and isotope analysis would help to elucidate this issue by differentiating between migratory and non-migratory individuals. Thus far; however, isotopic studies have indicated that ancient female northern fur seals fell into three distinct non-migratory geographic groupings (with year-round occupation in either Californian, North Pacific/ Gulf of Alaska/ Eastern Aleutians, or Western Aleutian waters (Burton *et al.* 2001; Burton *et al.* 2002).

Different patterns in ancient northern fur seal behaviour may be revealed if nuclear DNA (microsatellites or sex-specific markers) are considered, particularly if there is different sex-specific behaviour with regards to site philopatry. For instance, modern northern fur seals currently have differences in sex-specific migrational behaviour, with the males remaining in high latitude waters over the winter, while juveniles and females migrate south (Burton *et al.* 2001; Burton *et al.* 2002; Gentry 1998; Ream 2002; Ream *et al*. 2005), so it is possible that there may also have been behavioural differences with respect to rookery philopatry. There has been some evidence of 'straying', or a lack of site fidelity, in northern fur seals, though this is more common in younger individuals, with site fidelity increasing with age (Gentry 1998; Ream 2002). It is important to note that this study only examined mitochondrial DNA, and thus only traces the maternal lineages. While breeding with non-natal island males would not reduce patterns of mtDNA differentiation (provided that pups were born on and then exhibited philopatry to their maternal natal islands), the potential of sampling of both males and females from non-natal rookeries due to such visits may obfuscate patterns of differentiation. For future analysis, sex-specific markers which can identify patterns of northern fur seal genetic transmission in both modern and ancient populations will allow for investigation of whether paternity/genetic transmission follows the modern observations of behavioural philopatry and whether there were past differences between male and female northern fur seal philopatry.

6.5. Summary

With a handful of statistically significant differences at the level of site by site comparisons, and overall ancient F_{ST}/Φ_{ST} values significant at the 0.05 cut-off, it appears that there may be some evidence for population structure/subdivision among northern fur seals. The two clusters suggested by the D-loop networks may indicate ancient diversity that has been maintained until the present. Both the nucleotide mismatch distributions and networks suggest population expansion in the modern population and perhaps some degree of expansion in the ancient.

The phylogenetic trees did not reveal any clear evidence of patterning, though the haplotype diversity indicates a high number of unique D-loop haplotypes. The

cytochrome b diversity calculations are not robust due do the number of alignment gaps, as well as the very limited modern subset of this (only 5 modern northern fur seal *cytochrome b* sequences were available in the dataset) and, therefore, few interpretations could be made. It was, however, sufficient to clearly show that the D-loop diversity was higher than in this coding gene, as expected. Haplotype diversity was slightly higher in the ancient D-loop sequence data than in the modern, while the nucleotide diversity was similar between modern and ancient sets. The slight decline in D-loop haplotype diversity may potentially reflect a loss of diversity due to population bottlenecks, or it may be due to the other factors discussed (i.e. sampling of ancient material may artificially increase the diversity due to sampling over a large time scale, through DNA degradation, and the small sample sizes). The similar D-loop nucleotide diversity does not reveal a difference between past and present northern fur seal populations, and thus does not support evidence of greater structure among archaeological populations.

The high nucleotide diversity, combined with the paraphyly in the phylogenetic tree topologies as well as the mismatch distribution (both of which have an overall bell shape of recent expansion) are all features that are attributed to a generally panmictic population with exponential growth (Ingman and Gyllensten 2003). Together, this suite of features does not suggest much evidence for genetic patterning, either in the past or in the present. Despite this, the F-statistics do show some slight evidence of partitioning of variance in ancient northern fur seals. This partitioning can be seen in both the overall F_{ST}/Φ_{ST} values (5% among population variance, $P \leq 0.05$) and in the handful of significant island by island comparisons. Given that few studies have been undertaken on northern fur seal diversity using ancient DNA, and that of these, only Pinsky *et al.* (2010) conducted an AMOVA analysis, this thesis may be the first report of evidence suggesting some partitioning of variance in ancient northern fur seals.

To summarize, ancient diversity appears to be fairly similar to that of the modern sequence data, with high diversity values for all the D-loop data, consistent with other studies. While the proportion of unique haplotypes decreased, with fewer unique in the modern sample than the ancient, haplotype diversity remained high and nucleotide diversity values were within the same range. This suggests either the retention of high

genetic diversity, or the recovery of northern fur seals following recorded dramatic population declines. As well, there was no real geographic patterning of diversity in either the past or present data sets. This, combined with the phylogenetic tree data, showed no evidence of different populations in different locations. The nucleotide mismatch analysis, for both ancient and modern data sets appear to show populations with recent expansions, with perhaps a bit more trend towards stability in the ancient set. The two clusters or 'haplogroups' in the network analysis which are preserved over time may suggest that a good proportion of the diversity may have been preserved in northern fur seal, despite being intensive commercial hunting during the 1900s. This may reflect the resilience of northern fur seals. For the AMOVA analyses, the modern data showed no overall evidence of population subdivision, though two population pair comparisons were significant (one for each analytical method at the less stringent cutoff) suggesting a nearly panmictic population with little to no subdivision. The ancient data, in contrast, showed significant, but low population subdivision suggesting perhaps some greater structure in the past.

Chapter 7.

Conclusion

7.1. Introduction

This thesis has examined previously collected DNA sequence data from northern fur seal populations along the Pacific Coast of North America in order to investigate, through genetic evidence, the hypothesis of local subpopulations. This hypothesis was part of the broader investigation into the presence and nature of possible local rookeries along the coast of North America. The data included ancient as well as contemporary samples. The null hypothesis of this thesis was that no genetic difference would be observed between sampling sites. The data presented here tentatively rejects this null hypothesis with evidence of some partitioning of genetic data (F_{ST}/Φ_{ST} significant differences at $P \leq 0.05$, no overall significant difference between ancient and modern populations at $P \leq 0.005$). This may indicate some support for local rookeries. This chapter summarizes the importance of ancient DNA analysis of northern fur seal remains and the challenges faced by such analysis. An outline of the specific thesis objectives is then provided. This is followed by a summary of the approach undertaken, and the key findings relative to the hypothesis tested here and how these relate to the broader research questions. The chapter concludes by posing a series of research directions that can advance further research in this field.

7.2. Ancient DNA Analysis of Northern Fur Seal

Ancient DNA provides a unique opportunity to study genetic diversity and population structure. It allows for a way to look at the genetics of past organisms by providing a snapshot of information that may be lost by later events such as bottlenecks

and population expansions (Foote *et al.* 2012; Hofreiter, Serre, *et al.* 2001; Speller *et al.* 2012). Understanding these past populations can inform modern conservation decisions by providing a long-term view of a species' genetic diversity, as well as improving resolution of human interactions with this species in the past. For instance, if there is genetic evidence of structure (and therefore evidence of population subdivision) in northern fur seal populations in the past, this would support the archaeological hypothesis of local breeding colonies (Burton *et al.* 2001, 2002; Moss *et al.* 2006; Newsome *et al.* 2007; Pinsky *et al.* 2010). In turn, this would indicate that the remains found in coastal archaeological sites likely came from breeding colonies rather than from the energetically inefficient source of far offshore hunting of foraging northern fur seals. Support for local breeding colonies through the existence of local genetic populations would therefore provide insight into both past human hunting behaviour and pre-modern northern fur seal distribution and migration. The presence of local breeding colonies would lend support to the hypothesis that the breeding and migration patterns of northern fur seals seen today is quite different from that of the past, and would suggest a greater degree of behavioural plasticity. In addition, changes between past and present breeding and migrational behaviours may lend further insight into the ability of northern fur seals to recover from population depletion (Pinsky *et al.* 2010). This latter point is especially important as historic over-hunting of this species is well documented (Burton *et al.* 2001; Newsome *et al.* 2007; Pyle *et al.* 2001; Starks 1922; Sydeman and Allen 1999). Greater insight into past population sizes and population genetic structure at various points in time would enable assessment of the ability of northern fur seals to recover from dramatic declines.

There are a number of challenges associated with the analysis of northern fur seal population structure. First, this study utilized sequence data for ancient northern fur seals. As discussed in the literature section and expanded on in Appendix A, some of the challenges associated with ancient material include DNA damage, low copy number, contamination, and other post-mortem DNA damage such as deamination that may result in sequence modification (Brown and Brown 2011; Cox 2005; Nelson and Hebsgaard *et al.* 2005; Pääbo *et al.* 2004; Willerslev and Cooper 2005). In particular, deamination can lead to C→T and G→A misincorporations (see Appendix A for more discussion). The research in this thesis confirmed that these are the dominant types of

mutations in the aDNA set. Thus, it is likely that these may be the result of post-mortem damage rather than reflecting ancient diversity. Given the added difficulties associated with using aDNA, it is much harder to acquire a robust set of data to use. This analysis in particular was forced to use small data sets from archaeological sites. The data set was both small in total number of sequences and, as seen in the *cytochrome b* data, often sequencing reads were quite poor – particularly at the ends of amplicons – which resulted in a number of alignment gaps. The *cytochrome b* data comprised 67 modern and ancient sequences, while the D-loop set included only 66 ancient sequences compared to over 300 modern sequences. In addition, as other studies thus far have not found much evidence of population structure (Dickerson *et al.* 2010; Pinsky *et al.* 2010), it is unlikely that a smaller study such as this would show much evidence of structure. Despite this, there were significant differences apparent in the ancient D-loop DNA dataset. Further challenges are associated with the phylogenetic trees and determining relationships between species. Additional challenges associated with the phylogeny of northern fur seals include rapid speciation, high mutation rates and even sample misidentification or use of hybrid individuals which may make the phylogenetic relationships obscure (Wynen *et al.* 2001). Low bootstrap values suggest little difference between sequences, which could explain the poor resolution of the tree.

7.3. Approach

This thesis drew upon two sources of DNA sequence data for the northern fur seal population analysis. The first was generated by Moss *et al.* (2006) who utilized archaeological remains from three excavated sites to obtain aDNA sequences. This data was supplemented with sequence data obtained from GenBank to situate the data obtained by Moss *et al.* (2006) into a broader geographic context. The thesis reviewed the context and challenges with each of these data sources and their value to aDNA analysis.

The analysis component of this thesis drew upon a number of methods that compared the relationship between genetic sequences and the measures of nucleotide and haplotype diversity between them. Phylogenetic trees and networks were generated to confirm species identity, examine the relationships between haplotypes, and assess

haplotype frequency (section 5.2, Appendix C, Section 5.3.1). Measures of diversity, including haplotype (Hd) and nucleotide diversity (π), were calculated using DnaSP. An analysis of molecular variance (AMOVA) was utilized to assess F-statistic variables (F_{ST} and Φ_{ST}) so that the partitioning of genetic diversity could be examined. These techniques were also reviewed and described in detail in the methodology and literature review sections, though a brief overview is presented here.

A variety of methods were employed to read the chromatogram data, align and sort the sequences, generate phylogenetic trees and median-joining networks (Bandelt *et al.* 1999), calculate measures of haplotype and nucleotide diversity (Nei 1987; Nei and Tajima 1981), generate mismatch distributions, and conduct AMOVA analyses. The numerous programs and techniques used included ChromasPro, MEGA 6, DnaSP, NETWORK 4.6, PopART 1.7, GenAlEx 6.5, and Arlequin 3.5. The first program, ChromasPro (Technelysium Pty Ltd), was used to read the chromatogram data. The program MEGA 6 (Tamura *et al.* 2013) was then used to edit sequence ambiguities and trim the primers from analysis, import published sequence data, align the sequences with Clustal W algorithm, and generate phylogenetic trees. These resulting phylogenetic trees were used for both phylogenies/species identity confirmation and for assessing haplotype relationships. DnaSP 5 (Rozas 2009) was used to calculate haplotype and nucleotide diversity as well as generate nucleotide mismatch distributions. Median-joining networks (Bandelt *et al.* 1999) of ancient and modern D-loop data were generated using both NETWORK 4.6 (http:// www.fluxus-engineering.com) and PopART 1.7 (http://popart.otago.ac.nz). Finally, AMOVA analyses were conducted using both GenAlEx 6.5 (Peakall and Smouse 2012) and Arlequin 3.5 (Excoffier and Lischer 2010). While some of these methods may appear redundant (networks by both NETWORK and PopART, or AMOVA analysis by GenAlEx and Arlequin), this repetition using alternate programs was important for confirming the validity of the findings and exploring the methods available for assessment of population genetic structure change. For instance, the program NETWORK 4.6, the more commonly employed methodology, allowed for colour-coding of the nodes to show the relative proportions of ancient and modern haplotypes. The resulting network generated was highly reticulated with a number of nodes that were very complex and difficult to parse out to view haplotype connections, while PopART 1.7 more clearly separated the nodes automatically (http:// www.fluxus-

engineering.com, http://popart.otago.ac.nz). As discussed in the methodology section, Arlequin is the more commonly employed methodology for academic papers and is regarded as the 'better' method to employ (personal communication with Dr. Brent Murray, UNBC); it is thus an important methodology to incorporate in the analysis. However, GenAlEx 6.5 has the benefit of automatically generating helpful, clear figures that show the important data regarding the partitioning of variance. This contrasts with the long text format generated by Arlequin where the relevant data is less evident. That is, there are benefits to using both AMOVA analysis methods.

7.4. Null Hypothesis

The main purpose of this thesis was to test the hypothesis of whether or not there was genetic evidence of local subpopulations. This could provide support to the broader question of whether there is evidence for local northern fur seal rookeries along the coast of North America in the past. Using Moss *et al.*'s (2006) unpublished original mitochondrial DNA sequence data for northern fur seals, the null hypothesis of no difference in population genetic structure between past and present populations was tested using a variety of methods.

The general observation is that there is some evidence suggestive of possible structuring in the past. A few analyses generated statistically significant differences, though given the challenges of ancient DNA further investigation is required. The median-joining network analysis in both ancient and modern sequence data may indicate a continuity of diversity over time for/with two possible haplogroups, despite the dramatic northern fur seal population declines. This split, along with the pairwise AMOVA comparison findings of a handful of sites that may be more distantly related to one another, may be suggestive of potential structure. The high D-loop haplotype diversity shows that the majority of haplotypes are 'unique' in both ancient and modern populations, while the high nucleotide diversity may suggest high substitution rates. Thus, the network analyses and diversity values do not appear to be dramatically different between past and present northern fur seals. The AMOVA analyses suggested some difference in overall population genetic variance, with greater partitioning of the variance in the past, though these results were only significant at the $P \leq 0.05$ cut-off. The

AMOVA analyses results showed three significantly different site-by-site pairwise comparisons at P≤0.005, though only one of these comparisons was significantly different in analysis by both Arlequin and GenAlEx. Both Arlequin and GenAlEx found Cape Addington and Netarts to be significantly different. Only GenAlEx found significant differences between Netarts and Umpqua, while only Arlequin found significant differences between Cape Addington and Ts'ishaa. At the cut-off of P≤0.05, seven comparisons between archaeological sites were found to be significantly different, with identical support from both GenAlEx 6.5 and Arlequin 3.5. The AMOVA analyses therefore show some partitioning of variance and thus have some support for local-populations. As neither analytical approach to AMOVA analysis found any significant genetic patterning of variance in the modern sequence data, this therefore indicates a difference between past and present diversity.

It is possible the AMOVA analyses may have been skewed by DNA degradation and/or may have been biased by sampling issues. The null hypothesis was 'tentatively' rejected as only the P≤0.05 found an overall significant difference between ancient and modern datasets, while only a single site by site comparison was found to be significant for both F_{ST} and Φ_{ST} at the P≤0.005 cut-off (this was the Cape Addington/Netarts comparison). Further, there was no clear evidence of partitioning /past and present diversity differences in the DnaSP 5 calculations, or in the phylogenetic trees or networks. It therefore appears that the null hypothesis may be rejected, though further support would strengthen this argument.

7.5. Other Observations

The analysis and research also yielded several other interesting results. The first of these was the poor resolution of the D-loop phylogenetic tree, the high level of cycles in the network and the presence of multiple alleles at some of the sites in the D-loop sequences. Based on logic of phylogenetic trees, one would not expect the use of a different outgroup to affect the relationship of branches to one another within a tree. However, this was the case in this thesis. The poor phylogeny may be a reflection of the rapid divergence between the lineages or it may be due to the data and methods used. Other tree-building methods or parameters may improve the phylogeny. Regardless, the

species identification was supported by all trees. Second, the multiple polymorphisms seen at some D-loop sites may represent mutational hotspots, as SNP markers tend to be bi-allelic due to the independent probability of mutation occurring. In contrast, STR (short tandem repeat) loci/microsatellites tend to have more mutations at existing microsatellites due to slippage at the repetitive regions. Finally, the high number of cycles in the network analysis is interesting. It could be a reflection of the high diversity or the multiple alleles, but it is not typical for networks of mtDNA. It does not reflect the pattern of unilinear mitochondrial inheritance and lack of recombination.

7.6. Objectives

This section summarizes the research findings related to the research objectives. The specific objectives of this thesis were three-fold. First, to further analyze mitochondrial DNA from three excavated sites that had been extracted, amplified, and sequenced by Moss *et al.* (2006) but had not been published. The second objective was to integrate the unpublished DNA sequence data set from Moss *et al.* (2006) with previously published sequence data to identify whether there were any broad geographic patterns and determine whether the data fit with a broader perspective. The third was to explore and use multiple methods to enhance the ability to detect population structure change with the goal of attempting to determine whether there was any evidence of population genetics structure/population subdivision.

Regarding the first objective, to analyze this data set for patterning, this study found high haplotype diversity and few sequences per haplotype made identification of any patterning difficult. Among these three sites, there was little evidence of population genetics structure. Phylogenetic trees revealed that the *cytochrome b* sequence data from these three sites segregated into only two haplotypes, and each haplotype contained sequences from each of the three sites (Figure 5.1). For the D-loop region, there were a large number of haplotypes that often included one to three sequences, often from only one of these three sites, though these were interspersed amongst other haplotypes from other sites (Figure 5.2).

The second objective was to integrate this dataset with previously published sequence data to observe any broad geographic pattern, and how this data fits in with a broader perspective. When the data from this thesis was integrated with previously published sequence data to observe how it fit into a broader geographic pattern, there was little evidence of patterning visible in the phylogenetic trees (Figures 5.1 and5.2), nor when measures of nucleotide and haplotype diversity were considered across latitude (Figure 5.5 and 5.6). An AMOVA analysis was conducted to further investigate the partitioning of genetic data and to search for significant differences between archaeological and modern rookery sites. The AMOVA analysis results revealed a few, but significant differences in F_{ST} /Φ_{ST} values for both ancient and modern D-loop sequence data using both Arlequin 3.5 and GenAlEx 6.5. Overall, there is some statistical support ($P \leq 0.05$) for structure/subpopulations in ancient northern fur seals. Specifically regarding the integration of this data set with the published sequence data, most of the significant comparisons involved at least one of the sites from Moss et al. (2006). Neither analytical approach to AMOVA analysis found any significant genetic patterning of variance in the newly added modern sequence data. The addition of the modern data did allow for temporal analysis, with the finding of a change from 5% among population variance in the ancient to 0% in the modern (though only the 5% variance was significant, and only at $P \leq 0.05$). The addition of new data also allowed for a median-joining network to assess haplotype differences between the past and present, and found that there were a number of unique haplotypes in both data sets, but that the overall network form (with grouping into two clades) was preserved over time.

The third objective was to explore and use multiple methods to enhance the ability to detect population structure change. As discussed previously, the approach used in this thesis was to use a variety of methods. Phylogenetic tree building was conducted using an assortment of tree-building methods (neighbour-joining and maximum likelihood) and multiple substitution models. It is clear that given this data set (short sequences, rapid speciation) that the phylogeny of northern fur seals to other pinnipeds is difficult to determine and poses many challenges. Despite this, northern fur seal species identification was supported by all methods and substitution models employed. The settings and options for DnaSP were cross-checked with manual calculations and the resultant diversity values were able to be analyzed to compare

between both ancient and modern and between sites of different latitudes. Networks were generated by two programs, with both having utility. NETWORK enabled easy generation of pie-chart nodes to compare modern and ancient haplotypes, while PopART allowed for clear resolution between haplotypes. Particularly, those methods specifically designed to assess how genetic diversity is partitioned, in this case Arlequin 3.5 and GenAlEx 6.5, were quite useful in the discussion of population structure change over time as they both allowed for statistical analysis of both past and present data. The two analyses were mostly consistent, though differed slightly at the level of pairwise comparisons between archaeological sites at the $P \leq 0.005$ significance level. Again, the utility of multiple methods was illustrated. This third objective was met through the use of a variety of methods. Of course, this can always be expanded upon, with many more methods available to explore population genetics.

Multiple methods were used to examine the patterning of genetic data for past northern fur seal populations. Collectively, these methods provided some evidence for patterning of genetic data for past northern fur seal populations, with little evidence to support local genetic structure/subdivision in contemporary populations. Further, there is little detectable difference in genetic diversity of northern fur seals between the past and present, with both exhibiting high diversity values and little to no population genetic structure. The strongest support for ancient diversity difference comes from the AMOVA analyses at $P \leq 0.05$. At this significance level, the analyses revealed statistically significant partitioning of overall ancient variance well as significant differences between a handful of archaeological site comparisons. This suggests differences between the past and present diversity, and may indicate potential subpopulation divisions in the past. This warrants further investigation using more sensitive approaches and more robust samples.

7.7. Thesis Contributions

Several contributions were made by this thesis. Most importantly, this study identifies a level of structure/population subdivision in ancient northern fur seals. Thus far, it appears that this is the first study on northern fur seals using ancient DNA to report evidence of significant F_{ST}/Φ_{ST} values ($P \leq 0.05$), lending further support to the position

taken by other northern fur seal population genetics studies that assert that evidence of structure is limited in modern populations. Together this suggests a difference between past and present diversity in northern fur seals. The significant differences seen in the AMOVA analysis suggest the null hypothesis of no change in population genetic structure should be rejected. This analysis also made a slight improvement on the sample identifications made by Moss *et al.* (2006) by identifying an additional sample (NF19) to the species level. Additionally, the unusual effect of adding different outgroups to the phylogeny has been pointed out. This observation may advise other researchers to exercise caution and perhaps also use a variety of outgroups when generating a phylogenetic tree with extremely low support for the branches/nodes.

In terms of implications for archaeology, the finding of some evidence of structure in ancient northern fur seals lends support to the broader question of the existence of local rookeries. This may, therefore, have potential implications for location of human settlements as these are often coincident with resource locations and local rookeries would have represented a predictable and abundant resource. Second, it would also have implications for the type of artifacts expected at sites, given the differences in technology needed to harvest northern fur seals at sea versus on land, and those for specialized or general purpose. Not directly connected with archaeology, this study also has implications for modern wildlife management and conservation with regards to the ability of northern fur seals to retain or rebound to pre-harvest diversity levels and the related potential ecological impacts.

7.8. Future Research

During the course of this work, several potential research options and questions were identified that may help to improve northern fur seal population genetics research. These questions relate to the need for more robust genetic data from ancient northern fur seals (more sequences, better dated remains for DNA extraction, more sites, longer sequences, inclusion of other genetic markers etc.), investigations into the nature of observed changes (whether these are the result of DNA polymorphism or due to DNA degradation which is a challenge of ancient DNA research), and expanded analyses (from simple analyses like testing for isolation-by-distance with the F-statistic data or

more complex analyses utilizing Bayesian statistics). These will provide opportunities for other researchers to continue or extend the investigation into the population genetics of ancient northern fur seal, their migratory behaviour, their genetic diversity, and their ability to recover from dramatic population declines.

Given the small sample sizes at a number of archaeological sites, additional genetic data is required for more robust analyses. This includes the extraction and DNA sequencing of additional archaeological northern fur seal remains or analyzing other genetic markers (such as nuclear DNA SNPs or microsatellites). Additional sequences from each site would be especially useful for improving the AMOVA analysis which, as a statistical measure, would be greatly improved with larger sample sizes. Additionally, obtaining more sequence data from each site and having precise dating may allow for a clearer temporal resolution of patterns beyond the simple crude classification of 'ancient' and 'modern.' Improved dating of these archaeological samples could allow for samples to be grouped into narrower time frames. Shorter time frames with more samples would help to remove the complication of accumulated genetic changes over time that could artificially inflate measures of diversity. It would also allow for a more precise investigation into specific events that may have affected population sizes. These more precise timeframes would thus allow for an improved temporal resolution. Given the high number of alignment gaps in the *cytochrome b* sequence data, re-sequencing of some of these samples may be useful. The sequencing of additional archaeological sites may further improve investigation into geographic patterns and possible structure/population subdivision in northern fur seal populations. In addition, the use of other markers, such as microsatellites or nuclear DNA SNPs, could assist in illuminating northern fur seal population structure by providing another source of information beyond just maternal lineages. Since Dickerson *et al.*'s (2010) study of modern northern fur seal microsatellites revealed no population differentiation with seven loci, any future study should include more than seven loci. However, the various limitations of these markers should be taken into consideration. For instance, while microsatellites have been used in aDNA studies and show the power to identify differentiation at a finer scale, there is a chance that DNA degradation may increase the amount of dropout (Speller *et al.* 2012). It may be worth investigating these markers for the data samples used in this study. In particular, given the differences in migrational behaviour between male and female

northern fur seals (Newsome *et al.* 2007) it may be of use to use a sex-specific marker such as the nuclear amelogenin (*AMG*) gene (Alonso *et al.* 2004). Incorporating zooarchaeological information such as sex (or age) may allow for investigation into sex or age specific behaviours, such as differences in male vs. female migration patterns or the hypothesis of some haul-outs as training grounds/overflow areas (Gifford-Gonzalez 2011) for sub-adults who may not yet be at their natal rookeries.

Another potential area of future research is the assessment of whether observed 'polymorphisms' are in fact reflective of past diversity or a result of DNA degradation. One way to identify whether proposed mutations are the result of polymorphism or a result of DNA degradation is the use of the enzyme uracil-DNA-glycosylase which cleaves out the uracils resulting from cytosine deamination. Given that this method typically requires more starting template (as cleaved uracils result in abasic sites which prevent DNA amplification), the use of next generation sequencing would be beneficial to use with this technique (Briggs *et al.* 2010). This type of analysis would clarify whether much of the polymorphism seen in the past is truly the result of past genetics, or whether it is merely an artefact of the antiquity and preservation conditions of the samples.

Another simple future research direction is further analysis of the results from the AMOVA analyses/F-statistics. The variables F_{ST} and Φ_{ST} (which measure both genetic distance and haplotype frequency) are commonly used to assess within and among population genetic differences (Dickerson *et al.* 2010; Pinsky *et al.* 2010). Further analysis of the F_{ST} and Φ_{ST}, can be used to test for isolation-by-distance (Dickerson *et al.* 2010; Wright 1943). Isolation-by-distance is designed to distinguish between ongoing gene flow and recent population separation (Beebee and Rowe 2008). Ongoing gene flow would be expected to result in a relationship between geographic distance and F_{ST}/Φ_{ST} values between populations, with obviously less gene flow (and thus greater F_{ST}/Φ_{ST} values) occurring between populations that are more distantly separated. The relationship between estimates of FST/ Φ_{ST} against distance can reveal patterns of population distribution. Such a graph could show evidence of whether there is equilibrium of gene flow with genetic difference increasing with increasing geographic distances; whether there has been a recent separation in populations with no correlation between genetic difference and geographic distance; whether there is no gene flow and

there are no correlations between geographic distance and genetic difference; or a whether there is a combination of gene flow and genetic drift that may indicate a fragmented population (Beebee and Rowe 2008).

Another possible future research direction for further investigating population structure/subdivision in northern fur seals is to use Bayesian approaches. The program STRUCTURE (Pritchard *et al.* 2000) can be used to assess whether northern fur seal sequence data represents different populations. This program can reveal the probabilities of various hypotheses for number of populations and calculate their probabilities using a Bayesian algorithm (Berlin *et al.* 2008; de Thoisy *et al.* 2006). For instance, the program has been applied to the study of modern northern fur seal microsatellite data and found that the highest probability was that all of the northern fur seals belonged to a single population (Dickerson *et al.* 2010). Using this method to analyze ancient northern fur seal mitochondrial data would require some modifications, as STRUCTURE is primarily optimized for nuDNA data as it uses the assumption that loci are independent within populations, which is not the case for the non-recombining mitochondrial (Pritchard 2010). Another Bayesian approach is the use of an Approximate Bayesian Computation framework which can combine modern and ancient DNA, as well as other prior information, to gain a view of the dynamics of population structure in northern fur seals (Pinsky *et al.* 2010).

In summary, the majority of studies on northern fur seal genetics have suggested there may be some low level of population structure. The research carried out as part of this thesis supports these findings. However, the results described above also indicate that further investigation is needed.

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Appendix A.

Opportunities and Challenges in Ancient DNA Research

Ancient DNA provides a unique opportunity for looking at evolutionary processes and events in the past. Studies of modern DNA only provide indirect evidence of evolutionary processes, and more recent events may erase signals of past ones (through mechanisms like replacement or dilution) (Leonard *et al.* 2002; Willerslev and Cooper 2005). However, by looking at aDNA it is possible to investigate the direct evidence of various changes to population size and structure, and to study relationships between extinct species and populations (Pääbo 1989). Ancient DNA can contribute to the exploration of many questions. This essay will begin by discussing some potential applications of aDNA to various fields of study and examining some of the major developments in the study of aDNA. Next, some of the important characteristics of DNA will be summarized, including how they affect DNA post-mortem degradation, one of the greatest challenges in aDNA research. It will then discuss how to overcome the challenges in ancient DNA research to ensure the results are authentic. Finally, this essay will conclude with a brief discussion of some sources of ancient DNA.

Potential Applications of Ancient DNA Studies

Ancient DNA (aDNA) can allow greater insight into extinct species and populations. For example, it can contribute to enhancing our understanding of past interactions of organisms through analysis of genetic relatedness between individuals and/or species (Cooper *et al.* 1996; Pääbo *et al.* 2004; Willerslev and Cooper 2005). Genetic origins of species can be traced to explore questions of domestication, trade and population dispersal (Hansson and Foley 2008). Adding aDNA to the repertoire of tools available can provide greater insight into palaeopathology (Drancourt *et al.* 1998; Papagrigorakis *et al.* 2006; Schuenemann *et al.* 2011). Ancient DNA can even enable paleoenvironmental reconstructions in the absence of physical remains through the use of sediment DNA (Pääbo *et al.* 2004; Willerslev *et al.* 2003). Through aDNA, we can also investigate the phylogenies and phylogeography of past populations or groups.

Ancient DNA can be used to infer phylogeography or the distribution of genealogical lineages across the landscape and the process affecting this distribution. The data from aDNA can be used to examine changes in genetic diversity of species through time and make inferences of past climate and environmental changes (Chan *et al.* 2005; Dalén *et al.* 2007; Orlando *et al.* 2002). As there is a strong relationship between paleoenvironment and species distribution, paleoenvironmental reconstructions (even in the absence of macroscopic organismal preservation) can provide insight into ecological conditions and potential range limits for species for which absolutely no trace remains (Pääbo *et al.* 2004; Willerslev *et al.* 2003). Another common topic of phylogeographic interest is that of anthropogenic influence on distribution of organisms. The origins, patterns, and timing of events regarding domestication can be investigated, including identification of genes that were selected (as in the study of maize) or the origin of populations (such as the domestication of dogs or cattle, or the spread of farming)

(Bailey *et al.* 1996; Balter 2005; Brown 1999; Haak *et al.* 2005; Jaenicke-Després *et al.* 2003; Larson *et al.* 2007; Leonard *et al.* 2002; Willerslev and Cooper 2005).

Ancient genetic studies may also have additional cultural significance beyond the migration of people and domesticates by providing further data on life histories. Ancient DNA can be used to look at past diets and behaviours of humans or other animals through coprolites or food remains (Pääbo *et al.* 2004; Poinar *et al.* 2001, 1998; Willerslev and Cooper 2005). Alcohol fermentation can be detected through indicators such as yeast (*Saccharomyces cerevisie*) DNA (Cavalieri *et al.* 2003), and it has been suggested that geographic location of origin can be traced through chloroplast DNA (Hansson and Foley 2008). Other lifestyle indicators such as palaeopathology can also be investigated, even in the absence of morphological indicators of disease (Drancourt *et al.* 1998; Papagrigorakis *et al.* 2006; Schuenemann *et al.* 2011).

Ancient DNA and phylogenetic analysis can also allow greater insight into humans and their relationships with other hominids, such as Neanderthals (Krings *et al.* 1997; Stoneking and Krause 2011; Weaver and Roseman 2005). One major phylogenetic debate is the relationship between modern and archaic *Homo sapiens* (Gutiérrez *et al.* 2002; Hofreiter, Serre *et al.* 2001; Krings *et al.* 1997; Relethford 2001), particularly whether the transition from archaic to modern forms is best represented through a model of multiregional evolution (continuity) (Wolpoff *et al.* 2001), replacement (Green *et al.* 2006; Hodgson and Disotell 2008; Hofreiter, Serre *et al.* 2001; Krings *et al.* 1997), or replacement with limited gene flow (Wall and Hammer 2006); though genetic data provides more support to the latter two models. This question is additionally complicated by both the choice of analytical model used and the degradation of DNA, which can result in modifications and skew the results of genetic analyses (Briggs *et al.* 2007; Gutiérrez *et al.* 2002). The various models that can be used to study genetic relationships were discussed in more depth in Chapter 3: Analysis of Genetic Data.

Major Developments in aDNA Studies

This section will provide background information on some of the major developments in aDNA research. This will cover the span of time from the first successful extraction of aDNA to analysis of whole ancient genomes and population studies.

The earliest ventures into aDNA research began after it was discovered that some macromolecules could remain intact for very long periods of time, such as proteins (Pääbo *et al.* 1989). This was followed by immunological studies based upon proteins to sort out evolutionary lineages (Pääbo *et al.* 1989). The earliest attempts at aDNA actually began with cloning of aDNA into vectors (Pääbo *et al.* 1989; Pääbo *et al.* 2004). Higuchi *et al.* (1984) began work on 140 year old quagga DNA from a museum specimen, cloning the aDNA into bacteriophages (lambda phage and M13mp11) which were then used to infect *E. coli* cells to replicate the bacteriophages containing the aDNA inserts. This has since been repeated using the same methodology and identifying cytochrome oxidase I and NADH dehydrogenase I sequences (Higuchi *et al.* 1987; Willerslev and Cooper 2005). Cloning was also applied to amplify DNA from an Egyptian mummy in 1985, targeting repetitive DNA; however, because the testing used so much sample, it was not able to be replicated and the authenticity of this data remains in doubt (Pääbo *et al.* 1989; Pääbo *et al.* 2004; Willerslev and Cooper 2005).

Kary Mullis' invention of the polymerase chain reaction (PCR) using thermostable *Taq* resulted in a significant change in the field of aDNA (Pääbo *et al.* 2004; Pääbo *et al.* 1989; Willerslev and Cooper 2005). PCR could generate enough DNA to be studied from only a single molecule. In addition to this ability to replicate large amounts of sample DNA, it also resulted in some improvements in that replication errors were far fewer than errors from cloning (Pääbo *et al.* 1989; Willerslev and Cooper 2005). For instance, PCR enabled the identification that two positions from the earlier work done on quagga DNA were the result of artefacts (Pääbo *et al.* 2004; Willerslev and Cooper 2005). PCR also required less DNA, thereby allowing the study of DNA from far smaller samples. The use of smaller samples and the ability to amplify the amount of DNA present allowed for replication of experiments to verify or reject early findings, the importance of which is evident from a number of early claims that were later refuted by independent replication of experiments (Austin, Ross *et al.* 1997; Austin, Smith *et al.* 1997; Pääbo *et al.* 2004; Willerslev and Cooper 2005).

The field of aDNA has expanded to studies of ancient populations, targets of phenotypically important nuclear loci, developments in methodologies, new discoveries, and next generation sequencing, all of which have greatly expanded the horizons of aDNA (Travis 2010; Willerslev and Cooper 2005). For instance, a draft of the complete Neanderthal nuclear genome (38,000 years old) was recently completed. There is also new evidence suggesting that in some circumstances, such as in preserved seeds, ancient RNA may survive for substantially long periods of time, perhaps as long as 2,000 years (Travis 2010).

Characteristics of DNA

Ancient DNA can be difficult to work with due to post-mortem DNA degradation. In order to understand the impact of degradative mechanisms on DNA or how contamination or errors may impact the study of aDNA, background knowledge of the structure of DNA and the mechanisms of DNA replication are necessary. This section also describes the specific features of nuclear DNA, mitochondrial DNA and chloroplast DNA which can influence the decision to analyze one type of DNA over the other.

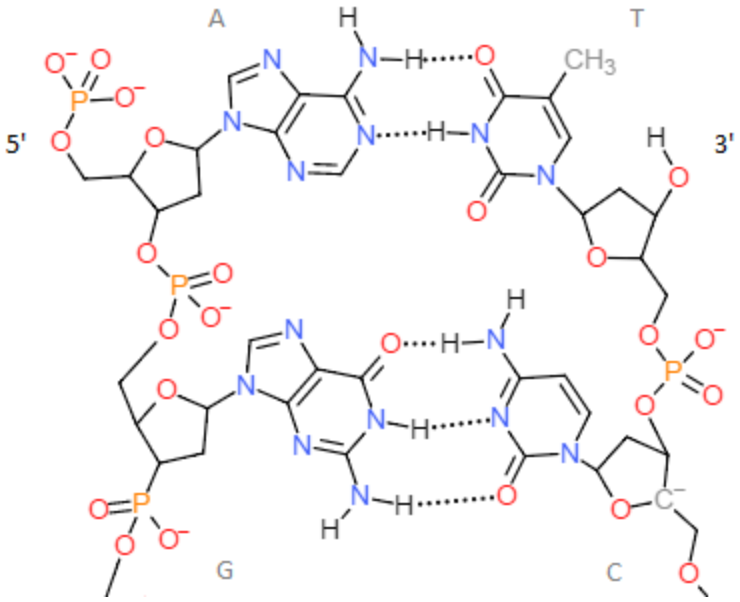

Figure A.1. Illustration of DNA nucleotide base pairing and nucleotide structure generated using ChemDoodle.
Solid lines represent chemical bonds (covalent bonds), while dotted lines represent hydrogen bonds. Carbon atoms are represented by black points, while phosphorus is shown in orange, oxygen atoms in red, and nitrogen in blue. This diagram shows the nucleotides adenine paired with thymine (A=T), followed by guanine paired with cytosine (G≡C). The two nucleotides on the left are the purines and the two on the right are pyrimidines. Image drawn with ChemDoodle 2D Structure demo (http://web.chemdoodle.com/demos/sketcher).

DNA is a macromolecule made up of linked subunits known as nucleotides. One nucleotide is composed of a sugar ring (in the case of DNA, this sugar is deoxyribose), with a base (nucleobase) attached to the 1' carbon of the sugar ring by a β-N-glycosidic bond (see Figure A.1) (Brown and Brown 2011; Nelson and Cox 2005). There are many different bases but in DNA, the main ones found are adenine (A), guanine (G), cytosine (C) and thymine (T) (Brown and Brown 2011; Nelson and Cox 2005). Attached to the 5' carbon of the sugar ring is a phosphate group. In a strand of DNA, this phosphate would then be linked to the 3' hydroxyl of the preceding nucleotide in a phosphodiester bond (Brown and Brown 2011; Nelson and Cox 2005). DNA tends to form a double helix shape (generally right handed, as in the case in the "A form" and the "B form", though on occasion sections of DNA may take on the left handed "Z form" helix), with two strands of DNA running antiparallel with one another and held together through hydrogen bonding of the nucleobases (Brown and Brown 2011; Nelson and Cox 2005). This hydrogen bonding tends to be quite specific, with G pairing with C through three hydrogen bonds, and A pairing with T through two hydrogen bonds. These bases can be classified as purines (those with a double ring structure such as A or G) or as pyrimidines (those with a single ring structure, such as C or T) (Brown and Brown 2011; Nelson and Cox 2005).

Because the hydrogen bonds are weaker than the covalent bonds, the two antiparallel strands of DNA can separate, and each can be used as a template for the synthesis of a new strand of DNA through the action of enzymes called polymerases (Brown and Brown 2011; Nelson and Cox 2005). This semi-conservative replication is the reason why DNA is able to pass on genetic information; this same mechanism can be used in the study of DNA, in particular, it is essential to the field of aDNA (Brown and Brown 2011; Nelson and Cox 2005). The enzyme polymerase will add a nucleotide triphosphate to the 3' end of the preceding strand, releasing a pyrophosphate. The nucleotide is added because it is complementary to the template strand (though polymerase errors do occur where the wrong base is added; however, the system has fairly high fidelity) (Brown and Brown 2011; Nelson and Cox 2005). The polymerase chain reaction (PCR) is currently the main mechanism for amplifying DNA and it works by cyclically repeating three steps, each at a different temperature (melting/denaturing, annealing, and extension). Because this synthesis of a new strand adds the nucleotide to the 3' end of another nucleotide, in an *in vitro* system short oligonucleotides called primers are needed. These short pieces of DNA will bind to a complementary region of the template strand and will demarcate the region of the DNA to be amplified. Nucleotides are then added to the 3' end of the primer and growing strand until the polymerase falls off by random chance, or by the start of a new cycle of denaturation/melting (Brown and Brown 2011; Nelson and Cox 2005).

DNA includes both coding and non-coding regions (Brown and Brown 2011; Nelson and Cox 2005). The specific definitions for the coding and non-coding regions can vary depending on how regulatory elements or RNA products are being considered. In general, coding DNA "codes" for a product (the definition used in this thesis includes both protein and RNA products), while the non-coding region may be composed of pseudogenes, repetitive elements, regulatory elements, and other 'junk DNA' which does not code for a protein or RNA product (Brown and Brown 2011; Nelson and Cox 2005). Variation in a gene or non-coding region is referred to as polymorphism (again, the definitions are fairly diverse with some restricted to protein-coding regions only, while other definitions, such as an STR polymorphism or polymorphism in a hypervariable region, may refer to parts of the 'junk DNA') (Brown and Brown 2011; Nelson and Cox 2005). This is important when considering constraints on DNA mutation – a mutation in a non-coding area may have little or no effect on an organism's 'fitness', while a mutation in a coding gene may be lethal or have its frequency in a population affected by selection (this is discussed in more depth in Chapter 3, section on "Evolutionary Concepts") (Allendorf *et al.* 2012).

The sum of an organism's genetic information is called its genome. An organism's genome may contain DNA in many different cellular locations, such as nuclear DNA (nuDNA) that comprises the chromosomes (in eukaryotic cells, this DNA is restricted to the nucleus, thus giving it the term nuDNA), mitochondrial DNA (mtDNA) (found in the mitochondria), or plastid/chloroplast DNA (cpDNA) (found in the chloroplasts of plants; plastid refers to a larger grouping of related organelles found in plants)(Beebee and Rowe 2008; Brown and Brown 2011; Nelson and Cox 2005; Pyke 1999). Bacterial and archaeal DNA has some unique features, such as the lack of a nucleus. Bacterial chromosomal DNA is located within the cytoplasm and lacks some of the features of eukaryotic DNA, such as histones; instead, the chromosomal DNA is typically circular and packaged tightly via supercoiling. For bacteria, extra DNA may be present as plasmids; these are small auxiliary pieces of DNA that can independently replicate from

the chromosomal DNA (Beebee and Rowe 2008; Brown and Brown 2011; Nelson and Cox 2005). Different information can be gained by looking at different type of DNA. In humans, the nuclear genome has around 3,200 million base pairs (bp), or is 3,200 megabase pairs (Mbp) in total, with this distributed among 23 pairs of linear chromosomes. The human mitochondrial genome is 16,569 bp long in a circular molecule (Beebee and Rowe 2008; Brown and Brown 2011; Nelson and Cox 2005). The mitochondrial genome is quite separate from the nuclear genome and bears much similarity with the genomes of bacteria, as would be expected from the endosymbiont hypothesis (Van Der Kuyl *et al.* 1995). There are many mitochondria per cell, with many copies of the mitochondrial genome per mitochondria (Beebee and Rowe 2008; Brown and Brown 2011; Nelson and Cox 2005). Among plants, the nuclear genome of various plants can range from around 466 Mbp (rice) to 16,000 Mbp (wheat), with mitochondrial genomes significantly larger than those of animals. The chloroplast genomes of plants are around 120-160,000 bp long (Beebee and Rowe 2008; Brown and Brown 2011; Nelson and Cox 2005). Among animals, even though the nuclear genome has far more information, it may be preferential to first try to obtain mtDNA. As aDNA has undergone some degree of degradation, the mitochondrial genome, with a much higher copy number, can increase the probability that at least some of the target sequences remain intact (Beebee and Rowe 2008; Dawnay 2007). In addition, it is also possible that factors such as small size, circular supercoiled structure, cellular location (in the mitochondria, rather than in the nucleus), and other factors may give mtDNA additional protection against shear breakage and nucleases (Foran 2006; Sinclair and Stevens 1966). An additional benefit of using mitochondrial genes is the lack of recombination and unilinear (maternal) inheritance. These traits make the analysis of mtDNA simpler than that of nuDNA where recombination and the Mendelian inheritance (random assortment of alleles) provide substantial variation. Mitochondrial DNA is optimal for tracing maternal lineages, though the trade-off is that less information is available using this data source (Beebee and Rowe 2008; Dawnay 2007). The increased probability of successful amplification and the simpler analyses make mtDNA a good starting point for aDNA analyses.

A brief sample of the different types of information that can be gleaned from mitochondrial, nuclear, and chloroplast DNA studies is provided below. Past work with mitochondrial genomes has looked at questions such as human migration patterns (i.e. Neolithic migration, history of Chinese populations), population diversity, and tracing the origin and spread of domesticates (i.e. horse, dog) (Haak *et al.* 2005; Leonard *et al.* 2002; Melchior *et al.* 2010; Vilà *et al.* 2001; Yao *et al.* 2003). Nuclear DNA has been used to examine a variety of questions, such as sex determination, tracing paternal lineages or comparing sex-bias in population admixture through Y-chromosome analysis; examining correlations between phenotype and domestication using expressed genes (such as those responsible for coat colouration); and using microsatellites to examine population genetics and population diversity (Allentoft *et al.* 2011; Dickerson *et al.* 2010; Huynen *et al.* 2003; Ludwig *et al.* 2009; Stoneking and Krause 2011; Zhao *et al.* 2011). Chloroplast DNA has been used to look at the history and geographic distribution of plants, including plant domestication (such as oak distribution or chenopod domestication), and has also been used to identify archaeological plant remains (Dumolin-Lapègue *et al.* 1999; Kistler and Shapiro 2011; Yano *et al.* 2004).

DNA Degradation

Post-mortem DNA degradation is inevitable. There are many mechanisms by which DNA degradation can occur. Handt *et al.* (1994) estimate that between 2,000 and 10,000 lesions appear in a person's genome per day. After death, the cell repair mechanisms no longer function and damage is no longer repaired (Handt *et al.* 1994; Pääbo *et al.* 2004; Willerslev and Cooper 2005). This section describes the various mechanisms by which DNA can degrade and the effect degradation has on the study of aDNA from enzymatic degradation to oxidation, hydrolysis, and crosslinking/alkylation (Bollongino and Vigne 2008; Burger *et al.* 1999).

After death, the cellular components begin to break down (autolysis) as enzymes begin to degrade DNA, such as the nucleases that had been sequestered in the lysosomes (Binladen *et al.* 2006; Brotherton *et al.* 2007; Pääbo *et al.* 2004; Pickering and Bachman 2009; Yang and Watt 2005). Bacteria, fungi and invertebrates also provide post-mortem enzymatic degradation (Binladen *et al.* 2006; Hofreiter, Serre *et al.* 2001; Pääbo *et al.* 2004; Yang and Watt 2005). Strand breaks result primarily due to the cleavage of phosphodiester bonds (Pääbo *et al.* 2004; Nelson and Cox 2005). It is thought that rapid dehydration, salt conditions, or the attachment to the mineral matrix of bone may help to reduce the impact of the enzymatic stage of DNA degradation (Brown and Brown 2011; Pääbo *et al.* 2004; Willerslev and Cooper 2005).

Oxidative damage occurs as a result of free radicals, such as hydrogen peroxide, peroxide and hydroxy radicals, causing blocking lesions (Pääbo *et al.* 2004). Oxidation tends to occur at double bonds, leading to ring saturation (and possibly to ring opening) (Brown and Brown 2011; Hebsgaard *et al.* 2005; Lindahl 1993). The result is a blocking lesion, such as 8-hydroxyguanine or 5-hydroxy-methylhydantoin, from oxidation of guanine and thymine respectively, or from the opening of the sugar ring (Brown and Brown 2011; Le Bihan *et al.* 2011; Lindahl 1993; Pääbo *et al.* 2004; Willerslev and Cooper 2005). Oxidative damage can also lead to Maillard products (Brown and Brown 2011).

Hydrolytic damage is a frequent occurrence in aDNA. Hydraulic deamination can lead to the conversion of cytosine to uracil (and to a far lesser extent, the conversion of adenine to hypoxanthine, guanine to xanthine, and 5-methyl-cytosine to thymine) (Hebsgaard *et al.* 2005; Lindahl 1993; Nelson and Cox 2005; Willerslev and Cooper 2005). When these templates are amplified, the modified bases incorrectly pair, resulting in miscoding in the resulting sequence. One method of resolving this problem involves the use of uracil-DNA-glycosylase, which will cleave the deaminated sites that have a uracil (Pääbo *et al.* 2004; Willerslev and Cooper 2005). Comparisons between treated and untreated samples reveal those positions where the conversion occurred. Hydrolytic damage can also occur at the β-N-glycosidic bond, resulting in baseless (abasic) sites. This occurs more frequently for the purines (leading to apurinic sites). These abasic sites may undergo chemical rearrangement of the sugar ring which can lead to a strand breakage (Brown and Brown 2011; Lindahl 1993; Nelson and Cox 2005).

In addition, there are other forms of DNA damage and modification which can impact aDNA studies. DNA crosslinking, Maillard products, and alkylation all serve as blocking lesions in DNA. UV radiation can generate thymine dimers between adjacent thymines. The resulting cyclobutane thymine dimers or 6-4 photoproducts can create kinks in the

DNA and act as a blocking lesion (Nelson and Cox 2005; Pääbo *et al.* 2004; Winters *et al.* 2011). Maillard products are the result of the reaction between a sugar and the nitrogen of an amino acid (Nelson and Cox 2005; Willerslev and Cooper 2005). These crosslinks can be broken by N-phenacylthiazolium bromide by a yet unknown mechanism (Lindahl 1993; Nelson and Cox 2005; Willerslev and Cooper 2005). Another type of blocking lesion which may occur in DNA is alkylation, such as methylation by methyltransferases in combination with S-adenosylmethionine, though this does not have a very significant impact in aDNA (Feil and Fraga 2012; Lindahl 1993; Pääbo *et al.* 2004; Willerslev and Cooper 2005).

The methods of enzymatic, hydrolytic, and oxidative degradation can be accelerated by the soil's pH, humidity, pressure, biological activity, airflow, and particularly by temperature (Bollongino and Vigne 2008; Burger *et al.* 1999). Unfortunately, with respect to predictions of DNA survival, not much is known about the relative rates at which these various types of damage occur and the degree to which these factors can alter the rate of DNA degradation (Willerslev and Cooper 2005). Some patterns have emerged, however, including the concentration of miscoding lesions in localized 'hotspots' where evolutionary changes tend to accumulate as well (Willerslev and Cooper 2005). In addition, it has recently been suggested that inter-strand crosslinks may occur faster than single-strand nicks, which has an impact on how aDNA may be treated in the future (Nelson and Cox 2005; Willerslev and Cooper 2005).

Challenges in Ancient DNA Studies

There are many challenges to working with aDNA. Low copy number, DNA damage/post-mortem modifications, amplification artefacts, and contamination are several of the interrelated problems that plague aDNA work. The biggest challenge is verifying the authenticity of the ancient sequences, as there is never a guarantee that the sequences are authentic target DNA. This section will describe the challenges of working with aDNA, DNA artefacts, and contamination; then discuss the criteria used to ascertain whether or not the DNA is of ancient origin.

As DNA damage accrues, the amount of amplifiable templates decreases (Brown and Brown 2011). As discussed above, mtDNA begins with more copies per cell. As a result, it is more likely to have more amplifiable template copies than nuDNA (mtDNA> nuclear repetitive sequences> single copy nuclear sequences). Specifically, low copy number in particular refers to DNA which is below the 'stochastic threshold' where the reliability and reproducibility of such experiments is based upon random chance; thus this limit can vary with the sensitivity of the equipment available (Alonso *et al.* 2003). Practically, however, template DNA less than 100 pg can be considered to be low copy number DNA (Alonso *et al.* 2003; Gill *et al.* 2000). Due to the features of aDNA, low copy number is a frequent problem that can result in failed detection of DNA/failed PCR amplification, though the ability of PCR to amplify even a single template molecule makes it very sensitive and suitable for the study of aDNA (Alonso *et al.* 2003). Due to the sensitivity of the PCR (as well as the low template copy number), even a small amount of contaminant DNA from exogenous sources (sources other than the specific sample itself) can have a significant effect (Brown and Brown 2011; Willerslev and Cooper 2005). Given the exponential amplification power of PCR, preferential amplification of one template molecule over another may occur (as in allelic dropout or hypo-

amplification) as a result of stochastic effects (Allentoft *et al.* 2011; Brown and Brown 2011; Findlay *et al.* 1995; Gill *et al.* 2000; Yang *et al.* 2003). DNA damage also increases the chance for PCR artefacts, such as jumping PCR or misincorporations (Hagelberg and Clegg 1991). As one would expect, low copy number also exacerbates the problems of PCR artefacts and negatively impacts reproducibility. Willerslev and Cooper (2005) suggest that when starting with template less than 1000 molecules, reproducibility becomes difficult and PCR artefacts have a greater effect.

"Jumping PCR" is one of many PCR artefacts which may occur as a result of post-mortem modification (Hagelberg and Clegg 1991; Handt *et al.* 1994; Pääbo *et al.* 1989). Jumping PCR occurs when the synthesis of a new strand is not completed as a result of lesions or strand breaks. During the next PCR cycle, this partially synthesized strand can then anneal to a new template molecule and continue to extend (unless it encounters another lesion/break) (Handt *et al.* 1994; Pääbo *et al.* 1989). This cycle of partial extension may occur several times. If this occurs within a 'homogenous system,' it can be beneficial by allowing extension beyond the limits of each individual template molecule in the original sample (Handt *et al.* 1994). However, when the system is not homogenous, then the result may be chimeric sequences (Austin, Smith *et al.* 1997; Handt *et al.* 1994; Pääbo *et al.* 1989; Salo *et al.* 1994). These chimeras may be formed from aDNA, contaminant DNA, or some combination of the two (Austin, Smith *et al.* 1997). Other errors that may occur include those due to polymerase error, misincorporations due to template damage, or post-mortem mutation (Kolman and Tuross 2000; Orlando *et al.* 2002). Polymerase errors can be minimized using a high fidelity polymerase such as *Pfu* or *Taq HiFi*, however, this cannot eliminate them completely (Willerslev and Cooper 2005). While polymerase errors are not restricted to aDNA, because of the limited amount of starting sample, the errors may have a greater impact. The numerous errors that can occur in aDNA necessitate that care be taken when analyzing aDNA (Hagelberg and Clegg 1991).

Contamination is the most significant problem in aDNA studies. It may occur any time after death of the organism – either during handling, burial, excavation, DNA extraction or PCR – with contaminants coming from all exogenous sources such as other specimens, microorganisms, soil, laboratory personnel, and many other sources (Austin, Smith *et al.* 1997; Hebsgaard *et al.* 2005). One of the greatest sources of contamination is from PCR products. PCR products are previously amplified DNA. These PCR products can spread by aerosol droplets that can contain millions of copies of amplified template. The number of potential copies of amplified sequence present in these droplets can potentially far exceed the number of copies found in aDNA extractions (Willerslev and Cooper 2005). In addition to the potential for contaminant molecules to outnumber authentic aDNA before amplification, as a result of the damage inherent in aDNA such as strand breaks, abasic sites, miscoding lesions, and crosslinks, the comparatively undamaged molecules of contaminant DNA may also preferentially amplify (Willerslev and Cooper 2005). In fact, Wayne *et al.* (1999) was extremely critical of reports of ancient human DNA, noting that when experiments were replicated under careful contamination protocols, there was no DNA, suggesting that earlier accounts were the result of contamination.

There are some features of aDNA which can be used to indicate that the sample is of ancient origin. Due to the fact that aDNA tends to be of a fairly short length (100-500 bp or less), contamination may be detected if long sequences are amplified (Cooper and

Poinar 2000; Hansson and Foley 2008; Pääbo *et al.* 2004). In addition, the artefacts and misincorporations arising from DNA damage may also be used as indicators of DNA authenticity (Hagelberg and Clegg 1991). These features are both just suggestive, however, and more rigorous standards are required to verify that the DNA is authentic aDNA.

Determining the Authenticity of DNA Results

The biggest challenge for the field of aDNA is in authenticating that the DNA is of ancient origin (endogenous DNA) as opposed to the result of contamination (exogenous DNA) (Bandelt 2005; Cooper and Poinar 2000; Gilbert, Bandelt *et al.* 2005, Gilbert, Rudbeck *et al.* 2005; Hagelberg and Clegg 1991; Willerslev and Cooper 2005). There are several common recommendations for determining authenticity, primarily focused on repeatability and reproducibility by external sources. This section will examine Cooper and Poinar's (2000) nine criteria for authenticity, as well as some of the additions or modifications suggested by others.

Cooper and Poinar's (2000) nine criteria for authenticity are commonly cited as standards for DNA authentication (Bandelt 2005; Cai *et al.* 2009; Gilbert, Bandelt *et al.* 2005, Gilbert, Rudbeck *et al.* 2005; Hansson and Foley 2008; Lalueza-Fox *et al.* 2004; Römpler *et al.* 2006; Winters *et al.* 2011). First, they recommend having a physically isolated work area dedicated to aDNA research located separate from where DNA amplification takes place (Cooper and Poinar 2000; Haynes *et al.* 2002). In their second and third criteria, Cooper and Poinar recommend the use of control extractions and amplifications that avoid the use of positive controls that may contaminate the samples. The resulting product should exhibit the appropriate molecular behaviour, including sizes typically less than 500 bp, with the PCR amplification efficacy displaying an inverse relationship with the product size. If single copy DNA is available, then one would expect that mtDNA should be able to be amplified as well (Cooper and Poinar 2000). The fourth criterion is reproducibility for the PCR amplifications and DNA extractions from the same specimen as a means of looking for contamination. The fifth criterion is that cloning of the amplified products and sequencing of clones should be used to identify the endogenous from exogenous sequences from one another. Sixth, the authors recommend independent replication by another laboratory in case contamination occurs in one of them. Seventh, Cooper and Poinar (2000) suggest using biochemical preservation of other organic residues such as amino acids as a marker for the degree of DNA survival to expect. They also suggest that quantitation of the template, particularly for human samples, should be done using competitive PCR and there should be less than 1,000 templates to exclude contamination (Criterion 8). Finally, the authors suggest that other material at the site be used as a negative control, such as the use of faunal samples from the same site (Cooper and Poinar 2000). Several of these criteria are debated. In particular, the primary debates centre on the fifth, seventh and eight points; the need for cloning, the use of biochemical preservation of other organic residues as a proxy for DNA survival, and the need for quantitation.

The necessity of using cloning as a means of validation has been debated. Some researchers followed Cooper and Poinar's (2000) recommendation of using clones as part of the authentication criteria, including Di Benedetto *et al.* (2000), Handt *et al.* (1996), Hofreiter, Jaenicke *et al.* (2001), Hofreiter, Serre *et al.* (2001), and Hofreiter *et al.*

(2002). However, other researchers such as Yang *et al.* (2008), Lambert *et al.* (2002), and Speller *et al.* (2010) did not use clones, though all three of these studies used dedicated aDNA laboratories, multiple extractions/amplifications, blanks, and independent replication by another laboratory to validate their results. On this debate, Winters *et al.* (2011) investigated whether the use of clones was actually necessary. As their ~3,500 year old northern fur seal samples did not generate different sequence data for direct sequencing compared to the sequence data generated using clones, they concluded that it is not always necessary to use cloning as an authentication criterion and that it should be done on a case by case. Winters *et al.* (2011) note that cloning is still essential for cases where the goal is to identify as many unique sequences as possible in the absence of next-generation sequencing.

There are a number of different suggestions for biochemical proxies in predicting DNA survival. The two most common are protein preservation (inferred by amino acid racemization- as changing ratios of enantiomers affect optical properties or by the products of flash pyrolysis) and bone preservation measures (such as crystallinity index).

Amino acid racemization is based upon the conversion of aspartic acid residues from the L to the D enantiomer, and the assumption that the kinetics involved are similar to those used for depurination (Austin, Smith *et al.* 1997; Bandelt 2005; Beja-Pereira *et al.* 2006; Hansen *et al.* 2006; Hebsgaard *et al.* 2005). Despite being used in studies such as Beja-Pereira *et al.* (2006), the degree of amino acid racemization does not always accurately reflect the degree of DNA survival because its use as a proxy was based on estimates of degradation by depurination, and therefore may not accurately represent DNA degradation occurring by other mechanisms besides depurination (Hebsgaard *et al.* 2005). Further, the premise that the kinetics of amino acid racemization are the same as for depurination may pose problems as most of these are based on depurination kinetics of DNA in aqueous solutions (Bandelt 2005; Hebsgaard *et al.* 2005; Willerslev and Cooper 2005). The utility of using amino acid racemization as a proxy was tested by Collins *et al.* (2009) who found no correlation between aspartic acid racemization and DNA amplification success. They suggested that as steric flexibility (particularly at the ends of collagen fibrils and under conditions where the collagen helix denatures to soluble gelatin) is important in allowing racemization of aspartic acid from the L (*levorotatory*) form to the D (*dextrorotatory*) enantiomer, the loss of soluble gelatinized collagen may skew the ratio of D to L enantiomers. Poinar and Stankiewicz (1999) suggest that flash pyrolysis with gas chromatography mass spectrometry could give an indication of protein preservation in a sample, and hence DNA preservation. If there are many pyrolysis products (such as 2, 5-diketopiperazines or proline-containing dipeptides) identified, then it is inferred that protein preservation, and hence DNA preservation, is high (Hofreiter, Jaenicke *et al.* 2001; Poinar and Stankiewicz 1999). However, Poinar and Stankiewicz (1999) suggest this method is only useful for identifiying those samples which contain longer peptides, and that this relationship between pyrolysis GC-MS/peptide surival and DNA preservation does not hold in hot climates.

For examining diageneic alterations of bone, the authors describe high performance liquid chromatography (HPLC) as a more sensitive method (Poinar and Stankiewicz 1999). Another argument against the use of pyrolysis GC-MS is a lack of empirical evidence supporting the correlation between the pyrolysis products and DNA preservation (Hofreiter, Jaenicke *et al.* 2001).

Finally, bone chemistry has also been used as a measure of preservation and for predicting DNA preservation. Bone preservation has been assessed using measures such as the crystallinity index of the hydroxyapatite, which can be determined using Fourier transform infrared spectroscopy (FTIR) or x-ray diffraction (Götherström *et al.* 2002; Schwarz *et al.* 2009; Sosa *et al.* 2013). The measures of crystallinity and amount of collagen remaining have been found to correlate with DNA success, suggesting that hydroxyapatite may play a role in DNA preservation (Götherström *et al.* 2002; Sosa *et al.* 2013). Studies that combine multiple factors may be of far greater use than those based on single factors to predict DNA survival (Schwarz *et al.* 2009; Sosa *et al.* 2013).

Regarding the criterion that the DNA template should be quantified, Alonso *et al.* (2004) suggests that real-time PCR may be the best method to do so because it has a higher sensitivity compared to hybridization techniques and it allows for the possibility of automation. Alonso *et al.* (2003) take a more in-depth look at several methods of quantifying DNA, including molecular hybridization (slot blot), end-point PCR, real-time PCR, competitive PCR (for mtDNA), and real-time PCR of mtDNA. They find that real-time PCR appears to be the best method.

The criteria presented by Cooper and Poinar (2000) serves as a starting point for a greater dialogue and increased rigour in authenticating aDNA. Some notable recommendations have emerged that expand beyond Cooper and Poinar's recommendations, including improved laboratory standards and techniques to eliminate cross-contamination. What follows below is a brief overview of some of these improved recommendations.

Willerslev and Cooper (2005) proposed several null hypotheses to help authenticate results as aDNA, including whether or not there was sufficient template, inhibition, false positives, and heterogeneous sequences (and if this was caused by deamination); whether or not the results were phylogenetically sound; and whether there is evidence of DNA preservation in the sample or in samples nearby. While the authentication process can never 'prove' that the sample DNA is ancient, these null hypotheses help to lend it support (Willerslev and Cooper 2005). In addition to recommending the use of a physically isolated, dedicated pre-PCR laboratory that has UV irradiation, as well as the use of blanks, reproducibility, cloning, and appropriate molecular behaviour as authentication standards, Willerslev and Cooper (2005) also provide more stringent recommendations to help reduce contamination. These include having isolated ventilation, arranging to have laboratory staff move unidirectionally from ancient to modern labs (so as to set up a 'concentration gradient' to avoid traversing from areas of high contaminant DNA to the aDNA laboratory), avoiding the use of positive controls (which may be a source of contamination), and using positive air pressure.

Recommendations were also made to improve laboratory standards. Lalueza-Fox *et al.* (2004) additionally recommend the use of facemasks and filter-tips. Willerslev and Cooper (2005) also argue that neither autoclaving nor a 'sterile' label are sufficient, and that all materials being used in the lab (reagents and tools) should be decontaminated by exposure to UV, extreme baking, acid, bleach (sodium hypochlorite), or some combination of these conditions. One of the improvements that Willerslev and Cooper (2005) made to Cooper and Poinar's (2000) recommendations is that they further clarify ambiguity with respect to what is meant by a 'physically isolated work area' for aDNA by emphasizing the importance of keeping aDNA work well away from amplified PCR

products and modern DNA work. For instance, Hansson and Foley (2008) note that for their work on ancient plant DNA, they used physically separated spaces between the pre- and post- PCR areas; however, the description of this setup is vague and may have been simply the use of different laboratory benches within the same room. This setup would not resolve the issue of aerosolized droplets that Willerslev and Cooper (2005) pointed out. A slightly improved, but still vague, application of authentication criteria is shown by Faerman *et al*'s (1998) work on approximately 15,000 year old human skeletons which used different hoods in different rooms and UV irradiation. Given that human DNA is particularly difficult to authenticate, it is surprising they did not also make use of bleach and filtered air, or provide information regarding the rooms the hoods were in (Malmström *et al.* 2007). Other setups have made further improvements on laboratory techniques and standards through using positive pressure, having completely isolated pre- and post-PCR work spaces (such as by having them in different buildings), using proper attire (disposable facemasks and foot coverings, gloves, laboratory coats or disposable gowns), as well as making different recommendations on decontamination procedures (Roberts and Ingham 2008; Speller *et al.* 2005).

As an added precaution when working with human DNA, Brown and Brown (2011) indicate that the DNA of the researchers handling the human remains should be compared with those of the sample to exclude them as sources of contamination. Several different projects have specifically excluded their own personnel as sources of contamination. Both Faerman *et al.* (2000) and Zhao *et al.* (2011) excluded contamination by laboratory personnel on the basis of ancestry or sex respectively. Melchior *et al.* (2010) took it one step further, obtaining the haplotype data for all laboratory personnel, archaeologists and anthropologists involved, and comparing the ancient data with this data base.

Given the importance of obtaining authentic results for aDNA, there is great discussion on best practices and criteria of authenticity. A number of key points were brought to light by Cooper and Poiner (2000), and there has been much debate on a number of their criteria leading to a number of further improvements. Unfortunately, as adherence to these criteria appears to be substantially lower than one might expect (Roberts and Ingham 2008), and given the need for authentic results, this discussion is still pertinent today.

Material That Can Be Used in Ancient DNA Studies

Ancient DNA can be derived from multiple sources. This section provides a brief overview of some of the sources from which aDNA can be isolated. Potential sources include faunal remains, plant remains, other objects, and hair.

For faunal remains, there is a general consensus that bones and teeth are better sources of aDNA than soft tissues, even among frozen samples (Brown and Brown 2011; Richards *et al.* 1995); however, there is much debate over the use of bone or teeth. Some researchers recommend the use of teeth due to the fact that DNA in teeth is more stable and will preserve longer, and that because teeth have a hard enamel coat (which also helps with decontamination procedures), there is far less contamination with the environment during diagenesis (Brown and Brown 2011; Drancourt *et al.* 1998; Papagrigorakis *et al.* 2006).

Regarding other materials from which aDNA can be obtained, the sources are quite diverse. As discussed earlier, soil and sediment cores may be the source of both plant and animal DNA (Andersen *et al.* 2012; Gugerli *et al.* 2005; Willerslev *et al.* 2003; Willerslev and Cooper 2005). Coprolites also represent another source of remains that may contain DNA from both faunal and floral sources; samples from 14,000 years old can give an indication of diet and paleoenvironment (Gugerli *et al.* 2005; Hofreiter, Jaenicke *et al.* 2001; Speller *et al.* 2010). Coprolites can even be used to indicate the presence of diseases; for instance, DNA from *Ascaris* eggs from coprolites has also been successfully extracted and amplified (Loreille, Roumat *et al.* 2001). Some work has also looked at recovering chloroplast DNA from ceramics obtained from a 2,400 year old Greek shipwreck, as well as from small pills found in a water proof container from a Roman shipwreck (Hansson and Foley 2008; Travis 2010).

Plant remains may come from seeds or other material such as corn cobs, or from inedible materials such as wood or other materials for construction (Brown and Brown 2011; Dumolin-Lapègue *et al.* 1999; Gugerli *et al.* 2005; Jaenicke-Després *et al.* 2003). The expanding study of ancient plant DNA includes investigation of both chloroplast DNA and nuclear genes (Gugerli *et al.* 2005; Hansson and Foley 2008; Jaenicke-Després *et al.* 2003; Travis 2010). Unfortunately, there has been less work so far on plant DNA as it tends to have much PCR inhibition, and chloroplast and mtDNA in plants tend to have very low polymorphism so there are fewer markers for analysis (Gugerli *et al.* 2005).

DNA isolation from hair is becoming much more common. It is a good source of DNA that is better than soft tissue and possibly better than bone and teeth as well (Brown and Brown 2011; Gilbert *et al.* 2004; Willerslev and Cooper 2005). Gilbert *et al.* (2004) suggest that hair may be largely resistant to contamination and can be easily decontaminated using sodium hypochlorite solution. In addition, the structure of hair and its low water content can help protect the DNA from hydrolytic damage. Gilbert *et al.* (2004) also suggest that nuclear inserts (numts) may be less of a problem for hair samples. DNA was been successfully recovered from 65,000 year old bison hair, as well as from 9,000 year old wool (Gilbert *et al.* 2004; Travis 2010).

Summary

This essay indicates that there are many potential applications for aDNA studies. Any attempt to understand DNA preservation equires an understanding of the history of the field of aDNA, the structure and organization of DNA in the cell, and the mechanisms by which DNA degrades. This degradation results in a number of challenges that are unique to aDNA, and that make authentication of results crucial. There are a number of recommendations and criteria for authenticating sequences as ancient, and though some of these are disputed, the need for authentication criteria is agreed upon (Roberts and Ingham 2008).

Appendix B.

DNA Extraction Protocol and Lab Methods

Sample preparation and DNA extraction and amplification of the samples in Moss *et al.* (2006) was undertaken by Dr. Yang and Dr. Speller in a dedicated ancient DNA laboratory at SFU. Sample preparation and extraction protocols were done as described in Speller *et al.* (2005), Yang *et al.* (1998), Yang *et al.* (2004), and Yang *et al.* (2005). The procedure for the amplification of the *cytochrome b* and D-loop regions was described in Moss *et al.* (2006).

Bone samples were first prepared by mechanical[27] and chemical decontamination. The first steps involved removing surface contamination by first abrading the surface with sandpaper to remove the outer layer, then immersing the samples in bleach solution (5-10% commercial)[28] for 5-10 minutes, followed by sequentially submerging samples in hydrochloric acid (1N), then in sodium hydroxide (1N) for approximately 30-60 seconds each (Yang *et al.* 2005). This step should be sufficient to destroy most external contaminating fungi, bacteria and DNA (Rogers *et al.* 2004). Samples were then rinsed with ultra-pure water to remove any traces of the bleach, hydrochloric acid and sodium hydroxide. The wet bone samples were then UV irradiated in a Crosslinker at 1200 nm for a minimum of 30 minutes on each side. The purpose of the Crosslinker is to generate thymine dimers in the exposed contaminant DNA to prevent their amplification.

DNA was extracted using the modified silica spin protocols of Yang *et al.* (1998) (Moss *et al.* 2006). This involved grinding the decontaminated bone to a powder, incubating in a lysis buffer, concentrating the sample using an Amicon filter, then extracting the DNA with a Qiagen silica-based spin column. For DNA extraction, the decontaminated bone samples were first ground into a powder. Three-five milliliters of lysis buffer (consisting of 0.5-0.25% SDS, 0.5 mg/mL proteinase K, and 0.5 M EDTA at pH8) were added to the powdered samples then left overnight at 50°C in a rotating incubator to dissolve the bone powder. Samples were centrifuged; then 1.5-2 mL of supernatant was transferred to an Amicon 10,000 NMWL centrifugal filter and spun until the volume was reduced to 100µL or less to purify and concentrate the sample (Yang 1998). The concentrated samples were then loaded into Qiagen QIAquick spin columns for purification. This was done following the manufacturer's recommendations for the QIAquick Nucleotide Removal Kit Protocol for use with a microcentrifuge, with the exception that the rinsing step involved two rinses with 400µL of buffer PE (QIAGEN, Hilden, Germany). Two separate elutions in 100µL were collected.

[27] Assuming sandpaper was used. References for Yang *et al.* (1998) and Yang *et al.* (2005) show use of sandpaper, but not the salmon papers (bones too small). Also Yang *et al.* (1998) paper (which used drilling) does not have bleaching, so I am assuming both were done.

[28] When we copied the northern fur seals we had 5.5% bleach. The Yang *et al.* (2005) paper cites 10%.

For *cytochrome b*, a 180 bp fragment was PCR amplified using the forward primer NFSF5-CtB (5'-CCAACATTCGAAAAGTTCATCC-3') and reverse primer NFS-R185-CtB (5'- GCTGTGGTGGTGTCTGAGGT-3'). For the D-loop/control region, a 199 bp fragment was amplified using the forward primer NFS-F99 (5'-CTCCCCCTATGTACTTCGTGCA-3') and reverse primer NFS-R301c (5'-GTACACTTTTCACAAGGGTTGCTG-3') (Moss *et al.* 2006). All PCR amplifications were carried out with 3 µL of extracted ancient DNA sample in a Mastercycler Personal (Eppendorf, Hamburg, Germany) thermocycler in 30 µL reaction volumes, with final concentrations of 2.25 U AmpliTaq Gold LD (Applied Biosystems), 50mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl2, 1.5 mg/mL BSA, 0.2 mM of each dNTP; each primer had a concentration of 0.3 µM. The PCR ran for 60 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds, with an initial 12 minutes of denaturing to activate AmpliTaq Gold LD. PCR products were separated by gel electrophoresis, running 5 µL of each sample on 2% agarose gel (visualized with SYBR-Green stain on a Dark Reader Box). PCR products were purified with Qiagen's MinElute. Purifed PCR products were sent to Eurofins MWG Operon for sequencing, with the majority of samples sequenced in both directions.

Appendix C.

Northern Fur Seal Taxonomy

The current taxonomic classification is based on morphological characteristics. However, several researchers have suggested that morphological traits are a poor choice for the taxonomic classification of Otariidae, as many genera exhibit convergence or may have morphological conservatism within some lineages, both of which can obscure differentiation (Arnason *et al.* 2006; Bininda-Emonds *et al.* 1999; Wynen *et al.* 2001). It has also been argued that the classification is based on too few traits and is not adequate for separating species lineages (Wynen *et al.* 2001). For instance, some lineages are only separated by a few traits, such as the presence or absence of underfur or a sixth upper canine tooth, to distinguish between the fur seals and sea lions sub-family classification. In addition, convergence in some traits (for example, the similar size, skull morphology and behaviour of *Arctocephalus pusillus* to sea lions) can obscure such taxonomic relationships (Wynen *et al.* 2001). This argument against the current classification system even extends through multiple levels of classification. These levels include debate at the sub-family division between sea lions and fur seals, with studies suggesting the current division of Arctocephalinae and Otariinae is not supported (Bininda-Emonds *et al.* 1999). Some of the debate has even extended to higher levels of taxonomic classification, such as whether the pinniped clade represent a monophyletic or diphyletic grouping (see Appendix C.2 for illustration of types of groupings) (Arnason *et al.* 2006). Phylogenetic trees attempting to group the Otariidae members by genetic relatedness can differ between studies based on the samples chosen and methods employed (Arnason *et al.* 2006; Wynen *et al.* 2001).

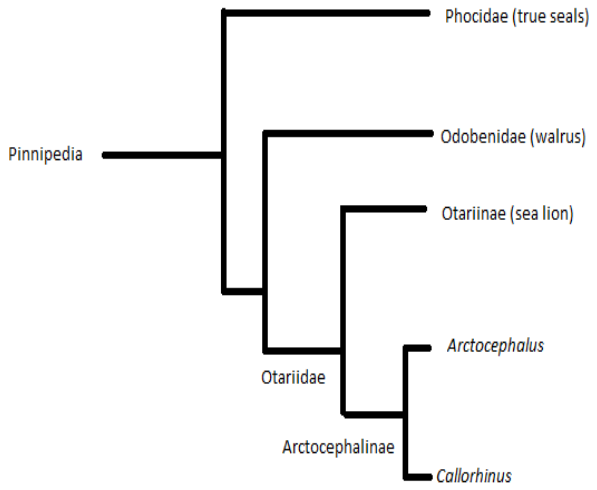

Figure C.1. Pinniped disputed taxonomy based on morphological characteristics showing the relationship of northern furs seals (*C. ursinus*) to other pinnipeds.
Graph drawn with the sub-family Arctocephalinae used to group the 'fur seals' (branch lengths have no meaning in this figure).

One of the species most strongly affected by the sea lion/fur seal classification debate is *Callorhinus ursinus*. Morphologically, this species is classified as a fur seal (sub-family Arctocephalinae); however, the phylogenetic analysis of both Bininda-Emonds *et al.* (1999) and Wynen *et al.* (2001) do not place this species in a monophyletic clade with other *Arctocephalus* species. Instead, other studies have placed *C. ursinus*, *Arctocephalus* species and the sea lion genera in a polytomy, or unresolved node on the phylogenetic tree (Bininda-Emonds *et al.* 1999) (though all three clades were monophyletic, or have placed *C. ursinus* as a lineage basal to the *Arctocephalus* and sea lion clade (Wynen *et al.* 2001). This latter representation does not separate *Arctocephalus* and the sea lion clades, as representatives of each 'clade' can be found interspersed amongst the other. Taken together, these studies suggest that even the sea lion/fur seal division may not be reflective of the evolutionary history of the species in these lineages. A study of ancient northern fur seal remains by Moss *et al.* (2006) also generated phylogenetic trees using *cytochrome b* and D-loop sequences. Like the *cytochrome b* phylogenetic tree of Wynen *et al.* (2001), the tree generated by Moss *et al.* (2006) using the same gene (albeit with a fragment half the size) also found a similar pattern of *C. ursinus* lineage diverging before the split between the sea lion and *Arctocephalus* lineages. In Moss *et al.* (2006) the sea lion species do form a monophyletic clade nestled within the *Arctocephalus* clade, while this is not the case in Wynen *et al.* (2001), where reciprocal paraphyly can be seen, with neither group forming a monophyetic clade (see Figure C.2 for examples of paraphyly and monophyly). Bininda-Edmonds *et al.* (1999) showed a monophyletic *C. ursinus* clade, though the relationship to *Arctocephalus* and sea lion species was unclear, as these three groups formed a polytomy. Thus despite being taxonomically grouped with *Arctocephalus* species, *Callorhinus ursinus* may be a distinct lineage. It may also be that there is no 'sea lion' clade (no monophyletic group descending from a common 'sea lion' ancestor), and instead the current term 'sea lion' may refer to a grouping based on convergent morphologic characteristics.

There are also numerous variations in taxonomic classification at the species level scale. For example, Wynen *et al.* (2001) found the genetic difference between two species (*A. philippii* and *A. townsendi*) to be quite low and similar to the values between the subspecies *A. pusillus pusillus* and *A. pusillus doriferus*, which therefore provides support that *A. philippii* and *A. townsendi* might be classed as related subspecies (Wynen *et al.* 2001). In contrast, other taxonomies based on skull morphology and other morphological traits suggest these are two separate species (Bininda-Emonds *et al.* 1999; Wynen *et al.* 2001). Another cause of difference in taxonomic groupings between studies may be due to sampling; for instance, a prior study may have sampled *A. tropicalis*/*A. gazella* hybrids, resulting in the two species appearing more closely related than in Wynen *et al.*'s (2001) own study.[29] These examples are part of a larger, general pattern of little statistical support for these groupings based on 'bootstrapping,' or repeat sampling of random subsets of the data used to lend statistical support for/against

[29] Bininda-Emonds *et al.* (1999) grouped *A. tropicalis* and *A. gazella* as being members of a paraphyletic clade, including a number of other *Arctocephalus* species; in contrast Wynen *et al*.'s (2001) study grouped *A. gazella* as part of a monophyletic clade with *A. philippii*, while *A. tropicallis* grouped with *A. pusillus* species.

groupings. This means that the support for these phylogenetic groupings is quite weak, and thus the phylogeny is difficult to resolve. The explanation for this poor resolution of fur seal/sea lion clades may be the result of rapid radiation following divergence (Wynen *et al.* 2001). Rapid radiation is often attributed as the cause of poorly resolved phylogenies since this results in great divergence of extant taxa compared to smaller divergence of ancestors, so long-branch attraction may become a problem (for more discussion see Chapter 3). Recent rapid radiations may also result in problems with incomplete lineage sorting, resulting in individuals with different ancestral alleles/haplotypes amongst the different descendants (Whitfield and Lockhart 2007). In addition, a high degree of character change can 'oversaturate' the analysis and may contribute to molecular homoplasies (Whitfield and Lockhart 2007). This hypothesis fits with the fossil evidence indicating that *Callorhinus ursinus* diverged before the sea lion/fur seal split approximately 6 million years ago. The poor resolution of sea lion and other fur seal species and inconsistent phylogenetic groupings indicate that sea lions may not form a monophyletic clade. However, if all sea lions do share a 'sea lion' common ancestor, then a rapid divergence following a sea lion/fur seal split around 3 million years ago could make it difficult to resolve the phylogeny (Wynen *et al.* 2001).

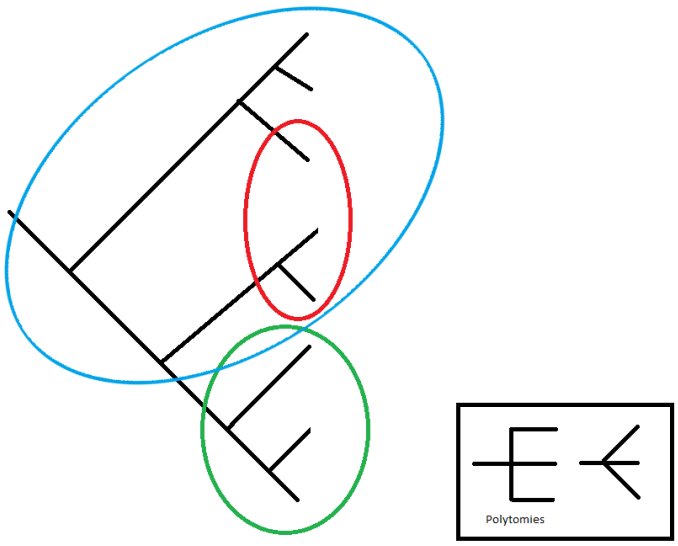

Figure C.2. Example cladogram illustrating the types of clades (monophyletic, paraphyletic and polyphyletic).
Monophyly (shown in green) includes a common ancestor and all descendants; paraphyly (shown in blue) refers to groupings containing a common ancestor and many but not all of its descendants; and polyphyletic groupings refer to groupings that include some species but not others, and are often based on homoplasies between lineages. Monophyletic groupings are the goal for classifying the relationship between organisms. Insert illustrates a polytomy, or unresolved node, that does not follow the typical bifurcating pattern (for more discussion on tree-building and phylogenetics, see Chapter 3).

The relationship of northern fur seals to other species is important when making species identifications of archaeological remains to ensure the remains under study belong to the desired species and to understand how the species in question is related to similar species. As discussed above, there is some debate on the taxonomy of northern fur seal.

Results of Phylogeny Testing

Phylogenetic trees were generated to confirm species identity (results shown in Table 4.1) and to test the phylogeny of northern fur seals, given that this is an area of som contentions. The phylogeny generated using the *cytochrome b* supported the hypothesis of *Callorhinus ursinus* diverging prior to the fur seal/sea lion split (Bininda-Emonds *et al.* 1999; Moss *et al.* 2006; Wynen *et al.* 2001) while the D-loop phylogeny is not so clear (Moss *et al.* 2006). Regardless, despite the issues with phylogeny, the trees all proved useful in species identity. Species identity was successfully confirmed using both genetic regions (see section 5.2). Northern fur seal sequences were used to investigate questions on population genetics (see section 5.3).

Cytochrome b

The *cytochrome b* phylogeny (Figure C.3) reveals that *Callorhinus ursinus* samples group into two branches, representing two separate haplotypes. For the most part, species form distinct clades which allow unambiguous species identification. This is especially clear for the *Callorhinus* sequences which form two lineages distinct from other clades. One exception is the *Arctocephalus tropicalis* species, which appears in multiple locations on the cytochrome b phylogeny. Rooting of the cytochrome b phylogenetic tree with *Phoca vitulina* (harbour seals) as anoutgroup revealed that the *Callorhinus* branch is basal to the polyphyletic sea lion/fur seal lineages (Figure C.4). While the position of *C. ursinus* in the phylogeny remained the same as in Moss *et al.* (2006), there was a substantial difference in how the remaining fur seal/sea lion clades grouped. The cytochrome b phylogeny in Moss *et al.* (2006) placed the sea lion clades as a monophyletic branch nestled within the *Arctocephalus* branches. However, the rooted cytochrome b phylogeny obtained in these results suggested a polyphyletic situation instead, where fur seal and sea lion clades were intermixed (Meyer and Paulay 2005). This finding is somewhat surprising as both this analysis and that of Moss *et al.* (2006) used the same initial raw sequence data supplemented with sequences obtained from GenBank to produce trees that used the neighbour-joining (NJ) method. The difference between these trees (Figures C.3 and C.4) and those generated by Moss *et al.* (2006) is that additional sea lion clades (*Neophoca*, *Phocarctos* and *Otaria*) were added to the species examined in this tree, and the single *Zalophus* sequence used by Moss *et al.* (2006) was not included in the GenBank data set used for tree-building. These three additional sea lion genera did not group with the *Eumetopias* branch and instead were distributed amongst the fur seal clades (Figures C.3 and C.4). Therefore, there are some significant differences between the trees generated below and those used by Moss *et al.* (2006) for the initial species identifications.

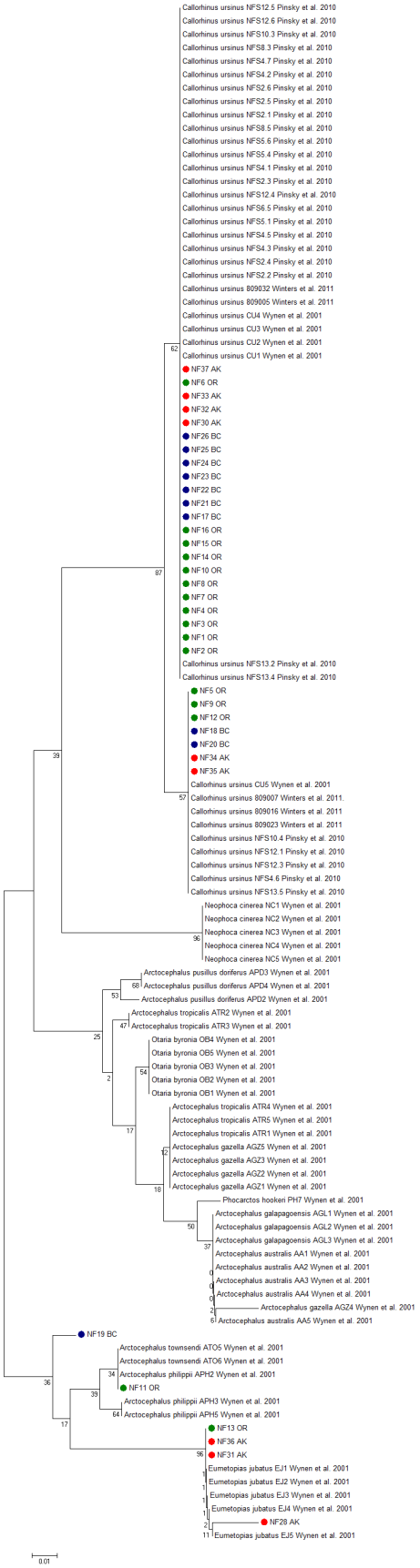

Figure C.3. Phylogenetic NJ tree of 139 bp cytochrome b sequences from Otariids. Generated using the Maximum Composite Likelihood model with 1000 bootstrap replicates. Filled circle indicators with the label “NF” designates those samples for which sequence data originated in the SFU aDNA lab. These samples are the ones being tested for species identity. These samples come from archaeological sites located from Cape Addington, Alaska (AK) coloured red, Netarts Oregon (OR) coloured green and Ts'ishaa site, and British Columbia (BC) coloured blue. Sample NF29 (which did not have successful PCR amplification for this gene) is not included in the data. *Not all bases were analyzed due to alignment gaps.

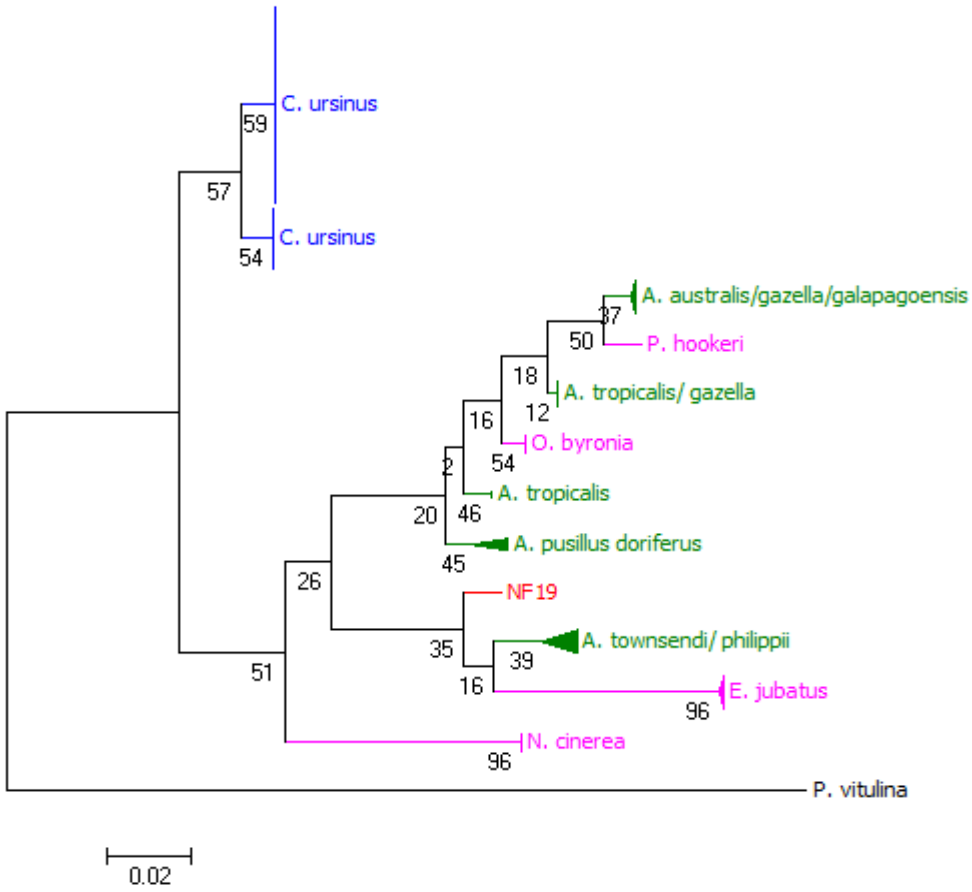

Figure C.4. Condensed, rooted NJ phylogenetic tree of 139 bp cytochrome b sequences from Otariids.
Figure generated using composite likelihood model, 1000 bootstrap replicates. Figure is a condensed, colour coded version of Figure C.3 above. Purple is used to identify “sea lion” genera, while green is used for Arctocephalus species and blue for Callorhinus species. Black is used to indicate the outgroup Phoca vitulina that was used to root the tree.

D-loop

The phylogenetic trees generated from the D-loop control region data (Figure C.5) generally support the cytochrome b species identification of the archaeological samples of Moss *et al.* (2006). The D-loop data did allow for more precise identification of several samples (Table 4.1, Figure C.5). For instance, NF11, which cytochrome b had generally placed with *Arctocephalus townsendi/A.philippi*, was resolved to group only with A. townsendi when the D-loop data was used instead. Sample NF19, which remained unknown after cytochrome b data due to its location basal to branches of both *Arctocephalus* and *Eumetopias jubatus*, grouped only with *Arctocephalus philippi* when the D-loop sequences were used. The specific species identifications determined using the cytochrome b phylogeny were supported by the D-loop phylogeny (Table 4.1).

Given that the D-loop/control is non-coding, it is not surprising that there are far more haplotypes among the C. ursinus samples. While the cytochrome b data revealed only 2 haplotypes in the phylogeny (Figure C.3), the D-loop tree shows 25 haplotypes (the algorithm that built the tree examined 116 out of the 160 sites, as sites with alignment gaps were excluded). The additional trees generated for haplotype analysis (Figures 5.1 and 5.2), the DnaSP 5 analysis30 conducted on a reduced D-loop data set, and the manual site-by-site comparison of both cytochrome b (see Table 5.1 in section 5.3.1) and D-loop (Table 5.2, in section 5.3.1) sequence alignments did reveal additional mutations/haplotypes that were not detectable due to the presence of alignment gaps that reduced the sites analyzed to build these trees (Figure C.3, Figure C.5).

Outgroups such as *Phoca vitulina* and *Otaria rosmarus* sequences were added to further investigate the D-loop phylogeny. In addition to the NJ trees, a maximum likelihood (ML) tree was also constructed to investigate the unexpected phylogenies that emerged when the trees were rooted. Results of these NJ trees are shown in Figure C.6a-c. The expected relationships shown in the cytochrome b tree (Figure C.4) were not supported by the D-loop trees (Figure C.6-c). It is also interesting to note that the choice of outgroup resulted in different phylogenetic trees

[30] The DnaSP 5 calculations of this D-loop data examined 130 out of 160 sites and found 26 haplotypes (results not shown, as alignment gaps made this a poor analysis). The data set was improved by reducing problematic regions by both trimming the ends to remove end alignment gaps and removing sequences with substantial alignment gaps. The result was a 157 bp sequence data set that was used to generate the DnaSP 5 data tables shown in Table 5.9 (Ancient D-loop) and Table 5.10 (Modern D-loop)

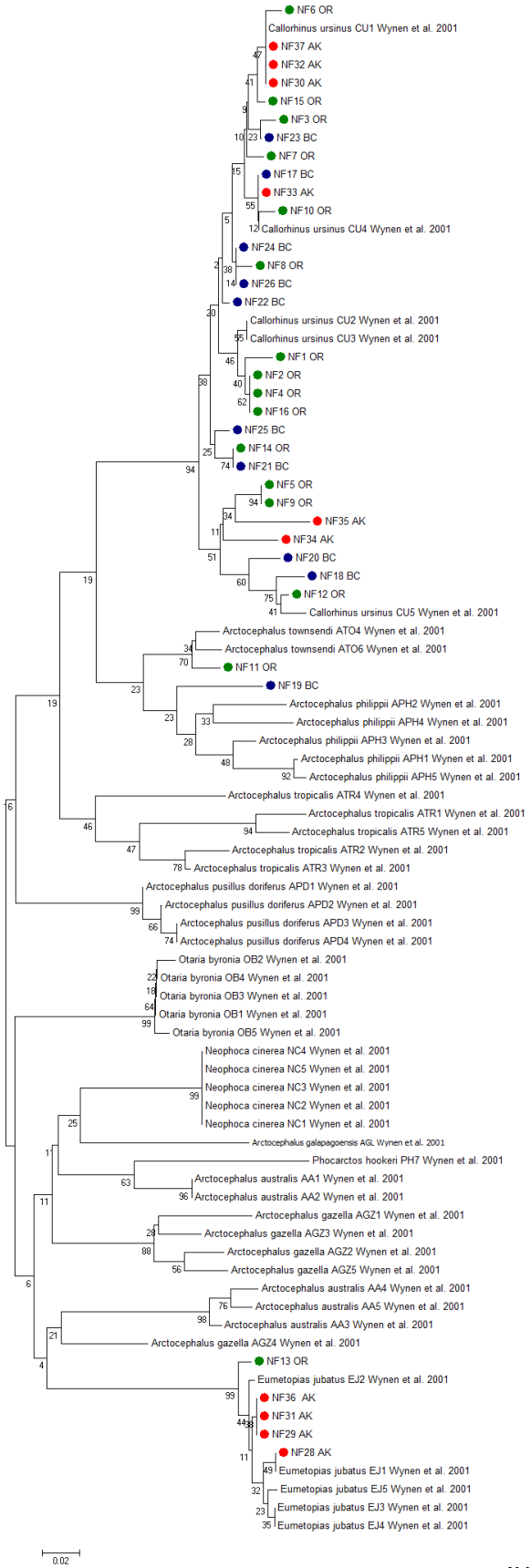

Figure C.5. Phylogenetic NJ tree of 160 bp control region/D-loop sequences from Otariids.
"NF" designates those samples for which sequence data originated in the SFU aDNA lab. Filled circle indicators with the label "NF" designates those samples for which sequence data originated in the SFU aDNA lab. These samples are the ones being tested for species identity. These samples come from archaeological sites located from Cape Addington, Alaska (AK) coloured red, Netarts Oregon (OR) coloured green and Ts'ishaa site, British Columbia (BC) coloured blue. Unrooted tree generated using the maximum composite likelihood model and bootstapped with 1000 replicates.

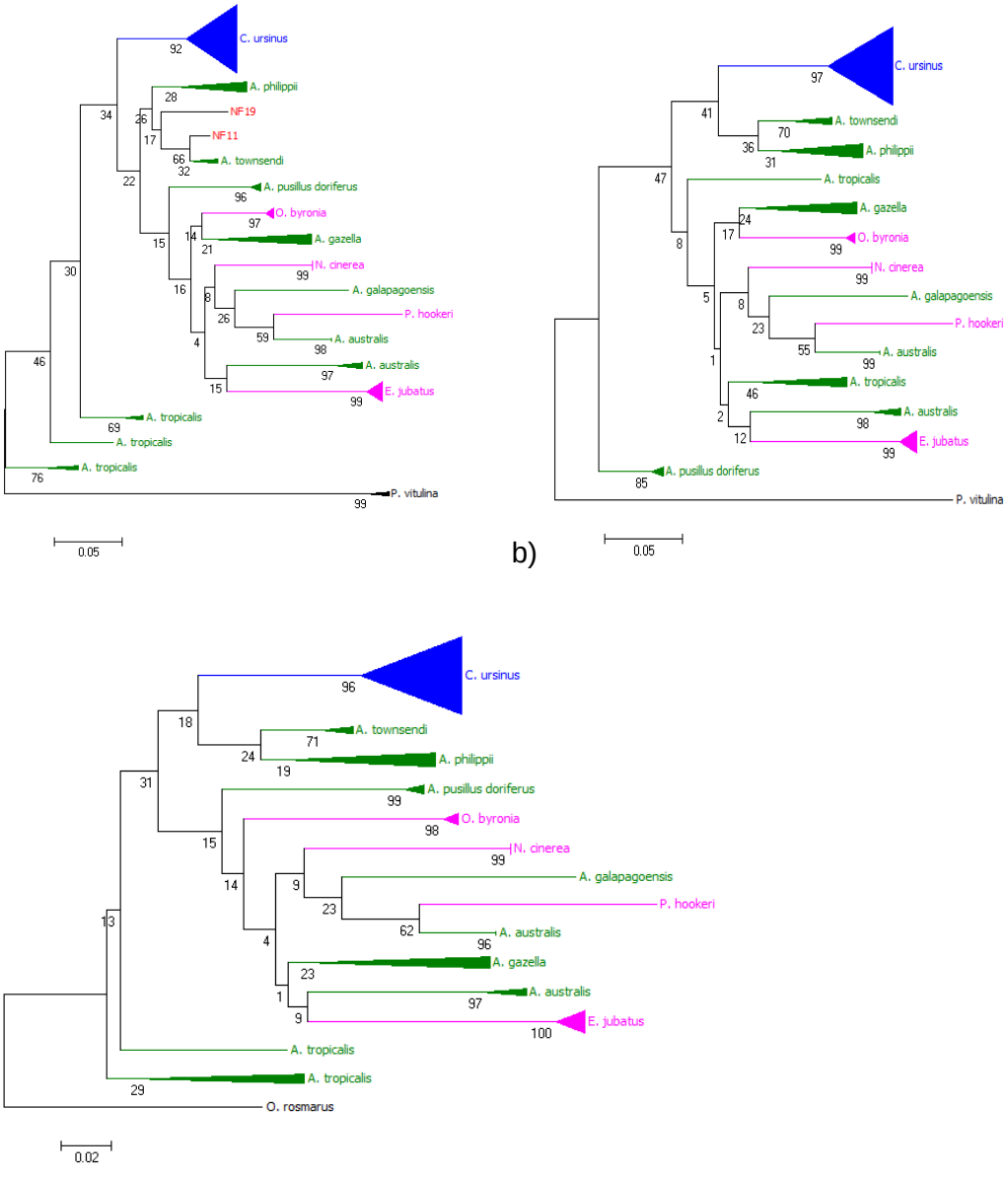

Figure C.6. Rooted minimized NJ trees generated using 160 bp control region/D-loop sequences from Otariids to show the impact of outgroup used. Trees generated using maximum composite likelihood model, with 1000 bootstrap replicates. a) Rooted with two *P. vitulina* (from AF522668) b) rooted with one *P. vitulina* sequence (from U36344.1) c) rooted with one *O. rosmarus* sequence (EU728531.1).

Because of the dramatic differences in phylogeny between these trees and those generated by Moss *et al.* (2006) regarding several clades, additional investigation into factors affecting tree topology were conducted. To examine the effect of tree-building methods and the use of different substitution models, ML trees (using different

substitution models) were also generated. The findings showed that changing the type of tree-building method did result in a different tree. However, neither the NJ trees nor the ML trees show the expected pattern of having C. ursinus branches as basal to the other Arctocephalus/Otariidae lineages (Figure C.6a-c, Figure C.7a-c). Between the NJ and first ML tree, both of which were rooted with *O. rosmarus* (Figure C.6c and Figure C.7a), the primary differences are the placement of the A. tropicalis, with the ML tree showing both A. tropicalis branches as more closely related to one another. The ML tree generated using the Jukes and Cantor model has the strongest support for the branch leading to northern fur seal (Figure C.7a), though even this branch shows very low support (boostrap value indicates it only groups here 29% of the time when the data is randomized). This tree also shows minor rearrangement of the *A. australis/P. hookeri/A. galapagoensis/N. cinerea* branch compared to the neighbour-joining counterparts (Figures C.6c, C.7a). Compared to the ML tree, in the NJ tree this *A. australis*/*P. hookeri/A. galapagoensis/N. cinerea* branch is closer to the *E. jubatus/A. australis/A.gazella* branch and O. byronia is also placed basal to the *A. australis/P. hookeri/A. galapagoensis/N. cinerea*. Additionally, the *E. jubatus/A. australis/A.gazella* branches in the NJ tree differ from the ML tree where it is located amongst the latter branch. Another difference between these two trees is the placement of the *A. pusillus doriferus* branch (Figures C.6c, C.7a). Finally, there is also a difference in the placement of the *A. townsendii/A. philippi* branch, with it being most closely related to the C. ursinus lineage in the NJ tree (Figures C.6c, C.7a).

Amongst the ML trees (Figure C.7a-c), the tree generated using Tamura and Nei's substitution model (b) and the tree generated with the Kimura-2-parameter model (c) show some particular similarities. Both of the latter models place the *A. tropicalis* and *A. pusillus doriferus* lineages as basal to the other sea lion/ fur seals, with the *A. tropicalis* branches forming a handful of separate branches, rather than a monophyletic clade expected for a species. The remaining Arctocephalus and sea lion branches are interspersed amongst one another. Again, there is no evidence of a monophyletic clade for either sea lions or fur seals present in the data.

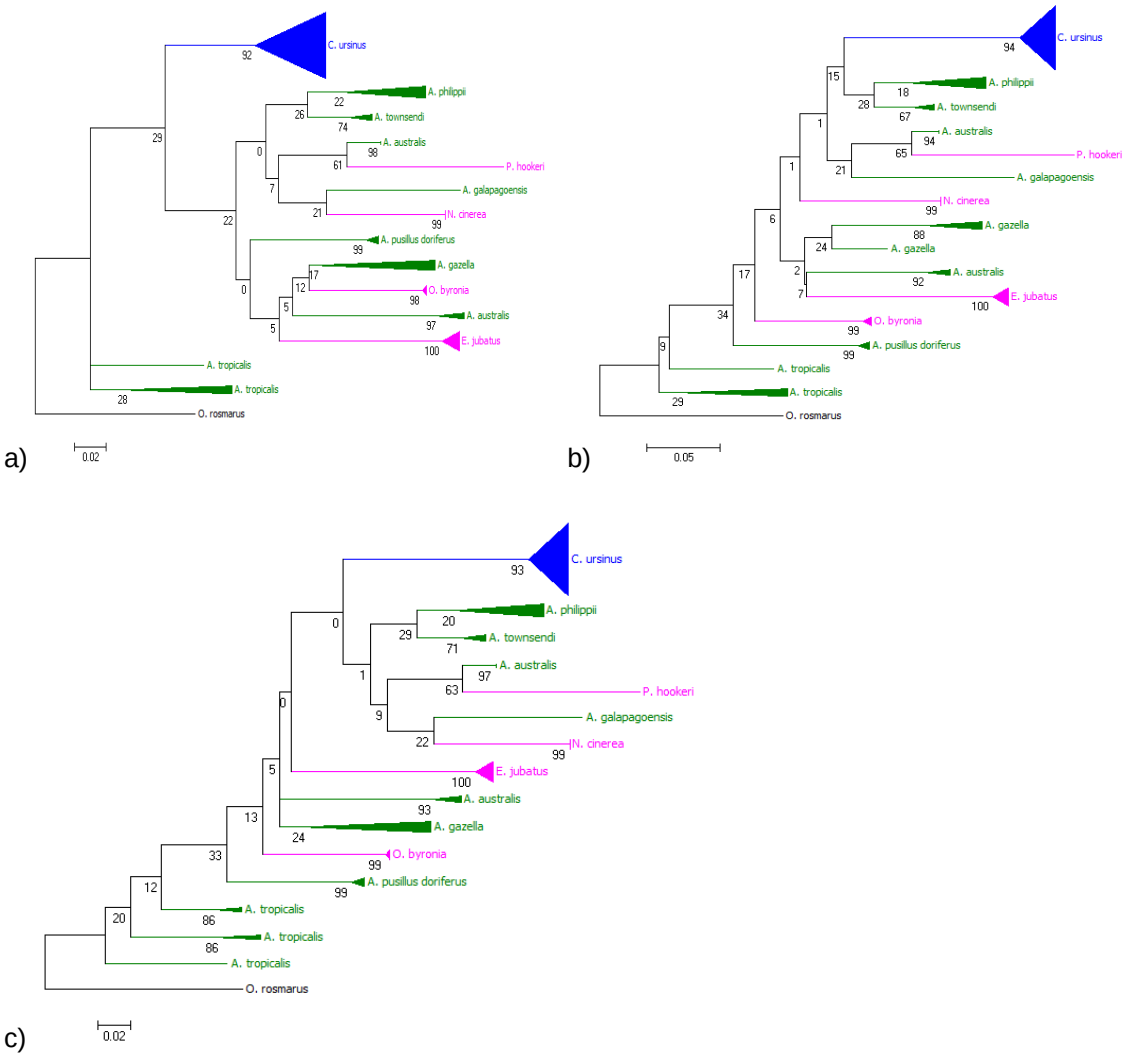

Figure C.7. Rooted minimized ML trees generated using 160 bp control region/D-loop sequences from various Otariid species using *O. rosmarus* to root the trees to show the impact of different substitution models.
Trees have been compressed to show species relations. Sea lion branches are shown in pink, *Arctocephalus* species branches in green, *Callorhinus* branches in blue, with the outgroup in black. a) Jukes and Cantor substitution model, 1000 bootstrap replicates; b) Tamura and Nei's substitution model, 1000 bootstrap replicates c) Kimura-2-parameter substitution model, 1000 bootstrap replicates.

Finally, despite the relationship between all of these branches being affected by outgroup, tree-building method and substitution model, and the very poor bootstrap values for the trees above, all of the trees generated showed that the northern fur seal sequences continuously grouped together. Despite the unclear phylogeny among otariid

species, species identity was solid for all northern fur seal sequences. The majority of other species also grouped together with the exception of *A. tropicalis* and *A. australis* whose sequences were routinely split into multiple lineages rather than grouping as a single species.

Discussion

Phylogenetic trees were of use for identifying the sample species, with both *cytochrome b* and D-loop data yielding similar results for species identifications. Species identifications were clear and identical for all samples with the exceptions of NF11 and NF19, which were unresolved on the *cytochrome b* tree but resolved using the D-loop phylogenetic. Additionally, these results supported the genetic species identifications by Yang and Speller in Moss *et al.* (2006). The resulting phylogenies exhibited some peculiar features. While the *cytochrome b* trees supported the general hypothesis of *C. ursinus* as basal to the fur seal/sea lion split (Moss *et al.* 2006; Wynen *et al.* 2001), the D-loop data did not support this. Instead it placed the *C. ursinus* sequences as nested within the tree, with various *Arctocephalus* sequences placed basal to the *C. ursinus* split. This lack of a clearly supported D-loop phylogeny highlights some of the difficulties in determining the phylogenetic relationship and taxonomic placing of the Otariid species, and is pertinent to the ongoing debate regarding northern fur seal taxonomy.

Taxonomic identity was consistent between D-loop and *cytochrome b* sequences. The majority of northern fur seal sequences were found to be *C. ursinus*, which is the species they had been assigned to based on morphological identity. However, samples NF13, NF28, NF29, NF31, and NF36 clearly grouped with *Eumetopias jubatus*. Two samples had unresolved phylogenies on the *cytochrome b* tree that were resolved with the D-loop analysis. Sample NF11 grouped with *Arctocephalus* (*A. townsendi*/ *A. philippii*) and was narrowed down as belonging to *A. townsendi* on the D-loop tree. For *cytochrome b* analysis, sample NF19 formed a separate branch off of the *Eumetopias jubatus*/*Arctocephalus townsendi*/*A. philippii* lineage. The questionable placement of NF19 was resolved through the D-loop data where the resulting tree placed this sample securely within the *A. philippii* group. As expected, these results generally correlate with the findings of Moss *et al.* (2006). However, using the additional sequence data did result in one improvement – clarification of sample (NF19) that had previously been identified by Moss *et al.* (2006) as belonging to *A. philippii* or *A. townsendi*. The D-loop data from this analysis confirmed identification of this sample as belonging more specifically to the *A. philippii group*.

When generating a phylogeny of Otariid species, the use of different mtDNA regions yielded different results. The coding *cytochrome b* sequence results yielded the expected phylogeny, with the *C. ursinus* lineage divergence preceding the split of other fur seal and sea lion lineages (found in both Moss *et al.* 2006 and Wynen *et al.* 2001). Surprisingly, the analysis of the non-coding D-loop sequence data yielded trees whose pattern varied depending on the analytical method and the sequences used for an outgroup. The phylogenetic trees generated in this study highlight the issues identified in Chapter 2 with regards to the current taxonomic classification system based on morphology.

The *cytochrome b* trees (Figures C.3 and C.4) show no clear separation of sea lion and fur seal clades, with members of each clade nestled amongst the other clade; this

indicates polyphyly in which Otariidae lineages *Neophoca*, *Phocarctos*, *Eumetopias* and *Otaria* distributed amongst the *Arctocephalus* lineages. This polyphyly suggests that the Arctocephalinae/Otariinae phylogenetic division is not supported. The use of morphological characters to distinguish fur seals and sea lion clades does not appear to represent the evolutionary history of these species. Convergent evolution and other mechanisms may make morphology a poor choice in taxonomic classification of Otariids. The finding of polyphyly among the fur seal/sea lion clades correlates with the findings of Wynen *et al.* (2001).

In contrast, the D-loop trees do not support the expected phylogeny that the *cytochrome b* data supports. This tree also does not agree with the data in Wynen *et al.* (2001) or the phylogeny in Moss *et al.* (2006), where *C. ursinus* is located basal to the Arctocephalinae/Otariidae branches. As discussed in the literature review section on Otariid phylogeny (section 2.2), the poor resolution of phylogenetic clades may be the result of rapid lineage radiation (Wynen *et al.* 2001). As the D-loop is a non-coding region, it is expected to show an increased number of DNA polymorphisms (Allendorf *et al.* 2012).

Given that a handful of the sites had more than one mutation, this D-loop data did not entirely conform to the typical pattern of SNP markers being bi-allelic (Allendorf *et al.* 2012). The neighbour-joining method can lose information due to the compression of character data into distance measurements (Holder and Lewis 2003); as a result, it seems possible that some of this polymorphism was not represented in the neigbour-joining trees. If a similar pattern of multiple mutations at a handful of SNP sites also occurs in other clades (such as the other sea lion and fur seal species), the neighbour-joining methods may not necessarily represent the best option for investigating phylogeny.

One of the common considerations when looking at phylogenetic trees and networks is that gene divergence is not the same as species divergence (Arnason *et al.* 1996; Patterson *et al.* 2006). There are a number of split lineages where one species may be found in separate branches on the phylogenetic trees, such as *A. gazella* appearing in branches with both *A. australis* and its own separate branch (Figure C.5), the two *A. australis* branches in Figure C.6a, or the multiple *A. tropicalis* branches in Figure C.6b. One possible explanation is that these isolated lineages may be the result of misidentified samples or species hybridization where one mitochondrial lineage may trace back to a hybrid ancestor carrying the mitochondrial genome of another species. However, if this was the case, because mitochondrial DNA does not recombine, all of the trees should be expected to similarly group this lineage with the same species (whether this is the correct species identification or the ancestral origin of the mitochondrial sequence). Since this is not the case and the split lineages are different for the different tree variables (Figure C.6a-c, Figure C.7a-c), this property must be the result of substantial homoplasy (see also results and discussion of the network analysis of the D-loop sequences).

Species Identification Summary and Discussion

All phylogenetic trees support the same identification of species (Table 4.1) as those identified by Moss *et al.* (2006). This indicates that while there are dramatic differences in tree topology and debate over relationship between various clades, the identification of northern fur seal remains is secure with reliable species identification. Sample NF11 was identified as belonging to *Arctocephalus townsendii* (though the *cytochrome b* tree additionally groups this with *A. philipppii*). A number of samples were identified as belonging to *E. jubatus,* including NF13, NF28, NF29 (D-loop data only), NF31, and NF36. Sample NF19 generated interesting results, as the *cytochrome b* data placed it as an unknown species, basal to both *Arctocephalus* and *Eumetopias jubatus* (Figure C.3, Table 4.1). The D-loop analysis of this sample instead grouped it clearly within *A. philippii*. These clearly non-northern fur seal samples were then excluded from further NFS population analysis. The D-loop species phylogeny presents a different topology than that found in Moss *et al.* (2006), despite using the same raw sequence data supplemented with GenBank sequences. The only difference between Figures C.6a-c is the particular sequence used as an outgroup, while the only difference between Figures C.7a-c is the model of substitution, yet all of these generated trees are different from one another. For a strong phylogeny, logically it would not be expected that the outgroup chosen would affect internal tree topology. While the use of different substitution models and tree-building methods may be expected to show some differences, the variety of different phylogenies generated reveals just how sensitive this data set is to the variables that go into tree building, indicating that the resulting phylogenies are quite tentative. This is further supported by low bootstrap values on many of the nodes. Additionally, it is expected that the D-loop data should support *the cytochrome b* data (Lau *et al.* 1998); however, this is not the case. The results of this study found no clear separation of sea lion and fur seal clades. The monophyletic sea lion clade identified by Moss *et al.* (2006) is not present in these phylogenies. Instead, the sea lion and fur seal clades appear polyphletic. This may be the result of poor support for a number of the branching nodes, which could be caused or contributed to by the homoplasy in the data set. Rapid population expansion/mutation rates may have contributed to this pattern.

Appendix D.

Sample Calculations

Artificial Dataset

500bp sequences, 5 individuals, 4 haplotypes

Position:	1	2	3	4	5	6…500
Haplotype 1 (N=2)	T	G	T	C	T	A…A
Haplotype 2 (N=1)	T	A	T	T	A	A…A
Haplotype 3 (N=1)	C	G	T	C	T	A…A
Haplotype 4 (N=1)	C	G	G	C	T	A…A

Proportion of polymorphic sites:

using equation:

$$\hat{P}_n = \frac{n_p}{n_t} = \frac{\#\,polymorphic}{\#\,total}$$

Polymorphic sites are #1, 2, 3, 4, 5; therefore 5 sites in total are polymorphic

$$\hat{P}_n = \frac{n_p}{n_t} = \frac{\#\,polymorphic}{\#\,total} = \frac{5}{500} = 0.01$$

Proportion of polymorphic sites= 0.01

Number of segregating sites

Measures the total diversity (under the infinite sites model) using the equation:

$$\theta = \frac{S}{\sum_{i=1}^{n-1} \frac{1}{i}}$$

Θ= measure of nucleotide polymorphism
S= proportion of segregating (polymorphic sites)
N= sample size

Numerator: S=0.01 (from previous calculation)

Denominator: N= 5 samples, therefore denominator is

$\sum_{i=1}^{5-1} \frac{1}{i} = 1 + \frac{1}{2} + \frac{1}{3} + \frac{1}{4}$ =2.083333333

$$\theta = \frac{0.01}{2.083333} = 0.0048$$

Nucleotide Diversity

Calculated using:

$$\hat{\pi} = \frac{n}{n-1} \sum_{i,j} x_i x_j \pi_{ij}$$

This is a measure of the average diversity, where:

π = nucleotide diversity= average of nucleotide differences per site between randomly chosen sequences
x_i= frequency of the *i*th type of sequence
π_{ij}= proportion of nucleotide differences between $_i$th and *j*th type of sequences

For the sample data set provided above, the number of pairwise differences can be identified between pairs of haplotypes:

# of pairwise differences between haplotypes:
(1,1) → 0 (same sequence)
(1,2) → 3 (positions 2, 4, 5)
(1,3) → 1 (position 1)
(1,4) → 2 (positions 1, 3)
(2,1) → 3 (see above)
(2,2) → 0 (same sequence)
(2,3) → 4 (positions 1, 2, 4, 5)
(2,4) → 5 (positions 1, 2, 3, 4, 5)
(3,1) → 1 see above
(3,2) → 4 see above
(3,3) → 0 same sequence
(3,4) → 1 (position 3)
(4,1) → 2 (see above)
(4,2) →5 (see above)
(4,3) →1 (see above)
(4,4) → 0 (same sequence)

Using the more conventional terms for allele frequencies (*p* and *q*) the equation can be rewritten as follows:

$$\hat{\pi} = \frac{n}{n-1}\Sigma_{ij}x_i x_j \pi_{ij} = \frac{n}{n-1}\Sigma_{ij}p_i q_j \pi_{ij}$$

Haplo-type 1	*p* (haplotype 1 frequency)	Haplo-type 2	*q* (haplotype 2 frequency)	π_{ij} (Proportion of nucleotide differences= 3 of differences/500bases)	$x_i x_j \pi_{ij}=p_i q_j \pi_{ij}$
1	(2/5)=0.4	1	0.4	0 (same sequence)	0
	0.4	2	0.2	3/500=0.006	0.4*0.2*.006=0.00048
	0.4	3	0.2	1/500=0.002	0.4*0.2*0.002=0.00016
	0.4	4	0.2	2/500=0.004	0.4*0.2*0.004=0.00032
2	(1/5)=0.2	1	0.4	3/500=0.006	0.2*0.4*0.006=0.00048
	0.2	2	0.2	0 (same sequence)	0
	0.2	3	0.2	4/500=0.008	0.2*0.2*0.008=0.00032
	0.2	4	0.2	5/500=0.010	0.2*0.2*0.010=0.00040
3	0.2	1	0.4	1/500=0.002	0.2*0.4*0.002=0.00016
	0.2	2	0.2	4/500=0.008	0.2*0.2*0.008=0.00032
	0.2	3	0.2	0 (same sequence)	0
	0.2	4	0.2	1/500=0.002	0.2*0.2*0.002=0.00008
4	0.2	1	0.4	2/500=0.004	0.2*0.4*0.004=0.00032
	0.2	2	0.2	5/500=0.010	0.2*0.2*0.010=0.00040
	0.2	3	0.2	1/500=0.002	0.2*0.2*0.002=0.00008
	0.2	4	0.2	0 (same sequence)	0
				$\Sigma\, p_i q_j \pi_{ij}$	0.00352

Going back to the original equation we have:

$$\hat{\pi} = \frac{n}{n-1}\Sigma_{ij}x_i x_j \pi_{ij}$$

$$\hat{\pi} = \frac{5}{5-1}(0.00352)$$

$$\hat{\pi} = 0.0044$$

Haplotype diversity

Is based on the equation (Nei and Tajima 1981)

$$H = \frac{N}{N-1}(1 - \Sigma_i x_i^2)$$

For this sample set, there are 5 sequences so N-5

$\Sigma_i x_i^2$ = (frequency of haplotype 1)^2+ (frequency of haplotype 2)^2+ (frequency of haplotype 3)^2+ (frequency of haplotype 4)^2

Therefore:

$$\Sigma\, x_i^2 = (2/5)^2+(1/5)^2+(1/5)^2+(1/5)^2$$
$$\Sigma\, x_i^2 = 0.28$$

Therefore:

$$H = \frac{5}{5-1}(1 - 0.28)$$

H= (1.25)(0.72) =0.9

Test of DnaSP

When the simulated data is run through DnaSP:

Output file: (DNA polymorphism)

Input Data File: F:\...\TEST Alignment Final.meg
Number of sequences: 5 Number of sequences used: 5
Selected region: 1-500 Number of sites: 500
Total number of sites (excluding sites with gaps/missing data): 500

Number of polymorphic (segregating) sites, S: 5
Total number of mutations, Eta: 5

Number of Haplotypes, h: 4
Haplotype (gene) diversity, Hd: 0.900
Variance of Haplotype diversity: 0.02592
Standard Deviation of Haplotype diversity: 0.161

Nucleotide diversity, Pi: 0.00440
T**heta (per site) from Eta: 0.00480**

Theta (per site) from S, Theta-W: 0.00480
  Variance of theta (no recombination): 0.0000092
    Standard deviation of theta (no recombination): 0.00303
  Variance of theta (free recombination): 0.0000046
    Standard deviation of theta (free recombination): 0.00215

Finite Sites Model
  Theta (per site) from Pi: 0.00443
  Theta (per site) from S: 0.00484
  Theta (per site) from Eta: 0.00483

Average number of nucleotide differences, k: 2.200
  Stochastic variance of k (no recombination), Vst(k): 1.341
  Sampling variance of k (no recombination), Vs(k): 0.762
    Total variance of k (no recombination), V(k): 2.103
  Stochastic variance of k (free recombination), Vst(k): 0.733
  Sampling variance of k (free recombination), Vs(k): 0.367
    Total variance of k (free recombination), V(k): 1.100

Theta (per sequence) from S, Theta-W: 2.400
  Variance of theta (no recombination): 2.290
  Variance of theta (free recombination): 1.152

Test shows values from DnaSP match those of manual calculations.

4 haplotypes, 5 sequences
Haplotype diversity (Hd)=0.9
Nucleotide diversity π =0.00440
Theta Θ= 0.0048

The analysis by DnaSP also shows a number of other variables, including other Theta values.

This analysis chose the “DNA polymorphism” setting (and there are also other variables available to chose from, such as Pi (JC-non-coding) - which was 0.00442 and did not match the manual calculations. Additionally, there are other settings available for use, such as the ‘Polymorphism and Divergence’ function)

This test, therefore, revealed which settings and which values were the desired ones that matched the manual calculations above.