Population substructure of harbor seals (*Phoca vitulina richardsi*) in Washington State using mtDNA

H.R. Huber, S.J. Jeffries, D.M. Lambourn, and B.R. Dickerson

Abstract: We examined the pupping phenology and genetic variation between the currently defined stocks of harbor seals, *Phoca vitulina richardsi* (Gray, 1864), in Washington's coastal and inland waters and looked in detail at genetic variation within the inland waters of Washington. We analyzed mtDNA variation in 552 harbor seals from nine areas in Washington State and the Canada–US transboundary waters. A total of 73 haplotypes were detected; 37 individuals had unique haplotypes. Pupping phenology and levels of genetic variation between the outer coastal stock (WA Coastal Estuaries, WA North Coast) and the inland waters stock (British Columbia, Boundary Bay, San Juan Islands, Smith/Minor Islands, Dungeness Spit, Hood Canal, Gertrude Island) corroborated the appropriateness of the present stock boundary. However, within the inland waters stock, Hood Canal and Gertrude Island were significantly different from the coastal stock, from the rest of the inland waters stock, and from each other. This indicates a total of four genetically distinct groups in Washington State, suggesting that managing the inland waters as a single stock may be erroneous.

Résumé : Nous examinons la phénologie de la mise bas et la variation génétique chez les stocks actuellement définis de phoques communs, *Phoca vitulina richardsi* (Gray, 1864), dans les eaux côtières et intérieures de l'état de Washington et étudions en détail la variation génétique dans les eaux intérieures du Washington. Nous avons analysé la variation de l'ADNmt chez 552 phoques communs de neuf régions des eaux de l'état de Washington et des eaux transfrontalières Canada–É.U. Nous avons identifié 73 haplotypes au total; 37 individus possédaient des haplotypes uniques. La phénologie de la mise bas et les niveaux de variation génétique entre le stock côtier du large (estuaires côtiers de WA, côte nord de WA) et le stock des eaux intérieures (Colombie-Britannique, baie Boundary, îles San Juan, îles Smith/Minor, flèche littorale de Dungeness, canal Hood, île Gertrude) confirment la justesse de la frontière actuelle entre les stocks. Cependant, au sein des stocks des eaux intérieures, les stocks du canal Hood et de l'île Gertrude sont significativement différents du stock côtier, du reste du stock des eaux intérieures et l'un de l'autre. Il y a donc un total de quatre groupes génétiquement distincts dans l'état de Washington, ce qui laisse croire que la gestion des eaux intérieures comme un seul stock constitue une erreur.

[Traduit par la Rédaction]

Introduction

Numbers of harbor seals (*Phoca vitulina richardsi* (Gray, 1864)) throughout the Pacific Northwest were severely reduced by bounty hunters in the first half of the 20th Century. In Washington, the state-run bounty program was begun because harbor seals were considered a significant competitor to commercial and recreational fishermen. The bounty was discontinued in the 1960s, and in 1972, the Marine Mammal Protection Act (MMPA) was passed. In the 1970s, it was estimated that only 2000 to 3000 harbor seals were present in the state (Newby 1973). In the mid-1980s, harbor seals in Washington were divided into two stocks based on differences in pupping phenology and contaminant concentrations (Boveng 1988), resulting in seals in the coastal waters (Oregon and Washington Coastal Waters Stock) being managed as one stock and seals in the inland waters (Washington Inland Waters Stock) as another (Barlow et al. 1995). By 1999, under the protection of the MMPA, numbers of harbor seals increased dramatically and both stocks of the Washington population were considered to be at an optimum sustainable population level (OSP) (Jeffries et al. 2003). Population structure of harbor seals in Washington and Oregon has been investigated in the past using differences in cranial morphology (Temte 1993), differences in pupping phenology (Bigg 1973; Jeffries and Johnson 1990; Temte 1991), movements of radio-tagged seals (Huber et al. 2001), and genetic variation (Bickham and Patton 1994; Lamont et al. 1996). All of these methods agree with the major stock boundaries defined in the late-1980s, although both the pupping phenology and genetic variation studies lacked resolution.

The pupping phenology at the interface of the two stock boundaries — the western Strait of Juan de Fuca from the coast east to Dungeness Spit (Fig. 1) — is uncertain. Be-

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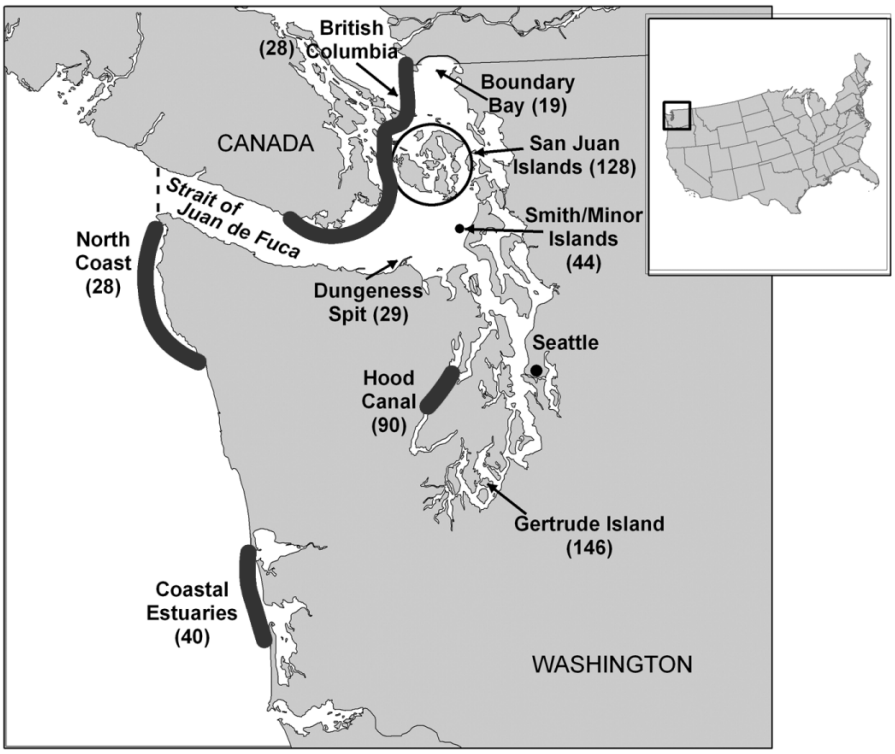
Fig. 1. Map of sampling locations of harbor seals (*Phoca vitulina richardsi*) in Washington State. Two sites are on the outer coast: North Coast and Coastal Estuaries. Seven sites are in the inland waters: British Columbia, Boundary Bay, San Juan Islands, Smith/Minor Islands, Dungeness Spit, Hood Canal, and Gertrude Island. Broken line at the mouth of the Strait of Juan de Fuca indicates putative boundary between coastal and inland water stocks. Sample sizes are indicated for each site.



cause of its remoteness and the low number of seals present, there have been few surveys in the area. Genetic variation between the two management units may be have been underestimated in the past (Bickham and Patton 1994; Lamont et al.1996) because of small sample sizes and reliance on samples collected from juveniles and adults throughout the year. This can lead to transient individuals being misidentified as belonging to a specific location. In the past, it was commonly believed that harbor seals do not migrate and exhibit strong site fidelity, generally moving <50 km to forage (Yochem et al. 1987; Thompson and Miller 1990; Suryan and Harvey 1998). But, with the increased use of satellite tags and aerial telemetry surveys of VHF tags, longer movements than previously suspected have been documented. In Alaska, Lowry et al. (2001) documented juvenile movements of 300–500 km. In Washington, harbor seals, mostly juveniles and adult males, have moved 100–325 km from place of tagging (National Marine Mammal Laboratory (NMML) and Washington Department of Fish and Wildlife (WDFW), unpublished data). Hardee (2008) found repeated movements of up to 100 km from point of tagging that occurred most frequently outside of the breeding season. A lack of samples has hampered any investigation into population structure within the inland waters of Washington. Seal populations in different regions within the Washington inland waters are increasing at different rates despite their close proximity (Jeffries et al. 2003), this could indicate genetically differentiated populations.

This study examines in detail the pupping phenology and genetic variation between the currently defined stocks and also within the inland waters of Washington. We collected samples only from unweaned pups to remove any bias caused by adult and juvenile movements outside of the breeding season. The objectives of this study are to determine how viable the present putative boundaries between the outer coast and the inland waters are and how much population substructure there is among harbor seals within the inland waters.

Materials and methods

Aerial surveys

The pupping phenology at the interface of the two stock boundaries — the western Strait of Juan de Fuca from the coast east to Dungeness Spit (Fig. 1) — was investigated in 2005. Aerial surveys of the Western Strait were flown every 2 weeks in July and August. The only age classes distinguishable from the air are pups and nonpups; pups are identified by color, size, and proximity to an adult female. Two to three surveys were scheduled for each 2-week low tide cycle. Surveys were flown from 2 h before to 2 h after low tide, in a single-engine plane at an altitude of 700–800 ft (1 ft = 0.3048 m) at 80 knots (1 knot = 1.852 km/h). All known sites were surveyed and new sites were looked for on each census. Data collected during surveys included date, time, location, a visual estimate of seal numbers, and photographs of all sites where more than 25 seals were hauled out. Photographs were taken with a digital camera (Nikon D100) with an 80–200 mm lens. The total number of seals (including pups) and the number of pups present at

each site were counted from digital images. Evidence of recent disturbances at known haul-out sites (haul marks on the beach or seals milling in the water off the haul-out site) were also noted.

Data collection and amplification of mitochondrial DNA (mtDNA)

Between 1994 and 2007, we collected skin samples from 552 unweaned pups from nine areas in Washington State and the Canada–US transboundary waters (Fig. 1, Table 1). Samples were collected during the process of tagging: skin from a 6 mm hole punched for tag placement was preserved in a 20% saline solution of dimethyl sulphoxide (DMSO) (Amos and Hoelzel 1991). Samples were stored at room temperature until processing.

Sample processing took place at Southwest Fisheries Science Center (SWFSC) Genetics Laboratory in La Jolla, California, USA, and the Molecular Ecology Research Laboratory (MERL) at NMML at the Alaska Fisheries Science Center (AFSC), Seattle, Washington, USA. For samples processed at SWFSC, DNA was extracted using the protocol specified for the FastDNA® kit (BIO 101, Inc., Carlsbad, California, USA). Primers TRO (5′-CCTCCCTAAGACTCAAGGAAG-3′) (R.L. Westlake, personal communication, indicated a slight modification from L15829 (Westlake and O'Corry-Crowe 2002)) and PvH00034 (5′-TACCAAATGCATGACACCACAG-3′) (Westlake and O'Corry-Crowe 2002) were used to amplify a 435-base-pair section of the control region of mtDNA. The target DNA was amplified in a PerkinElmer 9600 Thermocycler (PerkinElmer, Norwalk, Conneticut, USA) in 50 μL volume: 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.8 mmol/L deoxynucleotide triphosphates (dNTPs), 1.5 units *Taq*DNA polymerase, 0.3 μmol/L of each primer, and 150 ng DNA template. The polymerase chain reaction (PCR) amplification profile consisted of initial denaturation of 90 °C for 2.5 min, 37 cycles at 94 °C for 30 s, 48 °C for 60 s, and 72 °C for 60 s. A final cycle included 5 min at 72 °C, then cooling to 4 °C. Five microlitres of PCR product was electrophoresed on 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. The amplified PCR products were purified using QiaQuick (QIAGEN, Valencia, California, USA) columns. PCR products were sequenced by the direct dideoxy sequencing method of Sanger et al. (1977) using four-dye fluorescent technology of Applied Biosystems Inc. (ABI 1992). Sequencing was performed in 12 μL reactions containing 20 ng of purified PCR product, 0.10 μmol/L primer, and 2.5 μL PRISM DRhodamine dye-terminator mix (PE Applied Biosystems Inc., Foster City, California, USA). TRO and internal primer H16498 (5′-CCTGAAGTAAGAACCAGATG-3′) (Rosel et al. 1994) were used to carry out the cycle sequencing reaction in the PE 9600 with the following profile: initial denaturing at 96 °C for 4 min, 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and finally at 50 °C for 4 min. Excess dye-labeled terminators were removed by ethanol precipitation. Both strands were sequenced on an ABI 377 automated sequencer (Applied Biosystems Inc.) and edited and aligned with the SeqEd multiple-sequence editor program (Applied Biosystems Inc.).

For samples processed at MERL, DNA was extracted using the animal tissue protocol for DNeasy® kits (QIAGEN). Target DNA was amplified in MJ Research DNA engine in 25 μL cocktails using the SWFSC PCR protocol described above. PCR products were electrophoresed on 1.5% agarose gels at 100 V for 1.5 h. The gels were stained with SYBR® Green I nucleic acid gel stain (Molecular Probes™, Eugene, Oregon, USA) and viewed with a UVP Darkroom (UVP Inc., Upland, California, USA) with sizes verified with Hi-Lo DNA marker (Minnesota Molecular, Minneapolis, Minnesota, USA). To purify the amplified PCR fragment, the bands were excised from the gel and placed in 20 μL sterile water, stored overnight at room temperature, and then frozen. The subsequent cycle sequence PCRs were performed using the Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Biosciences, Division of GE Healthcare, Piscataway, New Jersey, USA) protocols in a MJ Research DNA engine (MJ Research, Inc., Waltham, Massachusetts, USA). Sequences were visualized using a LI-COR 4200 automated sequencer (LI-COR Biosciences, Inc., Lincoln, Nebraska, USA). Base calling, as well as sequence editing and aligning, were done with the associated E-seq software (LI-COR Biosciences, Inc.). A final 410-base-pair fragment of the control region was used in subsequent analyses. Sequencher version 4.7 (Gene Codes, Ann Arbor, Michigan, USA) was used to align forward and reverse sequences and to create a consensus for each sample. Sequences from all samples were aligned in Bioedit version 7.0 (Ibis Biosciences, Inc., Carlsbad, California, USA).

Analysis of mtDNA data

The number of variable sites, haplotypic diversity (H), and nucleotide (π) diversity were calculated with Arlequin version 3.01 (Excoffier et al. 2005). To investigate population structure between regional population groupings, an analysis of molecular variance (AMOVA) was performed in Arlequin using the outer coast (WA Coastal Estuaries, WA North Coast) and one to four groups within the inland waters (Dungeness Spit, British Columbia, Boundary Bay, San Juan Islands, Smith/Minor Islands, Hood Canal, Gertrude Island). F_{ST} and Φ_{ST} estimates were calculated for all population pairs in Arlequin. For all simultaneous probability tests, sequential Bonferroni adjustments were used to determine the statistical significance levels (Rice 1989). MODELTEST version 3.7 (Posada and Crandall 1998) was used to select the model of nucleotide substitution that best fit the data from among 56 models. The Kimura two-parameter distances model incorporating transitions, transversions, and gamma shape was the most appropriate model. Phylogenetic analysis of all haplotypes was conducted using this model and the neighbor-joining method implemented in MEGA version 4.0 (Tamura et al. 2007). The phylogenetic tree was rooted using an eastern Atlantic harbor seal (*Phoca vitulina vitulina* L., 1758) sample (GenBank accession No. U36354) (Stanley et al.1996). Bootstrap support for the tree topology was calculated after 1000 replicates. Isolation by distance was performed by regressing Φ_{ST} against geographical distance measured along the coastline using GENEPOP version 3.4 (Raymond and Rousset 1995). To investigate the phylogenetic relationship between haplotypes of Washington harbor seals, a minimum spanning network was created in Arlequin, viewed in Treeview, and drawn by hand. To ex-

Table 1. Sampling location, sample size (*N*), number of haplotypes, haplotypic diversity (*H*) and the respective standard deviation (SD), and percentage of nucleotide diversity (π) and the respective standard deviation (SD) for genetic samples of harbor seals (*Phoca vitulina richardsi)* in Washington State.

Sampling location	*N*	No. of haplotypes (unique)	*H* (SD)	π (SD) (%)
WA Coastal Estuaries	40	11 (5)	0.73 (0.05)	0.5 (0.3)
WA North Coast	28	11 (5)	0.87 (0.04)	1.5 (0.8)
Dungeness Spit	29	14 (0)	0.93 (0.03)	2.0 (1.1)
British Columbia	28	18 (8)	0.93 (0.03)	1.9 (1.0)
Boundary Bay	19	11 (1)	0.92 (0.05)	1.9 (1.0)
San Juan Islands	128	29 (7)	0.92 (0.01)	2.0 (1.0)
Smith/Minor Islands	44	14 (1)	0.91 (0.02)	1.7 (0.9)
Hood Canal	90	17 (5)	0.80 (0.02)	2.1 (1.1)
Gertrude Island	146	19 (5)	0.86 (0.02)	2.2 (1.1)
Total	552	73 (37)	0.92 (0.01)	2.0 (1.0)

amine the validity of our sample sizes, we created a rarefaction curve using the program Rarefaction Calculator (available from J. Brzustowski at http://www2.biology.ualberta.ca/jbrzusto/rarefact.php, accessed 23 November 2009). We used 522 individuals in the collection and considered subsamples from 10 to 500 in five sample increments.

Results

Aerial surveys of the western Strait of Juan de Fuca in 2005 showed the first harbor seal pup on 7 July. Pup numbers increased throughout the western strait through the survey on 23 August when 34 pups were observed.

A total of 410 base pairs of the mtDNA control region were analyzed for sequence variation in 552 harbor seals from nine sampling areas in Washington State and the Washington–Canadian transboundary waters (Fig. 1, Table 1). Fifty-two variable sites were identified with 56 substitutions (47 transitions and 9 transversions) and 2 indels. In total, 73 haplotypes were identified, 37 of which were represented by single individuals (Table 1). Haplotypic diversity was high (H = 0.92, SD = 0.01) and nucleotide diversity was moderate (π = 2.0%, SD = 1.0%), suggesting that most haplotypes were closely related (Table 1). All haplotypes have been submitted to GenBank (accession Nos. FJ472353–FJ472428).

There was significant genetic differentiation between the two current stock groupings representing the coastal and inland waters of Washington State (Coastal = WA Coastal Estuaries and WA North Coast; Inland Waters = British Columbia, Boundary Bay, San Juan Islands, Smith/Minor Islands, Dungeness Spit, Hood Canal, and Gertrude Island) (AMOVA, $F_{[1,7]}$ = 12, p = 0.03) (Table 2). This finding was further supported by the significant pairwise differences in F_{ST} values (Table 3) between the coastal area and all other areas. In addition, estimates of F_{ST} were significantly different between all sample pairs at the geographic extremes, the two sites on the coast (WA North Coast and WA Coastal Estuaries) and the two sites in southern Puget Sound (Hood Canal and Gertrude Island; Table 3, Fig. 1). Estimates of Φ_{ST} also showed significant levels of genetic differentiation for the two areas separated by the largest geographic distances, Gertrude Island and WA Coastal Estuaries (Table 3, Fig. 1). There was also a strong relationship showing genetic isolation by distance (R^2 = 0.5002, p = 0.032; Fig. 2).

Further investigation showed four significantly different areas (Coastal; Hood Canal; Gertrude Island; and northern Puget Sound = British Columbia, Boundary Bay, San Juan Islands, Smith/Minor Islands, and Dungeness Spit) (AMOVA, $F_{[3,5]}$ = 12, p = 0.03), rather than the originally defined split just between the coastal seals and those in the inland waters (Table 2). Within the inland waters, Hood Canal and Gertrude Island in southern Puget Sound were significantly different from each other (p < 0.006) and were significantly differentiated from most other populations (F_{ST}: 10 of 11 pairwise comparisons; Φ_{ST}: 6 of 11 pairwise comparisons), suggesting strong population subdivision (Table 3). Of the 10 pairwise comparisons between the five sites in close proximity in northern inland waters (Dungeness Spit, Smith/Minor Islands, San Juan Islands, Boundary Bay, and British Columbia), two of the F_{ST} estimates and four of the Φ_{ST} estimates were significant after Bonferroni corrections (Table 3). Dividing populations beyond four groups yielded no significant results (Table 2).

Only 14% of neighbor-joining tree nodes were supported by 60% bootstrap analysis. However, from these nodes, two patterns emerged: one clumping of haplotypes primarily from British Columbia (Fig. 3, C) and another primarily from Gertrude Island and Hood Canal (Fig. 3, D). The only large node separated by 97% bootstrap analysis consisted of samples primarily from Hood Canal and Gertrude Island (Fig. 3, D). The mtDNA phylogeny was more clearly represented using a minimum spanning network (MSN; Fig. 4), which also described these distinct clumpings. The resulting MSN shows four maternal lineages (A, B, C, D) generally characterized by an abundant central haplotype surrounded by rarer haplotypes. Contrasting geographic trends were apparent among the four maternal lineages. Matriline B contains over 90% of the haplotypes unique to the coastal sites, as well as inland haplotypes; matriline A consists primarily of inland haplotypes; matriline C is almost exclusively composed of haplotypes from British Columbia and matriline D consists mainly of haplotypes from Gertrude Island and Hood Canal (Fig. 4). The four most common haplotypes accounted for nearly half of the samples (268/552). None of the haplotypes were present in all nine sampling sites, but

Table 2. Results from analysis of molecular variance (AMOVA) for 2–5 sample groupings of harbor seals (*Phoca vitulina richardsi)* in Washington State.

Grouping scheme	Group	F_{ST}	F_{SC}	F_{CT}
(Coastal) (Inland Waters)	2	0.14143**	0.05507**	0.09139*
(Coastal) (NPS) (HC + GI)	3	0.08307**	0.04143**	0.04344*
(Coastal) (NPS) (HC) (GI)	4	0.08008**	0.03062**	0.05103*
(Coastal) (BC) (NPS – BC) (HC) (GI)	5	0.07683**	0.03557**	0.0550ns

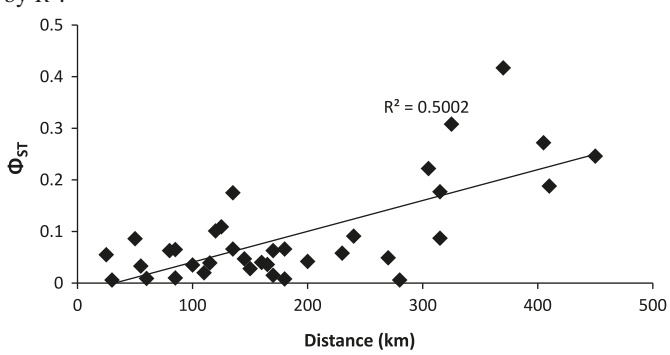
Note: NPS, northern Puget Sound (Boundary Bay, British Columbia, Dungeness Spit, San Juan Islands, Smith/Minor Islands); HC, Hood Canal; GI, Gertrude Island; BC, British Columbia; F_{ST} , variance of groups relative to total; F_{SC}, variance among samples within groups; and F_{CT}, variance of samples among groups. *, $P < 0.05$; **, $P < 0.001$; ns, not significant.

Table 3. Estimates of genetic differentiation (F_{ST} below diagonal and Φ_{ST} above diagonal) among sample areas of harbor seals (*Phoca vitulina richardsi*) in Washington State.

	WA Coastal Estuaries	WANorth Coast	Dungeness Spit	British Columbia	Boundary Bay	San Juan Islands	Smith/Minor Islands	Hood Canal	Gertrude Island
WA Coastal Estuaries		0.175[a]	0.222[a]	0.188[a]	0.417[a]	0.177[a]	0.308[a]	0.272[a]	0.246[a]
WA North Coast	0.071[b]		0.015	0.006	0.091	0.008	0.063	0.049	0.087[a]
Dungeness Spit	0.148[a]	0.069[a]		0.028	0.039	0.009	0.006	0.035	0.047
British Columbia	0.158[b]	0.055[a]	0.008		0.086[a]	0.010	0.101[a]	0.066	0.058[a]
Boundary Bay	0.166[a]	0.089[a]	0.051[b]	0.057[a]		0.033	0.063[a]	0.040	0.042
San Juan Islands	0.142[a]	0.056[a]	0.004	0.001	0.054[a]		0.055[a]	0.020[a]	0.036[a]
Smith/Minor Islands	0.177[a]	0.090[a]	0.001	0.020	0.061[b]	0.026[b]		0.065	0.109
Hood Canal	0.221[a]	0.109[a]	0.044[a]	0.023	0.132[a]	0.042[a]	0.062[a]		0.066[a]
Gertrude Island	0.188[a]	0.101[a]	0.051[a]	0.044[a]	0.102[a]	0.046[a]	0.050[a]	0.065[a]	

[a]Significant p value ($p < 0.006$) after Bonferroni corrections.
[b]p value approaching significance ($p = 0.009$).

Fig. 2. Isolation by distance of mtDNA of harbor seals (*Phoca vitulina richardsi*) in Washington State. Relationship between genetic distance (Φ_{ST}), pairwise comparisons of sampling locations, and the geographic distance (km) between sampling locations is indicated by R^2.



the four most common ones were widespread and each was present in six to eight of the sampling areas.

Discussion

This study, using pupping phenology and genetic analysis, confirms the boundary between Washington harbor seals on the coast and the inland waters. The pupping phenology at the interface of the two stock boundaries — the western Strait of Juan de Fuca from the coast east to Dungeness Spit — supports the current stock boundary with the seals east of the stock boundary line matching the timing of pupping in the inland waters (July–October), not the timing of pupping along the Washington Coast (May–June). It is unclear whether the timing of pupping is in response to genetic or environmental cues, but it is possible that there are environmental conditions which vary from one side of the boundary to the other that have resulted in selection for differences in pupping timing (conferring a reproductive advantage to the seals that pup early or late depending on location) which could have isolated the two populations beyond geographic distance alone (Temte 1991).

The genetic differentiation we found on either side of the putative line separating the coastal stock (WA Coastal Estuaries and WA North Coast) from the inland water stock (British Columbia, Boundary Bay, San Juan Islands, Smith/Minor Islands, Dungeness Spit, Gertrude Island, and Hood Canal) also supports the accuracy of the current stock boundary (Fig. 1.). However, we also found significant structure within the inland waters to separate what was thought to be one stock into potentially three stocks: northern Puget Sound (British Columbia, Boundary Bay, San Juan Islands, Smith/Minor Islands, and Dungeness Spit), Gertrude Island, and Hood Canal. These results are supported also by the isolation by distance analysis, which is consistent with a stepping stone model of population structure where mixing occurs mostly between neighboring subpopulations (Kimura and Weiss 1964).

The level of differentiation in our study was not reported in previous studies of harbor seals in Washington State for two possible reasons: earlier studies (1) had small sample sizes from a limited number of sites and (2) sampled adults and juveniles opportunistically throughout the year. Samples collected for the Lamont et al. (1996) study were limited to the WA Coastal Estuaries ($N = 22$) and Gertrude Island ($N = 32$) and did not include Hood Canal or any of the other in-

Fig. 3. The neighbor-joining tree was constructed for a 369-base-pair region of the mtDNA control region from 552 harbor seals (*Phoca vitulina richardsi*) in Washington State. An eastern Atlantic harbor seal (*Phoca vitulina vitulina*) was used as the outgroup (GenBank assession No. U36354). Evolutionary distances were computed using the Kimura two-parameter method with transitions, transversions, and a gamma distribution (shape parameter = 0.5). A, B, C, and D correspond to matrilines depicted in Fig. 4.

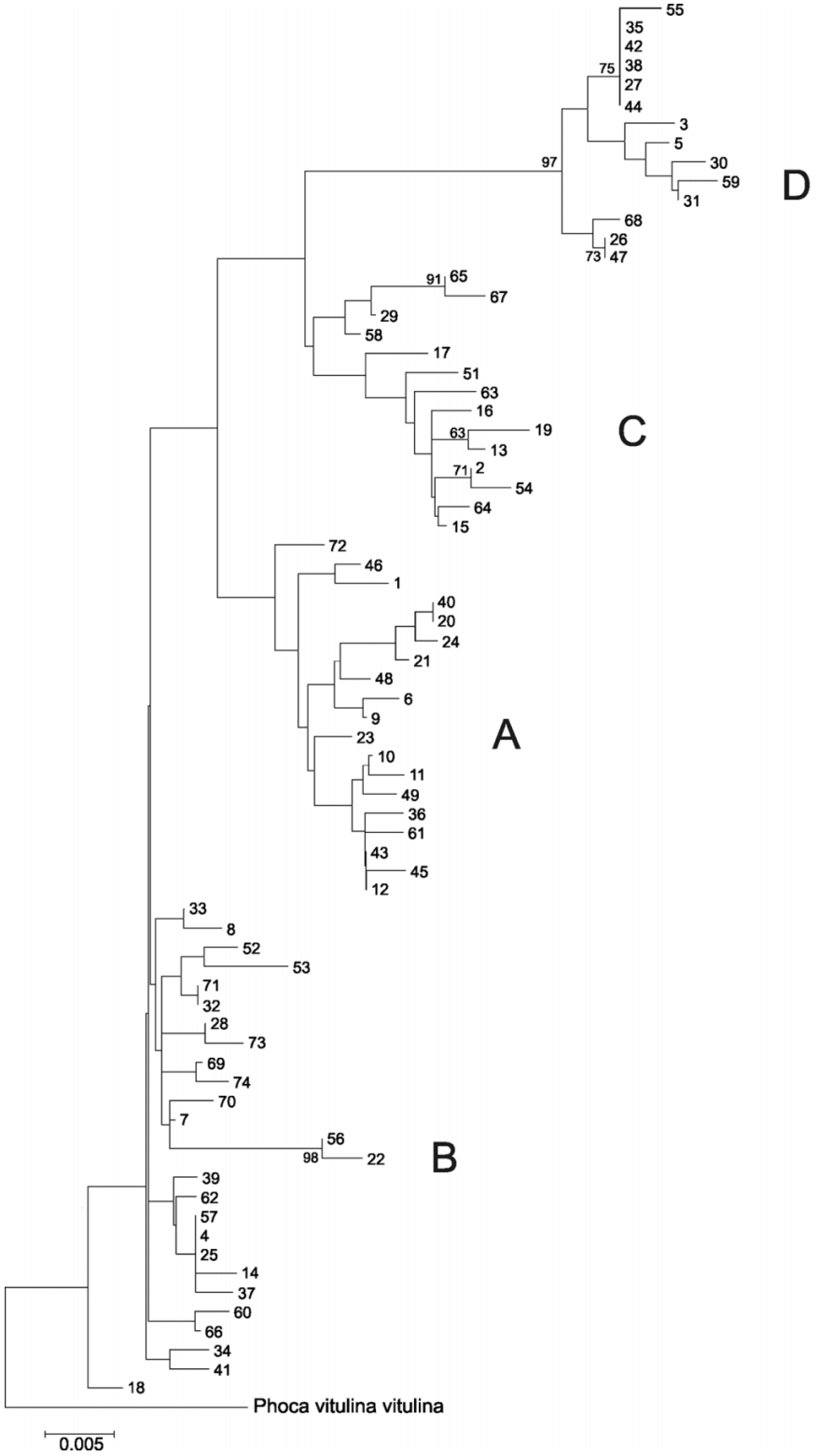
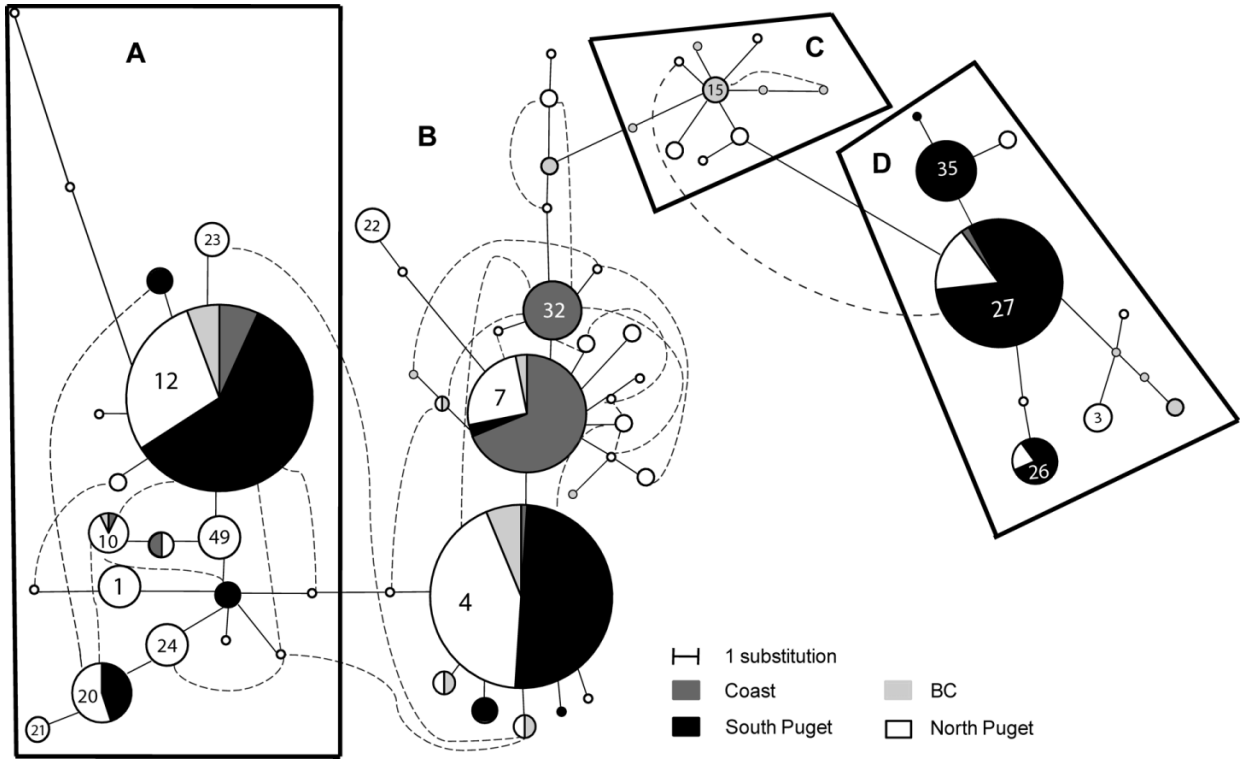


Fig. 4. Minimum spanning network (MSN) of harbor seals (*Phoca vitulina richardsi*) in Washington State showing 73 mtDNA haplotypes. Branch lengths are proportional to the number of substitutions between haplotypes. Circles representing individual haplotypes are proportional to abundance. The more abundant haplotypes are identified. Broken lines represent alternative relationships of haplotypes. A, B, C, and D refer to four matrilines described in the text.



land sites. The Bickham and Patton (1994) study had a better distribution of samples (WA Coastal Estuaries, Hood Canal, Gertrude Island, Neah Bay (western Strait of Juan de Fuca), and Protection Island (Dungeness Spit area)), but their sample size was small, with only 35 samples from all Washington sites combined. Sampling adults and juveniles, particularly outside of the breeding season, can confuse any analysis of population structure because harbor seals have been shown to make long-distance movements, primarily outside of the breeding season (Bonner and Witthames 1974; Boulva and McLaren 1979; Lowry et al. 2001; Hardee 2008), even though they are a nonmigratory species.

Although much less pronounced, we also found suggestion of further differentiation among the populations in northern Puget Sound (British Columbia, Boundary Bay, San Juan Islands, Smith/Minor Islands, and Dungeness Spit) particularly between Smith/Minor Islands and British Columbia, and Boundary Bay and the San Juan Islands. Like Burg et al. (1999), we found a clumping of samples composed primarily of seals from British Columbia (matriline C). The potential differentiation seen in northern Puget Sound in our study and by Burg et al. (1999) could be an artifact of small sample sizes in these areas. The rarefaction curve calculated for our study (data not shown) suggests that we need a minimum sample size of 80 individuals per area to adequately represent the haplotype composition. Also, unlike Hood Canal and Gertrude Island, locations in the northern inland waters have neither land barriers nor large geographic distances between them and the open waters of the Strait of Juan de Fuca. The lack of physical barriers to movement may facilitate gene flow among nearby harbor seal populations. Further evidence for the intermixing of animals in northern Puget Sound compared with southern Puget Sound is reflected in information from tagged and instrumented seals. In the Georgia Basin, Hardee (2008) found that male harbor seals tagged in the Belle Chain (northwest of the San Juan Islands) moved freely back and forth to the outer coast of Washington. In contrast, instrumented harbor seals (both males and females of all age classes) from Hood Canal were not detected outside of Hood Canal from June to October (Josh London, personal communication). Further investigations with larger samples sizes of unweaned pups are needed to elucidate genetic differentiation in northern Puget Sound.

Interestingly enough, similar genetic differentiation to that of harbor seals (separating populations in the Strait of Georgia from both Puget Sound and the coast) has been shown among several northwestern Pacific fish including copper rockfish (*Sebastes caurinus* Richardson, 1844) (Buonaccorsi et al. 2002), Pacific hake (*Merluccius productus* (Ayres, 1855)) (Iwamoto et al. 2004), and Pacific cod (*Gadus macrocephalus* Tilesius, 1810) (Cunningham et al. 2009). These studies attributed the differences in genetic population structure to postglacial colonization.

At its greatest extent, 14 000 years before present, the Cordilleran Ice Sheet completely covered the inland waters of Washington south to the present city of Olympia and extended 100 km west of the present Washington coastline near the Strait of Juan de Fuca (Booth 1987; Clague 1989: Booth et al. 2004). The full extent of the ice sheet lasted

only a few hundred years; as the ice retreated, melt water formed a glacial lake that drained to the coast through the Chehalis River (Booth 1987; Clague 1989; Booth et al. 2004). As the glacier continued to retreat, melt water increased until it created a northern spillway allowing sea water in through the Strait of Juan de Fuca. At this time, the ground level in the southern lowlands rose, cutting off the Chehalis spillway (Booth et al. 2004). It is possible that the current substructure seen in the inland waters is the result of harbor seals entering Puget Sound from the coast in two separate groups: one group entering the Strait of Georgia through the marine waters of the Strait of Juan de Fuca early in the glacier's retreat (then moving into Hood Canal and Gertrude Island), and the other group moving into the inland waters after the complete retreat of the Cordilleran Ice Sheet.

Conclusions

The results of our study corroborate the appropriateness of the current putative boundary between the coastal stock and the inland stock. In addition, we also found significant genetic differences within the inland waters. Managing the inland waters as a single stock may be erroneous; populations in different areas within the inland waters are increasing at different rates (Jeffries et al. 2003) and are genetically distinct. Because there are large genetic differences among harbor seals in Washington State in spite of the small geographic area that they represent, it is important to consider the implications of these differences when making management decisions.

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