Using Ancient mtDNA to Track Temporal Genetic Changes of Pacific Herring Populations in the Central Coast of British Columbia

by
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Abstract

Pacific herring (*Clupea pallasi*) are an important species of marine ecosystems, and to Coastal First Nations. Herring are now in decline across the Northeast Pacific, but there is much debate on the nature of this decline and its potential impacts on biological diversity of the species. This research project takes an in-depth look at mitochondrial DNA (mtDNA) of ancient herring bones recovered from stratified midden deposits at Namu, British Columbia to document changes in genetic diversity through time (7000 – 100 BP), and to explore the possibility of identifying region-specific herring populations. This study processed 60 samples with a success rate of 83.3% for mtDNA sequence analysis. Our data show that ancient DNA is generally well preserved in ancient herring remains as old as 7000BP, demonstrating the potential for retrieving genetic information about herring of the past. However, our mtDNA (D-loop and cytb) markers proved to be less informative in revealing changes of population diversity. Nuclear DNA markers and next generation sequencing technology are expected to make good use of the recovered herring DNA to better reconstruct natural history in the region.

**Keywords:** Pacific Herring; *Clupea pallasi*; ancient DNA; mitochondrial DNA; Namu, British Columbia; phylogeography; Heiltsuk Nation
Dedication

Thank you to my parents and brother, Mary Jane, Ed and Eric, who have always supported me and helped me though my education, both in and out of school. Thank you to my grandparents Ester, John, Margot, and Edward who had to foresight and love to plan so their grandchildren could go to university. This thesis is dedicated to my family.
Acknowledgements

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Chapter 1.

Introduction

Ancient DNA analysis is a powerful technique with applications in conservation genetics that have only begun to be actualized (DeSalle and Amato 2004; Haig 1998; Leonard 2008; Pääbo et al. 2004). For example, ancient DNA analysis has been used to assist with captive breedings, re-introductions, identification of hybridization, and even determining human impacts from exploitation; however, ancient DNA techniques have only relatively recently been applied to fish (Leonard 2008). Ancient DNA analysis is uniquely positioned to sample directly the past diversity of species without the inherent projection biases that modern analyses infer about past populations (Chan et al. 2006; Pauly 1995; Ramakrishnan et al. 2005).

Pacific herring (Clupea pallasi) are a resource that cannot be replaced for biological, cultural, and economical reasons (Herring School 2014; McKechnie et al. 2014; Stout et al. 2001; Fisheries and Oceans Canada and Department of Environment 1994). As an integral part of the entire coastal ecosystem, herring account for around 30% to 70% of predator diets off British Columbia’s coasts (Fisheries and Oceans Canada and Department of Environment 1994). In addition, Pacific herring have been culturally and socially vital to First Nation groups for thousands of years and continue to play a central role in their customs (Cannon 1991; McKechnie et al. 2014; Moss et al. 2015). Furthermore, the herring industry is also an important component of the broader Canadian economy, directly accounting for over 180 million dollars and six thousand jobs in 1993 alone (Fisheries and Oceans Canada and Department of Environment 1994).

Many researchers agree that herring stocks have been the victims of over-fishing in the mid-twentieth century (Fisheries and Oceans 2008; Fisheries and Oceans Canada and Department of Environment 1994). This over-exploitation is considered to be a
major factor in herring population decline and the closure of fishing stocks in 1967. It is however still disputed if over-exploitation of Pacific herring continues to be a problem today after the re-opening of fishing stocks in 1971 (Cleary et al. 2009; McKechnie et al. 2014). Over-fishing can weaken the genetic resilience of existing herring stocks as it has with other species thus making them more vulnerable to environmental changes, pollution, climate change, diseases, and predation (DeSalle and Amato 2004; Haig 1998; Hay et al. 2008; Leonard 2008). However genetic studies of Pacific herring have yielded conflicting evidence about stock structure (Hay and McKinnell 2002; Stout et al. 2001). This research aims to apply ancient DNA techniques to samples from Namu, BC that have been dated between 7,000 BP and 100 BP thus mitigating the lack of temporal depth issue present in wildlife conservation as well as potentially discovering a genetically distinct population before the modern era. This is the first study of long-term temporal shifts in genetic diversity of herring on the Central Coast of British Columbia. Providing baseline data on pre-industrial herring can be an invaluable tool for conservationists into the future.

**Research Objectives**

The larger goal of this study is to evaluate the genetic diversity of ancient Pacific herring populations as a baseline for the future conservation efforts. The specific objectives of this investigation are to analyze mtDNA from archaeological herring remains from the site of Namu, dating between 7000 years ago to 100 years ago, in order to:

1. Characterize the genetic diversity of ancient herring populations at Namu
2. Determine if Namu herring are philopatric
3. Investigate if mtDNA can reveal changes between Namu herring populations temporally and/or spatially
Chapter 2.

Background

Ancient DNA

There have been largely three waves of technological advancements in ancient DNA which have helped to shape the direction and scope of research in the field; pre-PCR, post-PCR, and next generation (Kaestle and Horsburgh 2002; Millar et al. 2008; Knapp and Hofreiter 2010). The methods of the second and third waves are still being actively published and thus relevant to contrast today. The application of the PCR method marks the second wave in the 1990’s which expanded the discipline by resolving the core barrier of low quantity of target DNA (Pääbo 1989). Second wave PCR methods are frequently typified by studies comparing mitochondrial DNA fragments that sample non-coding fragments. The third wave is next generation sequencing, which allows researchers with larger budgets to have the opportunity to study great numbers of nuclear fragments and even full genomes, as opposed to mitochondrial fragments (Hauser et al. 2008; Knapp and Hofreiter 2010).

Although the third wave is full of exciting new developments that have opened up a plethora of new research opportunities, older second wave methods utilizing primers and short amplified fragments are still used frequently in research due to restrictions such as cost, access to new machinery, and select ‘second wave advantages’ in investigating research questions such as large previously existing datasets (Cai et al. 2014; Røed et al. 2014; Shapiro et al. 2014). As very little third wave data has been collected for Eastern Pacific herring, we have chosen mitochondrial DNA for the benefits of lower cost, ease of extraction, and a greater body of previously published research for both coding and non-coding regions of DNA.
Pacific Herring

Pacific herring is a forage fish that is an ecological and cultural cornerstone (Herring School 2014; McKechnie et al. 2014; Stout et al. 2001). The archaeological evidence shows a substantial, abundant, and nearly consistent herring utilization in the coastal Eastern Pacific, currently dating back to 10,000BP (Cannon 2000; McKechnie et al. 2014; Moss et al. 2015). As a forage fish, it is a fundamental part of coastal food webs. Herring can live up to 15 years and can spawn every year after the age of four (Haegele and Schwegert 1985; Hay 1985). Females lay around 10,000 to 40,000 eggs on kelp or eel grass beds each year; however, due to heavy predation, pollution, viruses, spawn displacement by wave and egg anoxia, only one in every 10,000 eggs is estimated to survive to spawn (Bishop and Green 2001; Fisheries and Oceans, Department of Environment 1994).

An outstanding question in herring ecology is whether herring can exhibit philopatry (Beacham et al. 2008; Gao et al. 2001; Gustafson et al. 2006; Hay et al. 2001; Hay and McKinnell 2002; Hay et al. 2009; Speller et al. 2012; Ware and Tovey 2004). Philopatry is defined as the tendency of an organism to stay in or return to its home area. Many studies have been conducted to determine how individuals end up spawning at specific locations, and whether all stocks are migratory or if there are residential stocks; ultimately suggesting that philopatry is observed in a variety of marine species including Pacific herring and Orca (Gao et al. 2001; National Oceanic and Atmospheric Administration 2012). Some form of philopatry has been found in a number of herring research projects in small localized areas such as Cherry Point, Washington (Gustafson et al. 2006; Haegele and Schwegert 1985; Stout et al. 2001).

A variety of methods have been employed to determine which herring populations are philopatric. Methods include DNA (Beacham et al. 2008; Wildes et al. 2011), tagging of adult herring (Hay et al. 2001; Hay and McKinnell 2002), stable isotopes of otoliths (Gao et al. 2001), morphology (Rosenberg and Palmen 1982; Ryman et al. 1984), water temperature and salinity (Limborg et al. 2012), and spawning times (Jorgensen et al. 2005; Rosenberg and Palmen 1982; Small et al. 2005). Due to these in depth evaluations of populations in specific localities, distinct populations have been recognized based on timing (Hay et al. 2001), genetics (Wildes et al. 2011), and salinity
(Griffin et al. 1998). This creates the possibility that small populations could be—or already have been—eliminated unknowingly in locations that have not been studied, which could have long-reaching detrimental effects on herring stock strength.

Fitz Hugh Sound in the Central Coast of British Columbia

![Map of Fitz Hugh Sound and Namu, British Columbia](image)

*Figure 1. Locations of two sites investigated in this research.*

**Namu, British Columbia (E1Sx-1)**

The site of Namu (E1Sx-1) is located on the junction of the Fitz Hugh Sound and the Burke Channel, where the short Namu River stretches between Namu Lake and the
ocean (Figure 1). As unceded traditional Heiltsuk territory, the names Na’wamu or Ma’awas are also associated with the aboriginal settlement (Carlson 1995). The site of Namu has been more frequently investigated than most BC sites, with five field seasons over the last forty-five years, resulting in the excavation of over 100m³ of deposits (Cannon 2000; Carlson 1995; Cannon 1991). The site’s history is commonly divided up into six periods based on variations in artifact assemblages. Period 1 is the oldest era from 10,000 BP – 6000 BP, Period 2 has been dated to between 6000 BP to 5000 BP, Period 3 is from 5000 BP - 4500 BP, Period 4 is from 4500 BP - 3500BP, Period 5 is between 3500 BP -2000 BP and finally Period 6 reached from 2000 BP to contact (Cannon 1995). Zooarchaeological remains from Namu suggest herring have been utilized continuously for at least 7,000 years (Cannon 1991; Moss and Cannon 2011) making it an ideal location for this ancient DNA study. In the oldest era of Period 2, other than the virtual absence of shellfish, nearly the entire range of species recovered from later periods are present which include Pacific herring (Cannon 1991; Moss and Cannon 2011). Apart from the changes brought about by variations in the Namu River salmon productivity, the subsistence economy is consistent at the site (Cannon 1995).

**Hurricane Island (EITb-1)**

The site of EITb-1 located on Hurricane Island was major village site characterized by extensive deposits, remnant cedar plank houses on the surface, and a high density of fish remains in the zooarchaeological assemblage (Cannon 2000). Located 23 kilometers nearly due west from the site of Namu, Hurricane Island is separated from the much larger Hunter Island only by a narrow “Spitfire Channel” (Figure 1). The faunal remains sampled from four different bucket augers yielded low abundance of salmon, but high density of herring bones. This suggests occupation in at least the spring or late winter during spawning time; however, since herring were available year round, it could easily have been occupied at other times (Moss and Cannon 2011). The sites was first occupied around 2540 radiocarbon years BP, which corresponds to the hypothesized decline of the Namu salmon fishery in Period 5 (Cannon 2000). This date at the end of Namu’s Period 5 also corresponds to a variety of other sites in the greater Namu area initially being settled, including Kisameet Bay and Koeye (Cannon 2000). A regional bucket auger survey by Cannon (2000) was
undertaken to increase the region's sample of tested sites, which was further expanded by percussion coring techniques detailed in Martindale et al. (2009).
Chapter 3. Materials and Methods

Sample Selection

In this study, 60 herring vertebrae were selected for ancient mitochondrial DNA analysis from three different augers of a large shell deposit at the archaeological site of Namu (ElSx-1), and a single auger sampled from Hurricane Island (ElTb-1). The use of 60 samples was chosen based on the sample numbers tested in the only previous ancient herring investigation at the time (Speller et al. 2010) which was expected to reveal a detectable pattern between the two ancient sites of Namu and Hurricane Island British Columbia. The samples were selected from throughout each auger at roughly even intervals, to ensure bones did not come from the same individual.

The samples chosen for ancient DNA analyses were collected from the two sites by bucket auger in 1994 and 1996/1997 by Dr. Aubrey Cannon. The details and characteristics of these samples are summarized in Appendix A. Three of these bucket augers were collected at the site at Namu (F, D, J). Two of these (F and D) were collected next to a previous excavation (the “Rivermouth Trench”; Carlson 1995). Radiocarbon determinations from this adjacent trench excavation can be used as a proxy date to the samples used in this thesis. They date from 9720 ± 140 BP to 2720 ± 80 BP and are found at a surface depth of 390-395 cm to 64-87 cm, thus spanning Periods One to Five as described by Cannon (1991). Tree ring dating of samples from the adjacent trench additionally suggests these excavated deposits date from 10,676 to 2818 years ago.

Bucket auger J from Namu, was extracted by Cannon from about 7m south of the “Front Trench”, which was originally excavated in 1969 and 1970 by the University of Colorado (Hester and Nelson 1978). The contents of auger J were directly dated to 500-3500 BP, which includes both Period 5 and Period 6 (Cannon 2000; Cannon 1991). This is corroborated with both the original radiocarbon dates found in the adjacent trench and the averaged calibrated tree ring dates. These determinations fall within Cannon’s (1991) Period Six.
These samples were tested in a blind experiment system, specifically, the researcher was unaware of the ages and provenances of the samples until extraction and analysis was complete. The samples were acquired by Dr. Dongya Yang of Simon Fraser University from Dr. Aubrey Cannon of McMaster University. This ignorance of sample details avoids any possibility of unconscious bias stemming from expected results.

**Bone Preparation, Decontamination, and DNA Extraction**

To minimize researcher bias, I analyzed the samples without knowing their provenience or ages. Samples were divided up into groups of ten and processed according to previously established decontamination and extraction protocols in the dedicated Ancient DNA Laboratory at Simon Fraser University (Yang et al. 2004; Yang and Watt 2005; Yang et al. 1998). The first group of ten was divided and processed in two groups of five. Since every sample weighed less than 0.01 grams, only one test could be extracted from each bone. Thus, neither hydrochloric acid nor the bone saw normally used in Yang’s lab (Yang et al. 2004; Yang et al. 1998), were used in this research.

Decontamination involved a five-step process. First, sample bags were wiped with 100% commercial bleach, labelled with a lab code, and photographed. Next, individual samples were photographed in sample trays (Figure 2). A blank control tube was added to each batch of samples. Each vertebra was put into an individual 15 mL tube to be immersed in 100% commercial bleach for seven minutes, and finished by a 30 second rinse with UltraPure™ DNase/RNase-Free Distilled Water immediately followed by a 7 minute soak in the above mentioned UltraPure™ H₂O. The blank control was put through every protocol step with the same group of samples. Finally, the samples were subjected to UV irradiation in a cross-linker at 260 nm for 30 minutes on each side of the vertebrae before being added to 2mL of lysis buffer (0.5M EDTA pH8, 0.2-0.5% SDS, 0.5 mg/ml proteinase K). The upper tube and lid were then wrapped in Parafilm™, a small amount of paper towel, and covered with aluminium foil to control for possible leakage. Finally, these tubes were photographed and put into a rotating overnight incubator hybridization oven at 50°C.
A fragment of ancient salmon vertebrae was added to processing with herring vertebrae samples CPN 13 and 14 (Figure 2, bottom) to determine the potential for DNA leeching to inhibit extractions. The added salmon fragments were 0.36 grams and 0.20 grams each, however each outweighed their companion herring vertebrae by a factor of six. As the provenience of the herring samples was not known at the time, the bones used for the testing of DNA 'leeching' were chosen at random. If the provenience and age had been known, a younger sample would have been chosen for this test.
After the samples had incubated overnight and usually for no less than 15 hours, little or no bone powder remained in the tubes. Samples were photographed and a process based on the silica-spin column method was used for extraction (Yang et al. 1998). Specifically, tubes were centrifuged for 1.5 hours at 4.4 K-rpm. This short time was sufficient because the small amount of bone powder had formed a stable interface and 2 mL of the supernatant and could be pipetted into an Amicon Ultra-4 10k Centrifugal Filter Unit. Recommended procedure for the Amicon Ultra-4 10k Centrifugal Filter kit was followed according to kit instructions, resulting in a concentrated liquid of 100 µL. Samples were further purified using a QIAquick PCR Purification Kit with 100uL eluted from the column for PCR amplification. The first was for initial PCR amplification and the second was saved as a possible secondary source of DNA in case more was needed for subsequent tests. Both sample tubes and the original spin column were photographed before being transferred to the PCR set-up room for freeze storage.

**Polymerase Chain Reaction (PCR) Set-up and Amplification**

Following Speller et al. (2012), this research targeted fragments of the control region (D-loop) and the cytochrome b (cytb) gene of *Clupea pallasii* mtDNA genome. that was used in a two other herring studies, specifically pioneered in a poster presentation by Speller in July 2010 and later published in Speller et al. 2012. This allowed comparison of these samples mutations from both ancient and modern sources (Liu et al. 2011; Speller et al. 2012; Yang and Speller 2006). Two primer sets, one targeting 239 base pairs (bp) and the other 251 bp, with overlap for 112 bp, were used in the D-loop region. When combined, the D-loop fragment spanned a 266 bp region excluding primers. In addition, a single primer set targeting a 261 bp section in the cytb gene was utilized. The primers used by Speller et al. (2012) were also used for this study (Table 1). Additional attempts were made to create smaller primers to potentially increase the successful sequences for analysis. However, the variability in the regions selected, as well as the natural base pair composition prevented creating adequate primers. In addition, a variety of ancient salmon bones and primers were utilized for control groups. The salmon cytb primers (Cyt5/6) are published in Yang et al. (2004), and the salmon D-loop primers are published in Yang and Speller (2006) under the code smc7 (F) and smc8 (R).
To prepare ancient DNA for the highly sensitive polymerase chain reaction (PCR) amplification, DNA was added to a solution that includes a precisely measured amount of a forward and reverse primer pair, AmpliTaq Gold™ enzyme, and a previously mixed lab solution with dNTP and BSA. A final PCR set up of 30µL or 50µL was amplified through an Eppendorf Mastercycler. Samples CPN 1-41 were prepared to result in 30 µL set up, and samples 42-60 were prepared to result in 50 µL, with the PCR reaction solution containing 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl₂, 0.2 mM dNTP, 1.0 mg/mL BSA, 0.3 µM each primer, 3.0 or 5.0 µL DNA sample and 1.5–2.5 U/µL AmpliTaq Gold.

Both positive and blank negative controls were added at the beginning of the PCR set-up in addition to the blank negative which was first added in sample preparation and decontamination. Positive controls used were always ancient herring from this study or ancient salmon to prevent modern DNA contamination. Amplified products were sent through overnight shipping for sequencing to Eurofins MWG Operon, Inc. located in Huntsville, Alabama.

In addition to utilizing the primers as detailed in Table 1, a variety of methods such as cross species primers were employed for contamination and authenticity controls. A PCR series was run using herring samples CPN 1-5 with previously detailed salmon cytB primer pair 5/6. A PCR was also run using the mixed herring/salmon samples CPN 13 and CPN 14 running salmon cytB 5/6 primers in addition to a separate run with herring primers (Figure 2). This mixed herring/salmon attempt is further detailed in the following Contamination Controls and Authentication section.

---

### Table 1 Primers utilized for Herring PCR amplification

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Position</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-F6</td>
<td>CCACCCCTAACTCCCAAAGC</td>
<td>15601-15626</td>
<td>D-loop</td>
</tr>
<tr>
<td>CP-R289</td>
<td>CATSCATGTATCAATACATAGGTAC</td>
<td>15865-15889</td>
<td>D-loop</td>
</tr>
<tr>
<td>CP-F130</td>
<td>GTCTTCCATAACTGCAATATCAC</td>
<td>15730-15753</td>
<td>D-loop</td>
</tr>
<tr>
<td>CP-R425</td>
<td>GTGATTTTAATGATAGCTCG</td>
<td>16004-16025</td>
<td>D-loop</td>
</tr>
<tr>
<td>CP-F30</td>
<td>GCC TAC GAA AAA CCC ACC C</td>
<td>14380-14398</td>
<td>Cyt B</td>
</tr>
<tr>
<td>CP-R328</td>
<td>TAG TCC TCG GGC GAT ATG TG</td>
<td>14659-14678</td>
<td>Cyt B</td>
</tr>
</tbody>
</table>

Note: Position is based on Genbank accession NC009578. Primers are from Speller et al. (2012).
PCR conditions were 10 minutes at 94°C, followed by 60 cycles of 94°C at 45 seconds, 52°C for 30 seconds, and 72°C at 40 seconds, and concluded by a final extension step of 72°C for 10 minutes. After the PCR reaction, samples were stored at -18°C until removed from the machine. Successful PCR reactions were determined on 2% agarose gel with a 100 bp ladder (Invitrogen) using SYBRgreen (Invitrogen) as a dyeing element. All samples are stored at -20°C in a ziplocked bag and labeled in a laboratory freezer in the Post-PCR Laboratory at Simon Fraser University for future investigations.

**Contamination Controls & Authentication**

Controls to address potential contaminations were employed at every step of the extraction and amplification process (Cooper and Poinar 2000; Kaestle and Horsburgh 2002; Poinar 2003). During decontamination, extraction, and PCR set-up, protective clothing including Tyvek™ suits with hoods and foot covers, secured wrist covers between two layers of disposable gloves, and masks were worn over scrubs that are only utilized for ancient DNA procedures and shoes that do not leave the laboratory. The laboratory in which ancient sample preparation, extraction, and PCR set-up took place is a positive pressure facility in which stringent bleaching procedures are performed before and after each set of samples. To avoid contamination between samples or from external sources, all plastic tubes in the PCR set up stages were subjected to UV irradiation for 20 minutes prior to use and disposable filtered pipette tips were used on all pre-PCR pipetting. As briefly mentioned earlier, multiple blank extractions, negative controls, and positive controls were run with tested samples to help determine the possibility of systematic contamination, reagent failure, and human error.

The DNA extraction lab/PCR lab, also known as a post-PCR lab, is located in a different building than the ancient DNA lab. This includes a separate ventilation system, no overlap of equipment, and a protocol that includes a one way directional work flow, specifically from ancient to post-PCR laboratory, and a researcher cannot reverse that flow without taking a shower and changing clothes in the interim. In addition, no modern DNA has ever been extracted in the ancient DNA lab. All salmon positive controls processed with the ancient samples were recovered from archaeological sites.
(Rodrigues and Roth 2010) and all herring positive controls were from this same study with known sequences, none of which were repeated in subsequent amplifications.

As described previously, to determine the potential of DNA leeching and primer specificity, herring samples were run with salmon primers to determine if any DNA leeching could have happened before testing, either in storage or within the midden (Mitchell et al. 2008). Ancient salmon samples from a different site were run with herring primers to test the possibility of the herring primers mistakenly extracting similar non-herring DNA. In two samples, a fragment of ancient salmon bone was added as a control in order to simulate a ‘DNA leeching’ and briefly test control the possibility of an unexpected interaction that might occur if such a leeching were to occur.

Sequence validity was verified through overlapping primers, repeated sequencing, and repeated PCR amplifications. A chi-square test with Yates’ correction was conducted considering the rates of success between older and younger samples. Additionally, the Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution was ran using Mega 6.0 considering the transition and transversion bias for this data set and compared to Speller et al. (2012) modern Central Coast data, Speller et al. (2012) ancient British Columbia data, and select Liu et al. (2011) modern Pacific data (Tamura et al. 2013; Tamura et al. 2004).

**Species Determination**

Species identification was achieved by running each of the three fragments separately. All sequences were run through the GenBank library by way of the BLAST application search to determine their most likely species match (Altschul et al. 1990). Sequences were visually edited and examined using ChromasPro© 1.7.5 software (Technelysium Pty Ltd). Multiple alignments of the sample sequences were created using BioEdit software (Hall 1999). A species identification was considered valid if the sample matched 99% match or above for all reproducibility tests and no other closely-related species was indicated to be a match.
Haplotype Determination and Nucleotide Diversity

Combined D-loop 1 and 2 fragments as well as cytb fragments were aligned with previously published samples from Speller et al. 2012 (74 ancient, 53 modern) and fragments from Liu et al. 2011 (508 modern) using the software BioEdit v. 7.2.5 (Hall 1999) to determine which of this studies samples had previously identified as haplotypes as well as rates of potential new haplotypes. An additional dataset of the two D-loop fragments combined with the one cytb fragment as a single sample was analyzed. Due to a slight misalignment between my samples and the Speller and Liu studies, the sequences were truncated to a total of 351 bp in the D-loop, 210 bp in the cytb fragment, and 563 bp in the combined alignment. This is position 15654 - 16005 for the combined D-loop fragments and positions 14425 - 14635 for the cytb fragment based on the complete mtDNA genome of the Genbank specimen Clupea pallasii GI:148762534, AP009134.1. In the truncated region of D-loop, 28 base pairs were removed from my and Speller et al. (2012) samples. In the truncated region of cytb, 23 base pairs were removed from my and Speller et al. (2012) samples. There were zero variable base pairs in the truncated D-loop region, and one variable base pair removed in the cytb region, specifically located at absolute position 14639 on sample CPN 12, which was a C to T pyrimidine transition.

Any sequences identified in the above mentioned publications were given the previously assigned haplotype label according to Speller. If the remaining unassigned sequences polymorphic regions were confirmed by repeats or were confirmed by Liu et al. (2011), they were given ‘confident’ haplotypes. If the sequence did not contain any base pair ambiguities but was not repeated, the sequence was assigned a ‘tentative’ haplotype (Appendix B). Any sequences from samples that did not meet any of the above criteria were not labeled with a haplotype. Haplotype determination was achieved through use of FAbox, which is an online toolbox for fasta sequences (Villesen 2007). My sequences were uploaded with previously haplotype identified samples from both modern and ancient publications to keep sample names consistent and ran with the ‘DNA to haplotype collapse and converter’ tool.
Figure 3  Upper figure details the varied date range of each sample from the ancient Central Coast and which group each was assigned in a variety of analyses when a date range was not feasible. The upper figure also graphically represents the relative abundance of successful older to newer samples. Lower figure displays number of samples used in each respective group in this study. Groups 2, 3, and 4 are ancient samples from this study. Group 1 is a modern dataset obtained by Speller et al. 2012 from the Central Coast of British Columbia.

Samples were analyzed in two major categories, temporally and spatially. Spatially comparing two locations within the Central Coast, and secondly comparing the Central Coast to other regions in the Eastern Pacific. More complex temporal divisions were considered in the Central Coast samples, where samples were roughly grouped
into four categories (Figure 3). All sample groupings were analyzed for the percentages of unique singleton haplotypes. Haplotype (h) and nucleotide diversity values (π) were spatially calculated using Dna-SP v5.10 software (Librado and Rozas 2009). These h and π values were plotted and were compared to other ancient and modern site datasets calculated in Speller et al. 2012. In addition, Tajima's neutrality tests were conducted on each dataset individually and compared to each other with special and temporal identifications considered using MEGA 6.0 (Tamura et al. 2013).

**Network and Phylogenetic Analysis**

My data was run in Mega 6.0 for phylogenetic analysis after the best fit substitutional model was used in that same program. The phylogeny was reconstructed using the neighbour joining distance method, utilizing the Tamura 3-parameter method, bootstrap replicated 500 times, and setting the rates among sites as gamma distributed into 5 categories when considering any gaps or missing data to be a complete deletion resulting in 347 total sites. Gamma has been shown to better reflect realities in mtDNA (Yang 1993). A D-loop tree showing the relationship between specific ancient Namu samples considering their age groupings was created as well as a D-loop tree with all of the sample considered in this study, making special note of the relations between the ancient Namu samples, the ancient Hurricane Island samples, and the modern Central Coast samples.

In addition, network trees of the ancient D-loop haplotypes and select Pacific herring reference sequences were produced through Network (Bandelt et al. 1999, Polzin et al. 2003). Network software was utilized for the creation of median joining networks utilizing maximum parsimony (Bandelt et al. 1999). Suggested weight adjustments were followed according to the program user guide which included weighing transversions three times higher than transitions as transversions occur about 20 times less often than transitions in human mtDNA and down-weight hypervariable sites/characters from the standard of ten to five. The epsilon was kept at a default of ten. The ‘Connection Cost’ distance calculation method was used, and no additional contraction methods were used, such as star contraction or ‘frequency > 1 Criterion’ in order to keep the original high occurrence of singleton haplotypes visible.
Chapter 4.

Results

PCR Amplification and Sequencing

Fifty out of sixty herring bone samples yielded amplifiable DNA in at least one of the three fragments; this represents a success rate of 83.3%. Overall, a 74.2% fragment success rate was achieved with the combined D-loop sequences. When considering the two D-loop fragments separately, there was an 81.7% success rate for D-loop1 and a 66.7% success rate for D-loop2. A 78.3% success rate was achieved with the cytb sequences. The 32 samples from 100-2600 BP have a 90.6% success rate when considering Hurricane Island and Namu together, however when considered separately, of the 12 samples from Namu there is an 83.3% success rate, and of the 20 samples from Hurricane Island there is a 95% success rate. In addition, of the 13 samples from 2000-4000BP samples, they have a 92.3% success rate and the 16 samples from 4000-7000BP have a 62.5% success rate overall. To better determine if there is a correlation between success rates and age, a chi-square test with Yates’ correction for continuity was run. This resulted in a significant p-value of $X^2 = 3.846$ and $p = 0.0499$ (Preacher 2001). A complete record of all PCR attempts and success rate can be found in Appendix A and Table 2. The secondary 100μL elution of DNA from the QIAquick PCR Purification column for PCR amplification was not needed or used.

Throughout this study, all negative controls were blank, all positive controls were positive except for salmon cytb primers Cyt5/Cyt6 being used on in Series I086 in which the whole amplification failed. Herring bones ran with both D-loop and cytb salmon primers did not amplify any salmon DNA. Salmon bones ran with both D-loop and cytb herring primers did not amplify any herring DNA (Figure 4). In Series I083, where my study’s herring cytb primers were used on the combined herring and salmon samples (CPN 13 and CPN 14), salmon DNA was extracted once. In this instance, the DNA was sequenced and was correctly identified as Chinook salmon (*Oncorhynchus tshawytscha*), by the BLAST application search on ncbi.nlm.nih.gov (Altschul et al. 1990). When my herring D-loop primers were used on the same mixed sample, no
salmon or herring DNA were recovered in four different amplification attempts. This single cytb cross species extraction was unique as this was the only instance in which herring primers of any sort extracted salmon DNA. This extraction is significant as it indicates that there was no salmon DNA transfer to the CPN herring samples in this study and that this primer is salmon compatible. The species was determined by use of the previously described protocol utilizing BLAST search.

Figure 4  Electrophoresis gel image of PCR amplifications, no contamination, and no cross-species primer amplification. BK represents the blank extraction, NEG is for PCR negative control, and 100bp is for the 100 base pair ladder (InVitrogen, Varsibad, CA).

Ten of the samples (CPN 3, 9, 13, 14, 15, 33, 48, 51, 54, and 56) did not produce any of the three targeted fragments. They were relatively spread in ages as six were older than 4000BP and four were younger than 3500BP. All ten samples were re-run at least once in a set up targeting the highest success rate fragment, D-loop 1, and utilized
higher proportional quantities of Taq Gold as well as additional PCR run cycles. After this, no additional DNA extraction could be attempted, as no bone materials were left from these samples.

Overall, sequencing results were overwhelmingly ‘clean peaks’ with strong signal strength (Figure 5). Only four samples on the first amplification had weak, messy, or short sequences, however all but one, specifically CPN 52, were re-amplified successfully and found to have strong signals on all subsequent tests. CPN 52 could not be utilized in this study due to weak and ‘messy’ electrophoresis base pair peaks and short fragment length. CPN 52 could not be successfully re-amplified to a tentative or confident degree.

Forty-two of the fifty successful samples (84%) had at least one of their fragments re-sequenced from the same amplification, representing 47.7% of total successful fragments (Table 2). From these re-sequenced samples, there were three difference types found. First, different numbers of C base pairs within poly C regions in four samples were found in samples CPN 27, 31, 35, and 58. Second, the weak sequencing first found in samples CPN 1, CPN 2, and CPN 5 D-loop 1 was determined not to be related to the samples or amplification, but more likely the sequencing process itself as all three amplified strongly and without issue from the same PCR, lab code I080. The final difference is the D-loop 2 fragment of CPN 53 appeared on the agarose gel and sequenced well on the re-sequencing, when it did neither the first time. A total of 38 samples were verified and usable for tests on the combined D-loop fragments. A total of 45 samples were verified and usable for tests on the cytb fragment. And finally, a total of 36 were verified and usable for tests utilizing all three fragments combined into a ‘joint’ sequence.
Figure 5  Upper: Fragment of CPN 8 D-loop electrophoresis from 6000-7000BP sequence showing the high quality of CPN 8, one of the most ancient samples, which is typical in this study. Note at top how the numbers are between 479 – 895 in strength. Fragments from top to bottom include D-loop 1 reverse, D-loop 1 forward, D-loop 2 reversed, D-loop 2 forward.

Bottom: Segment of overlap and corresponding contig sequencing map showing the location and full contig of CPN 8 and the above electrophoresis image.
Table 2  
Successful extractions, re-sequencing from the same PCR amplification, and number of repeats.

<table>
<thead>
<tr>
<th>CPN SAMPLE</th>
<th>D-loop 1</th>
<th>D-loop 2</th>
<th>Cytb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>8</td>
<td>1.5</td>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>2.5</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>12</td>
<td>1.5</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
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<tr>
<td>17</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
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<tr>
<td>18</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>19</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table Key
0 = never worked
1 = successfully sequenced once
2 = successfully sequenced from two different PCRs
1.5 = successfully sequenced twice from the same PCR
2.5 = successfully sequenced from two different PCRs and once from the same PCR

Haplotype and Haplogroup Determination

D-loop Haplotypes

As generally expected for Pacific herring, high sequence variation was found in this study. Fifty-two variable base pairs sites were found on the D-loop fragment which resulted in 32 haplotypes among 38 D-loop samples (Figure 6). In a molecular diversity indices analysis performed on this dataset (Excoffier and Scheider 2005), 45 transitions and 8 transversions were observed, thus roughly a 5.6:1 ratio. Transitions are expected to be more prevalent than transversions in general, however a precise ratio has
previously not been published for either modern or ancient Pacific herring mitochondrial D-loop DNA.

After running D-loop fragments through FAbox (Villesen 2007), it was found that 36.8% of my sample’s haplotypes have been identified in previous ancient herring studies (Speller et al. 2012) and 60.5% have been found previously in either ancient or modern studies (Table 3). Thus, 63.2% of my D-loop haplotype data are new to ancient studies, and 39.5% of my samples are new to ancient or modern studies combined (Table 3). These percentages of unique haplotypes are higher than other ancient and modern Pacific herring studies, however the other ancient study’s sample size (Speller et al. 2012) is nearly twice as large than my dataset, and the major modern dataset used in comparison (Liu et al. 2011) is thirteen times larger than my dataset. This makes the comparisons hard to analyze by percentages as the percentage of unique haplotypes are less likely to be representative as dataset get smaller (Table 3). The higher level of unique haplotypes in comparably smaller datasets is also seen in temporal divisions for D-loop samples (Table 4). The only exception to this trend spatially, is in the modern Central Coast samples (Speller et al. 2012) which exhibit a significantly higher level of unique haplotypes overall at 60.4% in a dataset that is only 39% larger than my dataset. The spatial and temporal Central Coast analysis between Hurricane Island and Namu is harder to interpret confidently with the very low sample sizes in pre-4000BP datasets, and therefor is likely unreliable (Table 4). In summary, a larger dataset will produce more significant results spatially and temporally in the Central Coast.

Overall, fifteen new confident or tentative D-loop haplotypes have been identified and labeled with the codes D44 - D66 from this study. This expands on the system Speller et al. (2012) created, which identified and labeled haplotypes D1 – D43 (Appendix A). Comparisons between CPN and other ancient northeastern Pacific herring sequences displayed higher number of singletons in the CPN dataset (Table 3). The highest rates of singletons proportionally, considering all available datasets, are from the Central Coast modern samples (CM) at 60.4%. This number can be calculated from the Speller et al. (2012) data. This high number is considerably higher than the next highest singleton proportion; the ancient CPN data from the Central Coast in this study at that had a 39.5% unique singleton rate (Table 3), which is an unexpected result. The modern Central Coast dataset is potentially an inaccurate reflection of modern populations, or is
reflective of a new more diverse population that has migrated to the area in the modern era.

Table 3  Details of datasets utilized in relation to sample sizes, haplotypes and singletons.

<table>
<thead>
<tr>
<th></th>
<th>D-loop</th>
<th></th>
<th></th>
<th></th>
<th>Cytb</th>
<th></th>
<th></th>
<th></th>
<th>Joint</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Samples</td>
<td>Haplotypes</td>
<td>Unique Haplotypes %</td>
<td>Unique Haplotypes Considering Total Dataset</td>
<td>Samples</td>
<td>Haplotypes</td>
<td>Unique Haplotypes %</td>
<td>Unique Haplotypes Considering Total Dataset</td>
<td>Samples</td>
<td>Haplotypes</td>
<td>Unique Haplotypes %</td>
<td>Unique Haplotypes Considering Total Dataset</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Within Individual Dataset</td>
<td>(%) of singletons</td>
<td></td>
<td></td>
<td>Within Individual Dataset</td>
<td>(%) of singletons</td>
<td></td>
<td></td>
<td>Within Individual Dataset</td>
<td>(%) of singletons</td>
</tr>
<tr>
<td>CPN Central Coast (ancient)</td>
<td>38</td>
<td>32</td>
<td>84.1%</td>
<td>15 (39.5%)</td>
<td>45</td>
<td>17</td>
<td>37.8%</td>
<td>9 (20.0%)</td>
<td>36</td>
<td>33</td>
<td>91.7%</td>
<td>20 (55.6%)</td>
</tr>
<tr>
<td>CP Eastern Pacific (ancient)</td>
<td>74</td>
<td>43</td>
<td>58.1%</td>
<td>17 (23.0%)</td>
<td>73</td>
<td>16</td>
<td>21.9%</td>
<td>5 (6.8%)</td>
<td>70</td>
<td>42</td>
<td>60.0%</td>
<td>22 (31.4%)</td>
</tr>
<tr>
<td>Liu.E Eastern Pacific (modern)</td>
<td>508</td>
<td>212</td>
<td>41.7%</td>
<td>133 (26.2%)</td>
<td>93</td>
<td>19</td>
<td>20.4%</td>
<td>7 (7.5%)</td>
<td>93</td>
<td>65</td>
<td>69.9%</td>
<td>39 (41.9%)</td>
</tr>
<tr>
<td>CM Central Coast (modern)</td>
<td>53</td>
<td>45</td>
<td>84.9%</td>
<td>32 (60.4%)</td>
<td>38</td>
<td>12</td>
<td>31.6%</td>
<td>4 (10.5%)</td>
<td>37</td>
<td>33</td>
<td>89.2%</td>
<td>28 (75.7%)</td>
</tr>
<tr>
<td>Fragment Totals</td>
<td>673</td>
<td>280</td>
<td>67.2%</td>
<td>197 (29.3%)</td>
<td>249</td>
<td>41</td>
<td>27.9%</td>
<td>25 (10.0%)</td>
<td>236</td>
<td>140</td>
<td>77.7%</td>
<td>109 (46.2%)</td>
</tr>
</tbody>
</table>

Note: The column ‘Unique Haplotypes Considering Total Dataset’ refer to the number of haplotypes found within each dataset that was not found in any of the four total datasets. In contrast, the ‘Unique Haplotypes % Within Individual Dataset’ column details the percentage of haplotypes that are unique within the single dataset row. The bottom row gives the totals for the D-loop, cytb, and ‘joint’ fragments respectively. Note that the haplotypes column was not simply totaled as many of the datasets share haplotypes, thus would incorrectly inflate the total number of haplotypes found. Although the Central Coast is found within the Eastern Pacific, it is not duplicated in the Eastern Pacific row numbers. Data from ‘CP’ comes from Speller et al. (2012). Data from ‘Liu.E’ samples include all Eastern Pacific samples in Liu et al. (2011). ‘CM’ samples were referenced in Speller et al. (2012) however were unpublished on Genbank and thus were obtained from one of the authors in 2015.
Cytb Haplotypes

As expected, cytb variation was lower than D-loop variation in Pacific herring (Figure 6). Twelve variable base pair sites were found on the cytb fragment resulting in 17 haplotypes among 45 successful samples. Eight haplotypes were determined to be previously found in other modern and ancient datasets, resulting in nine unique singletons being identified. Eleven new haplotypes have been identified and labeled C20 – C31. This adds to Speller et al. (2012) previously identified C1 – C19 haplotype labeling system (2012). The relative diversity in the ancient Central Coast samples analyzed in this study is higher and the dataset is smaller than every other available dataset of Pacific herring (Table 3). The more homogenous nature of the cytb gene in Pacific herring samples suggest better tracking the rates of haplotypes though time than by D-loop fragments, however the issue of smaller sample sizes in pre-4000BP datasets is skewing in a species as diverse as Pacific herring (Table 4).

One discrepancy emerged in assigning haplotypes using FABox haplotype program verses previously defined haplotypes (Villesen 2007). Specifically, sample CP7 was originally the only sample labeled HapC4 in Speller et al. (2012), however it was grouped in with 34 other CP samples that those authors labeled as HapC1 by FABox (Appendix B). This indicates that different criteria was used between Speller et al. (2012) paper and this study in determining distinct haplotypes.
Figure 6  (Previous page) Total variable nucleotide sites of D-loop and cytb fragments. D-loops 1 and 2 are joined together in the left block, and ending with site 15974, and the variable sites of the corresponding cytb fragment, starting with site 14476. These sites are compared with the Genbank Reference Sequence AP009134.1 with the actual base pair location appearing at the top. Note the far more conservative nature of the cytb compared to the great diversity in the D-loop fragment selected.

Table 4  Central Coast ancient DNA samples temporally grouped, displaying unique haplotypes and singletons.

<table>
<thead>
<tr>
<th></th>
<th>Dloop</th>
<th></th>
<th></th>
<th>Cytb</th>
<th></th>
<th></th>
<th>Joint</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Samples</td>
<td>Haplotypes</td>
<td>Unique Haplotypes % Within Individual Dataset</td>
<td>Unique Haplotypes Considering Total Dataset (% of singletons)</td>
<td>Samples</td>
<td>Haplotypes</td>
<td>Unique Haplotypes % Within Individual Dataset</td>
</tr>
<tr>
<td>Group 1 Modern (CM)</td>
<td>53</td>
<td>45</td>
<td>84.9%</td>
<td>32 (60.4%)</td>
<td>38</td>
<td>12</td>
<td>31.6%</td>
</tr>
<tr>
<td>Group 2 100 - 2000 BP</td>
<td>23</td>
<td>19</td>
<td>82.6%</td>
<td>8 (34.8%)</td>
<td>26</td>
<td>8</td>
<td>30.8%</td>
</tr>
<tr>
<td>Group 3 2000 - 4000 BP</td>
<td>8</td>
<td>8</td>
<td>100%</td>
<td>4 (50%)</td>
<td>11</td>
<td>7</td>
<td>63.6%</td>
</tr>
<tr>
<td>Group 4 4000 - 7000 BP</td>
<td>7</td>
<td>6</td>
<td>85%</td>
<td>3 (42.9%)</td>
<td>8</td>
<td>6</td>
<td>75%</td>
</tr>
</tbody>
</table>

Note: The column ‘Unique Haplotypes Considering Total Dataset’ refer to the number of haplotypes found within each time period that was not found in any of the three time periods. The column ‘Unique Haplotypes % Within Individual Dataset’ is the percentage of unique haplotypes within each time periods, not considering the other time period datasets.

**D-loop and Cytb (Joint)**

As there are no other ‘joint’ haplotypic analysis combining both the commonly used D-loop with cytb fragments, there are no previously published haplotype labels for this joint analyses. This joint analysis resulted in a total of 126 haplotypes being identified out of 236 samples, 565 base pairs in length. The analysis increased the number of unique haplotypes identified and the proportional number of haplotypes in all four datasets analyzed (Table 3). The analysis of the CPN data resulted in 33 distinct haplotypes being labeled out of 36 samples.
Generally, the joint CPN haplotype divisions mirror the D-loop divisions as it the more diverse of the two DNA regions. There are three notable exceptions. For the CPN samples, the number of haplotypes decreased from the 38 D-loop samples by two, specifically CPN 38 and 45 could not be considered for the joint analysis. Furthermore, while CPN 7 was identified as D36 with CPN 5 and CPN 30 in the D-loop analysis, the addition of the cytb fragments kept CPN 7 with the other D36 samples however moved each CPN 5 and 30 into unique haplotype groupings individually. This also is the case with the sample CPN 26, which was identified as a D12 haplotype in the D-loop analysis along with CPN 17 and CPN 41. However in the joint analysis, CPN 26 was grouped individually when CPN 17 and 41 remained grouped as a D12, indicating a different methodology was used to determine haplotypes by Speller et al. (2012).

**Reproducibility, Validity, and Contamination**

Sequence validity was verified through overlapping primers, repeated sequencing, and repeated PCR amplifications. Both forward and reverse fragments were sequenced. Of the 48 samples that had at least either the D-Loop 1 or D-Loop 2 fragment working, 41 of them have sequenced successfully, thus each of those fragments were replicated in the 112bp overlapping segment (Figure 5). In addition, 25 of the successful samples (52.1%) had at least one of their fragments successfully amplified for a second independent PCR. If considering fragments, of the 115 successful fragments, 28 were re-amplified, which represents 24.3% of total successful fragments in this study.

As previously mentioned, no contamination was detected in any of the blank controls. When this lack of contamination is considered with the extremely high rate of unique haplotypes within the CPN dataset (84.2%), it suggests no cross sample contamination. When compared to Liu et al. (2011) and Speller et al. (2012) data, 60.5% of my sample haplotypes were found in other locations, or in modern populations from the Central Coast (Table 3). This further supports that these findings are representative of true haplotypes and not post-mortem mutations or sequencing errors. Further, there is no evidence of salmon DNA being transferred to the herring bones studied. Finally, there
was no concurrent project being run in either the ancient or post-PCR labs that had any contamination discovered during the entire period of this project.

The Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution demonstrated roughly similar rates of transition and transversion bias for my data set compared to Speller et al. (2012) modern Central Coast data, Speller et al. (2012) ancient British Columbia data, and select Liu et al. (2011) modern Pacific data (Tamura et al. 2013; Tamura et al. 2004). CPN data indicates \( k_1 = 12.776 \) for purines and \( k_2 = 5.435 \) for pyrimidines as compared to Liu et al. (2011) which indicates \( k_1 = 15.62 \) and \( k_2 = 6.648 \) for the transition/transversion rate ratios. As expected, transitional substitutions are demonstrated to be a much higher likelihood than transversonal substitutions, as well as the G/A mutation being far more likely than the C/T (Nei 1987).

**Phylogenetic and Network Analysis**

The phylogenetic neighbour joining tree produced by Mega 6.0 shows there is a standard equal distribution of samples between haplogroups B and C with a few shared haplotypes between sites (Figure 7). The presence of two haplotype A samples in the modern Central Coast samples is notable in that haplotype A is not present in the ancient Central Coast samples, which could indicate a modern population. To more fully illustrate dataset diversity, I also present the ancient Central Coast samples in a maximum likelihood phylogenetic tree with gamma modifier utilizing a Tamura 92 model as grouped by temporal ages by themselves (Appendix D).
Figure 7    Phylogenetic tree with all datasets considered in this study produced by MEGA 6.0. Ancient Central Coast Hurricane Island and Namu samples are marked by different symbols as well as separately from modern Central Coast to help illustrate the non-correlative relationship present. Solid red circles indicate ancient Namu samples, solid blue triangles indicate ancient Hurricane Island samples, red circles with white interiors indicate modern Central Coast samples from Speller et al. (2012). The top bracket is haplogroup B, the middle bracket is haplogroup A, the lower bracket is haplogroup C.
The AMOVA fixation indices display a non-correlative relationship between populations spatially or temporally. The vast majority of differentiation was within each defined ‘population’ or FST, which accounted for 93% - 100% of diversity (Table 5). Overall, cytb fragments are more indicative of divergent genetics temporally than their D-loop counterparts as those fragments were the ones to show more differentiation among populations within groups or between groups. This is most likely because clearer divisions can be made upon a population that does not have such a high percentage of divergence.

Small sample sizes prevent further explorations into the possible patterning between temporal groups and makes findings less likely to be representative (Lower Table 5). For instance, group four had only seven (D-loop) or eight (cytb) samples in it. Ideally, each age group would be tested against each other however due to AMOVA requirements, this ideal wasn’t possible. Specifically, it wasn't possible to test group four by itself against group three, thus requiring the two groups to be combined. Assuming a low sample size did not skew the results, it could be suggested that the combination of groups three and four did not result in a homogenous population due to the relatively high Fsc value of 2.16%. A high Fsc value indicates a non-homogenous populations have been grouped together inappropriately. The highest percentage of variations are contained in the temporal FCT results (Lower Table 5), which would suggest that the groups compared show genetic differentiation between them. However the higher percentage of differentiation consistently seen in the FCT cytb and D-loop analysis does not follow linear expectation. Specifically, the numbers which should be the highest with the assumption that the population gets more divergent over time (i.e. the two groups that are temporally the furthest apart, 3/4 and modern Central Coast) are actually shown to be the lowest. This either means that increasing diversity did not follow a linear pattern or it throws the other lower table FCT values into doubt.
Table 5  Results of hierarchical AMOVA on each mitochondrial DNA fragment region.

<table>
<thead>
<tr>
<th>Compared to</th>
<th>Ancient Eastern Pacific Coast/Modern Eastern Pacific Coast</th>
<th>Ancient Central Coast/Modern Central Coast</th>
<th>Ancient Central Coast/Ancient Eastern Pacific Coast</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-loop</td>
<td>% of variation</td>
<td>% of variation</td>
<td>% of variation</td>
</tr>
<tr>
<td>Among populations (Fst)</td>
<td>0.006 99%</td>
<td>-0.001 100%</td>
<td>0.002 100%</td>
</tr>
<tr>
<td>Among populations within groups (Fsc)</td>
<td>-0.002 0.15%</td>
<td>0.014 1.40%</td>
<td>0.005 0.47%</td>
</tr>
<tr>
<td>Between groups (Fct)</td>
<td>0.008 0.78%</td>
<td>-0.003 -0.29%</td>
<td>0.007 -0.68%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compared to</th>
<th>Ancient Central Coast: Group 3 and 4/Ancient Central Coast: Group 2</th>
<th>Ancient Central Coast: Group 2/Modern Central Coast</th>
<th>Ancient Central Coast: Group 3 and 4/Modern Central Coast</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-loop</td>
<td>% of variation</td>
<td>% of variation</td>
<td>% of variation</td>
</tr>
<tr>
<td>Among populations (Fst)</td>
<td>-0.018 101%</td>
<td>-0.005 100%</td>
<td>-0.006 100%</td>
</tr>
<tr>
<td>Among populations within groups (Fsc)</td>
<td>-0.042 -4.17%</td>
<td>0.002 0.20%</td>
<td>0.004 0.40%</td>
</tr>
<tr>
<td>Between groups (Fct)</td>
<td>0.023 2.35%</td>
<td>0.032 3.24%</td>
<td>0.025 2.55%</td>
</tr>
</tbody>
</table>

Compared in the upper table both spatially in the tan sections, and temporally in the gray sections of the graph. The lower table is compared exclusively temporally between different ages of populations in the Central Coast of British Columbia. In the upper table, ancient Eastern Pacific coast consists of CPN and CP samples, modern Eastern Pacific coast consists of CM samples as well as Liu et al. (2011) KI, YB, PI, and NO datasets. Ancient Central Coast consists of CPN samples and modern Central Coast consists of CM samples. In the lower table, all groups are from the Central Coast. Group 3 and 4 span 7000-2000 BP and group 2 spans from 2000 – 1000 BP. The group ‘modern central coast’, otherwise known as group 1, is from Speller et al. (2012).
Only two spatial analyses resulted in differences over 1%. First, the FSc of the cytb ancient Eastern Pacific coast compared with the modern Eastern Pacific coast at 1.4% suggests that a grouping that large is potentially missing a smaller outlying population. The smaller outlying population is most likely the population PI from Liu et al. (2011) paper, which was identified as an outlier in Speller et al. (2012) paper. The second population identified is the Fct between the ancient and modern Central Coast groupings at 1.27%, which could indicate a similarly unique population like PI from Liu et al. (2011). An ‘Exact Test of Sample Differentiation Based on Haplotype Frequencies’ (Raymond and Rousset 1995) confirmed the AMOVA results of non-significance in all cases except for one: the relationship between the ancient Central Coast samples and the ancient Burrard Inlet samples as presented in Speller et al. (2012) paper. In this one exception, the differences between the 38 CPN samples and the 28 CP Burrard samples were found to be significantly different at 0.01572 ± 0.0088, thus indicating populations that are significantly distinct statistically.

Dataset haplotype diversity and nucleotide diversity was determined both spatially and temporally for this ancient Central Coast dataset (Table 6). Spatially, results are very similar to previously published data (Liu et al. 2011; Speller et al. 2012). Much like AMOVA analysis of temporal comparisons, the small sample size of Group 3 and 4 was not adequate to be statistically representative (Table 6). However, there is some indication that some of Central Coast cytb data is slightly more diverse than previously published cytb data (Figure 8). This higher diversity in cytb over D-loop fragments is also supported in the AMOVA analysis as previously described (Table 5).

All datasets, except for CPN group 2, were found to have a significant negative value for Tajima’s neutrality test, indicating that rare alleles are present at low frequencies. Group 2 was found to not have a significant Tajima p-value. These significant negative values could potentially mean that there has been a population expansion after a recent bottleneck. In general cytb fragments were determined to have greater negative values than any of the D-loop fragments, which is expected as cytb is a gene coding region. Modern Central Coast samples were also indicated to have a greater negative D-loop value than ancient Central Coast samples with D values of -1.149 and -0.560 respectively. This difference could mean that a secondary bottleneck
has occurred between the sampling of CPN and CM, thus increasing the negative value in the modern sample.

All herring datasets were also analysed with the programs Network 4.6 and Network Publisher which showed a similar distribution to previously published datasets, however did not result in a particularly localized mutational grouping (Bandelt et al. 1999, Polzin et al. 2003). Figure 9 illustrates the complex nature of Pacific Herring DNA, and the high representation of singletons.

**Table 6**  
*Modern and ancient herring haplotype (h) and nucleotide (π) diversities based on 353bp mtDNA D-loop fragment.*

<table>
<thead>
<tr>
<th>Sampling Sites</th>
<th>Sample codes</th>
<th>n</th>
<th>Haplotypes</th>
<th>h</th>
<th>[SD]</th>
<th>π</th>
<th>[SD]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Namu</td>
<td>CPN 1-12, 32-57</td>
<td>20</td>
<td>19</td>
<td>0.995</td>
<td>[0.018]</td>
<td>0.02883</td>
<td>[0.01209]</td>
</tr>
<tr>
<td>Hurricane Island</td>
<td>CPN 16-31, 58-60</td>
<td>18</td>
<td>17</td>
<td>0.993</td>
<td>[0.021]</td>
<td>0.02567</td>
<td>[0.01169]</td>
</tr>
<tr>
<td>Namu and Hurricane Island Combined</td>
<td>CPN 1-60</td>
<td>38</td>
<td>32</td>
<td>0.989</td>
<td>[0.010]</td>
<td>0.0272</td>
<td>[0.01037]</td>
</tr>
<tr>
<td>Group 1 (Modern Central Coast)</td>
<td>CM 1-72</td>
<td>53</td>
<td>45</td>
<td>0.987</td>
<td>[0.007]</td>
<td>0.02838</td>
<td>[0.01252]</td>
</tr>
<tr>
<td>Group 2 (100 - 2000 BP)</td>
<td>CPN 16-31, 34, 39-41, 49-50, 58-60</td>
<td>23</td>
<td>19</td>
<td>0.98</td>
<td>[0.020]</td>
<td>0.02631</td>
<td>[0.01064]</td>
</tr>
<tr>
<td>Group 3 (2000 - 4000 BP)</td>
<td>CPN 2, 12, 35-36, 38, 42-43, 45</td>
<td>8</td>
<td>8</td>
<td>1.00</td>
<td>[0.063]</td>
<td>0.02827</td>
<td>[0.01281]</td>
</tr>
<tr>
<td>Group 4 (4000 - 7000 BP)</td>
<td>CPN 5-8, 10, 53, 55</td>
<td>7</td>
<td>6</td>
<td>0.952</td>
<td>[0.096]</td>
<td>0.03007</td>
<td>[0.01401]</td>
</tr>
</tbody>
</table>

Ancient samples grouped by location in italic font, modern samples are in bold, and ancient samples are grouped by time in standard font.
Figure 8  Plots of genetic diversity. Graph displays nucleotide diversity and haplotype diversity from this study (Namu, Hurricane Island, and both sites considered together) as well as ancient samples from Speller et al. (2012) and modern Pacific herring data from Liu et al. (2011). All NE Pacific sites group together with the Namu data, with Portage Inlet being the one outlying sample.
Figure 9  Spanning Network displaying Haplotype A (right most grouping), B (top left grouping), and C (bottom grouping). Legend depicts spatial and temporal aspects of samples. Size of pie illustrates the number of samples represented, differing from 1 sample for the smallest and 50 plus samples for the largest. CPN samples are distributed equally between Haplotype B and C, displaying no differences between younger and older samples. Figure was made with medium joining, maximum parsimony, with weights considering transitions, transversions, deletions, and the degree of variable sites differently. No star contraction or other simplifying algorithms were used to preserve the singleton distribution, and distance between samples being representative of genetic distances.
Chapter 5.

Discussion

Authenticity and Success Rate

A good overall rate of amplifiable DNA was found in Pacific herring in this study at 83.3%. This statistic, however, is better understood when broken down by age. As this is the first study analyzing Pacific herring samples older than 4000 years ago, the success rate for a sample bone that is soft and small was unknown. While the 32 younger samples ranging from 100-2600 BP success rate was high and in line with other similarly published data at 90.6%, the 16 older samples from 4000-7000 BP was much lower at 62.5%.

Further exploration indicates that there is a positive correlation between success rate and age (chi-square, Yates’ correction p= 0.0499; (Preacher 2001)). This correlation around age is also evident in the rates of samples with no successful extractions. Ten samples did not amplify at all, of those, six were older than 4000BP and four were younger than 3500BP. When considering the total numbers of these age groups, the older grouping had a higher percentage of failures (Older = 37.5%; Younger = 9% failure rate). For that reason, DNA degradation is likely a key cause of the failed extractions (Pääbo et al. 2004). Another possibility is that even though a gentler decontamination procedure was used, soft older bones could be more susceptible to the process of decontamination used (Kaestle and Horsburgh 2002). Some form of decontamination is necessary. However, future research into whether less destructive decontamination procedures are needed on small, older samples would be beneficial.

No salmon DNA was detected from any of the five herring samples in which salmon primers were used, suggesting that enough salmon DNA did not leech into the samples to be amplified (Willerslev and Cooper 2005). This conclusion is not effected by the single cross species extraction, as it was in the extraction in which salmon sample was added into the same extraction as the herring sample. This also suggests that the herring D-loop primers are specific enough to only target herring. Furthermore, none of
these primers extracted salmon DNA out of the mixed herring/salmon sample. This indicates that the cytb herring primers are not specific enough to target salmon or a variety of other organisms in the Actinopterygii class of ray-finned fish. This non-specificity does open the possibility of the primer being a useful ray-finned fish identification primer when species of the sample is uncertain -- a common issue in fish bone samples in archaeology. In addition, the fact that the cytb primer can potentially bind with other ray-finned fish suggests that the negative samples did not amplify because of contamination or other issues of degradation, not due to them being a different species such as sardine or anchovy.

No contamination was detected in any of the blank controls. This, combined with the extremely high rate of unique haplotypes within the CPN dataset (84.2%) suggest that this is a confirmed authentic dataset and that no cross sample leeching was present. Sequence validity was verified additionally though the repeating of at least one of three fragment in 52.1% of successful samples. These repeats were done with both forward and reverse fragments. Not included in the above repeat percentages is this study’s use of overlapping primers for the D-loop fragments. The use of these overlapping fragments was effectively repeating 46.9% and 44.6% of each the D-loop 1 and 2 fragments respectively, for all of the 38 samples used.

In an ancient DNA study, a number of other issues such as post mortem damages must be identified and overcome, if possible. Maximum Composite Likelihood Estimations of the Pattern of Nucleotide Substitution, specifically investigating the transition/transversion bias, was conducted in an attempt to find any irregularities. Upon comparing all populations separately, it was determined that not only the transition/transversion biases were similar, but the specific likelihood of substitution between each nucleobase to another nucleobase were similarly within a point of likelihood percentage of each other (Kumar 1995). Also, there was no spatial or temporal patterning of statistically distinct genetics among any of the populations. This finding could either suggest that post mortem damage has not radically altered the CPN or CP dataset, or that the likelihood of substitution test is not equipped to make determinations around post-mortem damage probabilities of substitution. Secondly, the likelihood of substitution test supports the conservative rate given to transition/transversion weights in this study’s Network analysis, as almost all substitution rates found in this MCL analysis
were between four and seven. Research indicates that nucleotide substitution can vary considering how many different diverse populations or species included (Kaestle and Horsburgh 2002). Future study should be not to rely on estimates for nucleotide substitution, but instead employ the interesting ‘single primer extension’ or SPEX method (Speller et al. 2012). The SPEX method suggests that the sole cause of authentic endogenous damage causing miscoding lesions are C to U type base mutations. This collaborates previous research into the nature of miscoding damage in ancient DNA (Brotherton et al. 2007; Gilbert et al. 2003; Pääbo et al. 2004). Further research in identifying causes and potentially fixing this miscoding lesion in sequencing could be an excellent step forward for ancient DNA research (Gilbert et al. 2007).

**Re-Sequencing Method**

In addition to traditional repeating samples with an independent PCR, re-sequencing samples from the same PCR was attempted for samples that were messy or failed, as well for successful sequences that could act as a control. Instead of identical results, three types of differences were found. In the first irregularity, four samples had a different number of C in a poly C region. This is understandable as it is largely considered a sequencing byproduct, although a minor example, of slippage (Nucleics 2014). Secondly, the D-loop 1 series I080 in which all successful sequences came out weak and messy in the first amplification, was strong and clear in the re-sequencing. This indicated that the first series sequencing data issue was not related to sample quality or PCR quality, but potentially due to some error during the sequencing set-up. This discovery saved not only reagent, but avoided additional use of limited original sample extract for a repeat amplification. Finally, the re-sequencing of the D-loop 2 fragment of CPN 53 not only appeared positive on the agarose gel, but resulted in a viable sequence when the first sequencing did not. As re-sequencing is much more affordable and easier to do than re-amplification, these preliminary differences show that cost can potentially be saved or a project improved by re-sequencing an individual PCR instead of using expensive reagents that either may not be available or possible in tight budgets. More research is recommended to explore the final cost analysis, and ultimately success of this method.
Sampling Procedures

A number of different strategies were employed to decrease the chance of analyzing multiple bones from the same individual and to prevent any biases in testing. However, it is difficult to determine the ultimate success of these strategies. The specially selected equally spaced vertical distribution of samples collected from a bucket auger results in a decreased chance of analyzing multiple bones from the same individual in this study. Further, not a single sample found in surrounding levels have the same haplotype. However, this vertical distribution can also be a confounding issue as it results in no clear temporarily clustered population, which limits this studies possible statistical analyses. For instance, some genetic statistical methods assume a temporally clustered dataset, more commonly seen in modern population studies, which is not always attainable in ancient DNA (Kaestle and Horsburgh 2002). As this study also sampled bones from sites with permanent settlements, it has the possibility of increasing the chance of sampling non-migratory herring populations that may have been utilized in non-spawning times. Another possibility is the Namu and Hurricane Island residents chose to not fish nearby, and the fish was collected from a non-local population. This dataset can be generally considered to not be a genetically unique population, but can be considered a regional population as tests run, such as AMOVA, have indicated that there is no significant difference in the ancient DNA of herring from the sites of Hurricane Island and Namu.

‘Joint’ Analysis

All three fragments were combined to determine if combining fragments would increase understanding and provide a greater magnitude of inquiry. The analysis of the combined CPN data resulted in 33 distinct haplotypes being labeled out of 36 samples. Generally, the joint CPN haplotype divisions mirror the D-loop divisions as it the more diverse of the two DNA regions. However the sheer number of different haplotypes and the number of unique haplotypes is not representative of the data, as they do not account for the decrease of total samples from 673 to 236. This high diversity increases the possibility for singletons and it does not reflect the possibility of shifting haplotype groupings.
The differences seen in the joint analysis compared to the D-loop or cytb analysis can be categorized in two ways. First, since all three fragments are needed, the number of haplotypes and samples decreased by two, thus shrinking the possible dataset. Secondly, when compared with the ancient Eastern Pacific CP dataset that already had assigned haplotypes, sample CPN 7 and CPN 26 were grouped differently in the D-loop analysis compared to the joint analysis. This ultimately resulted in two additional haplotypes, however within that number, two individual samples could not be considered, and four additional haplotypes were split from previously identified and grouped D-loop haplotypes. Use of this joint analysis has been beneficial in haplotypic and phylogenetic determination. It was not and would not be as beneficial in any analysis that was made more efficient that needed to be identified as a coding region or not, such as AMOVA, estimating the pattern of nucleotide substitution, Tajima’s neutrality estimates, or any future research into Bayesian Skyline Plots. The increased diversity shown in joint analysis underscores the importance of a large dataset in both an individual genome sense and a population sense, and the limitations of a traditional ancient DNA mtDNA study (Millar et al. 2008; Speller et al. 2012).

Phylogenetic and Network Implications

In phylogenetic studies today, there are many powerful software programs that have large numbers of adjustable variables that allow the researcher to closely fit conditions of a number of different scenarios. The programs Network and MEGA 6.0 were chosen in this study because they can consider non re-combining datasets, and also permit a variety of special settings appropriate for this study.

In Network, median-joining was determined to be the best algorithm as it allows multi-state data, which are positions in the fragment with numerous different mutations. Sample ‘weights’ were changed generally in a conservative way for computations considering sample relationships. The function of weighting is to increase weights on less likely events and decrease weights for more likely events. This is especially important for coding regions of the genome, and thus codon location was considered in the cytb fragment analysis. In both D-loop and cytb, hyper-variable sites were down-weighted from the default of ten as well. Transversions weights were increased by three
times, in relationship to transitions because transversions occur less frequently in
mtDNA. The exact ratio between the transitions and transversion varies depending on
species; the ratio has not yet been determined for Pacific herring. The rate of three times
weight change was chosen as recommended in the Network user manual, which is
considered conservative, especially considering the rate of transversions is about 20
times less often than transitions in human mtDNA (Bandelt et al. 1999; Nei 1987; Polzin
et al. 2003). The epsilon was kept at a default of ten, due to the highest weight being no
higher than 30, as recommended by the user guide. Despite this being a highly diverse
population, the user guide recommendations were not followed in relation to using
contraction methods. No contraction methods such as star contraction or ‘frequency > 1
Criterion’ were used. This decision was made in an effort to keep the original high
occurrence of singleton haplotypes visible.

When analyzing the Network results, a number of ‘reticulations’ and ‘cubes’ are
present in the central region and a few are in the peripheral regions of the D-loop (Figure
9). Presence of these structures is a common occurrence in central regions, however
existence of these in peripheral areas are not necessarily incorrect but can point to
problems in sampling or processing. Conversely, having no peripheral cycles in
peripheral areas does not guarantee a problem free dataset (Bandelt et al. 1999;

In MEGA 6.0, the Best-Fit Substitution Model test was run. This test suggests the
Tamura 3-parameter method is best fit if used with the discrete Gamma distribution with
five rate categories (Tamura et al. 2013). Two maximum likelihood phylogenetic trees
were created and bootstrapped 500 times. The D-loop tree utilized the Tamura 3-
parameter method with Gamma distribution. The cytb tree also utilized the Tamura 3-
parameter method with the Gamma distribution, but it also assumed some sites in the
fragment are invariable as cytb is a coding region of mtDNA.

The phylogenetic trees created of the CPN data fit previously published
expectations of Pacific herring datasets (Speller et al. 2012; Liu et al. 2011) and
furthermore, make phylogenetic sense. Within the D-loop dataset, CPN samples fall
equally between the established haplotype B and C. Within the cytb dataset, a traditional
phylogenetic tree was not clearly interpretable as no groupings clearly emerged. The
Network graph for cyt b, clearly shows one grouping encompassing the majority of samples (172), with only a few smaller groupings, the largest of which being only 26 in number (Appendix C). The current data does not allow us to make an inference about the presence of a unique population of Pacific herring at either Hurricane Island, Namu, or the sites combined. This inability to make an inference is due partially to the diverse nature of samples in the D-loop region as well as limitations in study sample size. A larger sample size would also increase the number of statistical programs available for analysis that are currently limited to modern studies (Waples and Yokota 2007). Speller et al. (2012) paper additionally suggests a low discriminatory power of mtDNA and recommends future investigations in SNPs and, if possible, microsatellites.

**Impact on Understanding: Sites**

In this study, Pacific herring genetics were analyzed for both spatial and temporal comparisons, and for the most part, no significant correlation could be found between the Hurricane Island and the Namu sites. The dataset available, did not suggest genetically unique populations at Hurricane Island and Namu, thus the two populations were most appropriately treated as one for the majority of this study. Due to the high number of singletons, tracking the rate of haplotypes through time was not possible with the current dataset sample size. However Liu et al. (2011) previously considered a population just 13.7% larger, which was sufficiently ample to suggest the Portage Inlet (PI) population has a unique genetic population today. Thus, the CPN dataset presented in this thesis is potentially large enough to find a unique population, however this dataset does not suggest a unique genetic population through AMOVA, \( h \) or \( \pi \) analysis as the PI population does in Liu et al. (2011). It is therefore reasonable to conclude that no philopatry is suggested by this data.

Interestingly, there was one distinct population that was distinct from nearly all Central Coast populations, both ancient and modern. The ancient Burrard Inlet samples in Speller et al. (2012) study when compared through an 'Exact Test of Sample Differentiation Based on Haplotype Frequencies' analysis, significantly differed from the Central Coast samples. The results of AMOVA and Network, however, do not suggest that either are a unique population when compared to all other available datasets of
Eastern Pacific herring. It is possible that for whatever reason, individuals that spawned in Burrard Inlet did not and currently do not spawn in the Namu/Hurricane Island sites, therefore suggesting some sort of spatial divide is present that existed both in the past and present.

Another interesting occurrence is the presence of an individual with haplotype ‘A’ mtDNA in the modern Central Coast and no ‘A’ haplotype in the ancient samples. This could be an issue of a small ancient sample sizes, a fluke individual in modern populations, or if it is an accurate representation, it could suggest a more recent migration from at least as far north as Alaska. This latter possibility could represent a repopulation movement from the north to the Central Coast area due to the over fishing that occurred in the Central Coast during the 20th century (Therriault 2007). If this ‘re-population from the north’ occurred, it would be difficult to test. It could be further researched with nuclear DNA studies, microsatellites, and SNPs. These tests could help illuminate finer details around the Pacific herring population, and potentially more specific locational markers that can be traced to a specific location. While it is more difficult to compare ancient to modern samples due to degradation, cost, and issues in software analyses, these results suggest that the effort and cost is advantageous.

Hay and McKinnell’s (2002) tagging analysis suggested that Pacific herring could stay in schools for years due to their strong tight schooling behaviour and not necessarily exhibit site fidelity to the original place of hatching. Populations have also been found to mostly stay within the general region of hatching, however there was some mixing of regions observed in the tagging study (Hay and McKinnell 2002). Small et al. (2005) noted a genetically unique population. When considering Small et al. (2005) study in context to Hay and McKinnell (2002) research, it is reasonable to hypothesize that Pacific herring is a species that can easily school with nearby hatch-mates for years and tend to move around in the surrounding area. This schooling pattern does not exclude the distinct populations that exhibit site fidelity due to remote location or unique conditions that require a different spawning time such as Cherry Point herring (Gustafson et al. 2006; Small et al. 2005). This schooling pattern seen in Hay et al. (2002) could form groups that spawn together for years in addition to exhibiting site fidelity. In the future, studies need to be on a site level to determine distinct populations
as these genetically unique populations can be formed by regional stochastic events (Hede et al. 2005; Liu et al. 2011; Small et al. 2005).

**Impact on Understanding: Herring and Wildlife Conservation**

Tajima’s neutrality test was conducted on each dataset individually and the results were then compared. All values were found to be statistically significant and suggest that the ancient Central Coast samples (CPN) is a population expansion after a recent bottleneck that occurred previous to 7000 BP. This pre-7000BP bottleneck is collaborated by other ancient population studies such as Grant et al. (2012) who estimated it to have occurred during the Pleistocene era. When analyzing modern Central Coast data collected in 2011, there is larger deviation from zero than in the ancient samples which have been dated from 7000 – 100 years ago. This could suggest that a secondary bottleneck has occurred between the sampling of the ancient and modern samples, which would make sense when considering the historical record of over-exploitation of Pacific herring in the late 19th and 20th centuries. This study is the first to suggest a secondary genetic suggestion of a secondary bottleneck between 100 years ago and 2011 is a first (Grant et al. 2012; Therriault 2007).

Due to the major bottleneck in the Pleistocene that is theorized to have erased previous population changes, Bayesian Skyline analysis is not an optimal method for Pacific herring (Grant et al. 2012). An excellent test of this hypothesis in future research would be testing the ancient DNA of samples older than the last Pleistocene. Unfortunately, acquiring such data would be near to impossible for Eastern Pacific populations due to the lack of known pre-Holocene archaeological sites.

Even though DNA is a relatively new and powerful method of determining discrete genetic stock structure, it is important to remember the limitations in our understanding of Pacific herring genetics and the commonly used non-coding markers. There are examples in the literature of known discreet herring stocks not registering as unique genetic populations due to the fragments selected for amplification (Small et al. 2005). This difficulty in determining genetically advantageous genes that define a population is not an error of the researcher as much as it is an avenue of future
research. This must also be tempered with the fact that not all behaviour or groups are simply defined by genetics (Hay and McKinnell 2002; Small et al. 2005). If spawning timing and location is a learned behaviour, it is equally important to protect the stocks of herring that exhibit site fidelity though learned behaviour as it is possible that only a proportion of the population is genetically similar, and may not appear to be a unique population.

Genetics is a powerful tool for determining discreet populations, however discovering these populations is hindered by the large range of Pacific herring. Although this particular analysis does not suggest the existence of discrete population on the Central Coast, it is possible that one will be indicated when a larger sample size or different methods are used in the future. Regardless, this study helps lay the groundwork for any future mtDNA studies utilizing next generation sequencing. This study should encourage researchers to use ancient nuclear DNA over traditional fragment based ancient mtDNA analysis.
Chapter 6.

Conclusions

For decades, researchers have debated whether the Pacific herring exhibited philopatry, with data seeming to point to many different conclusions. Distinct populations have been recognized in studies based on timing (Hay et al. 2001), genetics (Wildes et al. 2011), and salinity (Griffin et al. 1998). Additional studies have been run that have failed to find any distinct populations including studies based on timing (Hede et al. 2005), using stable isotopes of otoliths (Gao et al. 2001), genetics (Beacham et al. 2008), and morphology (Rosenberg and Palmen 1982; Ryman et al. 1984). Ancient DNA is a field that is rapidly advancing and becoming more interdisciplinary, and Pacific herring is an important species biologically, economically, and culturally. This research aimed to apply traditional fragment based ancient DNA methods to the question of philopatry and site fidelity by utilizing mitochondrial DNA, as well as investigate the population diversity and preservation in some of the oldest samples of the species.

Ancient DNA analysis was applied successfully from herring remains recovered from two sites located in the Central Coast region of British Columbia, Namu and Hurricane Island, for the purposes of discerning population patterns for wildlife conservation. The result of ancient DNA analysis of 60 herring remains suggested that: 1) DNA is well preserved at these sites from 100 – 4000 years BP and moderately well preserved in samples from 4000 – 7000 years BP; 2) all successful samples were indicated to be herring and not a similarly sized pilchard species; 3) no DNA ‘leeching’ from salmon was seen in any samples tested, nor other pilchards in cytb tests; 4) using a ‘joint’ alignment was useful in haplotyping, but was not useful in other statistical analyses; 5) re-sequencing the same PCR amplification in certain situations is a useful step to conserve original DNA extract and potentially save costs; 6) traditional mtDNA studies are not the most effective form to detect site fidelity or population changes in herring through time.

A high number of successful repeats, completely blank negative controls, and a low number of repeat haplotypes, suggest an authentic and positively confirmed dataset.
However, the great diversity present with few repeated haplotypes suggest that this population is not adequately sampled, thus significantly limiting the theoretical implications. While traditional ancient mitochondrial DNA sampling is more affordable than many more recent innovations in ancient DNA research and therefore could be more useful for wildlife conservation, these findings confirm that traditional fragment based ancient mitochondrial DNA is limited and less powerful for revealing population information around population patterns and composition of Pacific herring (Speller et al. 2012).

Although this mitochondrial DNA data failed to identify a genetically unique population of herring at Namu, future nuclear DNA studies hold the potential to more effectively determine unique populations. Unique populations have been found in locations that are geographically isolated, have conditions that support unique spawning timing, and/or have oral histories that suggest that the population is different in some way from other groups of herring (Hebe et al. 2005; Small et al. 2005; Stout et al. 2001). Future research should continue to look for and test sites that have a higher likelihood of unique populations as well as expanding into nuclear DNA studies that target advantageous genes.

Finally, in conjunction with each of these suggestions, obtaining a larger number of samples from more closely related time periods could assist in future research. Sampling evenly from augers were an affordable way to access deep deposits and ensure different individuals were not sampled multiple times without heavily disturbing the site. When more samples are made available, the range of the samples ages can be significantly reduced, making archaeological populations more equivalent to a ‘population’ that is defined in wildlife conservation and population statistics (Leonard 2008).

Hopefully this study and others like it will continue to help reinforce the growing role for ancient DNA in conservation management and may lead to ancient DNA becoming a standard aspect of governmental agency research and reports. Investigating individual herring sites is helping to ensure strong herring genetic diversity by discovering unique populations for protection. As a result, herring populations will be better able to handle challenges from the environment, the climate, and predators.
Through an improved understanding of ancient genetic diversity, modern genetic population loss can be better understood and managed both today and into the future.
References

Altschul, Stephen F.; Warren Gish; Webb Miller; Eugene W. Myers; & David J. Lipman.

Bandelt, Hans-Jürgen; Peter Forester; and Arne Röhl

Beacham, T. D.; J. F. Schweigert; C. MacConnachie; K. D. Le; and L. Flostrand

Bishop, Mary A.; and S. P. Green

Brotherton, Paul; Phillip Endicott; Juan J. Sanchez; Mark Beaumont; Ross Barnett; Jeremy Austin; and Alan Cooper

Cai, Dawei; Yang Sun; Zhuowei Tang; Songmei Hu; Wenying Li; Xingbo Zhao; Hai Xiang; Hui Zhou
2014 The origins of Chinese domestic cattle as revealed by ancient DNA analysis.  

Cannon, Aubrey  

Carlson, Roy  

Chan, Yvonne L.; Christian N. K. Anderson; and Elizabeth A. Hadly  

Cleary, Jaclyn S.; Jacob F. Schweigert; and V. Haist  

Cooper, Alan; and Hendrik N. Poinar  

DeSalle, Rob; and George Amato  
Excoffier, L. G. Laval; and S. Schneider


Fisheries and Oceans Canada, and Department of Environment


Fisheries and Oceans Canada


Gao, Y. W.; S. H. Joner; and G. G. Bargmann


Gilbert, M. T. P.; Anders J. Hansen; Eske Willerslev; Lars Rudbeck; Ian Barnes; Niels Lynnerup; and Alan Cooper


Gilbert, M. T. P.; Jonas Binladen; Webb Miller; Carsten Wiuf; Eske Willerslev; Hendrik Poinar; John E. Carlson; James H. Leebens-Mack; and Stephan C. Schuster

Grant, W. S.; Ming Liu; TianXiang Gao; and Takashi Yanagimoto


Griffin, Frederick J.; Murrali C. Pillai; Carol A. Vines; Juha Kaaria; Thea Hibbard-Robbins; Ryuzo Yanagimachi and Gary N. Cherr


Gustafson, Richard G.; Jonathan Drake; Michael J. Ford; James M. Myers; Elizabeth E. Holmes; and Robin S. Waples


Haegele, C.W.; J.F. Schweigert


Haig, Susan M.

Hall, Tom A.

Hauser, Lorenz; and James E. Seeb

Hay, D. E.

Hay, Douglas E.; Bruce McCarter; Kristen S. Daniel; and Jacob F. Schweigert

Hay, Douglas E.; P. Bruce McCarter; and Kristen S. Daniel

Hay, Douglas E.; and S. M. McKinnell

Hay, Douglas E.; K. A. Rose; Jacob Schweigert; and B. A. Megrey
2008 Geographic variation in North Pacific herring populations: Pan-Pacific

Hede Jørgensen, Hanne B.; Michael M. Hansen; and Volker Loeschcke

Herring School

Hester, J.J.; S.M. Nelson (editors)
1978 *Studies in Bella Bella Prehistory. Department of Archaeology, Simon Fraser University, Publication 5.* Burnaby, British Columbia: Simon Fraser University.

Kaestle, Frederika A.; and K. A. Horsburgh

Knapp, Michael; and Michael Hofreiter

Leonard, Jennifer A.
Lepofsky, Dana; and Ken Lertzman


Librado, P.; and J. Rozas


Limborg, Morten T.; Sarah J. Helyar; Mark De Bruyn; Martin I. Taylor; Einar E. Nielsen; Rob Ogden; Gary R. . . Carvalho FPT; and Dorte: Bekkevold


Liu, Jin-Xian; Andrey Tatarenikov; Terry D. Beacham; Victor Gorbachev; Sharon Wildes; and John C. Avise


Martindale, Andrew R.; Bryn Letham; Duncan McLaren; David Archer; Meghan Burchell; Bernd R. Schone

McKechnie, Iain; Dana Lepofsky; Madonna L. Moss; Virginia L. Butler; Trevor J. Orchard; Gary Coupland; Fredrick Foster; Megan Caldwell; and Ken Lertzman

Millar, Craig D.; Leon Huynen; Sankar Subramanian; Elmira Mohandesan; and David M. Lambert

Mitchell, Danielle; Patrick McAllister; Kurt Stick; and Lorenz Hauser

Moss, Madonna L.; and Aubrey Cannon (editors)

Moss, Madonna L.; Antonia T Rodrigues; Camilla F. Speller; Dongya Y. Yang

National Oceanic and Atmospheric Administration
Nucleics

2014 DNA sequencing troubleshooting. Electronic document,
https://www.nucleics.com/DNA_sequencing_support/DNA-sequencing-AT-slippage.html,

Pääbo, Svante


Pääbo, Svante; Hendrik Poinar; David Serre; Viviane Jaenicke-Despres; Juliane Hebler; Nadin Rohland; Melanie Kuch; Johannes Krause; Linda Vigilant; and Michael Hofreiter


Pauly, Daniel


Poinar, Hendrik N.

2003 The top 10 list: criteria of authenticity for DNA from ancient and forensic samples. *International Congress Series* 1239(0):575-579.

Polzin, T.; S V Daneschmand

Preacher, K.J.


Ramakrishnan, Uma; Elizabeth A. Hadly; and Joanna L. Mountain


Røed, Knut H.; Gro Bjørnstad; Øystein Flagstad; Hallvard Haanes; Anne K. Hufthammer; Per Jordbøy; Jørgen Rosvold


Rosenberg, R.; and L. E. Palmen

1982 Composition of herring stocks in the Skagerrak-Kattegat and the relations of these stocks with those of the North Sea and adjacent waters. Fisheries Research 1:83-104.

Ryman, N.; U. Lagercrantz; L. Andersson; R. Charkraborty; and R. Rosenberg


Shapiro, Beth; Russell W. Graham; and Brandon Letts

2014 A revised evolutionary history of armadillos (Dasypus) in North America based on ancient mitochondrial DNA. Boreas Early View Online.
Small, Maureen P.; Janet L. Loxterman; Alice E. Frye; Jennifer F. Von Bargen; Cherril Bowman; and Sewall F. Young

Speller, Camilla F; Lorenz Hauser; Dana Lepofsky; Jason Moore; and Antonia T. Rodrigues

Stout, H.A.; R. G. Gustafson; W. H. Lenarz; B. B. McCain; D. M. VanDoornik; T. L. Builder; and R. D. Methot

Tamura, Koichiro; Masatoshi Nei; and Sudhir Kumar

Tamura, Koichiro; Glen Stecher; Daniel Peterson; Alan Filipski; and Sudhir Kumar

Therriault, Thomas W.
2007 Area 9 Herring: a review of available information for stock assessment
Waples, Robin S.; and Masashi Yokota


Ware, D. M.; and C. Tovey


Willerslev, Eske; and Alan Cooper

2005 Ancient DNA. *Proceedings: Biological Sciences* 272(1558):3-16.

Yang, Dongya Y.; Aubrey Cannon; and Shelley R. Saunders


Yang, Dongya Y.: Barry Eng; John S. Waye; J. Christopher Dundar; and Shelley R. Saunders


Yang, Dongya Y.; and Kathy Watt

Appendices
<table>
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<th>CPN Sample</th>
<th>Date range (°C years BP)</th>
<th>PCR Analysis: Successful / Number of times tried (% Worked)</th>
<th>Depth (cm) Below Surface</th>
<th>Auger</th>
<th>Site</th>
<th>Site Name</th>
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<td>64 - 87</td>
<td>D1</td>
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<td>1/1 (100%)</td>
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Appendix B.

Assigned Haplotypes and Confidence

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Appendix C.

Network Analysis of CytB samples
Appendix D.

Phylogenetic Analysis of Ancient D-loop Central Coast samples by Median Age
Appendix E.

Ancient dloop and cytb sequences aligned from Hurricane Island and Namu (CPN)

Filename: final-alignments.zip