HISTORICAL POPULATION GENETICS OF *CALLORHINUS URSINUS* (NORTHERN FUR SEALS) FROM THE ALEUTIAN ISLANDS

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ABSTRACT

Historically, the Northern Fur Seal (*Callorhinus ursinus*) has been a staple food during human harvesting. While breeding localities exist primarily in Alaska today, archaeological rookeries once were found along the entire Eastern Pacific. Given this information, past genetic variation in *C. ursinus* may have been considerably higher than today. In order to place modern population genetic variation in a proper historic context, I obtained ancient DNA from the mitochondrial control region (157 base pairs) of 56 *C. ursinus* sampled from three geographically isolated Aleutian Islands (Kodiak, Unalaska and Shemya). Comparison of these data to unpublished data from extant individuals indicated high haplotype diversity (h=0.962–0.988), high nucleotide diversity (\( \pi = 0.034–0.051 \)) and low genetic structure (\( F_{st}=0.035 \)) in *C. ursinus* before and after the height of commercial sealing ~120 years ago. Lack of phylogeographic structure was observed in a combined data set comprised of modern and ancient individuals, further indicating that change in genetic diversity or structure has not occurred in the last 3000 years. Demographic analysis further indicated past growth in all populations with no evidence of a bottleneck, but populations did vary in timing and extent of expansion. Taken together, these results show that *C. ursinus* has not lost genetic variation or experienced increased population structuring due to the historical commercial sealing.
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The Department of Biology and Marine Biology, the Graduate School of UNCW and the National Science Foundation provided financial support for my research and studies. Dr. Seth Newsome at Carnegie Institution of Washington provided radiometrically dated samples for this project. People in Elizabeth Hadly’s laboratory at Stanford University taught me statistic skills and introduced fresh ideas, specifically Cheng Li, Malin Pinsky, and Dr. Elizabeth Hadly.

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CHAPTER 1

PAST GENETIC CHANGES IN *CALLORHINUS URSINUS* USING ANCIENT DNA

INTRODUCTION

Overview

*Callorhinus ursinus* (Northern Fur Seal) is a widespread pinniped species found in the North Pacific Ocean. It is considered a “vulnerable” species under the U.S. Endangered Species Act, with current populations of vital concern (Loughlin et al. 1994; NMML 2007). *C. ursinus* belongs to the family Otariidae, and the class Pinnipedia (Rice 1998). It is the only species in the genus *Callorhinus*, having diverged from the sister clades of the true fur seals (Arctocephalinae) and sea lions (Otariinae) at least 6 million years ago (Wynen et al. 2001). Being a high trophic level predator, it plays an important role in marine ecosystems. *C. ursinus* feeds opportunistically, primarily on a variety of fish, cephalopods and crustaceans (COSEWIC 2006). Their predators are Killer Whales, some sharks, foxes, and Steller Sea Lions (Gentry 1998).

*C. ursinus* has very thick fur that consists of with two layers--long guard hairs and a dense waterproof underfur. Furthermore, this species has a good sense of hearing, keen eyesight but lacks color vision. Males are much bigger than females (200-275 kg and 40-50 kg, respectively). They can live for more than 20 years (Gentry 1998; COSEWIC 2006). Growth rates of males and females are fairly similar until the fifth year of life, when males develop more rapidly (York 1987).
Northern Fur Seals have a pelagic distribution that ranges across the North Pacific including the east coast of Russia, Japan and the west coast of Canada and the United States (Ream et al. 2005). The Pribilof Islands in the east Bering Sea are home to the majority of the world’s breeding population today (Towell and Ream 2006). Additional small rookeries exist on the Commander Islands in the west Bering Sea, Kuril Islands in the north of Japan, Robben Island in the Sea of Okhotsk, and a few much smaller rookeries on Bogoslof Island in the Aleutian Island chain, and San Miguel Island off the coast of Southern California (Figure 1; Gentry 1998; Ream and Burkanov 2005). *Callorhinus ursinus* is a colonial breeder and exhibits strong site fidelity. Outside the breeding season, individuals spend nearly all their time in the open ocean to avoid mainland predators. Certain offshore islands have been used for pupping and breeding, where seals historically have been available to human exploitation (Gentry 1998; Ream and Burkanov 2005).

Based on the abundance of females and unweaned pups in archaeological sites (from ~5000 years ago to ~200 years ago), Newsome et al. (2007) confirmed that *C. ursinus* was one of the most common and broadly distributed pinniped species along the east Pacific Coast, stretching from the Pribilof Islands to southern California. Rookeries distributed continuously along the eastern Pacific coastline (eastern Aleutian Islands to California) indicated that *C. ursinus* once was more widespread than today (Figure 2).

The breeding and nursing behaviors of prehistoric and modern populations of *C. ursinus* are also known to be different. Modern *C. ursinus* on the Pribilof rookeries
aggregate in May. Adult males remain on the rookeries throughout the breeding season (Gentry 1998). Adult females give birth in late June. This pattern follows the “sub-polar” maternal strategy, where females wean their pups and move south with them (Gentry 1998). Being faithful to their natal site, most females will return to the Pribilof Islands the following March (Gentry 1998). Ream et al. (2005) monitored physical locations of 13 female *C. ursinus* from the Pribilofs during 2002-2003, and recorded their migration routes southward and back. Pups may remain at sea for 22 months before returning to their rookery of birth (COSEWIC 2006). Modern *C. ursinus* is weaned at about 4 months of age. This is known as a “short term” strategy, while almost all other otariids nurse their young for one to two years (Gentry and Kooyman 1986; Perrin et al. 2002; Newsome et al. 2007). However, a similar “long term” maternal strategy is thought to have been used prehistorically by Alaskan *C. ursinus* based on 5- to 12-month-old age profiles and isotope data (Newsome et al. 2007). The $\delta^{15}N$ isotope values that indicate nursing persisted longer (until 9-12 months) in Alaskan specimens than in extant specimens from these localities (Newsome et al. 2007).
Figure 1. The recent distribution of Northern Fur Seals. Black arrows indicate breeding localities. Yellow arrow and dotted line indicate Aleutian Islands. Map modified from http://commons.wikimedia.org/wiki/File:Bering_Sea_Location.gif#file, a composite of two maps on Wikimedia Commons: [http://commons.wikimedia.org/wiki/Image:Topographic90deg_N0E90.png and :Topographic90deg_N0W]

Figure 2. Map (courtesy of Seth Newsome, Carnegie Institution of Washington) showing three archaeological sites where C. ursimus specimens were collected (Shemya Island, Unalaska Island and Kodiak Island) and one modern site (Bogoslof Island) locates in the Aleutian Chain. Pribilof Islands are located north of Aleutian Islands. Other archaeological sites along the Pacific Northwest (Ozette, Hesquiat, Toquaht, Ts’ishaa, Seal Rock, Umpqua), and California (San Miguel, Point Mugu, Ano Nuevo, Moss Landing, Duncans Point) are also shown for completeness.
As demographic data reported, populations from the Pribilof Islands have experienced dramatic fluctuations in size (Loughlin et al. 1994; Gentry 1998; Balsiger et al. 2005; Newsome et al. 2007). The Aleut people who colonized Alaska about 8,000 years ago harvested *C. ursinus* as a staple food for millennia, but abundance did not significantly decline (Balsiger et al. 2005). Subsequent human exploitation led to three significant declines in *C. ursinus* numbers: the first one was from the late 1700s to the early 1800s; the second one was from the late 1800s to the early 1900s, and the third included the experimental harvest of females in 1956 (Ream 2002; Towell and Ream 2006). The fur trade started approximately about 220 years ago, and approached its height in the late 19th century, when the populations in Alaska declined dramatically with the arrival of Russian and European fur traders (Balsiger et al. 2005). Census size reached a low of 216,000 animals in 1912, close to a 90% population decline of the pre-exploitation size (NMML 2007; Macklin et al. 2008). As a result, the North Pacific Fur Seal Convention was signed in 1911 to limit hunting. Following this protection, the Alaska *C. ursinus* population grew again to 2 million individuals by 1950 (Macklin et al. 2008). Between 1956 and 1970, the population experienced a significant decline again, which was attributed primarily to an experimental harvest of females (Towell and Ream 2006). The population then began to increase to an estimated maximum population size of 1.25 million in 1974. By 1983 the population had declined to about 877,000 animals (Macklin et al. 2008). Following this period of decline, *C. ursinus* abundance on the Pribilof Islands was relatively stable until the mid-1990s (Towell and Ream 2006).
However, a new decline in the Pribilofs began in 2000. The total abundance on the Pribilof Islands was 636,000 during 2002 to 2006 (DFO 2007). Figure 3 shows changes in population size in the past 100 years. Reasons for the current decline are unknown but may include a decrease in reproductive rates, disease, natural predation, interspecies competition, climate changes, illegal killing, or pollutants (Adams et al. 2007). The species currently is officially considered as “threatened” under the Marine Mammal Protection Act of 1988 (Loughlin et al. 1994), and under the Species at Risk Act (SARA) by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) (DFO 2007). Overall, pup production on the Pribilof Islands declined by 38% over the last 30 years (DFO 2007). In contrast, a small population at Bogoslof Island (in eastern Aleutian Islands) has grown more than 12% per year between 1997 and 2005 (NMML 2007; Ream & Burkanov 2005). The number at Bogoslof is about 4.5% of the global populations (Ream & Burkanov 2005).
Figure 3. Fluctuations in population size of *C. ursinus* in the past 100 years on the Pribilof Islands. Data are cited from DFO 2007; NMML 2007; Macklin et al. 2008.
To better understand the cause and effect of the recent decline in *C. ursinus* populations in the Pribilof and contiguous breeding sites, genetic response to external stressors should be studied. For example, a comparison of genetic diversity before and after a population contraction can evaluate whether the species experienced a genetic bottleneck. Perhaps the most famous mammal bottleneck and corresponding inbreeding effects are described in studies of the cheetah. O’ Brien et al. (1987) compared allozymes from *Acinonyx jubatus raineyi* (east African cheetah) and *Acinonyx jubatus jubatus* (south African cheetah), and found low genetic distance between subspecies, and very low levels of heterozygosity in both of them, therefore affirming the cheetah as one of the least genetically variable mammal species. Recent inbreeding that resulted from loss of genetic variation after a Pleistocene bottleneck has inflicted considerable damage on cheetah’s survival rates and health.

In the context of my thesis on Northern Fur Seals, inter-specific comparison among pinniped species in genetic response to known population bottlenecks is especially informative. Several genetic studies have shown a correlation between historic population size reduction and genetic bottlenecks. For example, Weber et al. (2000) analyzed sequences of the mitochondrial DNA control region from *Mirounga angustirostris* (Northern elephant seal) that lived before, during and after a bottleneck in 1892, and found a sharp reduction in genetic variation after this bottleneck, which confirmed a significant reduction in population size. Similarly, *Arctocephalus townsendi* (Guadalupe fur seal) from the coast of California had experienced a loss of genetic
variability correlating with a bottleneck during the late 18\textsuperscript{th} and early 19\textsuperscript{th} centuries (Weber et al. 2004). Wynen et al. (2000) studied postsealing mitochondrial DNA variation in \textit{Arctocephalus gazella} (Antarctic fur seal) and \textit{Arctocephalus tropicalis} (Subantarctic fur seal) and suggested that both species recovered in population size through the recolonization of islands after the bottleneck in the late 18\textsuperscript{th} century.

Little is known about variation among current \textit{C. ursinus} populations except that the degree of geographical isolation is higher today than that in the past (Newsome et al. 2007). Furthermore, paleo-ecological data indicate that there may have been distinct dietary differences along the Aleutian chain from west to east (Newsome et al. 2007). This information derives from a study using isotopic ratios of carbon and nitrogen to help indicate the geographic distribution of the prehistoric populations (Newsome et al. 2007). The isotopic ratio of consumers reflects the isotopic composition fixed at the base of food webs (Burton et al. 2001). $\delta^{13}$C values inform about foraging location since $\delta^{13}$C values are higher in nearshore than open waters (Burton and Koch 1999). Based on $\delta^{13}$C values, prehistoric \textit{C. ursinus} foraged offshore across their entire range (Newsome et al. 2007). Nitrogen isotopes vary with both trophic level and latitude (Burton et al. 2001; Newsome et al. 2007). $\delta^{15}$N isotope values were higher in Southern \textit{C. ursinus} remains than in remains from Northern populations. Both $\delta^{13}$C and $\delta^{15}$N isotope values declined steeply from east to west along the Aleutian Islands, indicating that Aleutian populations on the west (Shemya Island) were ecologically distinct from eastern Aleutian populations (Unalaska Island, Kodiak Island; Newsome et al. 2007).
Objectives

The objectives of the first chapter in my thesis are to (a) provide historical context to population genetic variation in extant *C. ursinus*, (b) quantify genetic variation and structure in three Aleutian populations of differing ages, (c) present an intra-specific comparison of pre- and post-bottleneck genetic variation, and (d) present an inter-specific comparison of genetic response to population contraction in commonly harvested pinniped species. While modern genetic data can predict past genetic variation, ancient genetic data can test such predictions directly and determine lineage coalescences more exactly. Several studies on mammals show that ancient DNA tells more information than modern DNA by observing the past genetic parameters directly. Barnes et al. (2002) used 30 ancient specimens ranging from 45,000 to 14,000 years ago of *Ursus arctos* (Brown bear), and found no phylogeographical structure in the past compared with high structure in present. Consuegra et al. (2002) amplified DNA from 6 ancient samples of *Salmo salar* (Salmon) from 41,000 to 3,250 years ago, and suggested ancient salmon have a totally different haplotype. A higher genetic variation from past was confirmed by 9 ancient specimens of *Ovibos moschatus* (Musk ox) (MacPhee et al. 2005). 21 sequences of ancient *Ursus arctos* (Brown bear) collected from Spain confirmed no genetic structure of past populations (Valdiosera et al. 2007). Thus, I applied ancient DNA methodologies to a selectively neutral, highly variable genetic marker to document genetic variation, and geographic subdivision of populations in the Aleutian Islands of the past. According to other bottlenecked seals (Weber et al. 2000; Wynen 2001; Weber...
et al. 2004), *C. ursinus* likely experienced one or more population bottleneck(s). If true, genetic variation in populations predating these bottleneck events will exceed that of today. Secondly, Shemya seals differ in foraging strategy from other Aleutian populations (Newsome et al. 2008), hence I will test the null hypothesis of no interbreeding between Shemya seals and other populations. This project is part of a collaborative effort between UNCW, Stanford University, National Marine Mammal Laboratory (NMML), Simon Fraser University and Carnegie Institution for Science. My contribution in this effort was to investigate the Alaskan populations. Different teams targeted other ancient and modern populations: ancient Pacific Northwest by Simon Fraser University, ancient California by Stanford University, and modern populations by NMML.
METHODS AND ANALYSES

1. Sample selection

I examined sequence variation in the control region of the mitochondrial genome from 82 seals sampled at 3 archaeological sites. Radiometrically dated samples of individuals were selected and identified by colleagues from the Carnegie Institution for Science, and taken into the Ancient DNA laboratory at the Center for Marine Science of UNCW. I obtained 38 samples from Rolling Bay (Kodiak Island, ~1,000-500 years BP), 28 samples from Amaknak Bridge (Unalaska, ~3,300-2,700 years BP), and 18 samples from Shemya (western Aleutians, ~2,000-1,000 years BP). I had access to unpublished modern data (samples collected from 1993 to 1998; n=365) along the east Pacific sites (Pinsky et al. unpublished; Dickerson et al. unpublished). These DNA sequences derived from St. George Island (n=92), St. Paul Island (n=91) in the Pribilof Islands, Bogoslof Island (n=96) in the Aleutians, and San Miguel Island (n=86) in California. I compared my ancient DNA results to modern data from the Bogoslof population because of geographic proximity.

2. Ancient DNA decontamination and extraction

The ancient DNA technique is modified from modern DNA protocols to be more stringent. The major difficulty in working with ancient or degraded DNA samples lies in proving the authenticity of ancient amplified DNA. The high risk of contamination from modern sources is due to the degraded nature and low copy number of ancient DNA. The
Polymerase Chain Reaction (PCR) is extremely sensitive and can easily pick up contaminant DNA from many sources (Pääbo 1989; Wayne et al. 1999; Rawlence et al. 2009).

I performed all the molecular work in an isolated clean laboratory at the Center for Marine Science of UNCW, where we have never done any genetic work on pinnipeds. This laboratory consists of an outside sample preparation room and an inside ultra-clean room. Both laboratories were bleached and UV-irradiated overnight every time before use, and rooms were limited to few workers that were not allowed to re-enter the ultra-clean room for a period of 24 hours after PCR set up. Before DNA extraction, all surfaces were cleaned again with alconox detergent and a bleach solution. Labeled bones were stored in the sterile aDNA preparation room. The bones were pulverized, and small bits (< 500mg) were digested in extraction buffer (0.5M EDTA, 0.5% SDS, pH=8.0) with proteinase K (100 ug/mL) at 55°C for 24 hours (Yang et al. 2003). I used a Qiaquick PCR cleanup Kit (Qiagen) to perform the final DNA extraction on a maximum of five individuals per experiment, which always included a negative control. The DNA extraction was performed in a stand-alone workstation with positive airflow preventing additional contamination between samples and possible spread to the entire clean room.

3. PCR amplification and quantification

Contamination controls and detection are very important in ancient DNA studies (Yang et al. 2003; Newsome et al. 2007). Both negative and positive controls were set up along with ancient DNA samples for PCR amplification (Yang et al. 2003). Positive
controls were used to indicate whether PCR conditions were set up correctly and negative controls including blank extracts showed amplification products if contamination occurred. Positive controls were also used to indicate the sensitivity of individual PCR amplification and the level of contamination if it occurred, because the brightness of the stained DNA displayed its concentration. No PCR products were ever used when amplified along with a suspected contaminant. PCR was run under low-annealing temperature with high-fidelity DNA polymerase. PCR amplification of target DNA was performed in 50-ul reactions. I used the forward primer CalloCR1 (5’-CTCCCCCTATGTACTTCGTGCA-3’) and the reverse primer CalloCR2 (5’-GTACACTTTTCACAAGGGTTGCTG-3’) to amplify a 157 bp region of the mitochondrial control region (200 bp including primers). The PCR recipe with final concentrations was 0.2 uL (5U/uL) FirePol DNA polymerase (Solis BioDyne), 5 uL (10×) Reaction buffer B, 10 uL (25mM) MgCl₂, 5 uL (10mM) dNTPs, 5uL (20uM) primer for each, 5 uL (13mg/mL) bovine serum albumin (BSA) (1.3 mg/mL), 3 uL of DNA template and 11.8 uL sterile water. I used the following PCR conditions: after denaturation at 94°C for 10 minutes, DNA was amplified by 45 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 45 °C for 45 seconds, and extension at 72°C for 1 minute. A final extension period of 10 minutes at 72°C was followed by cooling of the PCR product to 4°C. PCR products and visualized on 2% agarose gels stained with ethidium bromide. postPCR cleanup was performed with EXO-SAP, which was made up of 1.7 uL Exonuclease 13.4 uL Shrimp Alkaline Phosphatase and 3.4 uL sterile water for 50 uL
PCR product.

4. Sequencing and Analyses

DNA sequencing was outsourced to Macrogen, Inc. in Seoul, Korea. Sequences were cleaned up and aligned with Sequencher 4.8. All unique haplotypes (distinct DNA sequences that occur in only a single individual) were verified by re-extraction, re-PCR and re-sequencing.

I performed several analyses of population genetic variation and structure using the programs Arlequin (version 3.1) (Excoffier et al. 2006) and DnaSP (version 5.0) (Rozas et al 2003; Librado & Rozas 2009). These programs assume that all samples are from extant individuals (Excoffier et al. 2006). I determined S (number of segregating sites), η (number of mutations), k (average number of nucleotide differences), h (haplotype diversity), and π (nucleotide diversity, per site) (Watterson 1975; Nei 1987). I estimated Tajima’s D, Fu & Li’s D, Fu & Li’s F, and Fu’s Fs in modern and ancient populations, as a test of selective neutrality or possible population expansion or contraction (Tajima 1989, 1993; Fu & Li 1993; Fu 1997). Finally, I estimated statistical separation of haplotype groupings through Analysis of Molecular Variance (AMOVA) (Weber et al. 2000; Wynen 2001; Excoffier et al. 2006). I used BEAUti (version 1.4.8) and BEAST (version 1.4.8) to calculate the α value (reflecting amount of rate heterogeneity among nucleotide sites) under the HKY+Gamma model, running with 2 independent MCMC simulations of 10,000,000 iterations in BEAST (version 1.4.8) (Drummond et al. 2005). The posterior output of α (=0.116) was applied to AMOVA in Arlequin, with the application of the
Kimura–two parameter distance model.

A rooted, time-measured Bayesian intraspecific phylogenetic tree using Monte Carlo Markov Chain (MCMC) simulation was built to show haplotype relationships within *C. ursinus* from modern and ancient populations. BEAST (Bayesian Evolutionary Analysis by Sampling Trees) accounts for serial sampling through time instead of assuming that all tips are of same age (Drummond et al. 2003). BEAST uses a MCMC Bayesian approach to find the best set of parameters and searches for the tree weighted with the highest posterior probability, and at the same time producing a credible sample of trees (Drummond et al. 2005). For the modern sample set, I used MEGA (version 3.0) to build a neighbor-joining tree and randomly picked 68 sequences that almost entirely represented all haplotype groups from the available 365 modern sequences. These included sequences from populations from St. George Island, St. Paul Island, Bogoslof Island and San Miguel Island. The modern samples plus the amplified ancient DNA sequences were taken together to build a tree.

I used BEAST (v 1.4.8) to build the intraspecific phylogenetic tree (Drummond & Rambaut 2007). To calculate the clock rate appropriately considering the problem of time inequality above and below species level, I designed and compared different sets of parameters, and picked up the most suitable rate for intraspecific clock (see Chapter 2). The first step was to determine the clock rate. I used 8.2 mya as the divergence time between *C. ursinus* and sea lions. The sea lions partially included *Eumetopias jubatus* (Steller sea lion), *Zalophus californianus* (California sea lion) and *Zalophus*
*californianus japonicus* (Japanese sea lion) (Slade et al. 1994; Higdon et al. 2007; Pinsky et al. unpublished). The data set included the 126 individuals of *C. ursinus* as the majority and one individual from each outgroup species. The sequences of outgroups were obtained from GeneBank and aligned with the 157 bp control region of *C. ursinus*. A nexus file of all 129 samples was imported in BEAUti. Rate was estimated under the HKY+Gamma+Invariant (HKY+G+I) substitution model with strict clock. Priors were set up as coalescent constant size, with population size as a uniform distribution from 0 to 60 million, and tree model root height as a normal distribution using 8.2 mya as the mean, and 2.1 mya as the standard deviation. Samples from the posterior were drawn every 20,000 MCMC steps over a total of 20,000,000 steps, with the first 10% discarded as burn-in for they were less trustable. The second step was plotting Bayesian skyline, a method for estimating past population dynamics through time from molecular sequences without a prespecified parametric model of demographic history (Drummond et al. 2005). The posterior output of the first step (intraspecific clock rate with 2.13E-7 per site per mya as the mean, and 1.36E-8 per site per mya as the standard deviation) was applied to build the tree. The intraspecific tree did not count the outgroup species. Bayesian analyses were performed using HKY+G+I model and a Bayesian skyline coalescence prior. The output tree file was applied to tree building. The third step used programs of TreeAnnotator and Figtree to make a maximum clade credibility tree, with the first 10% of the set of trees discarded as burn-in.
RESULT

I obtained a total of 58 sequences from 3 ancient populations, with 21 sequences from Rolling Bay on Kodiak Island, 18 sequences from Shemya Island, and 19 from Amaknak Bridge on Unalaska Island. Fifteen of the 58 sequences were obtained from Stanford University conducted by Cheng Li. Seventy-two of the 82 samples were successfully extracted and amplified, as shown by gel visualization, but of these, 14 out of the 72 individuals did not produce clean sequencing signals likely due to degraded bones and impurity of samples. The haplotype diversity (h) is high in all populations, ranging from 0.962 to 0.988. Similarly, nucleotide diversity ($\pi$) (the average number of nucleotide differences per site between any two DNA sequences chosen randomly from the sample population) has an overall high level (range from 0.034 to 0.051) but is considerably smaller in Unalaska ($\pi=0.034$) than other islands ($\pi=0.05$) (Table 1).
### a)

<table>
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<th>Population</th>
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</tr>
<tr>
<td>BOG modern</td>
<td>96</td>
<td>42</td>
<td>44</td>
<td>65</td>
</tr>
</tbody>
</table>

### b)

<table>
<thead>
<tr>
<th>Population</th>
<th>h ±SD</th>
<th>π ±SD</th>
<th>k</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kodiak</td>
<td>0.962±0.026</td>
<td>0.048±0.003</td>
<td>7.486</td>
</tr>
<tr>
<td>Unalaska</td>
<td>0.988±0.021</td>
<td>0.034±0.004</td>
<td>5.328</td>
</tr>
<tr>
<td>Shemya</td>
<td>0.980±0.024</td>
<td>0.051±0.004</td>
<td>7.941</td>
</tr>
<tr>
<td>Total ancient</td>
<td>0.986±0.006</td>
<td>0.045±0.002</td>
<td>6.967</td>
</tr>
<tr>
<td>BOG modern</td>
<td>0.988±0.004</td>
<td>0.048±0.002</td>
<td>7.595</td>
</tr>
</tbody>
</table>

### c)

<table>
<thead>
<tr>
<th>Population</th>
<th>Tajima’s D</th>
<th>Fu &amp; Li’s D</th>
<th>Fu &amp; Li’s F</th>
<th>Fu’s Fs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kodiak</td>
<td>0.138</td>
<td>0.127</td>
<td>0.152</td>
<td>-3.530</td>
</tr>
<tr>
<td>Unalaska</td>
<td>-0.596</td>
<td>-0.566</td>
<td>-0.667</td>
<td>-10.900 *</td>
</tr>
<tr>
<td>Shemya</td>
<td>0.046</td>
<td>-0.135</td>
<td>-0.095</td>
<td>-5.080 *</td>
</tr>
<tr>
<td>Total ancient</td>
<td>-0.345</td>
<td>-0.750</td>
<td>-0.717</td>
<td>-25.000 *</td>
</tr>
<tr>
<td>BOG modern</td>
<td>-0.359</td>
<td>0.648</td>
<td>0.294</td>
<td>-24.800 *</td>
</tr>
</tbody>
</table>

Table 1 Comparison of measures of genetic diversity and population changes in Alaskan *C. ursinus* populations of different age.

a), b) measures of genetic diversity; c) measures of population changes; n: number of individuals in each population; S: number of segregating sites; η: number of mutations; No. of hap: number of haplotypes; h: haplotype diversity; π: nucleotide diversity; k: average number of nucleotide differences; *P<0.02.*
Values of Tajima’s D, Fu & Li’s D, and Fu & Li’s F do not show significant deviation from the null (stable size) model (Table 1c; \( P > 0.10 \)). The direction of Tajima's D, Fu & Li’s D, and Fu & Li’s F is potentially informative about the evolutionary and demographic forces that a population has experienced (Tajima 1989, 1993; Fu & Li 1993; Fu 1997; Excoffier et al. 2006). For example, Tajima's test compares the total number of segregating sites of a set of sequences from a population versus the average number of mutations between pairs of sequences sampled from the same population. If these two numbers are the same, then the null hypothesis of neutrality cannot be rejected. If the average number of polymorphisms found in pairwise comparison minus the total number of polymorphic sites gives a negative value, it reflects an excess of rare polymorphisms in a population, which is consistent with either purifying selection or a recent increase in population size. Positive values indicate an excess of intermediate-frequency alleles and can result from a population decline (Tajima 1989, 1993). The tests are to identify whether sequences fit the neutral theory model with a null hypothesis of selective neutrality and population size constancy (Tajima 1989, 1993; Fu & Li 1993; Fu 1997; Excoffier et al. 2006). Here the values of Tajima’s D, Fu & Li’s D, and Fu & Li’s F do not indicate a signal of population expansion or contraction in any population.

Fu’s Fs statistic is very sensitive to population demographic expansion or contraction, which generally leads to large negative (or positive) Fs values (Fu 1997; Excoffier et al. 2006). Similar to Tajima’s D, the null hypothesis of Fu’s Fs test is
selective neutrality and constant population size. Fu’s Fs indeed appears more sensitive
to departure from population equilibrium than Tajima’s D (Table 1c). The Fs values of
populations from Unalaska, Shemya, “Total ancient” and Bogoslof all show significant
signs of population expansion ($P<0.02$; Fu 1997).

Analysis of Molecular Variance (AMOVA) is a method that evaluates the amount of
genetic structure in a data set, often indicative of multiple independent populations.
AMOVA results show that 96.54% of the variation in the total population is found within
populations, with only a 3.46% of the variation among populations (Table 2a). The
overall $F_{st}$ value of 0.0346 ($P=0.039±0.006$) indicates low structure and high gene flow
among populations. The population-specific $F_{st}$ indices that are low in the three
populations further denote lack of structure within single populations (Table 2b). The $F_{st}$
values are 0.029 (Kodiak), 0.028 (Shemya) and 0.048 (Unalaska). Population pairwise $F_{st}$
values range from 0.024 to 0.045, which are not significantly different from a total lack
of structure, while the P-values are not significant enough ($P> 0.05$ for all the
comparisons; Table 2c).

The Bayesian phylogenetic tree of *C. ursinus* control region sequences is shown in
Figure 4. The evolutionary relationships between individual haplotypes do not indicate
presence of significant structure between ancient and modern populations and highlight
the high level of genetic diversity before and after the fur trade, even in a single modern
population. Consistent with demographic expansion, the majority of nodes in the tree are
supported by low bootstrap values.
Table 2 Results from Analysis of Molecular Variance (AMOVA) on three ancient populations. a) AMOVA output and overall $F_{st}$; b) intra-population $F_{st}$ indices; c) inter-population pairwise $F_{st}$: $F_{st}$ values are below the diagonal, $P$-values are above.

Figure 4 (see next page). Intraspecific Bayesian phylogeny of 58 ancient and 68 modern samples. High bootstrap values (>0.50) are specified with error bars of 95% confident intervals. Time line shows age of each lineage. Individual samples are represented as ages, ybp stands for years before present. 0 (ybp) modern, 750 (ybp) Kodiak, 1500 (ybp) Shemya, 3000 (ybp) Unalaska.
DISCUSSION

Both the ancient and modern populations show high levels of haplotype diversity, high nucleotide diversity and low genetic structure. The mtDNA analyses bring out considerable genetic variation in *C. ursinus*, before and after historical commercial hunting. None of these analyses indicate a signal of a genetic bottleneck. The high degree of phylogenetic clustering of modern and ancient haplotypes together also indicates that little genetic variation has been lost. In addition, most of the variation was partitioned within rather than among populations, which demonstrates high gene flow among the Aleutian Islands. Population from Unalaska, rather than that from Shemya, appears somewhat genetically different from others. The demographic differences among the three populations are discussed in more detail in chapter 2. The genetic homogeneity among the three Aleutian Islands provides no clue of the foraging differences from the east to west Aleutian chain (as shown by paleoisotopic data).

**Bottleneck/Genetic variety**

A population bottleneck can sometimes lead to a genetic homogeneity and inbreeding, and gives rise to a high chance of extinction. Molecular markers are commonly applied to detect bottleneck-induced genetic change. High haplotype diversity in *C. ursinus* is not unusual compared to other historically abundant pinniped species, and is consistent with two unpublished data sets. Ream’s Ph.D. thesis (2002) documented extensive genetic variation within modern *C. ursinus* using 8 microsatellite loci. *C.
*ursinus* also showed high haplotypic diversity (control region) in both prehistoric (200-1500 ybp) and modern populations across the eastern Pacific range (Pinsky et al. unpublished).

Several studies estimated genetic diversity of pinnipeds after sealing in late 19\textsuperscript{th} century. Most of the studies utilized the mitochondrial DNA control region or nuclear DNA microsatellites because both molecular markers have high mutation rates, resulting in high resolution in population genetics studies. Fewer studies used other markers such as the slower evolving mitochondrial coding gene cytochrome b, the non-neutral major histocompatibility complex (MHC) genomic region or allozymic variation. Other than *C. ursinus*, in the family Otariidae, *Eumetopias jubatus* (Steller sea lion) (Bickham et al. 1996; Bickham et al. 1998; Trujilo et al. 2004; Harlin-Cognato et al. 2005 and Baker et al. 2005), *Arctocephalus gazella* (Antarctic fur seal) (Wynen et al. 2000), *Arctocephalus tropicalis* (Subantarctic fur seal) (Wynen et al. 2000), *Arctocephalus forsteri* (New Zealand Fur Seal) (Lento et al. 1997), *Arctocephalus pusillus pusillus* (Cape fur seal) (Matthee et al. 2006), *Otaria flavescens* (South American sea lion) (Freilich et al. unpublished), and *Arctocephalus philippii* (Juan Fernandez fur seal) (Goldsworthy et al. 2000) showed high levels of modern genetic diversity. The family Phocidae that includes *Halichoerus grypus* (Gray seal) (Graves et al. 2009), *Mirounga leonina* (Southern elephant seal) (Hoelzel et al. 1993; Slade 1997 & 1998), *Phoca vitulina* (Harbor seal) (Goodman 1998; Westlake and Ocorry 2002), *Phoca largha* (Spotted seal) (Mizuno et al. 2003) showed extensive modern genetic variation too. Similar results have also been
documented in other marine mammals, including *Balaena mysticetus* (Bowhead whale), a species that shows high post-bottleneck genetic variability (control region) with haplotype diversity of 0.949 (Borge et al. 2007). Table 3 summarizes diversity measures reported from some studies with high genetic variation in pinnipeds.
<table>
<thead>
<tr>
<th>Species</th>
<th>Marker</th>
<th>N</th>
<th>No. (h)</th>
<th>H</th>
<th>π</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arctocephalus forsteri</td>
<td>cytochrome b</td>
<td>56</td>
<td>NA</td>
<td>0.6812; Ht=0.7909(high)</td>
<td>NA</td>
<td>Lento et al. 1997&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Arctocephalus gazella</td>
<td>control region</td>
<td>145</td>
<td>26</td>
<td>NA</td>
<td>0.032</td>
<td>Wynen et al. 2000</td>
</tr>
<tr>
<td>Arctocephalus philippii</td>
<td>control region</td>
<td>28</td>
<td>13</td>
<td>0.905</td>
<td>0.030</td>
<td>Goldsworthy et al.2000</td>
</tr>
<tr>
<td>Arctocephalus pusillus</td>
<td>control region (prebottleneck)</td>
<td>106</td>
<td>57</td>
<td>0.975±0.006</td>
<td>0.011±0.006</td>
<td>Matthee et al. 2006</td>
</tr>
<tr>
<td>Arctocephalus townsendi</td>
<td>control region</td>
<td>26</td>
<td>25</td>
<td>0.997</td>
<td>0.055</td>
<td>Weber et al. 2004</td>
</tr>
<tr>
<td>Callorhinus ursinus</td>
<td>control region (750-3000ya)</td>
<td>58</td>
<td>42</td>
<td>0.986</td>
<td>0.045</td>
<td>This study</td>
</tr>
<tr>
<td>Callorhinus ursinus</td>
<td>control region (200-1500ya)</td>
<td>43</td>
<td>39</td>
<td>0.997</td>
<td>0.050</td>
<td>Pinsky et al. unpublished</td>
</tr>
<tr>
<td>Callorhinus ursinus</td>
<td>control region</td>
<td>365</td>
<td>186</td>
<td>0.987</td>
<td>0.046</td>
<td>Pinsky et al. unpublished</td>
</tr>
<tr>
<td>Callorhinus ursinus</td>
<td>Microsatellites</td>
<td>578</td>
<td>NA</td>
<td>~1.000</td>
<td>NA</td>
<td>Ream 2002 PhD thesis</td>
</tr>
<tr>
<td>Eumetopias jubatus</td>
<td>control region</td>
<td>1568</td>
<td>121</td>
<td>0.916±0.004</td>
<td>0.010±0.006</td>
<td>Baker et al. 2005</td>
</tr>
<tr>
<td>Eumetopias jubatus</td>
<td>control region</td>
<td>194</td>
<td>55</td>
<td>0.690 ~ 1.000</td>
<td>0.004 ~ 0.016</td>
<td>Trujilo et al. 2004</td>
</tr>
<tr>
<td>Eumetopias jubatus</td>
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<td>336</td>
<td>107</td>
<td>0.890 ±0.010</td>
<td>NA</td>
<td>Harlin-Cognato et al. 2005</td>
</tr>
<tr>
<td>Eumetopias jubatus</td>
<td>control region</td>
<td>224</td>
<td>52</td>
<td>0.927</td>
<td>NA</td>
<td>Bickham et al. 1996</td>
</tr>
<tr>
<td>Eumetopias jubatus</td>
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<td>36</td>
<td>13</td>
<td>0.850</td>
<td>NA</td>
<td>Bickham et al. 1998</td>
</tr>
<tr>
<td>Halichoerus grypus</td>
<td>Microsatellites, mtDNA</td>
<td>131&lt;sup&gt;1&lt;/sup&gt;, 114&lt;sup&gt;3&lt;/sup&gt;</td>
<td>63&lt;sup&gt;2&lt;/sup&gt;,&lt;sup&gt;5&lt;/sup&gt; 46&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.943 ~ 0.965&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.016±0.008&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Graves et al. 2009</td>
</tr>
<tr>
<td>Mirounga leonina</td>
<td>control region</td>
<td>48</td>
<td>26</td>
<td>0.944, 0.662</td>
<td>NA</td>
<td>Hoelzel et al. 1993</td>
</tr>
<tr>
<td>Mirounga leonina</td>
<td>mtDNA, nDNA</td>
<td>60</td>
<td>30&lt;sup&gt;4&lt;/sup&gt;</td>
<td>NA</td>
<td>0.029&lt;sup&gt;3&lt;/sup&gt;, 9*10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>Slade 1998</td>
</tr>
<tr>
<td>Otaria flavescens</td>
<td>control region</td>
<td>39</td>
<td>18</td>
<td>0.880</td>
<td>0.008 ~ 0.012</td>
<td>Freilich et al. unpublished</td>
</tr>
<tr>
<td>Phoca largha</td>
<td>control region</td>
<td>66</td>
<td>57</td>
<td>NA</td>
<td>NA</td>
<td>Mizuno et al. 2003</td>
</tr>
<tr>
<td>Phoca vitulina</td>
<td>Microsatellites</td>
<td>1029</td>
<td>68&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.6(±0.04) ~ 7.0 (±0.28)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>NA</td>
<td>Goodman 1998</td>
</tr>
<tr>
<td>Phoca vitulina</td>
<td>control region</td>
<td>778</td>
<td>225</td>
<td>0.975</td>
<td>0.015</td>
<td>Westlake and Ocorry 2002</td>
</tr>
</tbody>
</table>

Table 3. Studies showing high level of genetic variation in pinnipeds. N: number of animals, No. (h): Number of haplotypes, H: haplotype diversity, π: nucleotide diversity. 1 This rate is considered as high for survival of two divergent lineages found in interspecific comparisons of other species. 2. microsatellite; 3. control region; 4 nDNA; 5 number of alleles; 6 allelic diversity.
Despite many pinnipeds not showing any obvious reduction in genetic variation due to commercial sealing, several other species that were commercially hunted do indicate low genetic variability. Weber et al. (2004) used the mitochondrial control region to compare genetic diversity in *Arctocephalus townsendi* (Guadalupe fur seal) before and after a population bottleneck and documented a reduction in genetic variability. Lento et al. (2003) showed low diversity of a MHC Class II gene in *Phocarctos hookeri* (New Zealand sea lion). In family Phocidae, *Mirounga angustirostris* (Northern elephant seal) (Bonnell and Selander 1974; Hoelzel et al. 1993; Weber et al. 2000; Weber et al. 2004), *Monachus schauinslandi* (Hawaiian monk seal) and *Monachus monachus* (Mediterranean monk seal) exemplify well documented reductions in genetic diversity (Kretzmann et al. 1997; Aldridge et al. 2006; Harwood et al. 1996; Stanley and Harwood 1997; Pastor et al. 2004). Table 4 shows some of studies of pinnipeds with low genetic variation. It is important to note that low diversity in one marker need not imply loss of genetic variation, particularly if fast evolving markers have not been exploited yet. For example, in Otariidae, the two subspecies of Brown fur seal, *Arctocephalus pusillus doriferus* (Australian fur seal) and *Arctocephalus pusillus pusillus* (Cape fur seal) showed low diversity in the mitochondrial cytochrome b gene (Lento et al. 1997). While suggestive of a loss of genetic variation, another study (Table 3) using control region indicated high haplotype diversity after the recent sealing in *Arctocephalus pusillus pusillus* (Matthee et al. 2006). A similar trend exists in *Zalophus californianus* (California sea lion) with no cytochrome b diversity but high control region variation (Maldonado et al. 1994&1995).
<table>
<thead>
<tr>
<th>Species</th>
<th>Marker</th>
<th>N</th>
<th>No. (h)</th>
<th>H</th>
<th>Π</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arctocephalus. pusillus doriferus</em></td>
<td>cytochrome b</td>
<td>17</td>
<td>3</td>
<td>0.157</td>
<td>0.004</td>
<td>Lento et al. 1997</td>
</tr>
<tr>
<td><em>Arctocephalus. pusillus.pusillus</em></td>
<td>cytochrome b</td>
<td>25</td>
<td>8</td>
<td>0.728</td>
<td>0.006</td>
<td>Lento et al. 1997</td>
</tr>
<tr>
<td><em>Arctocephalus townsendi</em></td>
<td>control region</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(postbottleneck)</td>
<td>32</td>
<td>7</td>
<td>0.798</td>
<td>0.025</td>
<td>Weber et al. 2004</td>
</tr>
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<td>control region</td>
<td>149</td>
<td>2</td>
<td>0.410</td>
<td>0.007</td>
<td>Weber et al. 2000</td>
</tr>
<tr>
<td></td>
<td>control region(ca.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000-1800)</td>
<td>5</td>
<td>4</td>
<td>0.900</td>
<td>0.007</td>
<td>Weber et al. 2000</td>
</tr>
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<td>control region</td>
<td>8</td>
<td>2</td>
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<td>0.009</td>
<td>Weber et al. 2000</td>
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<td>2</td>
<td>NA</td>
<td>0.004</td>
<td>Hoelzel et al. 1993</td>
</tr>
<tr>
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<td>alozymes</td>
<td>67</td>
<td>NA</td>
<td>0*</td>
<td>NA</td>
<td>Hoelzel et al. 1993</td>
</tr>
<tr>
<td><em>Monachus monachus</em></td>
<td>microsatellites</td>
<td>52</td>
<td>3</td>
<td>NA</td>
<td>NA</td>
<td>Pastor et al. 2004</td>
</tr>
<tr>
<td></td>
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<td>18</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
<td>Harwood et al. 1996; Stanley</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Harwood. 1997</td>
</tr>
<tr>
<td><em>Monachus schauinslandi</em></td>
<td>control region</td>
<td>50</td>
<td>3</td>
<td>NA</td>
<td>0.007</td>
<td>Kretzmann et al. 1997</td>
</tr>
<tr>
<td><em>Phocarctos hookeri</em></td>
<td>SSCP³</td>
<td>39</td>
<td>2</td>
<td>NA</td>
<td>NA</td>
<td>Lento et al. 2003</td>
</tr>
<tr>
<td><em>Zalophus californianus</em></td>
<td>control region</td>
<td>40</td>
<td>11</td>
<td>NA</td>
<td>NA</td>
<td>Maldonado et al. 1995</td>
</tr>
<tr>
<td><em>Zalophus californianus</em></td>
<td>cytochrome b</td>
<td>40</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
<td>Maldonado et al. 1995</td>
</tr>
</tbody>
</table>

Table 4 Studies showing reduced genetic variation in pinnipeds.

N: number of animals, No. (h): Number of haplotypes, H: haplotype diversity, π: nucleotide diversity. * No detectable variation
Most studies listed above showed the same tendency of higher genetic diversity being maintained in populations with larger post-bottleneck abundance. Nearly all pinniped species were hunted during the sealing from the 17th to the 19th century. Species with high modern genetic diversity had survived from sealing, and thereafter maintained a comparatively large population size, ranging from hundreds of thousands to millions of individuals. For example, demographic data show the size of *C. ursinus* on the Pribilofs reached a low of 216,000 animals in 1912, which corresponds to a 90% population decline of the pre-exloitation size (NMML 2007; Macklin et al. 2008), but the species recovered to a stock size of 636,000 individuals during 2002 to 2006 (DFO 2007). On the contrary, species that proved to experience a significant bottleneck and failed to recover in abundance most often showed loss of genetic diversity. Thus, low levels of current population size corresponds with decreased genetic diversity: *Arctocephalus townsendi* (Guadalupe fur seal) had a recovered population of 10,000 by the late 1990s; *Phocarctos hookeri* (New Zealand sea lion) had a population of 15,000 in the mid 1990s; *Mirounga angustirostris* (Northern elephant seal) had a recovered size of 175,000 animals with a lower genetic diversity of today; *Monachus schauinslandi* (Hawaiian monk seal) and *Monachus monachus* (Mediterranean monk seal) are two species that are critically endangered with 1200 individuals and 350-450 individuals remaining respectively (Kretzmann et al. 1997; Pastor et al. 2004). Thus, it may be that despite major reduction in abundance during the fur trade, some species did not reach low enough numbers for genetic drift to act. Secondly, some species naturally vary in abundance, reflecting
variation in reproductive rates. Thus, species are expected to vary in overall abundance and population recovery rate.

A relationship between extent of geographic range and population size of pinnipeds is unclear, however. There is a trend that widely dispersed species are resilient to external stressors. Broadly dispersed pelagic species such as *Callorhinus ursinus*, *Eumetopias jubatus* (Steller sea lion) and *Phoca vitulina* (Harbor seal) have decent population size today (Goodman 1998; Westlake and Ocorry 2002; Trujilo et al. 2004). Almost all critically endangered or extinct pinnipeds are/were limited regionally and thus vulnerable: critically endangered *Monachus schauinslandi* (Hawaiian monk seal) and *Monachus monachus* (Mediterranean monk seal) are limited to Hawaiian waters and Mediterranean Sea/Eastern Atlantic waters respectively (Kretzmann et al. 1997; Pastor et al. 2004). Extinct *Zalophus japonicus* (Japanese sea lion) spread in the Sea of Japan (Sakahira and Niimi 2007), while *Monachus tropicalis* (Caribbean monk seal) lived in the Caribbean Sea and the Gulf of Mexico (Leboeuf et al. 1986). Though these correlations may be due to many causes other than geographic distribution, habitat management and species competition need careful consideration for conservation.

To evaluate genetic variability, it is also important to determine the strategy to choose appropriate molecular markers. Commonly used markers (e.g. mitochondrial DNA control region and nuclear DNA microsatellite) have high mutation rates that result in considerable resolution in genetic analyses. However, it is necessary to interpret data differently by taking into account the nature of every marker and specific species. For
example, the mtDNA control region is maternally inherited, so it is limited to the female component of a population to identify the level of genetic diversity. Suppose the sex ratio is balanced within *C. ursinus*, the maternal information can be indirectly applied to the total population. Important reasons for choosing the mitochondrial control region are the abundance of female fossils spread in archaeological sites, and ancient DNA techniques being more amenable to high copy number mitochondrial genes than to single copy nuclear markers. To modify this analyses, amplifying a longer length of sequence will improve resolution.

**Structure/natal fidelity/gene flow**

The low $F_{st}$ values among the three ancient populations of *C. ursinus* suggest a lack of genetic structure (in time and space) and high gene flow. Genetic structure is affected by habitat continuity, geographic distribution, population densities, level and direction of drift, migration etc. (Davis et al. 2008). To properly interpret the level of genetic structure found in *C. ursinus*, I compared previous genetic studies of pinnipeds that mentioned (lack of) global or regional genetic subdivision.

The low structure among populations of *C. ursinus* confirms two previous studies that used microsatellites and mtDNA control region data respectively (Ream 2002; Pinsky et al. unpublished). Neither of the two studies found significant structure in *C. ursinus* among breeding sites. Further studies might apply mitochondrial cytochrome b as a marker to test the level of structure. Similar to *C. ursinus*, some pinnipeds show high level of genetic diversity and low genetic structure. Mizono et al. (2003) found high level
of genetic diversity and low level of structure in *Phoca largha* (Spotted seal) using the mitochondrial control region. Matthee et al. (2006) used the same marker and suggested no geographic structure in *Arctocephalus pusillus pusillus* (Cape fur seal). The same pattern exists in *Cystophora cristata* (Hooded seal) (Coltman et al. 2007). *Pusa hispida* (Ringed seal), a species that occurs throughout the Arctic Ocean and can be found in the Bering Sea, had little genetic differentiation between main breeding areas although populations are clearly geographically subdivided (Palo et al. 2001; Palo et al. 2003). In a comprehensive study on six ice-breeding seals, *Pusa hispida* (Ringed seal), *Hydrurga leptonyx* (Leopard seal), *Ommatophoca rossii* (Ross seal) and *Lobodon carcinophagus* (Crabeater seal) exhibited low structure while two other ice seals (*Leptonychotes weddellii* Weddell seal and *Erignathus barbatus* Bearded seal) showed significant structure (Davis et al. 2008). Critically endangered *Monachus monachus* (Mediterranean monk seal) is a species with low genetic variability and no structure (Pastor et al. 2004). Table 5 lists some pinnipeds with low genetic structure.
<table>
<thead>
<tr>
<th>Species</th>
<th>Marker</th>
<th>N</th>
<th>$\Phi_{st}$</th>
<th>F$_{st}$</th>
<th>R$_{st}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arctocephalus pusillus. pusillus</em></td>
<td>control region</td>
<td>106</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
<td>Matthee et al. 2006</td>
</tr>
<tr>
<td><em>Callorhinus ursinus</em></td>
<td>microsatellites, control region</td>
<td>578</td>
<td>NA</td>
<td>0.0004 (P=0.273)</td>
<td>-0.004 (P=0.321)</td>
<td>Ream 2002 PhD thesis, Coltman et al. 2007</td>
</tr>
<tr>
<td><em>Cystophora cristata</em></td>
<td>microsatellites</td>
<td>123, 300</td>
<td>-0.001 (P=0.49)</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td><em>Hydrurga leptonyx</em></td>
<td>microsatellites</td>
<td>150</td>
<td>NA</td>
<td>0.001 (P=0.001)</td>
<td>NA</td>
<td>Davis et al. 2008</td>
</tr>
<tr>
<td><em>Lobodon carcinophagus</em></td>
<td>microsatellites</td>
<td>303</td>
<td>NA</td>
<td>0.003 (P=0.045)</td>
<td>NA</td>
<td>Davis et al. 2008</td>
</tr>
<tr>
<td><em>Ommatophoca rossii</em></td>
<td>microsatellites</td>
<td>90</td>
<td>NA</td>
<td>0.006 (P=0.221)</td>
<td>NA</td>
<td>Davis et al. 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.003-0.006</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phoca largha</em></td>
<td>control region</td>
<td>66</td>
<td>(P&gt;0.05)</td>
<td>NA</td>
<td>NA</td>
<td>Mizuno et al. 2003</td>
</tr>
<tr>
<td><em>Pusa hispida</em></td>
<td>microsatellites</td>
<td>149</td>
<td>NA</td>
<td>0.017</td>
<td>0.002</td>
<td>Palo et al. 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>78</td>
<td>NA</td>
<td>0.023 (P=0.007)</td>
<td>NA</td>
<td>Palo et al. 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>303</td>
<td>NA</td>
<td>0.005 (P=0)</td>
<td>NA</td>
<td>Davis et al. 2008</td>
</tr>
</tbody>
</table>

Table 5 Studies showing pinnipeds with non-significant genetic structure.

NS. Not significant
Comparatively more pinnipeds exhibited genetic structure than not. *Eumetopias jubatus* (Steller sea lion) shares a similar geographic range with *C. ursinus* (Ream 2002) but, compared to *C. ursinus*, it maintains high haplotype diversity after sealing and high genetic structure. It has been debated whether 2 or 3 populations (subspecies) of *Eumetopias jubatus* exist globally (Bickham et al. 1996; Baker et al. 2005), and a recent study suggested a phylogenetic break between eastern stock and Asian/western stock (Hoffman et al. 2006). *Phoca vitulina* (Harbor seal), as the most wide-ranging pinniped in the North Atlantic and Pacific Oceans, showed extensive subdivision among geographic populations by several studies (Stanley et al. 1993 & 1996; Kappe et al. 1997; Goodman 1998; Burg et al. 1999; Westlake and OCorry 2002). Wynen et al. (2000) inferred the population genetic history of *Arctocephalus gazella* (Antarctic fur seal) and *Arctocephalus tropicalis* (Subantarctic fur seal), and found extensive genetic structure in *Arctocephalus tropicalis* but less structure in *Arctocephalus gazella*. Other species that showed population structure include: *Arctocephalus forsteri* (New Zealand fur seal) (Lento et al. 1997), *Otaria flavescens* (South American sea lion), *Arctocephalus australis* (South American fur seal) (Tunez et al. 2006), *Halichoerus grypus* (Gray seal) (Allen et al. 1995; Boskovic et al. 1996; Graves et al. 2009), *Mirounga leonina* (Southern elephant seal)(Gales et al. 1989; Hoelzel et al. 1993), *Leptonychotes weddellii* (Weddell Seal), *Erignathus barbatus* (Bearded Seal) (Davis et al. 2008), and *Odobenus rosmarus* (Walrus) (Goodman 1998; Born et al. 2001). In other studies, the existence of genetic structure was dependent on which marker was chosen. For example, *Arctocephalus pusillus pusillus*
(Cape fur seal) showed no structure based on the control region (Matthee et al. 2006), but did so with cytochrome b (Lento et al. 1997). Graves et al. (2009) suggested geographic separation based on microsatellites, but no structure using the control region in *Halichoerus grypus* (Gray seal). *Monachus schauinslandi* (Hawaiian monk seal) presented structure by using multilocus fingerprinting but no structure in the control region (Kretzmann et al. 1997). Table 6 lists some of pinnipeds with high genetic structure.

The majority of phocids have a reproductive strategy of slight polygyny where breeding females are sparsely distributed, and a considerable proportion breed on ice (Cassini 1999; Coltman et al. 2007; Davis et al. 2008). All otariids breed on land and females aggregate to territories for mating (Cassini 1999). Land-breeding otariids commonly present significant population structure as a result of natal site fidelity (Coltman et al. 2007; Crockford & Frederick 2007). However, *C. ursinus* provides an exception. In contrast to *C. ursinus*, the land-breeding pinnipeds *Eumetopias jubatus* (Steller sea lion), *Arctocephalus gazella* (Antarctic fur seal), *Arctocephalus tropicalis* (Subantarctic fur seal), *Arctocephalus forsteri* (New Zealand fur seal), *Otaria flavescens* (South American sea lion), *Arctocephalus australis* (South American fur seal) all exhibit significant genetic structure. Some phocids breeding on land also display high level of structure consistent with strong natal site fidelity. They are *Phoca vitulina* (Harbor seal), *Mirounga leonina* (Southern elephant seal) and *Halichoerus grypus* (Gray seal).
<table>
<thead>
<tr>
<th>Species</th>
<th>Marker</th>
<th>N</th>
<th>$H_{st}$</th>
<th>$\Phi_{st}$</th>
<th>$F_{st}$</th>
<th>$R_{st}$</th>
<th>$G_{st}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arctocephalus forsteri</em></td>
<td>cytochrome b</td>
<td>56</td>
<td>0.191</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Lento et al. 1997</td>
</tr>
<tr>
<td><em>Arctocephalus gazella</em></td>
<td>control region</td>
<td>145</td>
<td>NA</td>
<td>0.074</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Wynen et al. 2000</td>
</tr>
<tr>
<td><em>Arctocephalus tropicalis</em></td>
<td>control region</td>
<td>103</td>
<td>NA</td>
<td>0.190</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Wynen et al. 2000</td>
</tr>
<tr>
<td><em>Erignathus barbatus</em></td>
<td>microsatellites</td>
<td>119</td>
<td>NA</td>
<td>NA</td>
<td>0.064</td>
<td>NA</td>
<td>NA</td>
<td>Davis et al. 2008</td>
</tr>
<tr>
<td><em>Eumetopias jubatus</em></td>
<td>control region</td>
<td>1568</td>
<td>NA</td>
<td>0.081-0.339</td>
<td>0.046-0.084</td>
<td>NA</td>
<td>NA</td>
<td>Baker et al. 2005</td>
</tr>
<tr>
<td><em>Eumetopias jubatus</em></td>
<td>control region</td>
<td>224</td>
<td>NA</td>
<td>NA</td>
<td>0.050±0.030</td>
<td>NA</td>
<td>NA</td>
<td>Bickham et al. 1996</td>
</tr>
<tr>
<td><em>Halichoerus grypus</em></td>
<td>microsatellites</td>
<td>131</td>
<td>NA</td>
<td>NA</td>
<td>0.0089-0.0967</td>
<td>0.0082-0.226</td>
<td>NA</td>
<td>Graves et al. 2009</td>
</tr>
<tr>
<td><em>Leptonychotes weddellii</em></td>
<td>microsatellites</td>
<td>893</td>
<td>NA</td>
<td>NA</td>
<td>0.030</td>
<td>NA</td>
<td>NA</td>
<td>Davis et al. 2008</td>
</tr>
<tr>
<td><em>Mirounga leonina</em></td>
<td>control region</td>
<td>48</td>
<td>NA</td>
<td>0.570</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Hoelzel et al. 1993</td>
</tr>
<tr>
<td><em>Monachus schauinslandi</em></td>
<td>fingerprinting</td>
<td>50,22</td>
<td>NA</td>
<td>NA</td>
<td>0.130-0.200</td>
<td>NA</td>
<td>NA</td>
<td>Kretzmann et al. 1997</td>
</tr>
<tr>
<td><em>Otaria flavescens</em></td>
<td>cytochrome b</td>
<td>70</td>
<td>NA</td>
<td>-0.210-0.610</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Tunez et al. 2006</td>
</tr>
<tr>
<td><em>Phoca vitulina</em></td>
<td>microsatellite</td>
<td>1029</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.181</td>
<td>0.187</td>
<td>Goodman 1998</td>
</tr>
<tr>
<td><em>Phoca vitulina</em></td>
<td>control region</td>
<td>227</td>
<td>NA</td>
<td>0.772</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Stanley et al. 1996</td>
</tr>
<tr>
<td><em>Phoca vitulina</em></td>
<td>control region</td>
<td>778</td>
<td>NA</td>
<td>0.004-0.405</td>
<td>0.004-0.168</td>
<td>NA</td>
<td>NA</td>
<td>Westlake and O’ corry 2002</td>
</tr>
</tbody>
</table>

Table 6 Studies showing pinnipeds with significant genetic structure.
A large proportion of ice-breeding phocids exhibit little genetic structure. Ice-packed seals most frequently show low genetic structure, e.g. *Cystophora cristata* (Hooded seal), *Phoca largha* (Spotted seal), *Hydrurga leptonyx* (Leopard seal), *Ommatophoca rossii* (Ross seal) and *Lobodon carcinophagus* (Crabeater seal). The reason for high level of gene flow in ice-packed seals may be the dynamic and unstable nature of the breeding habitat that does not assist natal site fidelity or polygyny (Coltman et al. 2007; Davis et al. 2008). Though expected to show elevated genetic differentiation because of ecological stable territories, the fast-ice-breeding seal *Pusa hispida* (Ringed seal) displays little structure, where fast-ice means stable ice attaches to land (Davis et al. 2008). Unlike most ice-breeding phocids, *Leptonychotes weddellii* (Weddell seal) and *Erignathus barbatus* (Bearded seal) displayed high genetic structure (Davis et al. 2008), and *Odobenus rosmarus* (Walrus) breeding on packed ice represents large population differentiation (Goodman 1998; Born et al. 2001).

The pattern of higher population differentiation in land-breeding pinnipeds and lower structure in ice-breeding seals is consistent, albeit with few exceptions. In contrast to ice-breeding seals whose breeding sites are dynamic, land-breeding seals mate on stable continent or islands where they develop sexual dimorphism, polygyny, and natal site fidelity for both males and females (Davis et al. 2008). The limit of gene flow among isolated rookeries leads to population partition in land-breeding seals. The inability to detect population structure in *C. ursinus* may seem astonishing considering the structure displayed in other land-breeding pinnipeds with high polygyny. However, several
possible reasons could explain this phenomenon. First, despite the high level of philopatry, even small amounts of gene flow can sometimes lead to genetic homogeneity (Ream 2002). Second, the large abundance of *C. ursinus* compared to other endangered species (*e.g.* Mediterranean monk seal) provides enough resilience to environmental disturbance and sustains high levels of genetic exchange. Third, the degree of migration is high in both males and females in *C. ursinus*. Males live in the open ocean year-round except for the breeding season, while females travel a long distance south to return the next year. These migratory behaviors may result in more extensive movement throughout rookeries, settling away from natal rookeries, and flexibility in mating. Lastly, the inequality in age-specific natal site fidelity improves the chance of gene flow. It is not well documented if pups return to their original natal areas with their mothers. One study suggested that young seals locate their natal areas better with age (Baker et al. 1995), so the larger span of rookeries settlement makes for a higher level of gene exchange.
CONCLUSION

In summary, mtDNA analyses of ancient and modern seals indicate that *C. ursinus* has not experienced a loss or reduction of genetic diversity. The high haplotype diversity in *C. ursinus* is not unusual compared to other pinnipeds that were historically abundant. There are other seals that do show a decrease in population size and genetic variability after a major population bottleneck. As a land-breeding pinniped with natal site fidelity and polygyny, *C. ursinus* however exhibits low level of genetic structure between the three studied Alaskan populations. This result indicates extensive gene flow among the geographically distant populations to prevent genetic differentiation.
LITERATURE CITED


population genetic structure in the South American sea lion (*Otaria flavescens*).


vagile marine mammal, the Steller’s sea lion (*Eumetopias jubatus*) Molecular Ecology. 15: 2821-2832.


genetic structure of the Spotted seal *Phoca largha* along the Coast of Hokkaido, based on mitochondrial DNA sequences. Zoological Science. 20: 783–788.


CHAPTER 2

DEMOGRAPHIC HISTORY OF CALLORHINUS URSINUS FROM ALEUTIAN ISLANDS

INTRODUCTION

*Callorhinus ursinus* is the only species in the genus *Callorhinus* (Wynen et al. 2001), and it belongs to the family Otariidae, and the class Pinnipedia (Rice 1998). *Callorhinus ursinus* has a pelagic distribution that ranges across the North Pacific (Ream et al. 2005). The Pribilof Islands in the east Bering Sea have served as primary breeding sites for millennia, and accommodate the largest population worldwide (Towell and Ream 2006). As one of the classic examples in pinnipeds showing strong polygyny, *C. ursinus* also presents natal site fidelity, where females from the Pribilofs wean their pups and move south with them, and return to the same islands the following March (Gentry 1998).

For the past hundreds of years with an apex during the late 1700s, *C. ursinus* has experienced several population collapses and recoveries. The Pribilofs population, in particular, practiced dramatic fluctuations in size (Loughlin et al. 1994; Gentry 1998; Balsiger et al. 2005; Newsome et al. 2007). Human exploitation in the last centuries led to further declines in *C. ursinus* abundance. The most serious one was in the late 19th century, resulting in a low of 216,000 animals in 1912, and corresponding to a 90% population decline of the pre-exploitation size (Balsiger et al. 2005; National Marine Mammal Laboratory 2007; Macklin et al. 2008). After this decline a law was signed to
limit hunting, and the population recovered in abundance. However, the population declined again by 50-60% from 1974-2004 (COSEWIC 2006; Adams et al. 2007) for unknown reasons. The species currently is officially considered as “threatened” under the Marine Mammal Protection Act of 1988 (Loughlin et al. 1994), and under Species at Risk Act (SARA) by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) (DFO 2007).

Because of the drastic population contraction in the late 19th Century, *C. ursinus* may have suffered from a genetic bottleneck, similar to other pinnipeds. In Chapter 1, I used ancient DNA techniques to compare ancient and modern populations from four Aleutian Islands adjacent to the Pribilofs (see Figure 2 in Chapter 1). The archaeological sub-fossils were collected from Kodiak (samples were dated as old as 750 ybp; ybp stands for years before present), Shemya (1,500 ybp), and Unalaska (3,000 ybp) while the modern samples were collected from Bogoslof Island. Both ancient and modern populations showed similar high level of haplotype diversity, high nucleotide diversity and low genetic structure. The high genetic variation within *C. ursinus* before and after the serious historical commercial hunting thus implied no genetic impact of the most recent population bottleneck (see also Ream 2002; Pinsky et al. unpublished). However, unlike other land-breeding pinnipeds with natal fidelity and polygyny, *C. ursinus* exhibited genetic homogeneity that demonstrated a high extent of gene flow among the geographically distant islands.

No populations showed significant difference in estimates of genetic diversity,
except Unalaska, which contained lower nucleotide diversity and fewer nucleotide
differences (see Table 1 in Chapter 1). Understanding of past demographic responses will
allow us to better predict for future changes. Thus, taking a closer look at each island
independently, I wanted to evaluate demographic history of individual Aleutian
populations by testing the size and timing of population expansion/contraction.

The direction and degree of population changes can be inferred from mismatch
distribution tests (Rogers and Harpending 1992) and statistics such as Tajima’s D and
Fu’s Fs tests (Tajima 1989; Fu 1997; Excoffier et al. 2006). To approach particular time
depth of demographic expansion or contraction, the Bayesian Skyline Plot recently was
developed to infer past changes in population size (Drummond et al. 2003; Finlay et al.
2009). Under coalescent theory, which demonstrates the relationship of demographic
history and root probabilities in a genealogical tree, the probability of lineages coalescing
at a particular time is inversely proportional to the population size at the same time
(Drummond et al. 2003). A Bayesian skyline therefore estimates effective population size
during the intervals between coalescences based on interval lengths (Pybus et al. 2000;
Drummond et al. 2003). BEAST (Bayesian Evolutionary Analysis by Sampling Trees) is
the program that constructs Bayesian skyline plots (Drummond et al. 2003), and it uses
Markov chain Monte Carlo (MCMC) sampling to explore the likelihood of parameter
estimates and genealogies fitting the data. BEAST provides a posterior distribution of
most likely genealogies and parameters, as well as population size ($N_e$) at different time
points (Drummond et al. 2003; Drummond et al. 2005).
Molecular sequences either sampled simultaneously (synchronously) or serially (heterochronously) through time, can be used to reconstruct demographic histories (Drummond et al. 2003). BEAST accounts for serial sampling through time instead of assuming that all tips are of the same age so ancient DNA can be applied heterochronously, with sufficiently measurably sampling ages (Drummond et al. 2003). Modern genetic data can predict past molecular evolutionary processes, while ancient genetic sequence can observe such predictions and determine lineage coalescences in action with rate and magnitude estimation (Drummond et al. 2003; Chan et al. 2006). Ancient DNA has the ability to estimate the timing and severity of population declines (Chan et al. 2006), formation or cessation of gene flow (Hadly et al. 2004; Hofreiter et al. 2004; Depaulis 2009), and even has been utilized to estimate substitution rate (Lambert et al. 2002; Ho et al. 2007).

In order to obtain a Bayesian skyline, BEAST requires reasonable priors on population size and genetic parameters because the Bayesian posterior is a function of these priors (Drummond et al. 2005). For example, the mutation rate of the molecular marker serves as a critical prior for interpreting the relationship between coalescence event and population size. Ho et al. (2005, 2007, 2008) hypothesized the time dependency of substitution rate, which suggested interspecific calibrations versus intraspecific calibrations may lead to disparate evolutionary scenarios. Deeper calibration points result in slower observed rates and older coalescences (Ho et al. 2008). If an evolutionary rate is calibrated from an external (deeper) calibration point, the
intraspecific rate will be underestimated (Ho et al. 2008). Therefore, employing deep fossil calibrations or canonical substitution rates (e.g., broadly applied rate of 1% substitution per site per million years for mammals) are not proper to apply to population-level data (Ho et al. 2008). The idea of time-dependent substitution rate has not been unanimously accepted (Bandelt et al. 2006; Emerson 2007; Bandelt 2008). For example, Bandelt (2008) argued that Ho used inadequate data in his study, and not every data set supports the time dependency theory. Therefore, it is best to verify on a case-by-case basis, whether disparity exists in intraspecific vs. interspecific substitution rate. In my case, strategy of selecting proper parameters for rate calculation should be carefully considered. Therefore, distinguishing inter- from intra- specific substitution rates is desirable for demographic studies when rate differences are suspected.

Rates of mutation and substitution show variation among lineages and genes. Nucleotide changes within a population or species are often transient and can be removed by genetic drift or selection (Penny 2005; Woodhams 2006), while in the long term segregating sites of sequences taken among species are fixed with higher probability (Penny 2005; Woodhams 2006). Because not all mutations can be fixed, calculating a rate from only fixed substitutions will result in lower long-term rates compared with that estimated from all short-term mutations incorporated. Analyses of the rapidly evolving non-coding mitochondrial control region have yielded great uncertainty in the rates of nucleotide substitution across both nucleotide positions and genealogies (Alter & Palumbi 2009), because of such features as rate heterogeneity across positions
(mutational hotspots), bias in nucleotide composition, and substitutional saturation (Ho et al. 2005, 2007; Woodhams 2006; Alter and Palumbi 2009). In modern DNA studies, mutation rates of control region are often higher than those of mitochondrial protein coding regions such as cytochrome b gene. The mutation rate of control region varies considerably among species with documented rates ranging from as high as 5-10% substitutions/site/my in pinnipeds (Slade et al. 1994), 43% substitutions/site/my in brown bears (Drummond et al. 2003), 93% substitutions/site/my in Adélie penguins (Lambert et al. 2002), 83%-234% substitutions/site/my in Tuatara (Hay et al. 2008) and 32%-260% substitutions/site/my in humans (Parsons et al. 1997; Sigurdardóttir et al. 2000; Howell et al. 2003). In contrast, rate variation of the slower cytochrome b gene is less drastic among species. For example, Hawaiian drepanidines show 0.016 sequence divergence per million years (Fleischer et al. 1998), which is similar to the traditionally recognized substitution rate of 1% substitutions per site per million years for protein-coding mitochondrial DNA (Ho et al. 2008; Hay et al.2008). Because of a slower substitution rate, back substitutions accumulate more gradually, which makes the cytochrome b gene appropriate especially for interspecific rate calibration. Therefore, a combination of markers appropriate below (control region) and above (cytochrome b) the species level is a valid strategy to better understand the interplay of mutation rate and population size changes.

Effective population size is another important parameter in the Bayesian skyline plot. Under the neutral theory (e.g. no selection, no migration, no mutation), genetic
diversity within a population is a formula of $\theta = 2Ne_f \mu$ in mitochondrial DNA, where $Ne_f$ is the female genetic effective population size, and $\mu$ is the mutation rate per site per generation (Slade et al. 1998; Pavlova et al. 2005), applied from $\theta = 4Ne \mu$ for an autosomal gene of a diploid organism (Fu and Li 1993). Total $Ne$ is composed of male and female, where $Ne_m$ is the number of male parents and $Ne_f$ is the number of female parents, and effective population size is given by $Ne=4Ne_mNe_f/(Ne_m+Ne_f)$ when the sex ratio varies from the 1:1 ratio (Wright 1931; Crow & Denniston 1988; Caballero 1994). Although this $Ne$ does not exactly equal to the total observed population count, the direction of change in $Ne$ through time (e.g. population expansion or contraction) will likely reflect actual population size changes.

Thus, I propose in this article to analyze the past demographic history of Aleutian $C. ursinus$ using DnaSP, Arlequin and BEAST to compare the direction, degree and time depth of population changes among different islands. Specifically, I used DnaSP and Arlequin to access general population estimates such as Tajima’s D, Fu & Li’s and Fu’s Fs tests and mismatch distributions. To best appreciate possible variation in mutation rate with time depth, I jointly compared the inter- and intraspecific rates by grouping different outgroup species with $C. ursinus$, and confirmed these rates with pairwise measures of cytochrome b and control region distances.
METHODS AND ANALYSES

1. Sample selection

I used a total of 58 sequences of 157 bp mitochondrial control region from 3 ancient populations, with 21 sequences from Rolling Bay on Kodiak Island (750 ybp; ybp stands for years before present), 18 sequences from Shemya Island (1500 ybp), and 19 from Amaknak Bridge on Unalaska Island (3000 ybp). I had access to unpublished modern data (samples collected from 1993 to 1998; n=365) along the east Pacific sites (Pinsky et al. unpublished; Dickerson et al. unpublished). I compared my ancient DNA results to modern data from the Bogoslof population (96 sequences) because of its geographic proximity to other Aleutian islands. I used MEGA (version 3.0) to build a neighbor joining tree and randomly picked 68 sequences that represent the haplotype network from the available 365 modern sequences, which included populations from St. George Island, St. Paul Island, Bogoslof Island and San Miguel Island. The 68 modern DNA samples plus the 58 ancient DNA samples were combined together to calculate the intraspecific clock rate.

2. General population estimates using DnaSP and Arlequin

I performed several analyses of population genetic variation and structure using the programs DnaSP (version 5.0) (Librado & Rozas 2009) and Arlequin (version 3.1) (Excoffier et al. 2006). I estimated Tajima’s D, Fu & Li’s D, Fu & Li’s F, and Fu’s Fs in modern and ancient populations, as tests of selective neutrality or possible population
expansion or contraction by the program DnaSP (Tajima 1989, 1993; Fu & Li 1993; Fu 1997). I used Arlequin to plot mismatch distributions of populations from the four islands independently, and an additional plot with 3 ancient populations combined together.

3. Rate comparison between control region and cytochrome b region

I used Arlequin to plot mismatch distributions to compare the ratio of rates between the cytochrome b region and control region. I obtained 10 sequences of 157 bp cytochrome b region, corresponding with sequences of 157 bp control region from the same animals. I also obtained another 5 sequences of 121 bp cytochrome b region, coupled with 5 sequences of control region from the same animals. I obtained the means of all the pairwise distributions to compare the rate differences of the two molecular markers.

The fossil record was used to estimate the interspecific rate of cytochrome b region using BEAST. Calculations were performed using each prior independently and combining all priors (Arnason et al. 2006; Higdon et al. 2008; Koepfli et al. 2008). The coalescent prior used here was the Yule process coalescent (speciation model). Rate was estimated under the HKY+Gamma+Invariant (HKY+G+I) substitution model, with partition into codon positions as 1+2, 3. The molecular clock model was uncorrelated lognormal relaxed clock. Priors of root height were (mya stands for million years ago):

- Pinniped-Mustelid, uniform distribution (23-52 mya)
- Otariidae, uniform distribution (6-52 mya)
- Odobenidae-Otariidae, uniform distribution (18.2-52 mya)
Phocidae, uniform distribution (14.6-52 mya)

Miroungini, normal distribution (7 MYA mean; 0.5 mya standard deviation)

Monachini, normal distribution (8 MYA mean, 0.5 mya standard deviation)

*Mustela*, normal distribution (5.3 MYA mean; 0.5 mya standard deviation)

Mustelidae, uniform distribution (10-52 mya)

Pinnipedia, uniform distribution (23-52 mya)

4. Calculation of intraspecific clock rate

I used BEAST (v 1.4.8) to calculate the intraspecific clock rate within *C. ursinus* (Drummond & Rambaut 2007). The programs distributed as part of the BEAST package included BEAUti, BEAST, and LogCombiner. Specifically, BEAUti was used for specifying priors to run BEAST; LogCombiner was a program used to combine log and tree files from multiple runs of BEAST. Tracer (v 1.4.1) was used for analysing results from Bayesian MCMC programs. To calculate the clock rate appropriately considering the problem of time inequality above and below species level, I designed different sets of parameters, and picked up the most suitable rate of intraspecific clock. I used 8.2 mya as the divergence time between *C. ursinus* and sea lions, where the sea lions included *Eumetopias jubatus* (Steller sea lion), *Zalophus californianus* (California sea lion) and *Zalophus californianus japonicus* (Japanese sea lion), which was applied in many studies (Slade et al. 1994; Higdon et al. 2007; Pinsky et al. unpublished). The data set included the 126 individuals of *C. ursinus* as the majority plus one individual from each outgroup species. The sequences of outgroup species were obtained from GenBank and aligned
with the 157 bp control region of *C. ursinus*. Nexus file of the total 129 samples was imported in BEAUti, while the ages of samples were not input because they were not considered as sufficiently long compared with the long internal nodes of the genealogy (8.2 mya) (Drummond et al 2003). Rate was estimated under the HKY+Gamma+Invariant (HKY+G+I) substitution model with strict clock. Priors were set up as coalescent constant size, with population size as a uniform distribution from 0 to 60 million, and tree model root height as a normal distribution using 8.2 mya as the mean, and 2.1 mya as the standard deviation. Samples from the posterior were drawn every 20,000 MCMC steps over a total of 20,000,000 steps, with the first 10% discarded as burn-in. I assessed convergence of each MCMC run in Tracer and by checking for sufficient ESS values (>200).

5. Comparing inter- and intra- specific rates

I compared the inter- and intra- specific rates by grouping the 126 individuals of *C. ursinus* with different numbers from outgroup sea lions: *Eumetopias jubatus* (Steller sea lion), *Zalophus californianus* (California sea lion) and *Zalophus californianus japonicus* (Japanese sea lion). Rate was estimated under the HKY+G+I substitution model with strict clock in BEAST. Priors were set up as coalescent constant size, and tree model root height as a normal distribution using 8.2 mya as the mean, and 2.1 mya as the standard deviation. I grouped species in different ways such as: a) 126 *C. ursinus* plus one *Eumetopias jubatus*; b) 126 *C. ursinus* plus 30 *Eumetopias jubatus*; or c) 126 *C. ursinus*
plus 57 outgroup species which included 30 *Eumetopias jubatus*, 23 *Zalophus californianus* and 4 *Zalophus californianus japonicus*. Numbers of sea lion comparisons were based on all available Genbank sequences for these species. In general, the posterior output of a) represented intraspecific rate, and c) represented interspecific rate. As the number of outgroup species increased, the rate decreased and approached the interspecific rate. Without the basic knowledge of the root height within *Callorhinus*, I had to add in the interspecific node (8.2 mya) to simulate the intraspecific rate, but the data contained mostly *Callorhinus* sequences.

6. Bayesian skyline plot

I built skyline plots of Kodiak, Shemya, Unalaska and Bogoslof independently, without any outgroup species by BEAST. Here I added in the sample ages to directly measure population dynamics in the recent thousands of years. Bayesian analyses were performed using HKY+G+I model with strict clock and a Bayesian skyline coalescence prior, and prior of population size as a uniform distribution from 0 to 60 mya. The posterior output intraspecific clock rate from the first BEAST run (see step 4) was applied to plot the Bayesian skyline. Samples from the posterior were drawn every 20,000 MCMC steps over a total of 20,000,000 steps, with the first 10% discarded as burn-in. I used the program Tracer (v 1.4.1) to examine the posterior output of each population. The medians of skyline population sizes were plotted through time using posterior tree model root height, respectively, and the four Aleutian populations were
plotted together. The female effective population size can be obtained from the function of $N_e \times \tau$, where $\tau$ is the female generation time of 10 years (Lander 1981; Wickens 1997).
RESULT

1. Estimates of population changes using the program DnaSP

Values of Tajima’s D, Fu & Li’s D, and Fu & Li’s F do not show significant deviation from the null (stable size) model ($P>0.10$). The direction of Tajima's D, Fu & Li’s D, and Fu & Li’s F is potentially informative about the evolutionary and demographic forces that a population has experienced (Tajima 1989, 1993; Fu & Li 1993; Fu 1997; Excoffier et al. 2006). Negative values reflect an excess of rare polymorphisms in a population, which is consistent with either positive selection or an increase in population size. Positive values indicate an excess of intermediate-frequency alleles and can result from a population decline. The tests identify whether sequences fit the neutral theory model with a null hypothesis of selective neutrality and population equilibrium (Tajima 1989, 1993; Fu & Li 1993; Fu 1997; Excoffier et al. 2006). Here the values of Tajima’s D, Fu & Li’s D, and Fu & Li’s F are not informative enough to indicate a signal of population expansion or contraction in any population (Table 7).

Fu’s Fs statistic is very sensitive to population demographic expansion or contraction, which generally leads to large negative (or positive) Fs values (Fu 1997; Excoffier et al. 2006). Similar to Tajima’s D, the null hypothesis of Fu’s Fs test is selective neutrality and population equilibrium. Fu’s Fs indeed appears more sensitive to departure from population equilibrium than Tajima’s D (Table 7). The Fs values of populations from Unalaska, Shemya, “Total ancient” and Bogoslof all show significant
signs of population expansion ($P<0.02$).

2. Demographical history generated from mismatch distributions

Distributions of pairwise differences (mismatch distributions) have been extensively used to estimate the demographic parameters of past population expansions (Schneider & Excoffier 1999; Atarhouch et al. 2006; Curtis et al. 2009). Mismatch indices give the direction, time and rate of demographic changes. Opposed demographic changes (e.g. population expansion versus bottleneck) display recognizable disparate signatures in mismatch distributions (Harpending et al. 1998; Schneider & Excoffier 1999). I used Arlequin (version 3.1) to simulate the “expansion” model and DnaSP (version 5.0) to simulate the “constant” model. Figure 5 (a-e) shows mismatch distributions of 3 Aleutian ancient populations (Kodiak, Shemya and Unalaska), the combined ancient populations, and one Aleutian modern population (Bogoslof). The real data (observed) of all the populations fit “expansion” model better than “constant” model. Unalaska is the one showing most recent expansion while the other two ancient and the modern samples show earlier expansion, because the peak of the distribution (tau) for Unalaska is 4.125 (Figure 5 c), which is almost half of the other three Aleutian populations (8.662, 8.559, 8.475 respectively, see Figure 5 a, b, e).
Table 7 Comparison population changes in Alaskan *C. ursinus* populations of different age using DnaSP

*P<0.02. Significance of Fu’s Fs: Kodiak (*P*=0.069); Unalaska (*P*=0); Shemya (*P*=0.009); Total ancient (*P*=0); Bogoslof (*P*=0).
Figure 5 Mismatch distributions of ancient and modern Aleutian populations of *C. ursinus*.

a) Kodiak; b) Shemya; c) Unalaska; d) Ancient samples together (58 individuals); e) Bogoslof (modern). X-axis shows pairwise differences between each two sequences those are randomly picked, y-axis is the frequency of the differences. The “observed” are distribution of real data; “constant population” is a model of stable population size simulated by DnaSP (version 5.0); “simulated expansion” is a simulated model of population expansion by Arlequin (version 3.1), with the up bound and low bound 95% confidence interval. Means of pairwise differences: a) 7.729, b) 7.941, c) 5.327, d) 7.718, e) 7.595; taus (location of crest) of pairwise differences: a) 8.662, b) 8.559, c) 4.125, d) 7.145, e) 8.475.
3. Rate comparison between different mitochondrial genes

Mismatch distributions of cytochrome b region and control region are plotted using Arlequin (version 3.1). In figure 6, the mean ratio of control region to cytochrome b rate for 15 samples of 121 bp of control region ($p_{\text{mean}}=6.59$) and for cytochrome b region ($p_{\text{mean}}=0.61$) equals 10.8. Similar estimates are obtained from the 15 samples of 157 bp control region ($p_{\text{mean}}=7.66$) against 15 samples of 121 cytochrome b ($p_{\text{mean}}=0.61$); and from 10 samples of 157 bp control region ($p_{\text{mean}}=6.44$) against 15 samples of 121 cytochrome b ($p_{\text{mean}}=0.87$): 12.6 and 7.6 respectively. The estimated rate of control region is roughly ten-fold that of the cytochrome b rate (range=7.6-12.6). A ten-fold difference in rate is supported by the fossil calibration data that shows an interspecific rate of cytochrome b gene at 1% substitution/site/my, which is about $1/10^{th}$ that of the interspecific rate estimate for the control region.
Figure 6 Rate comparisons between control region and cytochrome b region.

a) 15 sequences of 121 bp cytochrome b region paired with 15 sequences of 121 bp and 157 bp control region; b) 10 sequences of 157 bp cytochrome b region paired with 10 sequences of 157 bp control region. Paired sequences are from same individuals of *C. ursinus*. X-axis shows pairwise differences between each two sequences those are randomly picked, y-axis is the frequency of the differences.
4. Heterogeneity of inter- and intra-specific rates

The result of comparisons of inter- and intra-specific rates of control region is demonstrated in Table 8. The prior parameters are identical except the strategy to group *C. ursinus* with different outgroup species. Number 1-5 represent intraspecific rates of *C. ursinus* with samples being primarily from *C. ursinus*. Number 9-11 stand for intraspecific rates within outgroup species using *C. ursinus* as the minority. Number 12 and 13 represent interspecific rates of *C. ursinus* with several outgroup species and individuals. The intraspecific clock rate of *C. ursinus* is estimated as 0.192-0.357 substitutions per site per million years, and the interspecific rate is computed as 0.097-0.105 substitutions per site per million years, about a two-fold difference. To investigate the effect of adding in priors on the sample ages, I performed the same analyses with the same priors and ages, and obtained a rate of 2.348 substitutions per site per million years, which I considered too high for control region.
Table 8 Comparisons of inter- and intra-specific clock rates of control region using *C. ursinus* and outgroup species

<table>
<thead>
<tr>
<th>Number</th>
<th><em>C. ursinus</em> + outgroup species</th>
<th>Output clock rate (substitutions per site per mya)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>126 <em>C. ursinus</em> + 3 outgroups</td>
<td>0.213</td>
</tr>
<tr>
<td>2</td>
<td>126 <em>C. ursinus</em> + 1 <em>Z. californianus japonicus</em></td>
<td>0.198</td>
</tr>
<tr>
<td>3</td>
<td>126 <em>C. ursinus</em> + 1 <em>Z. californianus</em></td>
<td>0.230</td>
</tr>
<tr>
<td>4</td>
<td>126 <em>C. ursinus</em> + 1 <em>Z. californianus</em></td>
<td>0.357</td>
</tr>
<tr>
<td>5</td>
<td>126 <em>C. ursinus</em> + 1 <em>E. jubatus</em></td>
<td>0.192</td>
</tr>
<tr>
<td>6</td>
<td>126 <em>C. ursinus</em> + 30 <em>E. jubatus</em></td>
<td>0.152</td>
</tr>
<tr>
<td>7</td>
<td>126 <em>C. ursinus</em> + 23 <em>Z. californianus</em></td>
<td>0.569</td>
</tr>
<tr>
<td>8</td>
<td>126 <em>C. ursinus</em> + 4 <em>Z. californianus japonicus</em></td>
<td>0.183</td>
</tr>
<tr>
<td>9</td>
<td>1 <em>C. ursinus</em> + 30 <em>E. jubatus</em></td>
<td>0.073</td>
</tr>
<tr>
<td>10</td>
<td>1 <em>C. ursinus</em> + 23 <em>Z. californianus</em></td>
<td>0.249</td>
</tr>
<tr>
<td>11</td>
<td>1 <em>C. ursinus</em> + 4 <em>Z. californianus japonicus</em></td>
<td>0.185</td>
</tr>
<tr>
<td>12</td>
<td>126 <em>C. ursinus</em> + 57 outgroups</td>
<td>0.097</td>
</tr>
<tr>
<td>13</td>
<td>68 <em>C. ursinus</em> + 57 outgroups</td>
<td>0.105</td>
</tr>
</tbody>
</table>

Number 1-8, 12: the 126 *C. ursinus* are made up of 58 ancient samples and 68 modern samples.  
Number 9-11: the one *C. ursinus* is randomly picked up from the 126 individuals (same individual in different runs).  
Number 13: the 68 *C. ursinus* are all modern samples  
Number 1: the 3 outgroups include one individual picked randomly from *Z. californianus japonicus*, *Z. californianus* and *E. jubatus* respectively.  
Number 2-5: one outgroup species is randomly picked in each run. Two samples of *Z. californianus* are picked separately and calculated independently (Number 3, 4).  
Number 6-11: Grouped outgroup species are combined with 126 *C. ursinus*  
Number 12, 13: the 57 outgroups include 30 *E. jubatus*, 23 *Z. californianus*, and 4 *Z. californianus japonicus*.
5. Demographical history generated by Bayesian skyline plot

Bayesian skyline plots summarize estimates of timing of population changes and effective population size through time (Figure 7, 8). The medians of skyline population sizes were plotted through time (Figure 8a), the clock rate was 0.213 substitutions/site/mya. All four populations show signals of population growth, though starting at different times. Of the three ancient populations, the Kodiak population expanded 250,000 ybp and practiced a population contraction after the size reached an apex at 110,000 ybp. The Shemya population expanded later at 158,000 ybp and remained stable from 67,800 ybp onwards. The Unalaska population started to grow most recently (113,000 ybp) and consistently kept expanding. The modern population from Bogoslof started a rapid growth 208,000 ybp until it stayed at constant size at 34,700 ybp. From the skyline plot, the current female effective population sizes are 28,200 (Kodiak), 69,200 (Shemya), 188,000 (Unalaska) and 219,000 (Bogoslof) seals respectively, assuming the generation time for females (Tau) is 10 years (Lander 1981; COSEWIC 2006). Using the breeding ratio of adult females to males is 9:1 (Gentry 1998), the male and female effective population sizes applied to the function of $Ne=\frac{4Ne_mNe_f}{Ne_m+Ne_f}$ give rise to $Ne$ of 11,280 (Kodiak), 27,680 (Shemya), 75,200 (Unalaska) and 87,600 (Bogoslof) animals from each island. To compare the output from inter- and intra-specific rates, I also used the interspecific rate (0.097 substitutions/site/mya), and obtained approximately double the numbers of effective population sizes and twice the time span (Figure 8b).
a)

![Graph a)

b)

![Graph b)

Legend:
- Kodiak high rate median
- Kodiak 95% HPD higher
- Kodiak 95% HPD lower

Legend:
- Shemya high rate median
- Shemya 95% HPD higher
- Shemya 95% HPD lower
Figure 7 Bayesian skyline plots of independent populations of Aleutian *C. ursinus*

Vertical axis is $Ne \cdot \text{Tau}$. $Ne$ is effective population size of females, and Tau is the generation time (10 years). Horizontal axis stands for years before present. The medians, 95% confidence intervals of population sizes are shown. a) Kodiak; b) Shemya; c) Unalaska; d) Bogoslof.
Figure 8 Bayesian skyline plots showing the demographic history of Aleutian *C. ursinus*. Vertical axis is Ne*Tau*. Ne is effective population size of females, and Tau is the generation time (10 years). Horizontal axis stands for years before present. a) uses the high rate (intraspecific rate) within *C. ursinus* and b) uses the low rate (interspecific rate) among *C. ursinus* and outgroup species. The rates are 0.213 substitutions/site/mya and 0.097 substitutions/site/mya respectively.
DISCUSSION

This study jointly analyzed the inter- and intra- specific clock rates of mitochondrial control region to estimate the past population dynamics of Aleutian *C. ursinus*. To certify the control region rate, fossil record and phylogenetic computations were combined to examine the clock rate of mitochondrial cytochrome b region, with approval of rate comparison using sequence mismatch distribution from the two genes. The root height within *C. ursinus* was difficult to estimate for the lack of prior fossil information, so adding in external calibrations placed the root of the tree deeper. Adding in closely related species produced more prior knowledge for the data set but, if not analyzed properly, would yield interspecific rates that are two-fold lower than population rates. BEAST assessed the intraspecific rate of control region ranging 0.192 to 0.357 substitutions per site per million years, and the rate above the species level was computed as 0.097-0.105 substitutions per site per million years under the same prior parameters. The interspecific rate was confirmed by mismatch distribution tests and phylogenetic computation, where the substitution rate of cytochrome b gene was ~0.01 per million years, and the control region rate was ~10 times higher. The results confirmed that rates of substitution vary among lineages and genes but in *C. ursinus* also support the time dependency theory (Ho et al. 2005, 2007, 2008).

Bayesian analysis proved especially suitable for ancient DNA study. BEAST can be applied to large datasets, is faster than the regular evolutionary sampling methods using
maximum likelihood, counts samples with different ages, especially proper for ancient DNA sequences where aging serves as additional prior knowledge, and gives a set of parameters coalescent phylogenies and distribution for final comparisons. A Bayesian skyline plot assesses the particular time depth of population changes, which reconstructs demographic history. Bayesian approaches require strict prior parameters to input accurate information. It can be difficult to draw strong conclusions if the associated estimation error is taken into account. Considering the idea of measurably evolving populations (Drummond et al. 2003), I used different strategies: for the first step of calculating the clock rate, I did not add ages of samples as prior because they were not considered as old enough to be informative about mutation rate, so the sequences can effectively be treated as isochronous (Drummond et al. 2003); for the second step of plotting Bayesian skyline which did not require the root height as the prior knowledge, I added in sampling ages that provided an opportunity to see population dynamics information that was otherwise unattainable from modern sequences.

All analyses in my thesis are consistent in showing no genetic impacts of a recent population bottleneck. In Chapter 1 I proposed that both ancient and modern populations had similar high level of genetic diversity and low genetic structure. In this chapter, Fu’s Fs test, mismatch distributions and Bayesian skyline plots indicated a population expansion in Shemya, Unalaska and Bogoslof populations that started tens of thousands of years ago and continued to recent times. The Kodiak population experienced a contraction (though not drastic) after initial population growth. Correspondingly, Fu’s Fs
test showed no significant expansion in Kodiak population, and mismatch index of this population presented as “jagged” as probably multiple expansions instead of a single one. The other potential explanation for the “jagged” shape could be lack of sufficient sampling. Unalaska is the island showing most recent expansion by both mismatch distribution and Bayesian skyline plot, but still long before the arrival of humans.

It is worthwhile to take into account recent climatic events to evaluate the demographic changes, especially with the availability of documentation of climate events. Glacial cycles probably influenced the distribution of *C. ursinus* (Gentry 1998). Crockford and Frederick (2007) presented that sea ice extended further south to Unalaska Island about 3,500 to 2,500 years ago (the height of the Neoglacial period), and persisted longer into the summer. The expansion of sea ice in the Bering Sea would have prevented fur seals from using the Pribilof Islands as a summer breeding rookery (Crockford & Frederick 2007). In that case, fur seals’ ecological reaction may have differed at this time. Interestingly, my samples from Unalaska were collected from Amaknak Bridge, and were dated as 3,000 years old. The rapid population expansion (especially during the most recent time) in Unalaska could potentially be an evolutionary effect to the climatic events, but is not strongly supported by the Bayesian skyline plots, since the expansion started at an earlier time of hundreds of thousands years ago. The ecological responses of *C. ursinus* need to be further studied. At this point, it is unclear what type of ecological response could result in reduced genetic diversity and expansion time, while low structure is being maintained.
Reconstruction of demographical history of *Callorhinus ursinus* from Aleutian Islands does not indicate a genetic bottleneck. Instead, population expansions were observed in Unalaska, Shemya and Bogoslof islands. Fur seals from Unalaska started to increase in abundance most recently but still long before human arrival. More data are needed to address a possible impact of the Neoglacial on Northern Fur seal’s demographic history.
LITERATURE CITED


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