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Genetic Substructure of Pacific Harbor Seal (Phoca vitulina richardsi) Populations Along the Coasts of Oregon and Washington

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by

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Abstract

It has been assumed that the considerable dispersal ability of many marine species would prevent genetic divergence in the absence of geographic isolation. However, recent work has shown that many marine species often develop differentiation among areas within their known dispersal range. This ‘paradox’ of marine divergence is particularly important among marine mammal species where behavior can restrict gene flow. To investigate genetic substructure within such a species, I used tissue samples collected from 63 stranded Pacific harbor seals (*Phoca vitulina richardsi*) in Washington and Oregon between 2006 and 2010 for population stock analysis. DNA was extracted from frozen tissues, and a 551 bp fragment of mtDNA control region sequence and eight microsatellite loci were amplified to investigate localized genetic structure. Minimum spanning network and haplotype frequency analyses of mtDNA sequences indicated that while haplotype lineages are not isolated within a sampling region, there is some evidence of regional differentiation. On the other hand, microsatellite data suggest a lack of substructure among the animals sampled, with only a weak signal of limited gene flow between Puget Sound and coastal areas. Biased dispersal among age classes was also suggested, with juveniles showing less differentiation among areas than adults. Regardless of the historical scenario which has led to this complex pattern of genetic structure in Pacific harbor seals across the Pacific Northwest, my results suggest higher levels of exchange among areas than previously suspected, and will have important consequences for future management considerations for these stocks.

Dedication

This thesis is dedicated to Dr. Nina Karnovsky and Dr. Diane Thomson of the Claremont Colleges, whom I must thank for introducing me to biology research, believing that I belong here, and tirelessly encouraging excellence in their students. They are both amazing examples to young women in science.

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Chapter 1: Introduction

1.1 Genetic Techniques and Wildlife Conservation

The successful protection and management of wildlife populations relies on accurate descriptions of the genetic and demographic structure of a population. The ability to quantify immigration or emigration in a population, and therefore the overall levels of gene flow among populations of concern, is integral to identifying units within a region or species that warrant protection (Moritz 1994a, Paetkau 1999). The genetic variability within a population is an important gauge of fitness and the likelihood the population will persist in the event of a selective sweep (such as a disease epidemic), and thus is an important metric guiding the management of a population (Mitton 1994). Previously, wildlife conservation entities were determined largely by convenient geographic barriers or other supposed limitations to gene flow, but recently the use of genetic markers has been essential in describing the relative connectivity or isolation of wildlife populations, and has become central to wildlife management practices (Mitton 1994, Moritz 1994a, DeYoung and Honeycutt 2005).

The definition of intraspecies groups that warrant conservation effort and protection has been evolving with the increased use of molecular markers to indicate differentiation. Such groups are currently recognized under two classes: 'Evolutionarily Significant Units' (ESUs) and 'Management Units' (MUs) (Ryder 1986, Moritz 1994a). The intent of identifying ESUs with unique evolutionary histories/trajectories for management is to preserve the evolutionary heritage and potential among a set of ESUs for a species in the face of future selection (Moritz 1994a). For a species group to be considered an ESU,

isolation and divergence from other conspecifics must have been maintained long enough for 'meaningful genetic divergence' to have occurred and be concordant with divergence shown by non-molecular traits (Ryder 1986, Moritz 1994a, Paetkau 1999). Meaningful genetic divergence can be recognized as a pattern reciprocal monophyly for alleles at mitochondrial DNA (mtDNA) loci and significant divergence of allele frequencies among nuclear DNA loci (Moritz 1994b). However, considering that it could take thousands of years to establish reciprocal monophyly of mtDNA alleles, even in populations of only a few hundred individuals, it has been proposed that genetic divergence alone is not adequate to define ESUs (Paetkau 1999). Given that genetic changes in small, isolated populations can progress more quickly than predicted (Templeton 1980), but that waiting until markers are fixed at monophyly could take longer than an unprotected population may persist in the face of anthropogenic changes, it is appropriate to also include other types of data (e.g. morphological or ecological divergence, estimated divergence time, etc.) in the absence of complete genetic divergence to define an ESU (Ryder 1986, Paetkau 1999).

Unlike ESUs, which are delineated to preserve the long-term evolutionary potential of a species, the purpose of MUs is to identify populations that are currently experiencing low levels of gene flow which make them functionally isolated (Moritz 1994a). The persistence of a MU is much more likely to be determined by demographic trends rather than evolutionary processes, and therefore the conservation objective for these groups is to maintain functional persistence through short-term management given current population structure (Moritz 1994a). A MU, which can be considered synonymous with a management 'stock', can be identified by patterns of allele frequency divergence at

nuclear or mtDNA loci in the absence of allele monophyly within groups (Moritz 1994a). However, because the detection of such patterns are very sensitive to the type and number of loci used and it is not clear precisely how much differentiation in allele frequencies indicates a functionally isolated group, the addition of movement data can enhance the identification of isolated MUs (Paetkau 1999). Thus, the ecological and demographic dynamics of species and intraspecific groups continue to be important to conservation management when evaluating genetic data. For determination of MUs to lead to successful management, the characteristics of genetic markers used as well as the ecological setting, demographic history, and current genetic structure of the study system must be considered.

1.2. Molecular Markers for the Study of Wildlife Populations

Many types of molecular markers have been identified for the study of genetic structure among wildlife populations. These include allozymes, restriction fragment length polymorphisms (RFLPs), single nucleotide polymorphisms (SNPs), sequence data, and short tandem repeats (STRs or 'microsatellites'), among others (DeYoung and Honeycutt 2005). Each of these approaches has its strengths as well as limitations for the detection of genetic structure, however further discussion of these characteristics will be limited to the markers used in this study; mitochondrial DNA sequences and microsatellites.

1.2.1 Mitochondrial DNA Sequences

Animal mitochondrial DNA (mtDNA) is a maternally inherited, duplex covalently closed circular DNA molecule about 16-20 kilobases long (Avise et al. 1987, Moritz et al.

1987). There are several genes in this genome that appear to be highly conserved, including two ribosomal RNA genes, 22 transfer RNA genes, and 13 genes which code for electron transport or ATP synthesis enzyme subunits. There are few intergenic sequences, but there is a “control” region that lacks structural genes and is instead important in the initiation of replication and transcription (Moritz et al. 1987). Within the control region there are three recognized domains; the central domain and the two flanking (ETAS and CSB) domains. The central domain is known to be conserved among and within species, while higher mutation rates in the flanking regions account for the species-specific variability in control region mutation rates (Pesole et al. 1999). In particular, the displacement loop (D-loop) of the vertebrate control region is an important replication structure which is nontranscribed and considered selectively neutral, allowing mutations to accumulate rapidly in this region (Avise et al. 1987). Over the whole mtDNA genome, mutational differences are known to accumulate at a much faster rate than comparable single-copy nuclear DNA for vertebrates (Pesole et al. 1999). While the rate of mutation is generally considered to be one to ten times faster than single-copy nuclear DNA, the relative rate of mtDNA mutation is known to be variable even among closely related taxa (Avise et al. 1987, Pesole et al. 1999). The majority of these changes are single nucleotide substitutions in either the control region or silent third codon positions, though a smaller number of insertions or deletions (collectively 'indels') of variable size are sometimes seen, particularly in the control region (Avise et al. 1987, Moritz et al. 1987).

Mitochondrial sequences are effectively haploid for an individual. Their maternal mode of inheritance precludes recombination, so these sequences represent a single

inherited locus with a large number of possible character states among individuals defined by sequence changes (Avise et al. 1987). Changes in mtDNA sequence are known to accumulate quickly in early stages of population and species differentiation, followed by a reduction in rate that eventually leads to a plateau of nucleotide substitution due to sequence saturation and reversals (Avise et al. 1987). This relatively brief period of high differentiation rate confines the usefulness of highly variable mtDNA regions (such as the control region) to the study of conspecific populations and closely related species (Avise et al. 1987). However, population-level studies which take advantage of these features of neutral mtDNA regions have successfully established population structure for a range of wildlife species (for reviews see Avise et al. 1987 and Moritz 1994b), including detecting genetic divergence in cryptic species such as the North American brown bear (*Ursus arctos*), mule deer (*Odocoileus hemionus*), and Pacific blueline surgeonfish (*Acanthurus nigroris*) (Waits et al. 1998, Latch et al. 2009, DiBattista et al. 2011).

1.2.2 Microsatellite Loci

The nuclear genome of almost all eukaryotes contains multiple regions of short, simple repetitive elements which are actually five to ten times more common in the genome than non-repetitive motifs of similar size (Bruford and Wayne 1993). The smallest of these elements, consisting of repeat units of less than 5 DNA base pairs (bp) in length, are known as 'short tandem repeats' (STRs) or microsatellites. The number of repeat units at such sites is highly variable, presumably due to slippage during the replication and transcription process, which leads to polymorphisms in the sequence length at a given locus. This slippage process is believed to fit a 'step-wise' mutational

model, in which alleles separated by a single repeat unit are more closely related than alleles separated by a multiple units in length (Bruford and Wayne 1993, Slatkin 1995). The prevalence of these loci, as well as their high levels of variability, short lengths, codominant inheritance, and ease of amplification and allele scoring has made microsatellites a widely-used marker in studies of human and wildlife populations (Bruford and Wayne 1993, Paetkau et al. 1995, Goldstein et al. 1999, Luikart and England 1999, King et al. 2001, Narum et al 2008; for reviews see Bruford and Wayne 1993 and Balloux and Lugon-Moulin 2002).

The utility of microsatellite loci in studies of rare species or populations for which we have little previous genetic information is augmented by the tendency of primers developed in one species to amplify polymorphic loci across many related species (Bruford and Wayne 1993, Coltman et al. 1996). In addition, amplification of eight microsatellite loci has been deemed sufficient to detect demographic changes such as recent population bottlenecks in experimental conditions, and to estimate severity of population size reduction (Spencer et al. 2000). Wildlife population studies using microsatellites have revealed fine-scale population structure within species and have identified historical trends likely to have shaped the evolution of species (Jordan and Snell 2008, Narum et al 2008). Studies of mating systems in pinniped populations have also capitalized on the high levels of polymorphism in microsatellites to conduct extensive paternity analyses which have revealed unexpectedly high variability in the reproductive success of non-dominant males in different pinniped species (Coltman et al. 1998a, Wilmer et al. 1999). These loci also appear to be important indicators of the overall health of a population as measured by genetic diversity (Coulson et al. 1998, Da

Silva et al. 2005). In pinnipeds, variation in microsatellite loci has been positively correlated with increased birth rate and neonatal survival (Coltman et al. 1998b, Kretzmann et al. 2006), as well as reduced parasite loads in Atlantic harbor seals *Phoca vitulina vitulina* (Rijks et al. 2008). The ability of microsatellite techniques to detect subtle population structure as described above have made it a marker of choice for studying intraspecific variation in wildlife populations (DeYoung and Honeycutt 2005).

The use of any single DNA marker to evaluate patterns of divergence and speciation among groups inherently assumes that the gene tree is congruent with the actual evolutionary history of the groups examined (a.k.a. the organism or species tree). This assumption is not always met due to lineage-specific differences in inheritance patterns among loci (Degnan 1993). The only way to detect such differences is through the use of more than one type of molecular marker, because the evolution of populations is more likely to be truly represented by genetic loci if the pattern can be confirmed through multiple different markers (Slade et al. 1994). As such, the most robust approach for studying population differentiation and intraspecific genetic variability is to combine the use of multiple markers with different modes of mutation and inheritance, such as the concurrent use of both mtDNA and microsatellite markers.

1.3. Population Divergence in Marine Species

The paradigm of allopatric divergence that dominates terrestrial speciation models assumes that patterns of geographic distribution and genetic differentiation among terrestrial organisms can often be understood as the result of historical and environmental factors (Avise 1994, Scribner et al. 2005). However, in a marine environment the

apparent paucity of physical barriers and the tendency of marine organisms to be highly vagile (at least in juvenile stages) would make speciation by these mechanisms seem unlikely (Palumbi 1992, Palumbi 1994, Leray et al. 2010). Still, in many species of marine fishes, invertebrates, and even mammals, analysis of population structure has revealed genetic subdivision within a population range (Palumbi 1992, Cronin et al. 1996, O'Corry-Crowe et al. 2003, Hoffman et al. 2006, Wolf et al. 2008, Leray et al. 2010). In response to the apparent contradiction inherent in these examples, several isolation mechanisms that are not driven by dispersal barriers in marine systems have been proposed (Knowlton and Jackson 1994, Palumbi 1994). Specifically, it has been suggested that this so-called 'marine-speciation paradox' (Bierne et al. 2003) can be understood if we consider life history characteristics of some marine species, which “may be the primary influence on the amount of genetic differentiation among populations of marine organisms rather than barriers to dispersal or geographic distance” (Stanley et al. 1996).

The behavioral ecology of certain marine species and populations has the potential to influence genetic structure by affecting gene flow (Chesser 1991a, Chesser 1991b, Palumbi 1994). For example, reproductive asynchrony has lead to restricted gene flow in many marine species, including fishes, corals and gastropods (Knowlton 1993, Palumbi 1994). In some marine mammal species, such as eastern Pacific harbor seals (*Phoca vitulina richardsi*), clines in the timing of the breeding season are offset by up to two months between breeding colonies in the same region (Temte et al. 1991). In addition, intra- and interspecific niche segregation in foraging ecology appears to have driven speciation in the Galapagos sea lion (Wolf et al. 2008). Female site-fidelity has

also been linked to genetic differentiation on a smaller scale than predicted for highly vagile marine species such as green sea turtles (*Chelonia mydas*), California sea lions (*Zalophus californianus*) in the Gulf of California, and Steller sea lions (*Eumetopias jubatus*) based on mtDNA studies (Chesser 1991a, Bowen et al. 1992, Maldonado et al. 1995, Bickham et al. 1998, Schramm et al. 1999). In other species of marine mammals, greater female philopatry restricts gene flow, and genetic mixing among populations is dependent on male-mediated gene flow (Escorza-Trevino and Dizon 2000, Herreman et al. 2009). Considering the potential for genetic differentiation to be driven by behavioral ecology, it is critical to evaluate possible genetic divergence even in widely dispersing marine species, and to consider the natural history of a species when predicting how such patterns may develop.

1.4. Study System

1.4.1. Natural history of *P. vitulina richardsi*

The eastern Pacific harbor seal is one of five recognized subspecies of harbor seal (*Phoca vitulina*, Linnaeus 1758), a species in the monophyletic family Phocidae (Berta et al. 2006). *P. vitulina richardsi* is one of the most common and widely distributed pinniped subspecies in the northern oceans, and maintains a nearly continuous range along the west coast of North America as far north as the eastern Aleutian Islands and the Bering Sea, and southward to several offshore islands of Baja California (Jeffries and Newby 1986). Pacific harbor seals only inhabit temperate, ice-free waters, and while they may occasionally haul out on ice floes in glacial bays they are primarily found hauled out on sandy and pebble beaches, sandbars, and intertidal rock outcroppings,

which are important habitats for resting, breeding, pupping and molting. These seals are frequently associated with shallow bays and estuaries, and may be found as far as 180 miles inland along major rivers (Jeffries and Newby 1986).

Most closely related to the other small-bodied arctic seals (i.e. the Ribbon seal (*Histriophoca fasciata*), Harp seal (*Pagophilus groenlandicus*), Baikal seal (*Pusa sibirica*), Caspian seal (*Pusa caspica*), Ringed seal (*Pusa hispida*) and Largha seal (*Phoca largha*); Arnason et al. 1995, Berta et al. 2006), all harbor seals have short, robust bodies with a broad head and snout, short foreflippers and pelage that ranges from light tan to silver-gray with variable density of dark spots or circles (Reeves et al. 2008). Adult males and females exhibit much subtler sexual size dimorphism than polygynous phocids, with males reaching 190 cm in length and weighing up to 170 kg, while females will reach about 170 cm in length and weigh up to 130 kg (Jeffries and Newby 1986, Reeves et al. 2008). There is a noticeable difference, however, in the average lifespan of Pacific harbor seals between males and females (25 years vs. 35 years, respectively). Although shorter-lived, males still take longer than females to become reproductively active, reaching sexual maturity at 4-5 years of age as opposed to females' 3-4 years. Harbor seals mate aquatically, and although males may display and vocalize to attract the attention of females, they do so without holding onto territories (Reeves et al. 2008). Males are serial monogamists, pairing with one female for breeding until she leaves estrus, and sequentially moving on to another estrus female throughout the breeding season. Pups are born from April to September, although there is an obvious latitudinal cline in local pupping seasons, with pupping beginning earliest at lower latitudes and starting in progressively later months at higher latitudes (Reeves et al. 2008). In

Washington State alone, there are three distinct 6-8 week pupping seasons for breeding populations within 100 miles of each other between Puget Sound and outer coastal areas. The precocial newborn pups have already shed their lanugo coat in utero, and although born weighing only 8-12 kg, they will nearly double their weight in the brief 4-6 week weaning period before beginning to forage on their own (Jeffries and Newby 1986). Pacific harbor seals are dietary opportunists, and have a highly varied diet that includes demersal and pelagic fish (such as flounder, sculpin, hake, and herring), octopus, squid, and occasionally clams or small crustaceans (Jeffries and Newby 1986, Reeves et al. 2008).

As solitary animals that may temporarily congregate during the breeding season, harbor seals are thought to be relatively sedentary and do not typically seem to travel long distances, particularly as adults (Thompson 1993, Reeves et al. 2008). While foraging, harbor seals will rarely travel further than 50 km, and in such cases will move to haul-out sites closer to feeding grounds (Brown and Mate 1983, Thompson 1993). Foraging and reproductive behavior can both influence foraging distances; pupping, lactation, and mating displays are known to restrict daily travel distances from haul-outs, and an increase in food availability can reduce the mean foraging range of individuals by 5-10 km (Thompson 1993). Within Prince William Sound, satellite-tagged adult animals moved less than 10 km between successively used haul-outs and had a mean foraging distance from haul-outs of only 5-10 km, whereas juvenile animals had mean distances over twice as great (Lowry et al. 2001). While harbor seal pups have commonly been reported to travel distances of over 200 km in the first year of life, the majority of pups will remain in their natal area (>80% within 50 km of a rookery, in some cases;

Thompson 1993). Therefore, site-fidelity and a lack of dispersal are believed to be characteristic of this species over all life stages throughout its range, and this may frequently lead to local communities with less exchange between neighboring stocks than commonly found for more vagile marine species.

Common causes of morbidity and mortality for harbor seals include infection by parasites (e.g. roundworms, anopluran lice, heartworms, and protozoans), viral infections, attacks by natural predators (i.e. orcas (*Orcinus orca*) and great white sharks (*Carcharodon carcharias*)), and injuries from human interactions (Jeffries and Newby 1986). While infections by common parasites are not usually fatal (particularly for adult animals), there is an increasing body of evidence that indicates heavy loads of organochlorines or metals in can lead to immunosuppression and increased vulnerability to infection (DeSwart et al. 1996, VanLoveren et al. 2000). This effect may also increase susceptibility to viral infections, which could be critical to this species as harbor seals are known to have experienced several viral epidemics in recent history; several hundred animals in the northeast Atlantic died from an influenza virus in 1979-1980, and in the late 1980s as many as 20,000 animals across the Atlantic Ocean died from a phocine distemper virus (Reeves et al. 2008). Increased exposure to pesticides can also directly affect survival for harbor seal populations as they can cause increases in birth defects and spontaneous abortion (VanLoveren et al. 2000). Previous studies have shown PCB tissue concentrations in Puget Sound harbor seals have been detected at levels as high as 400 ppm; well above the 3 ppm levels known to cause reproductive dysfunction, metabolic abnormalities and mortality in other mammals (Jeffries and Newby 1986). In addition to these indirect anthropogenic effects on the health of wild harbor seal populations, there is

also direct competition with fisheries that results in the reduction of harbor seal populations through limited food resources, incidental mortality from fishery by-catch, or injuries due directly to human interactions (Reijnders et al. 1993, Reeves et al. 2008).

1.4.2. *P. vitulina richardsi* in the Pacific Northwest

The eastern Pacific harbor seal is subdivided into several stocks along the west coast of North America for management by U.S. Government agencies (Carretta et al. 2009). While current stock definitions are generally based on abundance trends considered important to management, it is acknowledged that recent genetic work suggests these delineations may not be adequate, and that current boundaries are merely a convenient representation of a complex, continuous system (Carretta et al. 2009). For example, Pacific harbor seals in Alaska are currently managed as three separate stocks which together are not considered to be in decline, although there is evidence of very different rates of population growth or decline between stocks from Bristol Bay to Ketchican, with noted declines in Prince William Sound (Small et al. 2003). Given recent genetic data the current stock delineations are being re-evaluated in Alaska, and Pacific harbor seals have been listed as an Alaska Species of Special Concern due to population declines (Angliss and Outlaw 2008). In addition, because of differences in pupping season, pollution loads, fisheries and movement patterns there are three separate harbor seal stocks recognized in Oregon, Washington and California. Animals in California represent a population that increased rapidly after 1972 until the 1990s when growth rates began to slow (Carretta et al. 2009). In Washington State, animals found in inland waters (from the Straight of Juan de Fuca through Puget Sound to Gertrude Island) exhibit

unique pupping phenology and pelage patterns from coastal animals, and combined with evidence of unique genetic markers these inland animals are also considered an independent management stock (Small et al. 2003). This inland stock was heavily reduced during bounty programs of the 1940s-1960s, but has also increased in size since the 1970s (albeit slowly), and appears to have stabilized around 12,000 animals (Carretta et al. 2009).

The remaining coastal animals of Oregon and Washington are managed as a single entity, and population assessments of animals in these areas are assumed to reflect trends for a single panmictic population. Aerial surveys from 1999 conducted by the National Marine Mammal Laboratory (NMML) and Oregon and Washington Departments of Fish and Wildlife (ODFW and WDFW) estimated a total stock population of 24,732, after correcting for survey count error rates (Jeffries et al 2003, Carretta et al 2009). This could represent a trend of decline from 1991 and 1992 estimates of over 28,000 animals in the coastal Oregon and Washington stock, although this difference in estimates is not statistically significant (95% CI of 1991 estimates = 24,697 - 31,960) (Huber et al. 2001). Historical population sizes for the western Pacific harbor seal are unknown for Oregon and Washington, although animals were freely hunted under bounty incentive programs from 1925 to 1972. At least 3,800 animals were killed in Oregon over the duration of these programs, and in Washington approximately 17,133 are believed to have been killed between 1943 and 1960 (Carretta et al. 2009). Since the passage of the Marine Mammal Protection Act (MMPA) in 1972, total population size of the coastal Washington/Oregon stock has rebounded from counts of 6,389 in 1977 to 16,165 in 1999, increasing at an annual growth rate of 4-7% between

1983 and 1996 (Carretta et al. 2009). Behavioral observations indicate that the protection afforded by the MMPA allowed seals to reoccupy haul-out sites in bays and estuaries, allowing for rapid population growth in the intervening years (Harvey et al. 1990).

While maximum net productivity rates for this stock have been estimated at 18.5% for Washington animals, 10.1% for northern Oregon and 6.4% for southern Oregon (based on changes in abundance data since 1975), recent work has shown that both the Washington and Oregon animals in this stock appear to have reached their carrying capacity and population growth has leveled off (Jeffries et al. 2003). Instances of recorded human-caused mortality are relatively low for this stock, with seal deaths or serious injuries due to fishery interactions (incidental take or tribal fisheries) averaging over 13.6 animals per year, with an additional 1.6 animals killed or injured per year from non-fishery human interactions between 2000 and 2004 (Carretta et al. 2009). The potential biological removal for this stock based on the maximum net growth rate for pinnipeds (12%) has been calculated at 1,343 harbor seals annually, so although the estimate of 15.2 annual human-related deaths or serious injuries must be seen as a minimum value due to under-reporting of stranding cases, it is still less than 10% of the potential for removal and is considered an insignificant loss (Carretta et al. 2009). Therefore, because it is highly unlikely that current levels of mortality pose a threat to population persistence, the eastern Pacific harbor seal in Oregon and Washington is not listed for protection under the endangered species act, is not considered “depleted” under the MMPA, is not classified as a “strategic” stock for conservation, and is currently considered to be within its Optimum Sustainable Population level (Reijnders et al. 1993, Carretta et al. 2009).

1.4.3. Population structure of *P. vitulina*

The cosmopolitan distribution of harbor seals in the northern oceans has lead to an extensive geographic range of animals which show an ancient differentiation between the Atlantic and Pacific Oceans, followed by a pattern of regional differentiation of west-to-east colonization in each ocean basin (Stanley et al. 1996, Westlake and O'Corry-Crowe 2002). There are two clearly divergent monophyletic clades of harbor seals in the Atlantic Ocean (east and west populations), as well as in the Pacific Ocean between the northwest (Bristol Bay, AK and eastward) and eastern Pacific (Washington state south through California) (Stanley et al. 1996). Population genetic differentiation follows a pattern of isolation-by-distance in Pacific as well as Atlantic populations of harbor seals (Lamont et al. 1996, Stanley et al. 1996, Goodman 1998, Westlake and O'Corry-Crowe 2002). Significant genetic subdivision has also been detected in Atlantic harbor seals among subpopulations in the Baltic Sea, Scandinavia, and Scotland (Stanley et al. 1996, Goodman 1998), and in Pacific Ocean populations among animals east of British Columbia, animals near Vancouver Island/Puget Sound, and the coastal animals of Washington, Oregon, and California (Lamont et al. 1996, Burg et al. 1999, Huber et al. 2010). Additional genetic differentiation among animals from Alaska and the western Pacific confirms the appropriateness of the three Alaskan management stocks established based on differences in pupping phenology, pelage, and demographic trend (Westlake and O'Corry-Crowe 2002, Carretta et al. 2009, but see O'Corry-Crowe et al. 2003).

Given that harbor seals have migratory and dispersal patterns that appear to behaviorally restrict gene flow between neighboring areas (Brown and Mate 1983, Lowry et al. 2001), it is not surprising that molecular techniques have detected patterns of

genetic marker distributions indicating distinct subpopulations within larger management stocks (Lamont et al. 1996, Burg et al. 1999, O'Corry-Crowe et al. 2003, Huber et al. 2010, but see Herreman et al. 2009). Genetic differentiation among groups of *P. vitulina richardsi* has been detected on a scale of only a few hundred kilometers in the northeast Pacific (Lamont et al. 1996, Stanley et al. 1996, Burg et al. 1999, O'Corry-Crowe 2003, Huber et al. 2010). For example, recent work utilizing mtDNA and microsatellite markers indicate a meaningful differentiation between harbor seals in Puget Sound and coastal Washington, and analysis of mtDNA haplotype frequencies has suggested even more subdivision both within Puget Sound and among coastal animals found in Washington, Oregon and California (Lamont et al. 1996, Burg et al. 1999, Huber et al. 2010). While extensive sampling of animals in Puget Sound and Vancouver Island has resulted in the elucidation of these subtle relationship patterns, equivalent sampling and analysis of coastal animals from coastal Washington and Oregon has not yet been done. Therefore, genetic substructure of coastal Pacific Northwest harbor seals has not been described, and it is currently unclear whether patterns of isolation-by-distance occur on the same scale as elsewhere in this species' range.

1.5. Study Aims and Significance

My intent is to address the potential for local population differentiation in Pacific harbor seals along the coasts of Oregon and Washington. Behavioral observations of animals from this region indicate that animals from coastal Oregon are as philopatric as animals from Alaska and elsewhere. Harbor seals observed in central Oregon between 1978 and 1980 either remained in the same bay or commonly traveled only to a

neighboring bay approximately 25 km away (Brown and Mate 1983). There is thus good reason to predict genetic structuring on the same scale as has been detected for other Pacific harbor seal populations (O'Corry-Crowe et al. 2003, Huber et al. 2010). In addition, the health of local ecosystems depends on the success of harbor seal populations because of their trophic level status as apex predators (Reeves 2008). The combination of these factors makes adequate assessment of gene flow among populations critical for appropriate MU delineation and consequently determining the appropriate scale for local ecosystem management.

There is already conservation imperative for evaluating the population structure and levels of connectivity for harbor seals in the Pacific Northwest. The MMPA of 1972 criteria states that marine mammal populations "should not be permitted to diminish beyond the point at which they cease to be a significant functioning element in the ecosystem of which they are a part, and, consistent with this major objective, they should not be permitted to diminish below their optimum sustainable population" (16 U.S.C. 1361 Sec. 2). While there has been debate as to exactly how to define optimum sustainable populations, clearly the scale at which we consider populations to have their own unique behavior and evolutionary trajectory will impact management. To ensure that these apex predators have populations that remain functional elements in their respective ecosystems, the question of how to best delineate MUs becomes critical.

In addition, the IUCN Conservation Action Plan for pinnipeds has proposed that it is important for new research to determine levels of genetic variability even in populations which have not been through bottlenecks, and to investigate the use of molecular techniques for identifying pinniped subspecies and stocks. In the context of

whole ecosystems, the IUCN recognizes that for all pinniped species it is important to pursue research "to understand better the role that pinnipeds play in marine ecosystems and how population changes may affect the ecosystem" (Reijnders et al. 1993). Clearly, a better understanding of population structure and dynamics of Pacific harbor seal stocks will promote the effective management of the species, its prey species, and entire local ecosystems.

1.5.1. Specific Aims

The need for a thorough evaluation of population genetic structuring of Pacific harbor seals along the coasts of Washington and Oregon was deemed critical, not only because a fine-scale analysis of population structure has not been done in this range, but also because this species is an elegant model with which to examine genetic divergence in the marine environment. By studying movement and gene flow in this species, we stand to gain a much clearer understanding of how the behavioral and demographic characteristics of marine mammal species or populations that restrict gene flow can lead to differentiation. Therefore, my specific aims in this study were as follows:

Aim 1: Sample tissues from Pacific harbor seals across a large geographic range of the Oregon and Washington coast where the potential for local differentiation exists.

As a species that has been observed to be philopatric and exhibits local genetic differentiation in some parts of its range (Brown and Mate 1983, Lamont et al. 1996, Lowry et al. 2001, O'Corry-Crowe et al. 2003, Huber et al. 2010), I predicted similar levels of differentiation in animals along the coasts of Washington and Oregon. In

particular, I was interested in sampling across the current management stock boundary between inland Washington (Puget Sound) and Washington's outer coast from the Long Beach peninsula and northern and central Oregon. This boundary was established largely because of differences in population abundance trends and variation in the phenology of pupping season (Carretta et al. 2009), and my goal was to use genetic data to assess the adequacy of this current stock delineation. I predicted that I would find levels of differentiation in allele frequencies along this range that would identify two or more population groups as MUs as outlined above, based on the range of differentiation of previous studies (O'Corry-Crowe et al. 2003, Huber et al. 2010).

Aim 2: Use both mitochondrial and nuclear markers to assess population structure.

As described above, there are limitations to inferences made from the analysis of any single genetic marker, and these limitations can best be overcome by the use of multiple different markers. Ideally, the use of markers with different tempos of mutation and modes of inheritance can identify discordant patterns that individually could be misinterpreted, and will instead give a better overall view of genetic structure. The use of mitochondrial DNA sequences as well as multiple microsatellite loci can illustrate trends over very different timescales, with mitochondrial sequences mutating slowly enough to give an historical view of population structure while microsatellites reflect recent gene flow. Additionally, in the context of this work I expect the different modes of inheritance of mtDNA and microsatellites (maternal versus bi-parental, respectively) to generate a detailed depiction of differences in allele frequencies among geographic sampling areas, reflecting the gene flow among matrilines and in the overall population.

I specifically hypothesize the following for each marker:

mtDNA:

H1: Frequencies of unique sequences ('haplotypes') will differ among groups of animals across the regions sampled.

H2: Haplotypes will be most similar in animals sampled within the same area, and less similar among animals from regions separated by larger distances.

Microsatellites:

H1: Frequencies of alleles will differ among groups of animals across the regions sampled for multiple microsatellite loci.

The null hypothesis for both of these markers is that haplotype or allele frequencies will be the same among sampling regions. Together, this information will generate an expanded view of patterns of genetic structuring (e.g. isolation by distance, clines, etc.) in this species across a little-studied expanse of its range, and will allow me to suggest mechanisms that may be leading to genetic differentiation in the absence of geographic barriers to gene flow.

1.6. Suitability of Approach

In this study we used tissue samples collected from Pacific harbor seals which were recovered by regional Marine Mammal Stranding Networks along the coasts of Oregon, southern Washington and Puget Sound to analyze population structure. Marine mammals that swim or wash ashore and become ‘beached’ or stuck in shallow waters because they are disoriented, ill, injured, or have died are considered ‘stranded’ (NOAA 2011). The National Oceanographic and Atmospheric Administration (NOAA) National

Marine Fisheries Service (NMFS) Office of Protected Resources oversees the operation of several regional stranding networks composed of government agencies, academic institutions and volunteers to respond to reports of stranded marine mammals. These teams respond to calls of live animals on the beach that may be sick or injured, and will provide information to the public in cases of healthy animals about the regulations protecting marine mammals from human disturbance. In most cases, however, animals reported to the stranding network have either washed up dead or are found alive but in poor condition and die soon after the initial sighting.

One of the major functions of stranding networks is to collect valuable demographic and health data from these recovered animals and to determine as much as possible about the circumstances of the animal's death. As a routine part of this process, necropsies are performed and tissue samples collected for archival and histopathology analysis when appropriate. This makes tissues archived through this network valuable for population studies because all of the data concerning the condition of the animal can be tied to its genetic data in population studies, and evaluated for trends by genetic structure. Causes of morbidity and mortality can be described for populations over time, and trends in demographic structure can be evaluated in the context of documented morbidity and mortality data. Samples from stranded animals are also more easily obtained than samples from living animals, and are therefore a valuable resource for obtaining tissue samples from protected marine mammal species over multiple age classes. Although this sampling method is limited because the exact source of the animal (i.e., natal rookery) and distance traveled since death can rarely be determined, the high natal philopatry observed in Pacific harbor seals makes the approach useful in this case.

Chapter 2: Materials and Methods

2.1. Sample Collection

Tissue samples were collected from 63 stranded harbor seals recovered between 2006 and 2010 (Appendix A). Samples were collected in association with the Marine Mammal Stranding Networks of Northern Oregon/Southern Washington, Central Oregon, and the Washington Department of Fish and Wildlife. Tissues were collected from animals found as far south as Florence, Oregon north to Grayland, Washington (a range of over 430 kilometers of coastline), and from a subset of individuals from southern Puget Sound (a straight-line water distance over 500 km from the nearest coastal sample). Harbor seals which washed up dead or were observed to die on the beach within the range of the given stranding network branch were either processed on site or recovered and frozen for necropsy at a later date. At the time of necropsy, samples of blubber (including skin), muscle, blood and all of the major organs are routinely collected by the stranding networks based on the state of decomposition (i.e., organs are sampled only when they are not too degraded to recover DNA). I only used samples of skeletal muscle, cardiac muscle, and skin for DNA analysis. Tissue samples for DNA extraction were taken as a small portion (less than 1 gram) of the larger organ or tissue section available from routine sampling, either in the course of necropsy or after tissues had been parceled and stored. In both cases, tissues were frozen and stored at -20 ^{o}C until processed for DNA extraction.

2.2. DNA extraction and quantification

The DNA for both mtDNA and microsatellite analyses was extracted from tissues

according to the protocols set out in the DNEasy™ Blood and Tissue Kit spin column protocol (Qiagen, Valencia, CA). For each sample, approximately 25 mg of tissue were carefully macerated with a sterile scalpel blade on a clean surface before digestion with lysis buffer, and tissue lysis incubation times were extended from the Qiagen protocol to a minimum of 36 hours. Extracted DNA was then quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and associated NanoDrop 1000 Operating Software (version 3.5.1). The AE elution buffer from the Qiagen DNA extraction kit was used as a blank, and resulting DNA concentrations, A260/280 and A260/230 ratios were determined from the spectrometry absorbance curve. Detailed protocols are described in Appendix A.

2.3. mtDNA Analysis

2.3.1 PCR Amplification and Sequencing

A 551 bp region of the mitochondrial genome, including 51 bp of the tRNA-Pro gene and 500 bp of control region immediately downstream, was amplified using the following primers from Huber et al. (2010): TRO (modified L15829 from Westlake and O'Corry-Crowe 2002) 5'-CCTCCCTAAGACTCAAGGAAG-3'; and PVH00034 (Westlake and O'Corry-Crowe 2002) 5'-TACCAAATGCATGACACCACAG-3' (Integrated DNA Technologies, Coralville, IA). Primers were diluted with TE buffer to a final 25 µMolar concentration. Reactions were carried out using 1 µL of each primer, a volume of eluted DNA that contained approximately 150 ng of DNA, and a remaining volume of sterile water up to 25 µL total reaction volume, which were all added to illustra™ PuReTaq Ready to Go™ PCR Beads (GE Healthcare, Waukesha, WI).

Amplification reactions were run on either a PTC-100 Peltier Thermalcycler or PTC-150 MiniCycler (Bio-Rad, Hercules, CA) using the following program from Huber et al. (2010): 90 ^{o}C for 2.5 minutes, followed by 37 cycles of 1) denaturing at 94 ^{o}C for 30 seconds, 2) annealing at 48 ^{o}C for 60 seconds, and 3) extension at 72 ^{o}C for 60 seconds, followed by a final extension step at 72 ^{o}C for 5 minutes. Products were then held at 2-4 ^{o}C until purified. PCR products were purified using the QIAquick^{R} PCR Purification kit spin column protocol (Qiagen, Valencia, CA).

Sanger termination cycle sequencing reactions were carried out using purified PCR product as the amplification template, and following procedures recommended by the Oregon State University Center for Genome Research and Biocomputing (CGRB) Core Laboratories (Corvallis, OR). The target sequence was primed in separate reactions in each direction using TRO and H16498 (Rosel et al. 1994): 5'-CCTGAAGTAAGAACCAGATG -3'. Each reaction mixture included 2.5 µL of purified PCR product template, 0.25 µL of 25 µMolar primer stock, 2 µL Big Dye^{R} Terminator 3.1 reaction mix (Applied Biosystems, Carlsbad, CA), 1 µL 5x buffer, and 4.25 µL of sterile water, for a total reaction volume of 10 µL. Amplification reactions were run on the following program recommended by the CGRB: 96 ^{o}C for 5 minutes, followed by 25 cycles of 1) denaturing at 96 ^{o}C for 30 seconds, 2) annealing at 50 ^{o}C for 15 seconds, and 3) extension at 60 ^{o}C for 4 minutes. Products were stored at 2-4 ^{o}C until 10 µL of the reaction mixture was transferred to a 96-well plate and sealed with an airtight lid for transport. Samples were analyzed on an ABI 3730 capillary sequencer at the CGRB Core Laboratories at Oregon State University. Sequence traces were delivered electronically through the CGRB ordering website (http://weborder.cgrb.oregonstate.edu).

2.3.2 Statistical Analyses

Sequence reads were downloaded from the CGRB website as both .ab1 and .seq files. Complimentary sequence reads were aligned in the SeqMan II program within the Lasergene^{R} version 6.1 analysis package (DNASTAR, Madison, WI) and trimmed with default settings of peak tolerance. Consensus sequence conflicts were called by eye from the peak scan, and consensus sequences exported as text files. Consensus sequences were then aligned in ClustalX2 (Larkin et al. 2007), and ends were trimmed to standardize sequence length by hand in the Mesquite matrix editor (Maddison and Maddison 2010) so unique haplotypes could be identified. A 'haplotype' in the general sense is simply a collection of alleles at different loci that are inherited together, however in the context of haploid mtDNA, a haplotype refers to the same unique collection of single nucleotide polymorphisms found in a given segment of DNA sequence (DeYoung and Honeycutt 2005). To check for previously identified haplotypes among the animals sampled, the 76 sequences submitted to GenBank by Huber et al. (2010) were downloaded and included as a separate group in the program Arlequin version 3.11 (Excoffier et al. 2005) for comparison with the haplotypes that I identified. The 551 bp haplotypes that I used were trimmed by 2 bp on the 5' end of the positive strand and by 139 bp on the 3' end to limit all sequences to the length of Huber et al. (2010) GenBank sequences, and Arlequin was used to identify shared and unique haplotypes within and among groups. Sequences from this study which corresponded to a haplotype previously identified by Huber et al. (2010) were assigned the identification number given to that sequence in GenBank (numerical values 1-76, Accession # FJ472353-2428), whereas haplotypes unique to this work were

assigned alphabetical identifiers (A-I).

Sequences were then divided into four groups based on the geographic sampling location for initial descriptive analyses; 1) southern Puget Sound ("inland WA"), 2) coastal Washington from Grayland to the Columbia River ("coastal WA"), 3) coastal Oregon from the Columbia River south to Tillamook Bay ("northern OR"), and 4) coastal Oregon from Tillamook Bay south to Florence ("central OR"). Unique haplotypes were identified, and indices of genetic diversity, F-statistics, and a minimum spanning network (MSN) were calculated in Arlequin version 3.11 (Excoffier et al. 2005). A MSN was also generated in TCS version 1.21 (Clement et al. 2000), and a consensus network was drawn by hand from the TCS and Arlequin networks. The MSN is determined using the number of mutational steps separating each pair of haplotype sequences to construct the most parsimonious network of haplotypes which minimizes the number of steps between related sequences. A MSN is the most appropriate phylogeny to investigate gene genealogies at the population level because most assumptions of tree construction analyses (e.g. complete lineage sorting) are not met in early stages of population divergence (Clement et al.2000).

An Analysis of Molecular Variance (AMOVA) was used to calculate among and within population variation in haplotype frequency, as described by the F-statistic analogues Φ_{IS}, Φ_{IT} and $\Phi_{ST,}$ by calculating a pairwise distance matrix (Excoffier et al. 2005). An AMOVA analysis is analogous to a traditional ANOVA in that variation in the nucleotide diversity (the average distance between randomly selected haplotypes) is partitioned into within and among population components, so the level of organization contributing most of the diversity in the sample can be identified (i.e. variability within

groups vs. variability due to differentiation) (Excoffier et al. 1992). The Φ-statistics, while analogous to Wright's F-statistics and Weir and Cockerham's θ (Weir and Cockerham 1984), are more appropriate for the analysis of haplotypic sequence data than conventional F-statistics because they take advantage of the additional information given by mutational changes observed in sequence data, and incorporate pairwise mutational distances between sequences into calculations of differentiation among groups (DeYoung and Honeycutt 2005). Conventional F-statistics, on the other hand, consider all unique haplotypes to be equally independent and calculate the amount of diversity found within versus among groups based on allele frequencies only (DeYoung and Honeycutt 2005). Pairwise Φ_{ST} values were calculated for the four geographic sampling areas in an AMOVA analysis implemented in Arlequin, and traditional F-statistics were calculated for comparison. Simple pairwise difference calculations were used to estimate differentiation, because different models of nucleotide substitution (Jukes-Cantor 1996, Kimura 2-parameter, Tajima and Nei, Tamura, and Tamura and Nei) resulted in very similar group-wise and pairwise Φ_{ST} estimates (data not shown).

Indices of neutrality were also calculated for mtDNA sequences in Arlequin, which included Tajima's D test and Fu's F_S estimates. The Tajima's D test is a comparison of the nucleotide diversity (π), which is the mean pairwise number of nucleotide differences among sequences in a sample, with the expected number of segregating sites per sequence given the number of samples (Tajima 1989). Under neutral conditions where the effects of mutation and drift have produced allele frequency equilibrium the expected values of these estimates should be the same, and thus any difference between them (the D-estimate) would indicate a departure from neutral conditions. Along similar

lines, Fu's F_S is an estimate calculated using the probability of obtaining the number of alleles (or fewer) observed in the sample given the nucleotide diversity (Fu 1997). A high probability (strongly positive F_S) indicates a deficiency of alleles as seen following population bottlenecks, whereas a low probability (strongly negative F_S) indicates an excess of alleles as seen with recent population expansion. Both situations indicate violations of neutrality assumptions, thus it was important to rule out such conditions for loci used to estimate population divergence.

Mitochondrial sequence data were also used in conjunction with geographic distances among populations as calculated from the most direct water route between central sample point locations in ArcGIS 10 (ESRI, Redlands, CA) to analyze isolation by distance among populations using Isolation-By-Distance Web Service (IBDWS) version 3.16 (Jensen et al. 2005). IBDWS is a simple linear regression of pairwise genetic distance as a function of geographic distance between putative populations which can be used to describe how much of the variation in genetic distance among populations can be described by changes in geographic distance. Pairwise distance matrices from AMOVA Φ_{ST} analysis of four putative subpopulations were regressed against geographic distances and significance tested against a null distribution of 1,000 randomizations.

Geographic distance measurements were also used to investigate spatial autocorrelation and cluster assignment using the Geneland package version 3.2.4 (Guillot et al. 2005) in the R statistical environment (R Development Core Team 2011). This model is a Bayesian clustering algorithm that uses locus assignment to determine the posterior probability of different possible numbers of putative subpopulations. In such methods a Metropolis-Hastings algorithm is used to start with arbitrary parameter

estimates for number of putative populations and associated allele frequencies, and then proposes moving to another set of arbitrary parameter values. The likelihood of the proposed parameter set given the data is calculated, and if the proposed parameters are less likely than initial estimates then the move is rejected and another move is proposed. If the proposed set of parameters is more likely than the current set the move is accepted, and the process is repeated. This chain of steps with randomly proposed moves (including moves that were not accepted) is known as a random-walk Markov Chain. In this way the chain explores likelihood over a multi-dimensional space of parameter values and will tend to move to and remain in the areas of highest likelihood. The distribution of the parameter space the chain visits can be sampled randomly to estimate the posterior probability of parameter values; a process known as Markov Chain Monte Carlo (MCMC) sampling.

As implemented in Geneland, this MCMC algorithm assumes linkage equilibrium within subpopulations, and will generate estimates of the most probable number of populations (and their haplotype frequencies) before assigning individuals to these clusters. MtDNA haplotypes were treated as highly variable alleles at a single locus, and to incorporate possible effects of spatial autocorrelation the relative geographic location of each sample was included with its haplotype. Each part of geographic space in the sample range (i.e. each polygon in a Poisson-Voronoi tessellation model) is then assigned a population cluster given the assignment probability of the individual samples within it. The GPS coordinate locations for each animal were converted to planar Lambert azimuthal equal-area projection coordinates in R v.2.12.1 (R Development Core Team 2011) before use in Geneland. Three independent runs of 100,000 MCMC steps (after

20,000 burn-in steps) were sampled at a rate of 1 per 100 iterations, and posterior probability distributions calculated for each number of putative subpopulations k = 1-5. The correlated allele frequency model was used since recent divergence is assumed for these putative subpopulations, and posterior probability distributions were used to infer the most likely number of populations.

2.4. Microsatellite Analysis

2.4.1 PCR Amplification of Loci

Eight polymorphic microsatellite loci amplified for the Pacific harbor seal in previous work (Allen et al. 1995, Gemmel et al. 1997, Goodman 1997, Burg et al. 1999, Davis et al. 2002) were used to analyze genetic differentiation for populations sampled in this study. The primers used for each locus are given in Table 1. Amplification reaction mixes for each locus contained 0.5 µL of 25 µMolar primer stock for both forward and reverse primers (Pv11a stocks further diluted to 5 µMolar stock before use), and 6 µL of the organic compound trimethylglycine (or glycine betaine) which enhances PCR amplification by preventing the formation of secondary structures and optimizing purine binding thermodynamics (Henke et al. 1997). From 0.5-4 µL of template DNA was used in each reaction based on concentration of extracted DNA. Sterile water was added to a final volume of 25 µL in a 0.6 mL reaction tube with one illustra^{TM} PuReTaq Ready to Go^{TM} PCR Bead.

Initial investigations indicated that different primers optimized target sequence amplification under different programs, so amplification reactions were run on one of four programs based on the optimal conditions for each primer pair (Appendix B). After

amplification, 0.5 µL of the reaction product was diluted in 50 µL of sterile water, and 0.5 µL of the dilution was transferred to a 96-well plate and sealed with an airtight lid for transport. Samples were analyzed in an ABI 3730 capillary sequencer at CGRB Core Laboratories at Oregon State University. Output fluorescence traces of products with an internal lane standard were delivered electronically through the CGRB ordering website (http://weborder.cgrb.oregonstate.edu).

2.4.2 Statistical Analysis

Microsatellite reads were downloaded from the CGRB website and analyzed in PeakScanner™ v1.0 (Applied Biosystems). Peaks of allele sizes were scored against a standard ladder from the trace read. Because hetorozygotes may be mistakenly scored as homozygotes in the absence of strong signal from the second allele, scoring accuracy was calculated for a subset of samples which were re-analyzed at higher or lower concentrations. Raw allele scores for each locus were ordered by size and binned into size classes, with clusters of reads similar in size representing a single allele separated by at least two bp in length from the nearest cluster of similarly-sized reads. These bin allele labels were used as the allele input data for all subsequent analyses.

The program LOSITAN (Antao et al. 2008) was used to address selection on microsatellite loci using the F_{ST}-outlier method. This method simulates fixation indices among populations for each locus under assumptions of neutrality, and assumes that if observed fixation indices (i.e. F_{ST} estimates) are excessively higher or lower than would be expected based on the expected heterozygosity this excess or deficiency of homozygotes is the result of selection at those loci. Ten thousand simulations were run

under a step-wise mutational model with four putative populations, and any loci with excessively high or low F_{ST} values relative to the expected heterozygosity (i.e., potentially subject to negative or balancing selection) were identified for removal from further analyses.

All microsatellite loci were analyzed in the Bayesian assignment program STRUCTURE 2.3.3 (Pritchard et al. 2000) with no *a priori* assumptions about the number of populations present. The basic Metropolis-Hastings and MCMC sampling algorithm procedures are the same as those described for Geneland, with no explicit spatial assumptions. In this case, genotypic microsatellite data across eight independent loci were used to determine the most likely number of clusters based on allele frequencies and assuming Hardy-Weinberg equilibrium within subpopulations. Multiple stochastic sampling chains were run with one to five putative subpopulations (k = 1-5). MCMC chains were run under each scenario with 1,000,000 sample steps following a 500,000 step burn-in period. The admixture model of population structure using correlation of allele frequencies among populations was used because complete lineage sorting is not assumed for these populations. Chains were repeated to avoid sampling from any single run becoming trapped in local high probability maxima. Resulting likelihood estimates for each value of k populations were used to determine the most likely number of clusters within the sample.

Microsatellite data were also imported into Arlequin, and indices of allelic diversity and R_{ST}-like statistics were calculated given the same rules for *a priori* population assignment described for mtDNA analyses. The R_{ST} statistic is another analog of the classic F_{ST} but, like Φ_{ST}, it incorporates the additional information inherent in

genetic markers (in this case, pairwise distance based on relative allele sizes) to inform calculations of variance among populations. Assuming a step-wise mutational model for these loci implies that alleles separated by a single di-nucleotide repeat unit would be more closely related than alleles separated by multiple repeat units. The additional information about genetic distance incorporated into calculations of R_{ST} makes is a more accurate gauge of population differentiation assuming the step-wise mutational model is appropriate; however standard F_{ST} estimates were also calculated for comparison (Slatkin 1995). Garza-Williamson indices (i.e., number of alleles divided by allelic range) were also calculated for each locus to investigate whether a paucity of alleles exists given the allelic range, which would suggest a recent population bottleneck.

To analyze possible spatial autocorrelation among animals sampled along this continuous range, the Geneland version 3.2.4 package (Guillot et al. 2005) was implemented with microsatellite data as described above for mtDNA, with genotypic data of multiple alleles over eight independent loci using the correlated allele frequency model. Given that the presence of null alleles can cause overestimation of homozygosity and misrepresent the state of Hardy-Weinberg (dis)equilibrium, a parameter of null allele frequency was also allowed for each locus in the sampling algorithm to estimate the frequency of null alleles predicted for each locus.

Microsatellite data were also used to investigate biased dispersal within the sample using FSTAT v 2.9.3.2 (Goudet 2001, Goudet et al. 2002). To investigate sex-biased dispersal, animals within each of the sample groups were marked as either male or female and F-statistics as well as heterozygosity and relatedness indices were calculated independently for each group, and for the overall sample. The mean assignment index

(mAIc) is highest for the more philopatric of groups because it represents the probability of a genotype occurring in a given cluster, while the variance in assignment index (vAIc) is expected to be highest for disperser because alleles are being detected over a wider range of clusters. Within-group gene diversity (Hs) is expected to be largest for the group dispersing most, as is F_{IS} (indicating the relative heterozygote deficit), while F_{ST} is expected to be highest in the philopatric group in which we expect allele frequencies to be most differentiated. Animals within each population were also categorized as either 'adult' or 'young' (a classification encompassing subadults, juveniles, yearlings, and pups) to investigate whether dispersal was biased among age classes (age classes could not be further subdivided due to small sample size). A one-tailed test with 1,000 permutations was used for both age and sex-biased dispersal analyses.

2.5 Combined Analysis of mtDNA and Microsatellite Markers

The cluster assignment program Geneland has the unique ability to perform combined analysis of mtDNA and microsatellite data from the same individuals simultaneously. The same procedure and settings described for mtDNA and microsatellite analyses above were used to investigate geographic cluster assignment using both the mtDNA haplotype data and microsatellite data from the five neutral loci selected by LOSITAN.

Chapter 3: Results

Skeletal muscle, heart muscle or skin samples were collected from 63 stranded harbor seals from northern Oregon (n = 27), central Oregon (n = 14), the southern Washington coast (n = 10), and Puget Sound (n = 9). Sampling represented all major demographic groups (61% males vs. 39% females; 52% adults, 13% subadults, 8% yearlings, and 26% pups) (Appendix A). Of the 63 animals sampled, DNA was successfully extracted from 60 individuals.

3.1 MtDNA

Samples from which adequate quantities of DNA were recovered were amplified for the target 551 bp control region sequences. The four geographic areas sampled all showed high frequencies of unique haplotypes (Fig. 1). Twenty-seven unique haplotypes were identified in 60 animals (Appendix C and Fig. 2). Of these haplotypes, 16 are identical to a subset of the 73 harbor seal haplotypes deposited in GenBank by Huber et al. (2010). Nine of these haplotypes from animals collected on the Oregon coast were novel for this primer set. Only one haplotype found in Puget Sound was shared with a coastal population, and within coastal animals only four haplotypes were shared among regions.

There were 148 total nucleotide substitutions detected among the samples, all of which were transitions. There were only two indel substitution sites, both of which were found in Oregon haplotypes. Neither indel was seen in coastal Washington haplotypes and only one was seen in Puget Sound animals. There were more variable substitution sites which were unique to Puget Sound animals than any other sampling area (Table 2).

Nucleotide diversity and the number of variable sites were highly variable among sampling areas. However, neutrality indices were nonsignificant for all groups, and overall, indicated that this mtDNA marker did not appear to be under selection (Table 2). Haplotype diversity was also high but similar for all groups, with many unique haplotypes being detected in each sampling area and in only single individuals (Table 2 and Fig. 2).

Minimum Spanning Network

A minimum spanning network of the relationships among haplotypes, reproduced identically in both Arlequin and TCS, indicated that no haplotype lineages were monophyletic within a geographic area (Fig. 2). The most commonly observed haplotypes (ID# 7 and 32) were detected in all of the coastal sampling areas, but only one haplotype observed in more than one individual was detected in Puget Sound (ID# 4). Common haplotypes which were shared among geographic sampling areas were closely related to each other, separated by only single mutational changes in a 'star' phylogeny. However, two lineages of unshared haplotypes appeared to diverge from the main cluster, with the most distantly related sequences being separated by as many as 20 mutational steps from the main cluster and 11 steps from the nearest related haplotype. However, none of the lineages or star-shaped clusters of related haplotypes were consistently sampled from the same sampling area, indicating that there is no geographic basis for the patterns of relatedness among haplotypes.

AMOVA Φ_{ST} and F_{ST}

Significant among-group variation in haplotype diversity was detected in the

analysis of four putative geographic subpopulations (Φ_{ST} = 0.0590, p = 0.0325). Pairwise comparisons showed that while some sampling groups were not different from others, the variable patterns of significant difference among all sampling areas indicated that consolidation of these areas into fewer putative subpopulations could not be justified (Table 3). Specifically, the Puget Sound group was significantly different from coastal Washington and northern Oregon groups but it was not different from the central Oregon group. In addition, while individuals were significantly different between coastal Washington and central Oregon, northern Oregon animals were not different from animals in either of these areas.

To explore the possible effects of unequal sample size (n = 27 for Northern Oregon, n < 15 for all other groups) on haplotype frequency analyses among groups, 12 haplotypes within the northern Oregon group were randomly sampled and used in calculations of group-wise Φ_{ST} and F_{ST} and pairwise $\Phi_{ST.}$ This randomized resampling from the largest group was repeated ten times for calculations of both statistics, and these estimates were compared to those resulting from analysis of all haplotypes in a single sample (Appendices D.1 and D.2). Estimates of standard F_{ST} based on allele frequency counts alone detected higher levels of differentiation among populations than Φ_{ST} in all cases. Multiple re-sampling of the largest population produced results of pairwise Φ_{ST} comparisons that were partly inconsistent among iterations, and inconsistent with analysis including all samples (Appendices D.1 and D.2). In every iteration Puget Sound animals were significantly different from coastal Washington samples, and in most cases coastal Washington animals were significantly different from central Oregon individuals as well (Appendix D.2). However, in only two resampling instances were Puget Sound

animals significantly different from Northern Oregon animals as they were in the analysis including all samples.

The combined results of these re-sampling trials indicate that differentiation between Puget Sound and coastal Washington animals is highly significant and consistently supported. However, although the use of all samples in an AMOVA analysis suggests differentiation between Puget Sound and northern Oregon animals (Table 4) detection of this effect is highly variable based on the particular haplotypes sampled from northern OR, and is therefore not consistently supported. On the other hand, while analysis of the full sample using AMOVA did not indicate a significant difference between central Oregon and coastal Washington animals, resampling iterations detected a significant difference with fairly high consistency (80%), supporting the existence of a subtle differentiation between these coastal sampling areas.

Geneland

Analysis of mtDNA sequences in Geneland with associated geographic locations resulted in a model with two to three population clusters having the highest posterior probability (ln P(D) = 0.03 for both models). While the indication of two to three populations was similar to structure indicated by AMOVA analyses for mtDNA, the individuals assigned to these clusters were scattered over multiple geographic areas. In all three independent chains, all individuals sampled in Puget Sound were assigned to a single cluster, as were all individuals from coastal Washington, with no sharing between these groups (Table 4). However, animals from central Oregon were assigned to coastal Washington clusters (and in one iteration, Puget Sound) as well as to a third cluster

containing northern Oregon animals. Variable numbers of the northern Oregon animals were also assigned to the coastal Washington cluster (Table 4). This overall cluster assignment suggests that while differentiation between coastal Washington and Puget Sound is supported, there is no clear geographic pattern associated with haplotype clusters throughout the sampling areas. In this case the geographic proximity of haplotypes was not correlated with similarity in haplotype frequencies, supporting results of the minimum spanning network which indicate that closely-related haplotypes are not found in the same geographic areas.

Isolation-by-Distance

Pairwise genetic distance estimates regressed against a geographic distance matrix in IBDWS indicated that pairwise genetic and geographic distances were not significantly correlated, indicating no decrease in relatedness with increasing geographic distance among populations as had been weakly suggested by pairwise Φ_{ST} estimates ($Z = 170.596$, $r^2 = 0.006$, $p = 0.471$).

3.2. Microsatellites

All eight microsatellite loci were polymorphic, having from two to eleven alleles per locus (Table 5). A subset of 21 duplicate amplification reactions revealed that zero of nine heterozygote individuals and only one of 12 homozygote individuals were originally scored incorrectly when alleles were read from output scans, giving an overall allele scoring error rate of less than 5%. Allelic size ranges were similar among geographic sampling areas for most loci, and when size ranges were compared to the number of alleles for each locus (via the G-W statistic) there was no indication of a recent bottleneck effect (Table 5). Observed heterozygosity and gene diversity were similar among loci, and differences between observed and expected heterozygosity did not indicate departure from Hardy-Weinberg equilibrium overall, although alleles at loci LC26, Pv9, and M11A were found to be in disequilibrium for one or two populations (Table 6). Unlike mtDNA haplotype data, there were very few microsatellite alleles that were unique to a geographic sampling area. Allele frequencies appear to be similar among regions for most loci, although the distribution of alleles in Puget Sound animals at loci TB and Pv9 appear to diverge strongly from the allele distributions of coastal groups (Fig. 3 and Appendix E). These limited differences in allele frequencies among geographic sampling areas suggest that there is limited divergence among sampling areas for these nuclear loci outside of Puget Sound.

LOSITAN Selection Analysis

The LOSITAN selection simulations identified three microsatellite loci that may be under selection pressure based on the F_{ST}-outlier approach; Pv9, M11A, and LW11-2

(Table 7). Specifically, this method indicated that there was a heterozygote deficiency at locus Pv9 and heterozygote excess at loci M11A and LW11-2, indicating that these loci were under negative or balancing selection (respectively) and violated assumptions of neutrality. This result was congruent with findings of Hardy-Weinberg disequilibrium for loci Pv9 and M11A under analysis in Arlequin, although those cases were limited to only one or two sampling areas. Subsequently, further analyses of microsatellite data were conducted with and without the three loci identified by LOSITAN as under selection to address the sensitivity of estimates of heterozygosity and genetic differentiation to these potentially non-neutral markers.

STRUCTURE

Assignment analyses in STRUCTURE did not suggest any clustering within the population. Multiple chains run for k = 1-5 putative populations resulted in a model with a single population being the most likely (mean lnP(D) = -1443). Repeating these analyses after removing the loci identified by LOSITAN did not change the outcome.

F_{ST} and R_{ST}

Given four putative populations from the geographic sampling areas, pairwise R_{ST} estimates indicated that the Puget Sound group was significantly different from all other sampling areas, and that none of the coastal areas were different from each other (Table 8). To obtain a biologically relevant estimate of R_{ST} it is important that *a priori* putative population assignments reflect true population structure as much as possible, so a step-wise consolidation procedure was followed to remove artificial subdivisions within populations for comparison. Because the smallest pairwise R_{ST} was found between

northern and central Oregon these populations were combined and pairwise R_{ST} estimates re-calculated. Again, the only significant difference was between Puget Sound animals and coastal animals, so the undifferentiated groups were combined until only two putative populations remained (Puget Sound and coastal animals), and these groups were found to be significantly different (Table 8). Allelic counts, diversity, and heterozygosity indices for the two consolidated groups are shown in Appendix F.

The collapsing of sampling areas into fewer putative populations, while more biologically accurate, exacerbates the problem of unequal sample size when calculating population differentiation measures. To address this issue, ten individuals were randomly sampled from the putative coastal population for calculation of R_{ST} estimates with Puget Sound animals to compare to calculations made using all samples. Five such random sampling comparisons were made, resulting in consistently significant R_{ST} values between the two populations in all cases which were consistent with the results of using all sampled individuals (Appendix G.1). Calculation of R_{ST} estimates using all samples and the coastal animal subsets was repeated using only the five loci identified as neutral in LOSITAN to check for sensitivity to non-neutral loci. These comparisons resulted in an overall R_{ST} estimate that was not significant between Puget Sound and coastal sampling areas ($p = 0.108$, and a mean R_{ST} from subsampling that confirmed this relationship ($p = 0.1469$), although sampling iterations were a mix of significant and non-significant results (Appendix G.1). Standard F_{ST} statistics were calculated under the same conditions, and were found to be significant among these two putative populations in all cases when all loci were used, and in all but one sampling iteration when only five loci were used (Appendix G.2). This suggests that when all loci were used the difference in

sampling effort between the two putative populations did not have much of an impact on our estimate of differentiation between the populations, which was highly significant ($p = 0.009$). However, when only the five neutral loci selected by LOSITAN were used no significant differentiation was found when all samples were included and was only sporadically detected when subsets of equal sample size were used. Therefore, a conservative interpretation of these findings is that there is little if any differentiation suggested by neutral microsatellite loci alone, but when all loci are used there is consistent evidence for divergence between Puget Sound and all coastal sampling areas.

Geneland

Geographically explicit cluster assignment in Geneland indicated that a model of $k = 2$ clusters was the most likely for models run both with all eight loci and with only the five loci indicated as neutral by LOSITAN (Figure 4). Cluster assignment using all loci grouped all Puget Sound animals into a single cluster with no others in each independent run, with all coastal animals assigned to a second cluster. Repeating analyses with the five loci deemed neutral by LOSITAN again assigned all Puget Sound animals to a single cluster, but additionally assigned two animals from central Oregon to this cluster, while the second cluster contained all other coastal animals in every run (Table 9). The frequency of null alleles was estimated to be less than 0.06 for all loci in all cases, indicating that it is unlikely that null alleles are distorting our measures of homozygosity.

Biased Dispersal

Differentiation among populations was analyzed with two subsets of possible

dispersal bias groups in FSTAT, and F_{ST} estimates generated from 1,000 permutations were compared among dispersal groups. Analysis of sex-biased dispersal among the two putative populations (Puget Sound and coastal animals) did not indicate any difference in dispersal between males and females (Table 10a). However, this estimate is restricted by the fact that only one Puget Sound animal sampled was female, which severely limited our ability to detect bias between these groups. This analysis was therefore re-run using only coastal animals in three putative subpopulations (coastal Washington, northern Oregon, and central Oregon) to detect bias in dispersal over this range. Again, no differences among estimates indicating philopatry or dispersal were found. However, when dispersal bias was investigated by age class over the two main putative populations (Puget Sound and coastal animals) there was a significant difference in F_{ST} estimates between adults and younger animals (Table 10b). Other indices of dispersal or philopatry (i.e. assignment indices, gene diversity and F_{IS}) did not reinforce a difference in dispersal between age classes, but the significantly higher F_{ST} among adult animals across the geographic sampling areas still suggests stronger differentiation and higher philopatry in adults than in younger age classes.

3.3. Combined Marker Analysis

Results of Geneland cluster analysis with both the microsatellite loci and the mtDNA haplotype data resulted in identical group assignment, F_{ST}, and F_{IS} estimates for both the all loci and five loci microsatellite analyses (refer to Table 9). As with analysis of microsatellites alone, two subpopulation clusters were determined to be the most likely. These results are congruent with interpretation of the microsatellite-only analyses,

indicating weak but consistent differentiation between Puget Sound and coastal animals.

Chapter 4: Discussion and Conclusions

4.1 Genetic structuring of populations

4.1.1. MtDNA: Haplotype frequencies differ among regions, but lineages are independent of geography

Analyses of mtDNA haplotype frequency distributions among sampling areas suggests strong, significant differentiation between Pacific harbor seals in Puget Sound and coastal Washington, and weak but consistent differentiation between the most distant coastal sampling groups, coastal Washington and central Oregon. This result is consistent with previous work on Pacific harbor seals in this range which showed high haplotypic diversity and high frequencies of unique haplotypes within subpopulations (Burg et al. 1999, Huber et al. 2010). To address the sensitivity of differentiation estimates to unequal sample size, repeated sampling of 12 random individuals from the northern Oregon sample produced Φ_{ST} estimates that were very consistent with estimates made from using all animals with unequal sample size (mean resampling group-wise $\Phi_{ST} = 0.0605$, $p = 0.053$; all samples group-wise $\Phi_{ST} = 0.059$, $p = 0.033$). This consistency increases confidence in the value of this estimate, and I conclude that the unequal sample sizes from these sampling areas appear to have had little effect on estimates of mtDNA differentiation.

Because the null hypothesis of the AMOVA is that $\Phi_{ST} = 0$, it is important to consider not simply whether estimates are considered significantly different from zero, but the magnitude of the Φ_{ST} estimate itself. Previous studies have suggested that Φ_{ST} values of as low as 0.055 are consistent with isolated groups warranting consideration as

an isolated unit for management in this species (Huber et al. 2010). My estimates of pairwise Φ_{ST} between coastal Washington and central Oregon hovered around this threshold with variable significance, indicating only weak differentiation between these groups. The pairwise Φ_{ST} of 0.155 between Puget Sound and coastal Washington, however, is a very strong indication of significant differentiation between inland and coastal areas. This result is consistent with previous work indicating a split between Puget Sound and coastal Washington, which in combination with other considerations has led to the current management boundary between these regions (Lamont et al. 1996, Carretta et al. 2009, Huber et al. 2010). The additional indication of subtle differentiation among animals sampled in coastal Washington versus central Oregon (and rarely between Puget Sound and northern Oregon) does suggest that weak differentiation may occur over large geographic distances—a signal indicative of isolation by distance patterns of gene flow. The weak differentiation suggested in this study occurred on the scale of ~ 250 – 550 km, which is consistent with the spatial scale of subpopulation differentiation from previous work on Pacific harbor seals in Alaska (O'Corry-Crowe et al. 2003).

The minimum spanning network generated for all mtDNA haplotypes (Figure 2) supports a pattern of irregular geographic clustering, with no clear association of related haplotypes to geographic sampling area. It also indicates that haplotypes found in Puget Sound and coastal Washington are not found within single lineages, and do not appear to have the fewest mutational steps between them, but rather are scattered around the network lineages with variable degrees of distance between them. This lack of lineage sorting by geographic area is consistent with matrilines previously studied in Washington (Huber et al. 2010), but shows even less divergence in haplotype frequencies among

areas. The finding that haplotypes occur in very different frequencies among sampling areas (AMOVA analysis), but that these differences do not represent divergent lineages (haplotypes network) is surprising because there is no clear evidence for how haplotypes have come to be distributed in this pattern throughout the study range. The simplest explanations of differentiation among geographic areas (namely, a founder effect from limited migration/colonization events) must be rejected, and more complex combinations of effects must be considered to explain this result.

Although AMOVA Φ_{ST} results show consistent (albeit slight) differentiation among animals sampled from coastal Washington and central Oregon, isolation by distance was not supported directly from genetic and geographic distance matrices as examined by IBDWS. The regression of pairwise Φ_{ST} as a function of geographic distance between groups indicated that geographic distance does not explain variation in genetic distance ($r^2 = 0.00613$, $p = 0.471$). Some degree of spatial autocorrelation in genetic distance was suggested, however, in the clustering patterns revealed by Geneland. Cluster assignment maps clearly show that the spatial relationships associated with mtDNA haplotype frequencies are complex and non-linear. Assignment of all animals from a sampling area into a single cluster, as was the case with animals from both coastal Washington and Puget Sound, suggests that individuals in both of these areas share a higher degree of haplotype frequency similarity with each other than with animals from the other area. However, Oregon animals were also assigned to both of these clusters, and a mixture of Oregon animals was also assigned to a third cluster, making Oregon animals likely to be associated with each other as well as with both coastal and inland Washington animals. This result, when considered in light of haplotypes network analysis and

AMOVA Φ_{ST} estimates suggests that Oregon animals may represent a pool of haplotypes which are shared throughout this study area. The larger sample effort in northern Oregon could account for the apparent prevalence of these haplotypes throughout the sampling area by biasing local detection of rare alleles, however re-sampling of haplotype subsets from northern Oregon appears to confirm the lack of differentiation between northern Oregon and all other populations (Appendix B, Table B.3). Given these results we must consider the possibility that previous work reporting divergence between Puget Sound and coastal Washington populations, which did not detect as many of these shared or intermediate haplotypes found in Oregon, may have under-represented genetic exchange among these areas. The results presented here suggest that an Oregon population (or a larger panmictic ancestral population) may have served as the source of several distantly-related haplotypes for both Puget Sound and coastal Washington populations which now have limited exchange, accounting for the current differences in mtDNA haplotype frequencies.

4.1.2. Microsatellite Loci: Limited or no differentiation is suggested among regions

In contrast to patterns of mtDNA haplotype distribution, the paucity of rare alleles and similarity of allele frequencies in microsatellite loci among sampling regions suggests that there is very little differentiation among these groups. Still, of the eight microsatellite loci examined two loci (TB and Pv9) appear to show the greatest variation in allele frequencies among groups, and both indicate different allele frequencies between Puget Sound and the rest of the coastal groups. Estimates of R_{ST} indices using all loci appear to confirm this split, even when using only ten sub-sampled haplotypes from the coastal group. However, the use of only neutral loci as identified in LOSITAN did not

indicate significant differentiation (R_{ST} = 0.036, p = 0.108), and repeated sub-sampling gave highly variable results. Even in sampling iterations of only neutral loci where R_{ST} values were found to be significant, as with Φ_{ST} estimates, it is important to consider the value of the estimate as well as its significant difference from zero. While R_{ST} estimates were over 0.12 for all sampling iterations using all loci, the use of only five neutral loci resulted in a mean R_{ST} estimate of under half that value (mean R_{ST} = 0.052, range R_{ST} = 0.018 – 0.095). This difference could be due to reduced power, as too few loci can result in a reduction in signal, but it is most conservative to assume that selection leading to an excess or dearth of homozygosity in the three removed loci could have driven the differentiation signal among groups in estimates of R_{ST}. However, there are other reasons why estimates of heterozygosity may have been extremely high or low that are not due to selection (e.g. Wahlund effect). For example, the allele frequencies at locus Pv9 appear to be different between Puget Sound and coastal populations (Fig. 3) which could be why observed heterozygosity was, as measured by LOSITAN, excessively low at this locus. Therefore, without entirely discounting the signal from these F_{ST}-outlier loci, overall patterns of microsatellite differentiation indices suggest weak but consistent differentiation between Puget Sound and the rest of the coastal populations, with no support for differentiation on a smaller scale.

Bayesian assignment analysis in STRUCTURE further indicated a lack of clustering or differentiation among any of the animals sampled, suggesting that a single panmictic population was most likely given the microsatellite data. By contrast, the similar Bayesian assignment algorithm implemented in Geneland did indicate differentiation between two population clusters, suggesting a weak split between animals

sampled in Puget Sound and animals from the coast (similar to mtDNA Geneland analyses). Such apparently contradictory results may be obtained from programs with similar cluster assignment methods because of differences in the assumptions of each sampling algorithm. Analysis in STRUCTURE does not incorporate geographic autocorrelation into its clustering predictions, whereas the Geneland algorithm assumes complete lineage sorting among populations—a highly unrealistic assumption because animals are continuously distributed along, and up to the edges of, sampling areas. Simulation studies have found that while both programs assign individuals to the correct cluster with high accuracy even among populations with low differentiation (F_{ST} = 0.03-0.05), STRUCTURE performs better than Geneland when there is high geographical admixture. In addition, Geneland has been found to overestimate the true number of distinct clusters as compared to STRUCTURE (Chen et al. 2007). When we consider the value of the F_{ST} estimate itself, Geneland analysis of all microsatellite loci resulted in a mean F_{ST} estimate of 0.057, while analysis of the five neutral loci resulted in a mean F_{ST} estimate of only 0.027. Although two clusters were assigned that clearly divided out Puget Sound animals from coastal animals, the degree of differentiation is relatively weak when only neutral loci are considered, so between these two assignment programs there is little support for divergence among sampling areas. When considered in conjunction with conservative estimates of R_{ST} in Arlequin (using neutral loci), microsatellite data in this study indicate very little divergence among geographic sampling areas, with only weak differentiation between inland Washington and coastal animals being supported.

Analysis of biased dispersal did not indicate sex-biased gene flow among populations. However, when the sample was subdivided by age class, there was a

significant difference in the differentiation among populations ($p = 0.038$). Although microsatellite loci indicated little differentiation among sampling regions, differences that do exist are much stronger among adult animals than their younger counterparts. This finding is not surprising when we consider previous behavioral observations of long-distance migration events that have been recorded for juveniles (Lowry et al. 2001). It is still unclear, however, whether this juvenile movement is true dispersal (i.e. resulting in gene flow) or a transient movement preceding a return to natal breeding grounds. Regardless of the end result of these movements, the reduced differentiation in group-wise Φ_{ST} among juveniles detected in these dispersal bias analyses suggests that the *potential* for gene flow among these regions is due to juvenile movement.

4.2. Avoiding the dangers of single gene trees: A comparison of multiple markers

The main implication of conflicting patterns of genetic differentiation suggested by mtDNA versus microsatellite data is that male and female animals have different contributions to patterns of gene flow among the regions sampled. The finer geographic scale of differentiation detected by mtDNA than by microsatellites suggests that regional differences in haplotype frequencies were driven by restricted female gene flow, most likely due to female philopatry. While no sex-biased dispersal was detected by analysis in FSTAT, these estimates were highly constrained by the fact that only one female animal was sampled in Puget Sound, and we assume any real effect could not be detected with this sample. While mitochondrial data indicate strong differentiation among animals from Puget Sound and coastal Washington, as well as the potential for weak differentiation along the coast, results of microsatellite analyses were variable and only some lines of

evidence suggest weak differentiation among Puget Sound and coastal animals. These patterns indicate that although groups of harbor seals along the coasts of Washington and Oregon may appear distinct because of high levels of unique mtDNA haplotypes, there is little evidence to suggest restricted gene flow among groups. As for the relationship between animals in Puget Sound and coastal animals, while mtDNA haplotype frequency differentiation confirms previous findings of genetic divergence among these areas (Lamont et al. 1996, Huber et al. 2010) the addition of microsatellite data indicates that this differentiation is largely limited to mtDNA matrilines, and that gene flow of bi-parentally inherited markers among these areas is more common, possibly through biased dispersal of males. Indeed, even phylogenetic network analysis of mtDNA haplotypes indicates that while the current distribution of haplotypes may differ among sampling regions, there is no geographic lineage sorting. Therefore, I must conclude that there is more connectivity among sampling regions in this study than has been suggested from behavioral observations or the use of only mtDNA markers in other studies (Lowry et al. 2001, O'Corry-Crowe et al. 2003, Huber et al. 2010).

There are many possible historical scenarios that may have led to the surprising genetic structure observed among these regions. Clearly, a single colonization event or phylogeographic lineage splitting can not explain how the mtDNA haplotypes came to be distributed as they are. The answer may be that these haplotypes were not all contributed to geographic areas at one time or from one closely-related group. If historically there was a large panmictic population of harbor seals in the northeastern Pacific that had randomly contributed a subset of mtDNA haplotypes to different geographic areas, then more recent isolation may have caused the haplotypes to cease being moved among

groups and cause the differentiation in frequencies we see today. If this isolation was largely maintained by female philopatry but males continued to disperse among groups then we would not expect the signal from maternally and biparentally inherited genetic markers to be congruent, as was observed in this study.

Another explanation could be historical isolation of these populations during the last glacial maximum, which has been suggested as the cause for divergence of Puget Sound animals which are thought to have been trapped in inland waters by glaciers moving down from Vancouver Island and British Columbia (Burg et al. 1999). If this was the case, recent admixture since isolation ceased would have begun to erase signs of historical differentiation. Given that microsatellite loci appear to experience higher rates of gene flow among these areas, it is possible that microsatellite alleles have already become widespread in the population again while mtDNA haplotypes represent relic historical signals of isolation that will continue to homogenize over geographic areas given current rates of admixture. Certainly many other historical circumstances may have led to the current patterns of genetic differentiation, but regardless of the exact scenario it is important to recognize that admixture will continue to play an important role in the genetic structure among northeastern Pacific Ocean populations of this subspecies.

4.3 Study Limitations

The inferences that I have made are limited in a few critical ways because of the sampling opportunities available by this approach. Small sample sizes are an inherent problem to the study of endangered and protected species, particularly when sampling effort is expensive as is often the case with marine mammals. Clearly in some instances a

larger sample size would have lent more power to our statistical analyses (e.g., issues of unequal population size in Φ_{ST} and R_{ST} calculations). Moreover, many of the statistics used in this study assume that my sample is an adequate representation of the entire study population (in this case, many thousands of animals in each area). Therefore, I should not assume that sampling at the level of this study is adequate to detect rare alleles among recently diverged populations. However, a very high level of diversity among genetic markers was detected, particularly among mtDNA sequence haplotypes and nucleotide diversity. Haplotype and nucleotide diversity estimates in this study were actually consistent with or higher than estimates reported from previous studies among Pacific harbor seals over similar parts of their range (Westlake and O'Corry-Crowe 2002, Huber et al. 2010). Therefore, even under the assumption that the whole range of diversity may not have been captured, my results indicate highly diverse loci are common for mtDNA markers in this population and that high frequencies of unique haplotypes among areas support local differences in subpopulation haplotype composition.

I also must be cautious about conclusions drawn from the geographic sampling data because the stranded animals were not sampled at their natal location or observed alive in most cases. It is possible for stranded animals to be moved by ocean currents from the location where they died to the place they were recovered, limiting some of our spatial accuracy. In addition, because subadults and adults were sampled in this study there is the potential for us to sample transient migrant animals far from their natal site, which may or may not be contributing to gene flow in that area. Although these potential sources of spatial data error are of concern, we predict that the scale of behavioral philopatry observed in adult animals (Lowry et al. 2001) will limit the extent of post-

mortem movement and transient sampling effects for most animals relative to the scale at which this study was conducted. Seasonal variation in sampling of animals was also similar between adults and subadults in this study, which supports the difference in structuring among areas between adults and juveniles and indicates this result was not an artifact of seasonal variation in dispersal or genetic composition of the areas sampled.

The use of molecular markers in the study of evolution requires implicit assumptions about the evolution of these markers themselves. The mtDNA control region is believed to be selectively neutral and mutate at an exceptionally fast rate compared to nuclear DNA. However, comparisons among mammal groups indicated that marine mammals have lower overall mtDNA mutation rates than primates and other mammal orders (Pesole et al. 1999). This is a result of variability in the mutation rates of three domains within the control region (central and two flanking regions, ETAS and CSB). Harbor seals are known to have similar mtDNA mutation rates as other mammal orders in ETAS and central domains, while both cetaceans and pinnipeds have reduced mutation rates in the CSB domain compared to other orders, leading to an overall reduction in mtDNA mutation rate that may reduce our ability to detect divergence on the same time scale as other vertebrate taxa (Pesole et al. 1999). However, this would indicate that inferences of differentiation among groups in this study are actually conservative, suggesting differentiation has been established for some time under slower mutation rates. In addition, while microsatellite loci are predicted to mutate in a step-wise manner (inserting or deleting single di- or trinucleotide repeat units one at a time) it is possible for the slippage mechanism believed to be responsible for this variation to reanneal, and skip over multiple units at once. Such multiple unit jumps have been observed directly in

pedigree analyses of captive bottlenose dolphins (Duffield, pers. comm.). If the step-wise mutational model is insufficient or incorrect to describe the evolutionary behavior of these alleles, then my assumptions about the correlation between similar allele size and relatedness would misrepresent the genetic similarity within groups. The state of the science suggests that the genetic distance estimates used in this analysis (Φ_{ST} and R_{ST}) are the most appropriate of those currently available for the markers used in this study, however I should acknowledge that my inferences are limited by how accurately such models represent true biological phenomena.

4.4 Implications for management and wildlife health

Mitochondrial DNA analyses of haplotype frequencies among geographic sampling areas indicate that there are significant differences among some of the regions sampled in this study; a signal of differentiation which would identify MUs as defined by Moritz (1994a). Specifically, our results would suggest that under this definition there would be at least two, but possibly three management units; Puget Sound, and two coastal groups at the northern and southern extents of the Oregon and Washington coasts, because there were significant pairwise Φ_{ST} differentiation estimates among each of these comparisons using mtDNA. Considering the weaker differentiation signal which appears to be driven by distance among coastal sampling areas, it is more likely that these populations experience a 'stepping-stone' mode of gene flow between neighboring groups rather than a divergent break at a particular coastal location. However, it is not the regional distinction, but rather the connectivity among populations (as detected by, in particular, nuclear DNA) which was surprising given previous behavioral observations

for the species. I believe that it is this connectivity, and how it is mediated, that deserves further consideration in establishing policies for the management of this species.

Although Pacific harbor seals are not a species of concern in the Pacific Northwest, there are several threats to pinnipeds identified by the IUCN that could inhibit their ability to persist as a relevant, functional component of local ecosystems without proper management. Some of the greatest immediate threats to pinniped populations in general are incidental catch by commercial fisheries, and direct killing by fishermen (Reijnders et al. 1993). Incidental catch by commercial fisheries is known to occur in at least 20 species of pinnipeds worldwide, and is believed to have negative effects on Pacific harbor seals in Alaska. While the direct killing of harbor seals was reduced greatly after the elimination of local U.S. Bounty schemes in the 1960s and 1970s, there are still accounts of recreational and commercial fishermen killing seals to reduce their perceived effect in areas where they are frequently observed to feed on desirable fishery species (Reijnders et al. 1993).

Pinnipeds are also known to suffer from episodic mass mortalities as a result of viral epidemics and ENSO events, which have serious implications for the demographic structure of the surviving population if it is even able to persist (Reijnders et al. 1993). The increase in marine disposal of medical and domestic animal waste and international livestock transport is likely to increase the incidents of exposure of marine mammals to disease agents, making the study of epidemiology and consideration of episodic die-offs critical to the successful long-term management of this species. Increased exposure to environmental contaminants and consequent decreases in immunity and health status will also increase susceptibility to diseases and increase morbidity and mortality (Jonsson et

al. 1990). Pinniped species will also continue to face increasing habitat disturbance and degradation, competition with commercial fisheries for food resources, and changes in climate which are likely to exacerbate all of the issues already mentioned (Reijnders et al. 1993). Considering these risks, it is critical not only to recognize the importance of genetic variability in populations for their ability to persist, but to understand how that variability is maintained. The connectivity among animals from geographic areas analyzed in this study suggests that movement of animals among areas contributes to the genetic variability in each local group. This not only indicates that management of animals in any local area should consider the status of nearby groups that contribute to local genetic diversity, but that it should also consider the role of specific demographic groups in maintaining that genetic exchange. This study suggests that males and juveniles may have a higher contribution to genetic exchange among groups than females and adults, and therefore management should specifically address maintaining the potential for these demographic groups to disperse among areas.

4.5 Conclusions

The lack of clear divergence of these genetic markers by geographic region is important to our understanding of dispersal and gene flow among Pacific harbor seals of Oregon and Washington. While management stock boundaries have previously been established between inland Washington (Puget Sound) and coastal animals based on differences in pupping phenology, pelage and demographic trend (Carretta et al. 2009), the genetic markers in this study suggests a consistent low level of exchange between these areas. The mitochondrial data is consistent with previous work which showed high

levels of unique haplotypes in a sampling area, but some haplotypes within a matriline occurring in multiple areas (Huber et al. 2010). The nuclear data from this study are also consistent with work done on harbor seals in Alaska which suggested largely male-mediated gene flow among areas (Herreman et al. 2009), which would explain the lack of structure detected with these recombining microsatellite loci that are bi-parentally inherited, as opposed to the maternally inherited mtDNA markers. Taken together, these markers indicate that connectivity between these populations is higher than may be suggested by the consistent observations of philopatry in this species.

To date, this is the first detailed sampling of genetic diversity for Pacific harbor seals along the Oregon coast. While some haplotypes of Oregon animals were previously identified in Washington animals, many were novel for this set of primers and unique to Oregon populations. This high level of unique haplotypes and the variation in allele frequencies indicate that some local differentiation may be present, although patterns of shared alleles and spatial autocorrelation of clustering may be the result of complex historical processes and biased gene flow patterns. The level and mode of exchange between regions will obviously have important implications for local stock management. While we confirm differentiation at the level of current stock boundaries based on mtDNA haplotype frequencies used to define management units, the unexpected lack of divergence among several loci in this study suggests conservation concerns of local populations will affect populations on the regional level as well. Some infectious sources of morbidity and mortality for any of the local populations could quickly become important considerations in the management of harbor seals throughout this sampling range. Dispersal bias analyses also suggest that certain age classes may be more critical

to maintaining genetic diversity among regional subpopulations than others, and implies that changes in survival rates of these specific groups will directly affect the genetic structure of larger management stocks. Together, these data demonstrate that the demographics of dispersal and gene flow may have much greater consequences for the conservation and management of this species than previously assumed.

4.6 Recommendations for Future Work

Many of the implications of this work rely on the assumption that sampling has captured the range of genetic diversity of a population, and that it accurately reflects the frequencies of rare and common alleles within that population. The inferences about genetic structure that can be made confidently from this preliminary work was limited by our sample size as it likely did not meet the assumptions above. Therefore, the patterns of genetic structure presented here could be confirmed and nuances investigated through a more extensive sample, expanded both in sample size and ideally geographic range to determine with more certainty the geographic scale of local differentiation. The current stock boundary between coastal Oregon and California is admitted to be largely a delineation of political convenience (Carretta et al. 2009), so a thorough description of the scale at which to expect allele frequency changes is warranted for the entire west coast of the U.S. to address the appropriateness of this boundary.

One of the most pressing questions to come out of this work was how to interpret the movement patterns of juveniles; namely, are they non-reproductive transient visitors among areas, or do they contribute to gene flow? This question will be important to successful management of this species because if only a few demographic groups are

responsible for the majority of gene flow among populations (e.g. males or juveniles) then the management of these demographic groups will determine the scale of isolation and management for the whole species in this range. Further studies of genetic differentiation which also incorporate either pup tagging and re-sighting data over a two to three year period, or large scale sampling of males and pups at breeding rookeries to exclude or assign paternities may help address whether juvenile movement leads to gene flow.

Finally, I hope to see further use of MMSN data and tissues in analyses of population genetics, demographic structure, and health. The array of data collected in conjunction with tissue samples by regional stranding networks is incredibly valuable to the study of populations because it makes it possible to track causes of mortality, disease epidemiology, shifts in demographic structure, exposure to pollutants, and diet composition of these species non-invasively. Not only do tissues become available for a variety of analyses, but population structure can be analysed in the context of emerging health issues, climate-driven changes in food webs, and demographic composition of populations. Further studies which evaluate gene flow and dispersal among populations concurrently with prevalence of disease, for example, would be important investigations for the future management of the species if we hope to prevent mass mortalities in the case of an epidemic such as the phocine distemper virus which killed tens of thousands of harbor seals in the Atlantic Ocean only a few decades ago (Reeves et al. 2008). This valuable information is already being collected and made available to investigators, and can only enhance the ability of future studies to describe the range of factors impacting a population which will be critical to consider for effective management.

Table 1. Primer sequences and sources for all amplified microsatellite loci used in this study.

Primer	Sequence	Source
TBPv2	F: 5'-CTCTCCCATCCTCATATTA-3'	Burg et al. 1999
	R: 5'-GTACTACCCAATATAGAGAC-3'	
Pv9	F:5'-TAGTGTTTGGAAATGAGTTGGCA -3'	Allen et al. 1995
	R: 5'-ACTGATCCTTGTGAATCCCAGC-3'	
Pv11	F: 5'-GTGCTGGTGAATTAGCCCATTATAAG-3'	Goodman 1997
	R: 5'-CAGAGTAAGCACCCAAGGAGCAG-3'	
Hg6.1	F: 5'-TGCACCAGAGCCTAAGCAGACTG-3'	Allen et al. 1995
	R: 5'-CCACCAGCCAGTTCACCCAG-3'	
M11A	F: 5'-TGTTTCCCAGTTTTACCA-3'	Gemmel et al. 1997
	R: 5'-TACATTCACAAGGCTCAA-3'	
LC26	F: 5'-CTCAAGGGACTGAGCCACTCA-3'	Davis et al. 2002
	R: 5'-ACGGCAGGATTCTGAAACACT-3'	
LW11	F: 5'-CTCTCCCTCTCACCTTCC-3'	Davis et al. 2002
	R: 5'-GGCAAATGAGGTGATGTC-3'	

Table 2. Standard diversity indices and neutrality indices for mtDNA control region sequence data.

Index	Puget Sound	Coastal WA	Northern OR	Central OR	Total	Mean	Std. Dev.
Number Haplotypes	7	4	12	11	27	8.5	3.697
Substitution sites *(All transitions)*	27	3	15	15	31	15	9.798
Indels	1	0	2	2	2	1.25	0.957
Private subst. sites	12	0	2	1	15	3.75	5.56
Nucleotide diversity	8.944	0.911	3.362	5.198		4.604	3.385
Haplotypic diversity	0.917	0.733	0.883	0.956	0.91	0.872	0.097
Neutrality indices							
Tajima's D	-0.777	-0.507	-0.928	-0.164		-0.594	0.335
P-value	0.253	0.342	0.163	0.438		0.299	0.118
Fu's FS	0.050	-1.071	-3.066	-3.703		-1.947	1.740
P-value	0.458	0.105	0.084	0.032		0.170	0.195

Table 3. Results of pairwise comparisons from AMOVA analysis in Arlequin. Numbers above the diagonal are Φ_{ST} estimates, while numbers below the diagonal are associated p-values. Significant p-values are highlighted in bold print.

Sample Location	Puget Sound	Coastal WA	Northern OR	Central OR
Puget Sound	-	0.1555	0.0761	-0.0123
Coastal WA	**<0.001±0.000**	-	0.0170	0.1623
Northern OR	**0.036±0.015***	0.297±0.033	-	0.0381
Central OR	0.423±0.036	0.072±0.023**	0.117±0.027	-

*Significance of this pairwise comparison was *not* consistently supported in several re-sampling trials where a random subsample of northern Oregon haplotypes was used for comparisons.

**Significance of this pairwise comparison was consistently detected in several re-sampling trials (eight of ten) where a random subsample of northern Oregon haplotypes was used for comparisons.

Table 4. Three independent chains of Geneland cluster assignment of animals from four geographic sampling areas to three putative population clusters.

	Sampling region individuals assigned from:			
Cluster Assigned	Puget Sound	Coastal WA	Northern OR	Central OR
Iteration 1				
1	0	10	15	5
2	8	0	0	4
3	0	0	12	5
Iteration 2				
1	0	10	26	6
2	8	0	0	0
3	0	0	1	8
Iteration 3				
1	0	10	16	6
2	8	0	0	0
3	0	0	11	8

Table 5. Number of alleles, allelic size range, and associated Garza-Williamson statistics of microsatellite loci in four geographic sampling areas.

Number of Alleles

Locus	Puget Sound	Coastal WA	Northern OR	Central OR	Total	Mean	Std. Dev.
LC26	4	4	8	7	9.0	5.75	2.062
LW11	3	6	6	6	6.0	5.25	1.500
Pv9	2	3	5	4	6.0	3.50	1.291
Pv11	7	5	8	7	11.0	6.75	1.258
M11A	4	4	4	4	4.0	4.00	0.000
TB	5	6	7	8	8.0	6.50	1.291
LW11-2	2	2	2	2	2.0	2.00	0.000
Hg6.1	6	9	10	8	11.0	8.25	1.708
Mean	4.125	4.875	6.250	5.750	7.125		
Std. Dev	1.808	2.167	2.550	2.188	3.018		

Allelic Size Range

Locus	Puget Sound	Coastal WA	Northern OR	Central OR	Total	Mean	Std. Dev.
LC26	13	17	23	23	25.0	19.00	4.899
LW11	4	10	10	10	10.0	8.50	3.000
Pv9	12	9	14	17	19.0	13.00	3.367
Pv11	14	10	20	20	20.0	16.00	4.899
M11A	6	6	6	6	6.0	6.00	0.000
TB	10	17	19	19	19.0	16.25	4.272
LW11-2	2	2	2	2	2.0	2.00	0.000
Hg6.1	14	18	23	19	23.0	18.50	3.697
Mean	9.375	11.125	14.625	14.500	15.500		
Std. Dev	4.442	5.395	7.449	7.053	7.858		

Garza-Williamson Statistic

Locus	Puget Sound	Coastal WA	Northern OR	Central OR	Mean	Std. Dev.
LC26	0.286	0.222	0.333	0.292	0.283	0.046
LW11	0.600	0.545	0.545	0.545	0.559	0.028
Pv9	0.154	0.300	0.333	0.222	0.252	0.080
Pv11	0.467	0.455	0.381	0.333	0.409	0.063
M11A	0.570	0.571	0.571	0.571	0.571	0.001
TB	0.455	0.333	0.350	0.400	0.385	0.055
LW11-2	0.667	0.667	0.667	0.667	0.667	0.000
Hg6.1	0.400	0.474	0.417	0.400	0.423	0.035
Mean	0.450	0.446	0.450	0.429		
Std. Dev.	0.159	0.141	0.119	0.143		

Table 6. Expected heterozygosity, observed heterozygosity, and associated p-values of departures from Hardy-Weinberg equilibrium as calculated in Arlequin.

	Expected Heterozygosity (He)						Observed Heterozygosity (Ho)					
Locus	Puget Sound	Coastal WA	Northern OR	Central OR	Mean	Std. Dev.	Puget Sound	Coastal WA	Northern OR	Central OR	Mean	Std. Dev.
LC26	0.791	0.605	0.778	0.743	0.729	0.085	0.667	0.500	0.741	0.714	0.656	0.108
LW11	0.647	0.747	0.727	0.661	0.696	0.049	0.667	0.800	0.741	0.714	0.731	0.056
Pv9	0.425	0.574	0.642	0.577	0.555	0.092	0.556	0.700	0.556	0.857	0.667	0.144
Pv11	0.876	0.737	0.812	0.762	0.797	0.061	0.778	0.600	0.889	0.714	0.745	0.121
M11A	0.673	0.689	0.630	0.616	0.652	0.035	0.889	0.400	0.593	0.500	0.596	0.211
TB	0.745	0.800	0.825	0.793	0.791	0.033	0.667	0.600	0.815	0.786	0.717	0.101
LW11-2	0.503	0.479	0.425	0.495	0.475	0.035	0.333	0.300	0.444	0.357	0.359	0.062
Hg6.1	0.856	0.889	0.821	0.865	0.858	0.028	0.889	0.900	0.852	0.857	0.875	0.024
Mean	0.690	0.690	0.707	0.689			0.681	0.600	0.704	0.688		
Std. Dev	0.161	0.132	0.138	0.123			0.182	0.200	0.157	0.175		

H-W Equilibrium: p-values

Locus	Puget Sound	Coastal WA	Northern OR	Central OR	Mean	Std. Dev.
LC26	0.138	**0.025**	0.718	0.559	0.360	0.331
LW11	0.107	0.417	0.595	0.663	0.446	0.248
Pv9	1.000	0.520	0.293	**0.047**	0.465	0.406
Pv11	0.727	0.568	0.172	0.616	0.521	0.242
M11A	**0.002**	**0.035**	0.918	0.786	0.435	0.484
TB	0.726	0.070	0.699	0.401	0.474	0.307
LW11-2	0.493	0.481	1.000	0.571	0.636	0.246
Hg6.1	0.836	0.955	0.767	0.775	0.833	0.087
Mean	0.504	0.384	0.645	0.552		
Std. Dev	0.378	0.325	0.286	0.239		

Table 7. Results of LOSITAN F_{ST}-outlier selection analysis. Probabilities that simulated F_{ST} estimates are less than observed F_{ST} estimates which are very high or very low are likely to be under negative or balancing selection, respectively. Loci with p-values beyond the LOSITAN significance thresholds are highlighted in bold print below.

Locus	Het. (obs)	F_{ST}	P(sim F_{ST}<sample)
LC26	0.7581	0.0380	0.7353
LW11	0.6953	0.0010	0.2744
Pv9	0.7091	0.2182	**0.9999**
Pv11	0.7896	-0.0089	0.1073
M11A	0.6367	-0.0243	**0.0492**
TB	0.8187	0.0341	0.6939
LW11-2	0.4601	-0.0333	**0.0464**
Hg6.1	0.8571	-0.0010	0.1405

Table 8. Pairwise R_{ST} estimates for comparisons with varying numbers of putative populations used in the step-wise consolidation of groups which were not found to be significantly different. Numbers above the diagonal are R_{ST} estimates based on a step-wise mutational model, while numbers below the diagonal are associated p-values. Significant p-values indicating differentiation are highlighted in bold print.

Step 1	Puget Sound	Coastal WA	Northern OR	Central OR
Puget Sound		0.1928	0.1053	0.1498
Coastal WA	**<0.001±0.000**		0.0098	-0.0012
Northern OR	**0.009±0.009**	0.279±0.032		-0.0079
Central OR	**<0.001±0.000**	0.432±0.038	0.621±0.048	

Step 2	Puget Sound	Coastal WA	Coastal OR
Puget Sound		0.1928	0.1167
Coastal WA	**<0.001±0.000**		0.0076
Coastal OR	**<0.001±0.000**	0.342±0.049	

Table 9. Results from three independent chains of Geneland cluster assignment of animals from four geographic sampling areas to two putative population clusters using all eight microsatellite loci and only the five neutral loci, with associated mean within-group F_{IS} and mean among-group F_{ST}.

All loci	*Sampling region individuals assigned from:*					
Cluster	Puget Sound	Coastal WA	Northern OR	Central OR	F_{IS}	F_{ST}
1	0	10	27	14	0.024	0.057
2	8	0	0	0	0.022	

5 loci	*Sampling region individuals assigned from:*					
Cluster	Puget Sound	Coastal WA	Northern OR	Central OR	F_{IS}	F_{ST}
1	0	10	27	12	0.010	0.027
2	8	0	0	2	0.075	

Table 10a. Results of FSTAT analyses of biased dispersal using microsatellite data. Analyses of sex-biased dispersal do not indicate differences in these indices among sexes when all sampling areas are used or when only the three coastal sampling areas are included.

Sex-biased Dispersal

2 Populations - Inland and Coastal

	Male	Female	Overall	p-value
mAIc	0.00164	-0.0023		
vAIc	7.06199	4.5194		0.217
Hs	0.706	0.7239		
F_{IS}	0.0642	0.445	0.0433	
F_{ST}	-0.0132	-0.0519	-0.0095	0.936

3 Coastal Populations only

	Male	Female	Overall	p-value
mAIc	0.1371	-0.1987		
vAIc	6.4169	5.4609		0.391
Hs	0.6996	0.7226	0.7007	
F_{IS}	0.0511	0.0485	0.0389	
F_{ST}	-0.0129	-0.0354	-0.0095	0.92

Table 10b. Results of FSTAT analyses of biased dispersal using microsatellite data. Aanalysis by age-class indicates a significant difference in F_{ST} between young and adult animals (significant p-values are highlighted in bold print).

Age class-biased Dispersal

2 Populations - Inland and Coastal

	Adult	Young	Overall	p-value
mAIc	0.00694	-0.00586		
vAIc	9.7166	7.30851		0.742
Hs	0.6843	0.6949	0.6967	
F_{IS}	0.0595	-0.0231	0.0238	
F_{ST}	0.1385	0.059	0.0577	**0.038**

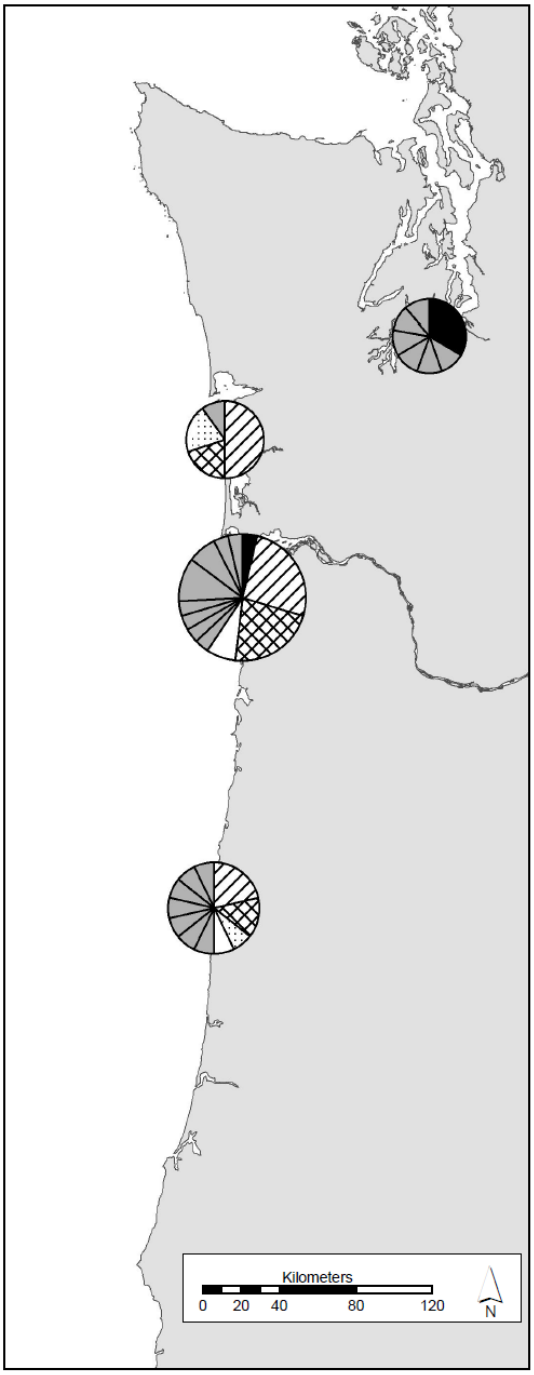

Figure 1. Haplotype frequency composition of the four geographic sampling areas. Shared patterns indicate a haplotype is shared among regions, whereas gray sections indicate a haplotype that was unique to that one single geographic are (many such 'singletons' have been grayed out for clarity; all are unique to a single sampling region.)

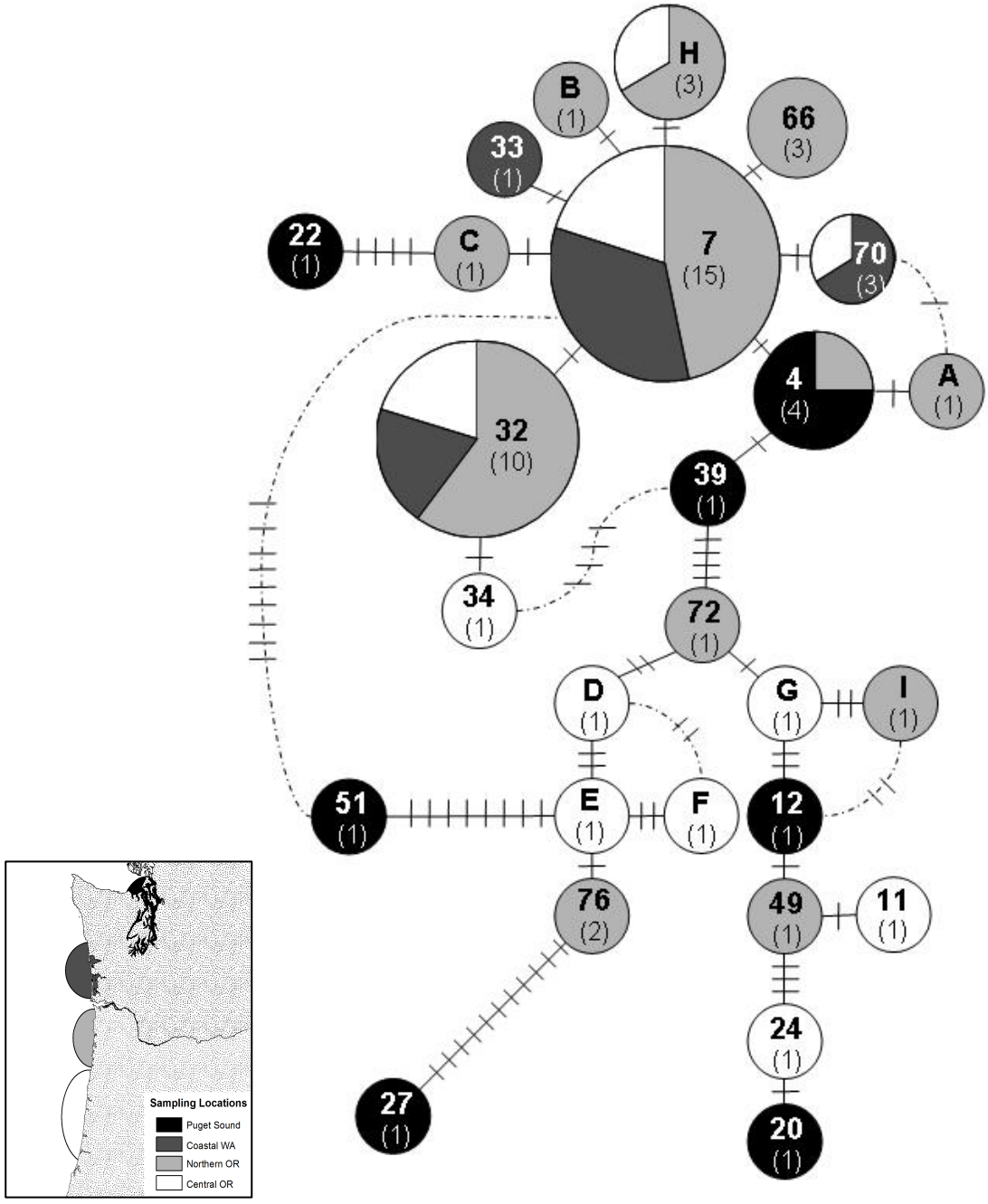

Figure 2. Minimum spanning network for all mtDNA haplotypes, as generated in Arlequin and TCS. Boldface type indicates the identification label of each haplotype; numbers indicate haplotypes previously identified by Huber et al. (2010), while letters indicate novel haplotypes for this primer set. The frequency with which each haplotype was observed is shown in parentheses, and represented by the relative size of each circle. Colors indicate the sampling location in which a haplotype was found (see inset map) Each hash mark represents a single mutational event between two haplotypes. Dashed lines indicated alternative network linkages supported in both Arlequin and TCS.

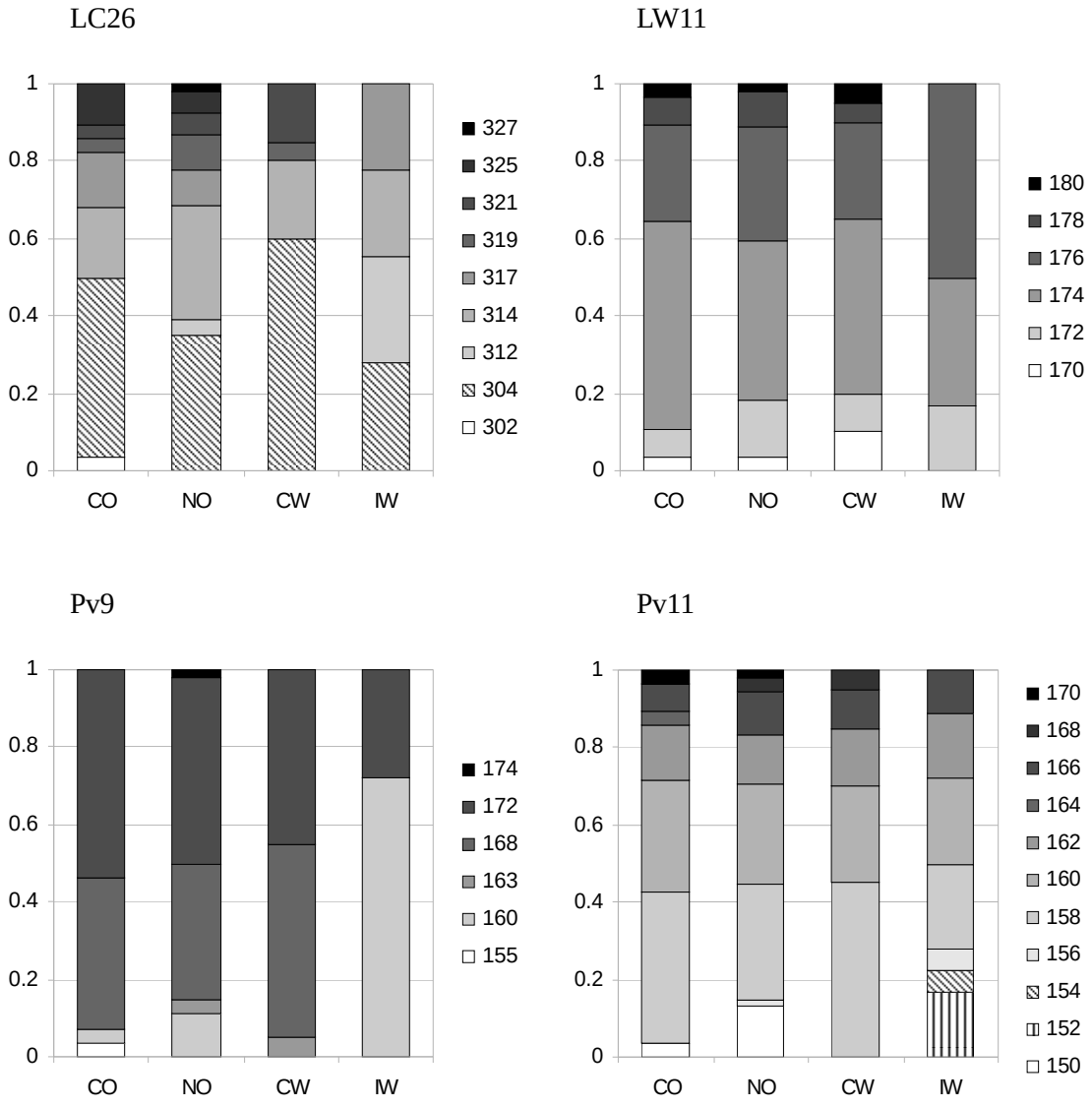

Figure 3. Relative allele frequencies among geographic sampling areas for each of eight polymorphic microsatellite loci. The x-axis indicates sampling region; CO = Central Oregon, NO = Northern Oregon, CW = Coastal Washington, and IW = Inland Washington (Puget Sound). Legend items describe individual allele lengths for each locus.

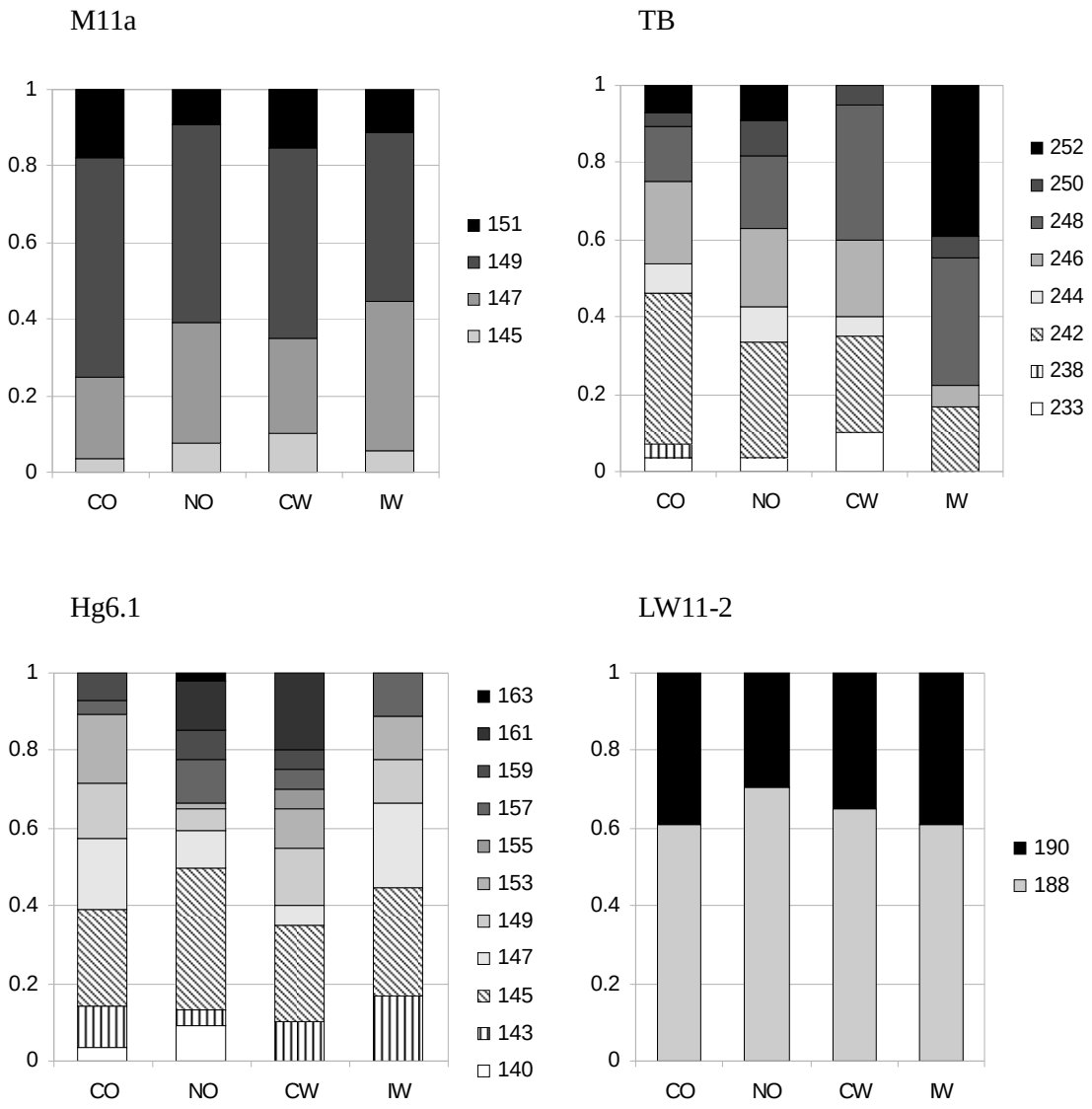

Figure 3 (continued). Relative allele frequencies among geographic sampling areas for each of eight polymorphic microsatellite loci. The x-axis indicates sampling region; CO = Central Oregon, NO = Northern Oregon, CW = Coastal Washington, and IW = Inland Washington (Puget Sound). Legend items describe individual allele lengths for each locus.

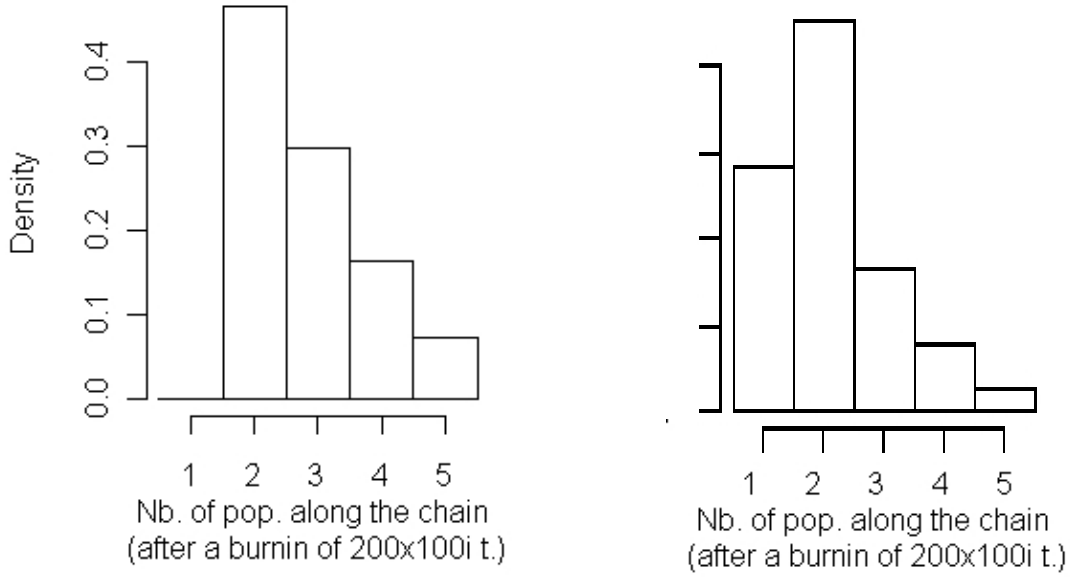

Figure 4. Posterior probability density distribution for k = 1-5 putative populations for data cluster modeling in Geneland using all microsatellite loci (left), and five selectively neutral loci (right). The model with the highest posterior probability in both cases, which is therefore the most likely, is k = 2 subpopulations.

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Appendix A: Sample Collection

Source, stranding identification number, collection location and demographic classes of all animals sampled in this study. Source abbreviations indicate stranding network affiliates that provided tissue samples; NOSW = Northern Oregon/Southern Washington Marine Mammal Stranding Program, HMSC = Hatfield Marine Science Center, and WDFW = Washington Department of Fish and Wildlife.

Sample Region	ID	Age	Sex	City	State	Source
Puget Sound						
	CRC-970	UNK	UNK	UNK	WA	WDFW
	EI-0901	Adult	F	McNeil Island	WA	WDFW
	EI-0903	Pup	M	Eagle Island	WA	WDFW
	GI-0907	Yearling	M	McNeil Island	WA	WDFW
	GI-0912	Adult	M	Gertrude Island	WA	WDFW
	GI-0945	Adult	M	Gertrude Island	WA	WDFW
	2009-050	Pup	M	Gig Harbor	WA	WDFW
	2009-069	Pup	M	Bremerton	WA	WDFW
	2009-074	Pup	M	Poulsbo	WA	WDFW
Coastal WA						
	080909	Subadult	M	Ocean Park	WA	NOSW
	2009-067	Subadult	M	Grayland	WA	WDFW
	2009-120	Adult	M	Grayland	WA	WDFW
	100321	Adult	F	Long Beach	WA	NOSW
	100629A	Adult	F	Long Beach	WA	NOSW
	090728	Subadult	M	Long Beach	WA	NOSW
	101001	Adult	M	Oysterville	WA	NOSW
	070313B	Adult	F	Oysterville	WA	NOSW
	2009-053	Adult	F	Seaview	WA	WDFW
	2009-061	Yearling	M	Westport	WA	WDFW
Northern OR						
	070602	Pup	F	Garibaldi	OR	NOSW
	070611	Adult	M	Warrenton	OR	NOSW
	071001	Adult	M	Warrenton	OR	NOSW
	071005	Subadult	F	Del Ray	OR	NOSW
	071012	Adult	F	Seaside	OR	NOSW
	080227	Subadult	M	Garibaldi	OR	NOSW
	080316	Subadult	F	Cannon Beach	OR	NOSW
	080407	Yearling	M	Seaside	OR	NOSW
	080510	Adult	F	Pacific City	OR	NOSW
	080630	Adult	M	Cannon Beach	OR	NOSW

Appendix A (continued): Sample Collection

Source, stranding identification number, collection location and demographic classes of all animals sampled in this study. Source abbreviations indicate stranding network affiliates that provided tissue samples; NOSW = Northern Oregon/Southern Washington Marine Mammal Stranding Program, HMSC = Hatfield Marine Science Center, and WDFW = Washington Department of Fish and Wildlife.

Sample Region	ID	Age	Sex	City	State	Source
Northern OR (continued)	081014	Adult	UNK	Rockaway	OR	NOSW
	090507	Adult	M	Gearhart	OR	NOSW
	090707	Pup	F	Gearhart	OR	NOSW
	090816	Adult	F	Gearhart	OR	NOSW
	090826	Pup	F	Nehalem	OR	NOSW
	090827	Adult	M	Cannon Beach	OR	NOSW
	090907	Yearling	M	Gearhart	OR	NOSW
	090921	Yearling	M	Cannon Beach	OR	NOSW
	100319	Pup	F	Seaside	OR	NOSW
	100323	Adult	F	Seaside	OR	NOSW
	100529	Pup	F	Gearhart	OR	NOSW
	100618	Yearling	M	Gearhart	OR	NOSW
	100620	Yearling	F	Gearhart	OR	NOSW
	100621	Adult	M	Cannon Beach	OR	NOSW
	100630	Adult	M	Gearhart	OR	NOSW
	100917	Yearling	M	Gearhart	OR	NOSW
	101111	Subadult	M	Tillamook	OR	NOSW
	090820A	Adult	F	Gearhart	OR	NOSW
	100615A	Pup	M	Seaside	OR	NOSW
	100629B	Adult	M	Cannon Beach	OR	NOSW
Central OR						
	060530	Adult	M	Yachats	OR	HMSC
	061025	Subadult	F	Florence	OR	HMSC
	070212	Adult	F	Bandon	OR	HMSC
	070416	Subadult	M	Lakeside	OR	HMSC
	070713	Pup	M	Charleston	OR	HMSC
	070721	Pup	M	Bandon	OR	HMSC
	071105	Pup	F	Newport	OR	HMSC
	080415	Yearling	F	Lincoln City	OR	HMSC
	080812	Pup	UNK	Florence	OR	HMSC
	080822	Subadult	M	South Beach	OR	HMSC
	090121	Adult	F	Waldport	OR	HMSC
	090227	Subadult	M	Lincoln Beach	OR	HMSC
	090507	Adult	M	Newport	OR	HMSC
	090508	Subadult	M	Newport	OR	HMSC

Appendix B: Method Protocols

B.1. DNA extraction, quantification, amplification, purification, and target locus sequencing or amplification reaction protocols.

DNA Extraction – Dneasy Blood and Tissue Kit (Qiagen)

Add 180 uL buffer ATL to 1.5 mL centrifuge tube.
Add 20 uL proteinase K to centrifuge tube.
Place <25 mg tissue in centrifuge tube and vortex to mix.
Incubate tissue samples at 56 ^{o}C 24-36 hours until completely lysed.
Vortex lysis product 15 seconds to mix.
Add 200 uL buffer AL and vortex to mix.
Incubate mixture at 56 ^{o}C for 10 minutes.
Add 200 uL ethanol and vortex to mix. Centrifuge mixture 1 minute.
Pipet supernatant into mini spin column in 2 mL collection tube. Centrifuge 1 minute.
Discard flow-thru and collection tube, and place column in a new collection tube.
Add 500 uL buffer AW1. Centrifuge 1 minute.
Discard flow-thru and collection tube, and place column in a new collection tube.
Add 500 uL buffer AW2. Centrifuge 3 minutes.
Discard flow-thru and collection tube, place column in a clean 1.5 mL centrifuge tube.
Pipet 50 uL buffer AE directly onto column membrane.
Incubate at room temperature 1 minute.
Centrifuge 1 minute. Pipet eluted DNA into a clean, labeled 0.6 mL centrifuge tube.
Pipet 50 uL buffer AE directly onto column membrane.
Incubate at room temperature 1 minute.
Centrifuge 1 minute. Pipet eluted DNA into a clean, labeled 0.6 mL centrifuge tube.
Store at 2-4 ^{o}C until use.

NanoDrop Quantification of Samples

DNA samples vortexed and centrifuged briefly before use.
Pipet 2 uL buffer AE in a single droplet on the Nanodrop crystal and lower cover.
Take a blank standardization read from the instrument.
Wipe AE buffer away with kimwipe, and pipet 2 uL sample onto Nanodrop crystal.
Lower the instrument cover, and take a measurement reading.
Repeat pipetting, taking readings, and removing samples for each sample.

mtDNA PCR Protocol

Pipet the following into each reaction mixture with PCR Ready to Go Bead:
1 uL 25 uMolar TRO primer stock
1 uL 25 uMolar PvH00034 primer stock
A volume of DNA between 1 and 23 uL equal to 150 ng or more of DNA

Vortex and spin reaction mix briefly before use.
Run samples in thermalcycler under program from Huber et al. (2010)

B.1 (continued). DNA extraction, quantification, amplification, purification, and target locus sequencing and amplification reaction protocols.

PCR Clean-Up—QIAquick PCR Purification Kit (Qiagen)

Add 125 uL buffer PB to PCR mixture.
Pipet mixture into spin column with collection tube and centrifuge 1 minute.
Discard flow-thru. Add 750 uL buffer PE to column.
Centrifuge 1 minute, and discard flow-thru.
Centrifuge 1 minute more, and discard flow-thru.
Transfer column to clean 1.5 mL centrifuge tube.
Add 50 uL buffer EB directly to membrane to elute DNA. Centrifuge 1 minute.
Store eluted DNA in a 0.6 mL centrifuge tube.

mtDNA Sequencing Reaction—CGRB Core Laboratories

Pipet the following into each reaction mixture:
2.5 uL PCR product
0.25 uL 25 uMolar primer stock
2 uL Big Dye Terminator sequencing mix
1 uL 5x buffer
4.25 uL sterile water

Vortex and spin reaction mix briefly before use.
Run samples in thermalcycler under CGRB program.

Microsatellite Amplification

Pipet the following into each reaction mixture with PCR Ready to Go Bead:
0.5 or 1 uL of forward primer (depending on locus)
0.5 or 1 uL of reverse primer (depending on locus)
6 uL Betaine
14 to 17.5 uL sterilized water (adjusted based on DNA volume)
0.5 to 4 uL template DNA (depending on locus)

Vortex and spin reaction mix briefly before use.
Run samples in thermalcycler under one of four microsatellite programs.

B.2. PCR thermalcycler programs for the amplification of microsatellite loci.

	Valsecchi and Amos (1996)	Valsecchi and Amos (1996)	Gemmel et al. (1997)	Burg et al. (1999)
Initial Step	95 ^{o}C for 5 min	95 ^{o}C for 5 min	2 min at 94 ^{o}C	2 min at 94 ^{o}C
Set 1 # cycles	5	5	8	8
1	60 sec at 93 ^{o}C	60 sec at 93 ^{o}C	30 sec at 94 ^{o}C	60 sec at 94 ^{o}C
2	60 sec at 52 ^{o}C	60 sec at 48 ^{o}C	30 sec at 48 ^{o}C	60 sec at 48 ^{o}C
3	50 sec at 72 ^{o}C	50 sec at 72 ^{o}C	40 sec at 72 ^{o}C	60 sec at 72 ^{o}C
Set 2 # cycles	9	9	25	25
1	45 sec at 90 ^{o}C	45 sec at 90 ^{o}C	15 sec at 94 ^{o}C	40 sec at 89 ^{o}C
2	60 sec at 60 ^{o}C	60 sec at 56 ^{o}C	15 sec at 52 ^{o}C	40 sec at 48 ^{o}C
3	60 sec at 73 ^{o}C	60 sec at 73 ^{o}C	40 sec at 72 ^{o}C	40 sec at 72 ^{o}C
Loci	LC26, LW11, Pv9	Pv11	Hg6.1	TBPv2, M11A, Hg6.1

Appendix C: MtDNA Haplotypes. Unique mtDNA haplotype nucleotides are shown over 33 variable sites. Blank spaces indicate the nucleotide at that site for a haplotypes is consistent with the sequence listed in the reference 'ID' row. Letters indicate nucleotide substitutions, and dash marks indicate indels. Site position numbering is based on Arnason and Johnsson (1992).

Site	16388	16411	16442	16444	16447	16449	16464	16470	16471	16472	16477	16479	16483	16484	16485	16491	16494	16518	16559	16568	16569	16578	16579	16580	16588	16594	16607	16613	16615	16619	16632	16694	16737	16745
ID	T	A	A	G	A	T	G	T	G	C	C	C	C	-		G	C	T	A	A	T	C	G	G	A	T	T	G	T	A	T	A	G	C
4									A																									
7																																		
11	C		G		-					T										G				A	G					G	C			
12	C		G		-					T										G				A							C			
20	C		G		-	C							T							G										G	C			
22																A		C	G									A				G		
24	C		G		-	C				T			T							G										G	C			
27	C	G			-		A			T	T	T									C		A		G	C		A	C		C			
32													T																					
33																				G														
34													T										A											
39									A	T																								
49	C		G		-					T										G				A						G	C			
51	C				-							T					T								G		C		C	G			A	
66																						T												
70				A																														
72	C		G		-				A	T																					C			
76	C		G		-							T								G						C					C			
A				A					A																									
B								C																										
C																A																		
D	C		G		-				A											G											C			
E	C		G		-							T								G											C			
F	C		G		-															G											C			T
G	C		G		-				A	T														A							C			
H														C																				
I	C		G		-					T				C										A							C			

Appendix D: MtDNA Re-sampling Estimates

D.1. Group-wise AMOVA results of random resampling from the largest population sample (northern Oregon) for population comparison iterations. Standard F_{ST} statistics are included, as well as differentiation estimates obtained from using all sampled individuals (i.e. allowing unequal sample sizes) for comparison. Significant p-values for overall Φ_{ST} are highlighted in bold print (all F_{ST} values were significant).

Iteration	Φ_{ST}	P-values	F_{ST}	P-values
1	0.0523	0.071±0.001	0.1154	<0.0001±0.000
2	0.0506	0.062±0.001	0.0993	<0.0001±0.000
3	0.0823	**0.017±0.001**	0.1194	<0.0001±0.000
4	0.0474	0.079±0.001	0.1074	<0.0001±0.000
5	0.0835	**0.015±0.001**	0.1194	<0.0001±0.000
6	0.0489	0.079±0.001	0.1033	<0.0001±0.000
7	0.0529	0.065±0.001	0.1074	<0.0001±0.000
8	0.0545	0.061±0.001	0.1074	<0.0001±0.000
9	0.0665	**0.037±0.001**	0.1315	<0.0001±0.000
10	0.0666	**0.039±0.001**	0.1556	<0.0001±0.000
Mean	0.0605	0.053	0.1123	<0.0001±0.000
All samples	0.0590	**0.033±0.001**	0.1209	<0.0001±0.000

D.2. Ten iterations of pairwise Φ_{ST} estimates for comparisons of four putative populations using random subsamples from the northern Oregon group (n = 12). Numbers above the diagonal are Φ_{ST} estimates based on a pairwise distance model, while numbers below the diagonal are associated p-values. Significant p-values indicating differentiation are highlighted in bold print.

1	Puget Sound	Coastal WA	Northern OR	Central OR
Puget Sound		0.1555	0.0217	-0.0123
Coastal WA	**<0.001±0.000**		0.0740	0.1623
Northern OR	0.216±0.045	0.198±0.042		-0.0079
Central OR	0.049±0.036	**0.036±0.020**	0.423±0.076	

2	Puget Sound	Coastal WA	Northern OR	Central OR
Puget Sound		0.1555	0.0026	-0.0123
Coastal WA	**<0.001±0.000**		0.0768	0.1623
Northern OR	0.360±0.049	0.081±0.032		-0.0018
Central OR	0.405±0.037	**0.045±0.020**	0.360±0.019	

3	Puget Sound	Coastal WA	Northern OR	Central OR
Puget Sound		0.1555	0.0913	-0.0123
Coastal WA	**<0.001±0.000**		-0.0153	0.1623
Northern OR	**0.027±0.014**	0.676±0.034		0.0981
Central OR	0.586±0.034	**0.036±0.015**	**0.036±0.028**	

4	Puget Sound	Coastal WA	Northern OR	Central OR
Puget Sound		0.1555	0.0061	-0.0123
Coastal WA	**<0.001±0.000**		0.0605	0.1623
Northern OR	0.369±0.057	0.189±0.029		-0.0127
Central OR	0.414±0.036	**0.036±0.015**	0.396±0.036	

5	Puget Sound	Coastal WA	Northern OR	Central OR
Puget Sound		0.1555	0.1020	-0.0123
Coastal WA	**<0.001±0.000**		-0.0009	0.1623
Northern OR	**0.018±0.012**	0.333±0.0360		0.0926
Central OR	0.468±0.063	0.072±0.023	**0.036±0.024**	

D.2 (continued). Ten iterations of pairwise Φ_{ST} estimates for comparisons of four putative populations using random subsamples from the northern Oregon group (n = 12). Numbers above the diagonal are Φ_{ST} estimates based on a pairwise distance model, while numbers below the diagonal are associated p-values. Significant p-values indicating differentiation are highlighted in bold print.

6	Puget Sound	Coastal WA	Northern OR	Central OR
Puget Sound		0.1555	0.0033	-0.0123
Coastal WA	**<0.001±0.000**		0.0846	0.1623
Northern OR	0.315±0.065	0.081±0.021		-0.0143
Central OR	0.477±0.045	0.054±0.020	0.505±0.043	

7	Puget Sound	Coastal WA	Northern OR	Central OR
Puget Sound		0.1555	0.0282	-0.0123
Coastal WA	**<0.001±0.000**		0.0032	0.1623
Northern OR	0.216±0.041	0.459±0.044		0.0140
Central OR	0.505±0.043	**0.045±0.020**	0.288±0.030	

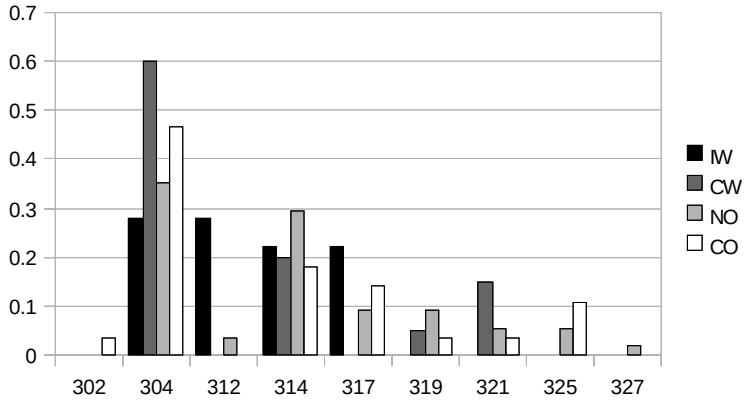
8	Puget Sound	Coastal WA	Northern OR	Central OR
Puget Sound		0.1555	0.0200	-0.0123
Coastal WA	**<0.001±0.000**		0.0771	0.1623
Northern OR	0.297±0.031	0.099±0.025		0.0009
Central OR	0.405±0.047	**0.045±0.015**	0.342±0.065	

9	Puget Sound	Coastal WA	Northern OR	Central OR
Puget Sound		0.1555	0.0677	-0.0123
Coastal WA	**<0.001±0.000**		0.0259	0.1623
Northern OR	0.117±0.033	0.288±0.038		0.0348
Central OR	0.460±0.064	**0.018±0.012**	0.216±0.024	

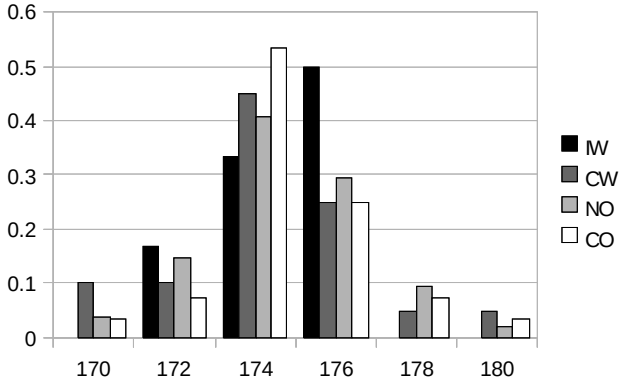
10	Puget Sound	Coastal WA	Northern OR	Central OR
Puget Sound		0.1555	0.0643	-0.0123
Coastal WA	**<0.001±0.000**		0.0290	0.1623
Northern OR	0.117±0.024	0.279±0.032		0.0345
Central OR	0.496±0.048	**0.045±0.015**	0.279±0.037	

Appendix E: Microsatellite Allele Distributions

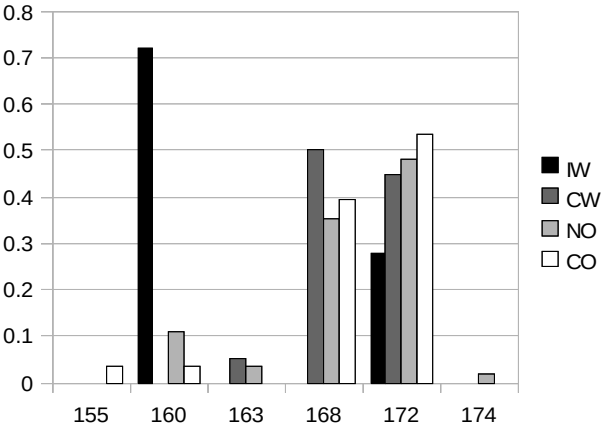
LC26



LW11



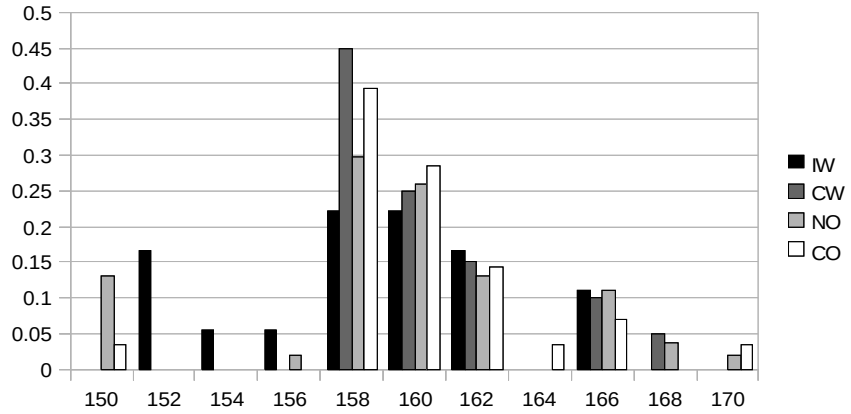
Pv9



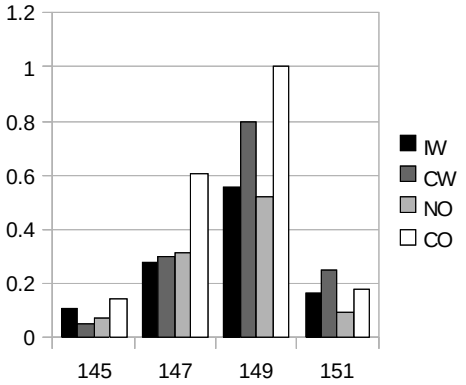
Allele size range frequency distributions for each microsatellite locus for the four geographic sampling areas. Numbers on the x-axis indicate allele size, while the y-axis indicates frequency. Shading indicated geographic sampling area as follows: black = Inland Washington (Puget Sound), dark gray = Coastal Washington, light gray = Northern Oregon, white = Central Oregon.

Appendix E (continued): Microsatellite Allele Distributions

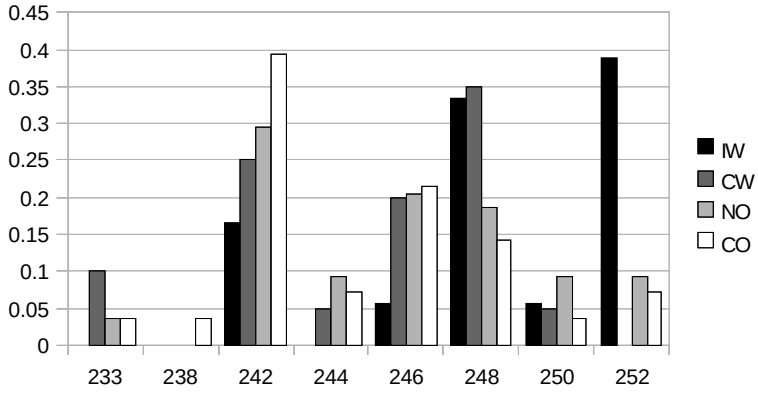
Pv11



M11A



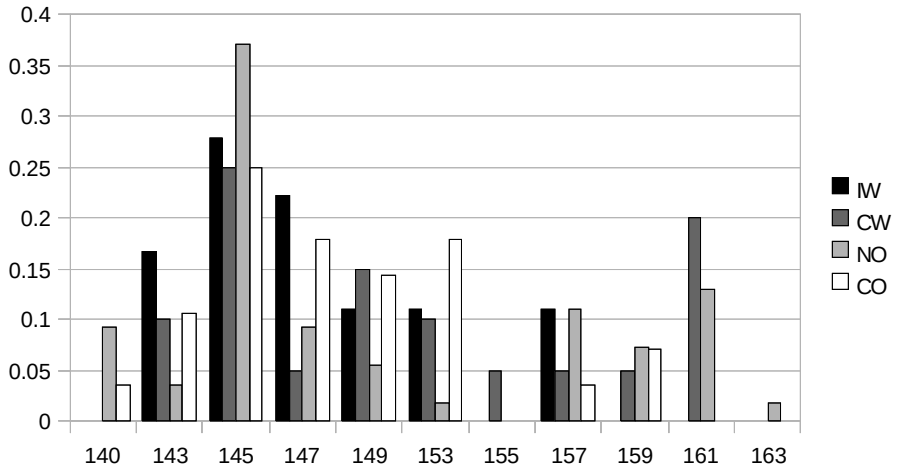
TB



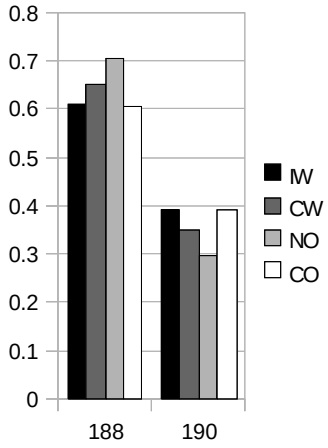
Allele size range frequency distributions for each microsatellite locus for the four geographic sampling areas. Numbers on the x-axis indicate allele size, while the y-axis indicates frequency. Shading indicated geographic sampling area as follows: black = Inland Washington (Puget Sound), dark gray = Coastal Washington, light gray = Northern Oregon, white = Central Oregon.

Appendix E (continued): Microsatellite Allele Distributions

Hg6.1



LW11-2



Allele size range frequency distributions for each microsatellite locus for the four geographic sampling areas. Numbers on the x-axis indicate allele size, while the y-axis indicates frequency. Shading indicated geographic sampling area as follows: black = Inland Washington (Puget Sound), dark gray = Coastal Washington, light gray = Northern Oregon, white = Central Oregon.

Appendix F: Microsatellite Indices for Two Populations

Allele counts and ranges, and Garza-Williamson indices (number of alleles divided by allelic range) for eight microsatellite loci in two consolidated putative subpopulations, as measured in Arlequin. A lack of low Garza-Williamson values (all >0.1) indicates it is unlikely any of these loci is influenced by a recent population bottleneck.

Number of Alleles

Locus	Coastal	Inland WA	Mean	Std. Dev.	Total
LC26	9	4	6.5	3.536	9
LW11	6	3	4.5	2.121	6
Pv9	6	2	4.0	2.828	6
Pv11	9	7	8.0	1.414	11
M11A	4	4	4.0	0.000	4
TB	8	5	6.5	2.121	8
LW11-2	2	2	2.0	0.000	2
Hg6.1	11	6	8.5	3.536	11
Mean	6.88	4.13	5.50	1.95	7.13
Std. Dev.	2.95	1.81	2.38	0.81	3.02

Allelic Size Range

Locus	Coastal	Inland WA	Mean	Std. Dev.	Total
LC26	25	13	19.0	8.485	25
LW11	10	4	7.0	4.243	10
Pv9	19	12	15.5	4.950	19
Pv11	20	14	17.0	4.243	20
M11A	6	6	6.0	0.000	6
TB	19	10	14.5	6.364	19
LW11-2	2	2	2.0	0.000	2
Hg6.1	23	14	18.5	6.364	23
Mean	15.50	9.38	12.44	4.33	15.50
Std. Dev.	7.86	4.44	6.15	2.42	7.86

Garza-Williamson Index

Locus	Coastal	Inland WA	Mean	Std. Dev.
LC26	0.346	0.286	0.316	0.043
LW11	0.545	0.600	0.573	0.039
Pv9	0.300	0.154	0.227	0.103
Pv11	0.429	0.467	0.448	0.027
M11A	0.571	0.571	0.571	0.000
TB	0.400	0.455	0.427	0.039
LW11-2	0.667	0.667	0.667	0.000
Hg6.1	0.458	0.400	0.429	0.041
Mean	0.465	0.450	0.457	0.010
Std. Dev.	0.115	0.159	0.137	0.031

Appendix F (continued): Microsatellite Indices for Two Populations

Observed and expected heterozygosity at each microsatellite locus by consolidated putative population as calculated in Arlequin.

Observed Heterozygosity (Ho)

Locus	Coastal	Inland WA	Mean	Std. Dev.	Total
LC26	0.686	0.667	0.676	0.014	0.757
LW11	0.745	0.667	0.706	0.055	0.701
Pv9	0.667	0.556	0.611	0.079	0.656
Pv11	0.784	0.778	0.781	0.005	0.792
M11A	0.529	0.889	0.709	0.254	0.633
TB	0.765	0.667	0.716	0.069	0.813
LW11-2	0.392	0.333	0.363	0.042	0.454
Hg6.1	0.863	0.889	0.876	0.018	0.851
Mean	0.679	0.681	0.680	0.001	0.707
Std. Dev	0.152	0.182	0.167	0.022	0.119

Gene Diversity (He)

Locus	Coastal	Inland WA	Mean	Std. Dev.
LC26	0.740	0.791	0.765	0.036
LW11	0.705	0.647	0.676	0.041
Pv9	0.606	0.425	0.515	0.128
Pv11	0.777	0.876	0.826	0.070
M11A	0.630	0.673	0.651	0.031
TB	0.807	0.745	0.776	0.044
LW11-2	0.449	0.503	0.476	0.038
Hg6.1	0.852	0.856	0.854	0.003
Mean	0.696	0.690	0.693	0.004
Std. Dev	0.130	0.162	0.146	0.022

Appendix G: Microsatellite Re-sampling Estimates

G.1: Step-wise mutation model Rst calculations and associated p-values for five random resampling iterations of population comparisons. Results from analyses including all eight microsatellite loci and only the five neutral loci are presented. Differentiation estimates obtained from using all sampled individuals (i.e. allowing unequal sample sizes) are also included for comparison. Significant p-values are highlighted in bold print.

Sampling Iteration	R_{ST} All loci	P-values	R_{ST} 5 loci	P-values
1	0.1281	**<0.001±0.000**	0.0331	0.207±0.033
2	0.1261	**<0.001±0.000**	0.0714	**0.045±0.028**
3	0.1254	**0.009±0.009**	0.0412	0.122±0.034
4	0.2152	**<0.001±0.000**	0.0953	**0.036±0.015**
5	0.1324	**0.009±0.009**	0.0181	0.324±0.041
Mean	0.1455	**0.0036**	0.0518	0.1469
All samples	0.1264	**0.009±0.009**	0.0358	0.108±0.029

G.2: Standard Fst calculations and associated p-values for five random resampling iterations of population comparisons. Results from analyses including all eight microsatellite loci and only the five neutral loci are presented. Differentiation estimates obtained from using all sampled individuals (i.e. allowing unequal sample sizes) are also included for comparison. Significant p-values are highlighted in bold print.

Sampling Iteration	F_{ST} All loci	P-values	F_{ST} 5 loci	P-values
1	0.0386	**0.027±0.014**	0.0201	0.090±0.030
2	0.0560	**<0.001±0.000**	0.0337	**0.018±0.012**
3	0.0498	**<0.001±0.000**	0.0155	0.135±0.028
4	0.0811	**<0.001±0.000**	0.0287	**0.027±0.014**
5	0.0719	**<0.001±0.000**	0.0371	**0.009±0.009**
Mean	0.0595	**0.0054**	0.0270	0.0559
All samples	0.0646	**<0.001±0.000**	0.0279	**0.036±0.015**