Genetic Analysis of Eastern Pacific Harbour Seals (*Phoca vitulina richardsi*) from British Columbia and Parts of Alaska using Mitochondrial DNA and Microsatellites

by

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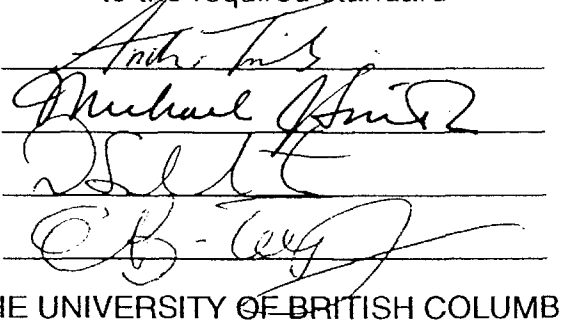
to the required standard



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Abstract

In British Columbia the population of harbour seals, *Phoca vitulina richardsi*, has increased from 9,000 to 135,000 since their protection 25 years ago. Differences in pelage patterns and pupping times suggest that more than one population of harbour seals may be present in the eastern Pacific.

Molecular analyses were used to investigate the genetic diversity and population structure of harbour seals along the B.C. coast and in parts of Alaska. The allele frequency at seven microsatellite loci and the haplotypic diversity of the mitochondrial control region (D-loop) were examined.

A 475 base pair fragment containing the tRNA proline and part of the mitochondrial control region was amplified and sequenced from 128 animals. Sixty variable sites defined 72 mtDNA haplotypes with pairwise nucleotide differences as high as 5%. Only 14 haplotypes were shared between two or more seals. Some of the more frequent haplotypes were unique to specific areas, while others were distributed over a broad geographic range. Three groups representing the southern Strait of Georgia, southern B.C. and northern B.C./southeast Alaska were observed using parsimony and distance based phylogenetic reconstruction. Additional analyses using sequences from Washington and California revealed the presence of another population comprising the outer coast of Washington, Oregon and California.

The order of the clades suggests that the Pacific Ocean was colonized twice. The first invasion occurred approximately 0.67 MYA and represents only a small portion of today's harbour seals in southern Vancouver Island. Seals from the second invasion, about 0.38 MYA, are distributed throughout the Pacific.

Analyses of five polymorphic microsatellite loci show that the allele frequency distribution is significantly different in southern British Columbia and northern British Columbia/Alaska. Average heterozygosity was similar for northern and southern populations, however the allelic diversity was higher in the southern population. The migration rate for males based on microsatellite data (3-7 seals/ generation) was higher than that obtained for females from the mtDNA (0.3 females/ generation). This suggests that although migration rates are low they are sufficient to allow gene flow between the two populations.

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Acknowledgements

Samples

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Chapter 1 General Introduction

The eastern Pacific harbour seal (*Phoca vitulina richardsi*) inhabits the coastline from Baja California to the Aleutian Islands in Alaska (Temte *et al.* 1991). A second subspecies (*Phoca vitulina stejnegeri*) ranges from Japan to the Commander Islands, in the western Pacific. Two other subspecies of harbour seals live in the western (*Phoca vitulina concolour*) and eastern (*Phoca vitulina vitulina*) Atlantic, while the fifth subspecies (*Phoca vitulina mellonae*) inhabits freshwater lakes in Québec. All five subspecies of harbour seals occur in the northern oceans (Figure 1.1). They are thought to have originated in the western Atlantic and migrated through the Arctic Ocean to colonize the Pacific less than 2 million years ago (MYA) (Stanley *et al.* 1996, Árnason *et al.* 1995).

It is not known whether the eastern Pacific harbour seals consist of more than one distinct population. However, differences in pelage colouration and timing of birth suggest there could be at least three separate populations between California and Alaska.

There are three forms of pelage colouration in the eastern Pacific: black, common, and muddy. Based on these patterns, there may be as many as three distinct populations of harbour seals consisting of Glacier Bay, Queen Charlotte Islands, and the Strait of Georgia (Stutz 1967, Figure 1.2). In the Queen Charlotte Islands, 56% of the seals have a black pelage compared to 19% in Glacier Bay. In the Strait of Georgia, 46% have the common pelage compared to 16% in the Queen Charlotte Islands.

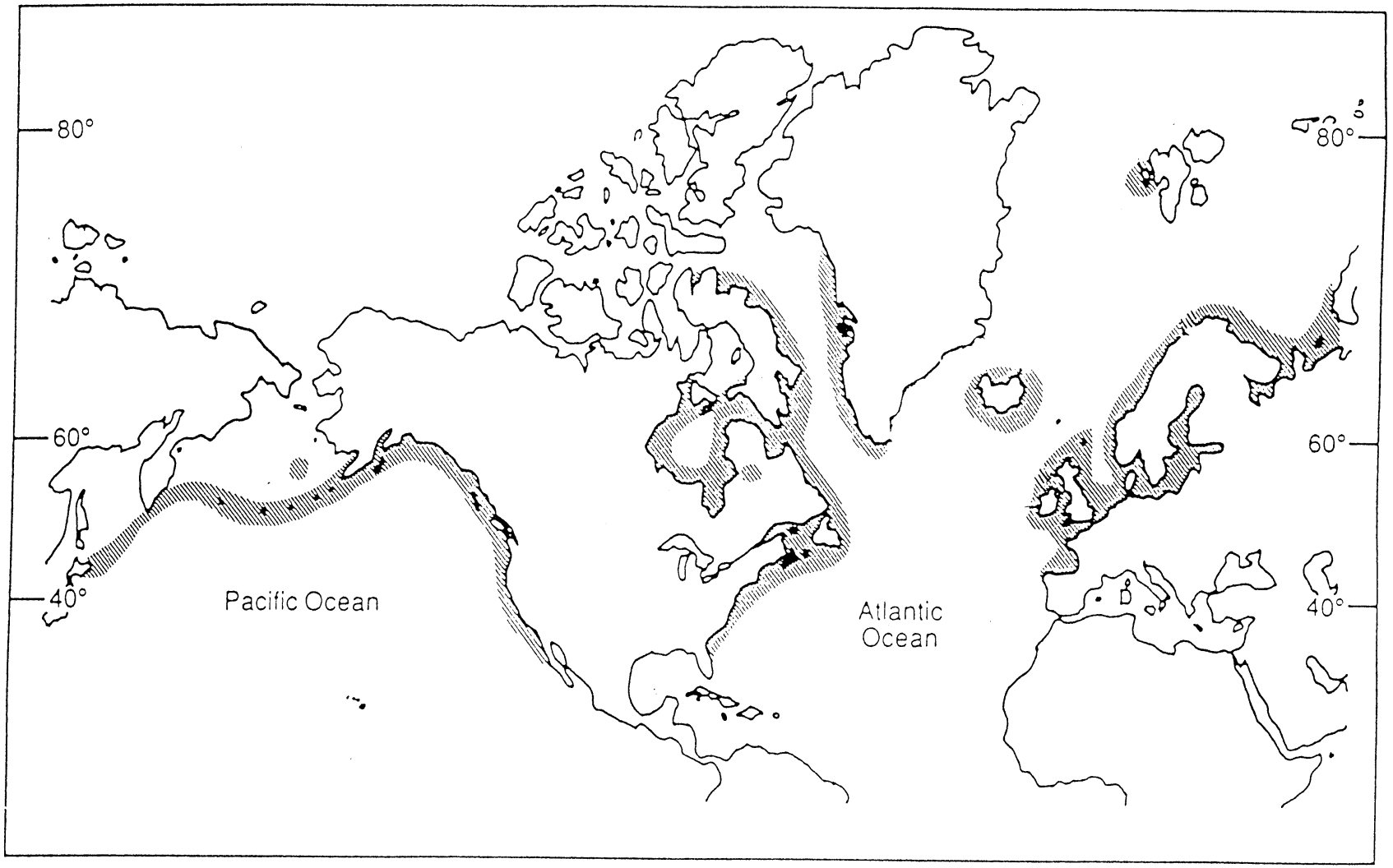

Figure 1.1 Worldwide distribution of the five subspecies of harbour seals: *P.v. stejnegeri* (western Pacific), *P.v. richardsi* (eastern Pacific), *P.v. mellonae* (lakes in Québec), *P.v. concolour* (western Atlantic) and *P.v. vitulina* (eastern Atlantic) (after Riedman 1990).

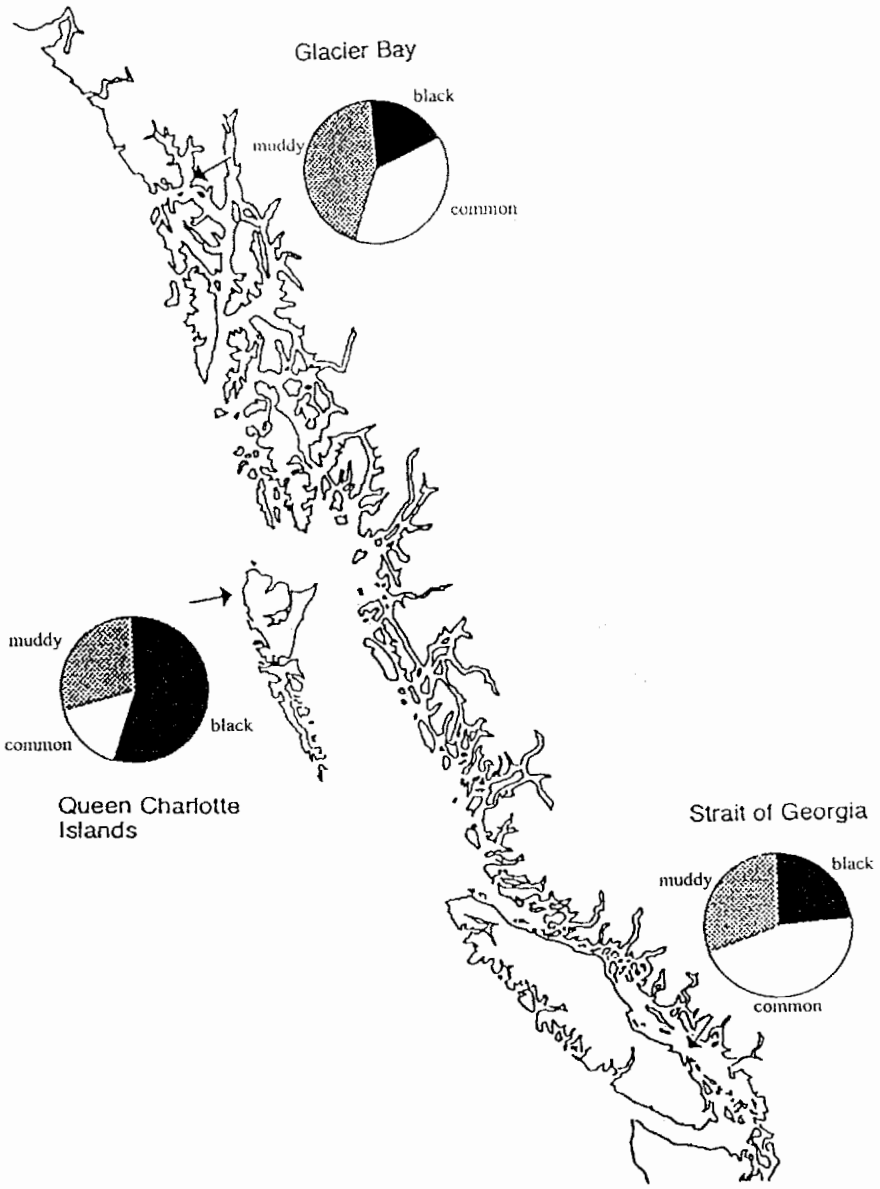

Figure 1.2 Pelage pattern variation in eastern Pacific harbour seals (from Stutz 1967).

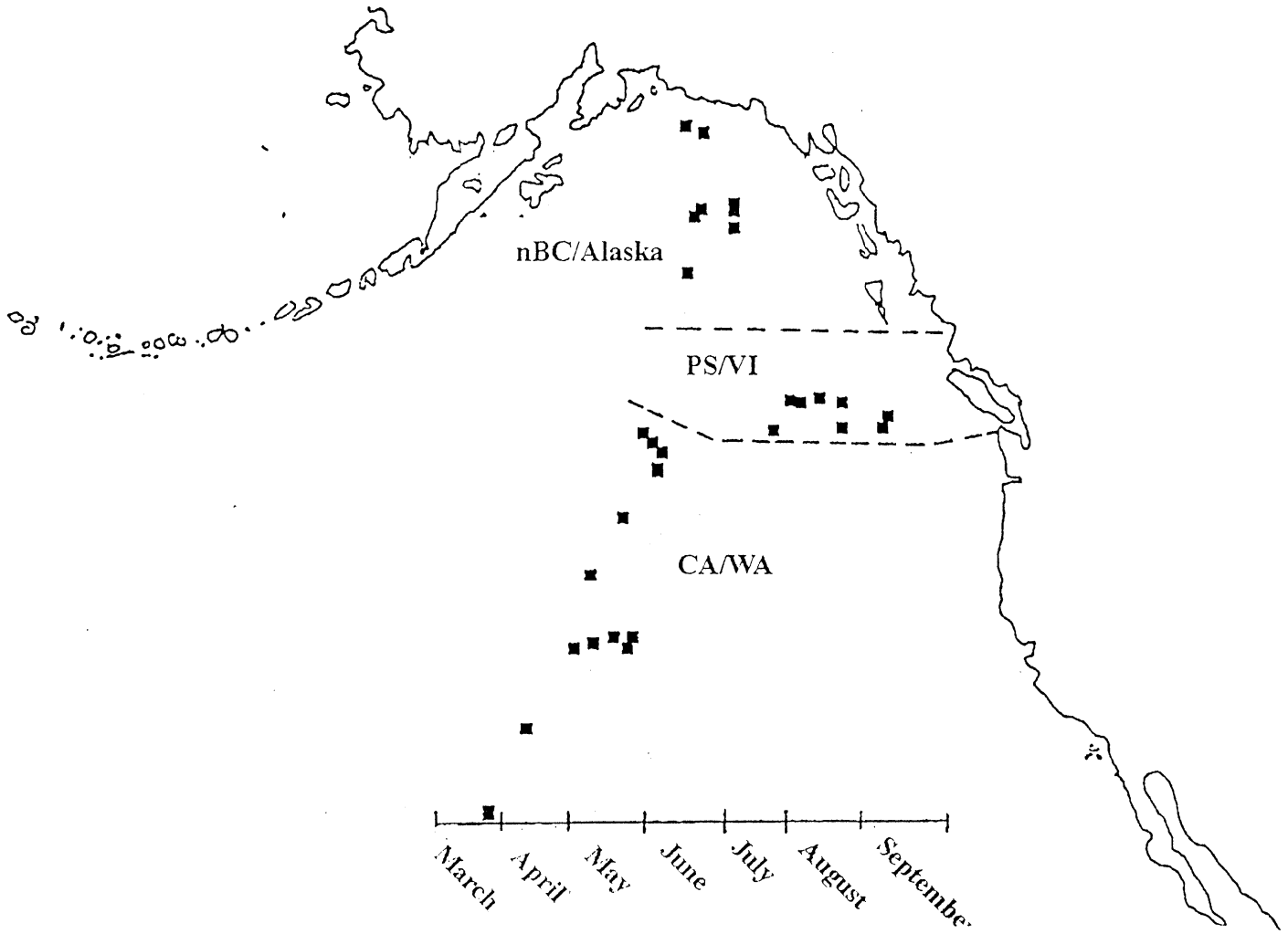

Figure 1.3 Latitudinal variation of pupping dates in eastern Pacific harbour seals. Three noticeable groups exist: California to Washington (CA/WA), Puget Sound and Vancouver Island (PS/VI) and northern B.C. and Alaska (nBC/AK) (after Temte *et al.* 1991).

Pupping times also suggest that there may be different populations in the eastern Pacific (Figure 1.3). A latitudinal cline in pupping exists between Baja California (where pupping starts in March) and the outer coast of Washington (where pupping occurs in May) and northern B.C. and Alaska (where pupping occurs in June). However, in the Puget Sound and Vancouver Island region, seals give birth in July and August. (Temte *et al.* 1991).

The total number of harbour seals in the eastern Pacific is estimated at more than 285,000 (Olesiuk *et al.* 1995, Small and DeMaster 1995, Jemison and Kelly 1995, Pitcher 1990, Barlow *et al.* 1995). The largest concentration of harbour seals appears to be in B.C. where 47% of the eastern Pacific seals are currently found (Olesiuk *et al.* 1995). Approximately 25% are in Alaska (Small and DeMaster 1995), 16% in Washington and Oregon and 12% in California (Barlow *et al.* 1995). In most regions of the eastern Pacific, the populations of harbour seals are either stable or increasing, with notable exceptions in parts of western Alaska. Many of the eastern Pacific populations were subject to bounties and extensive hunting prior to the mid 1970s.

Between 1913 and 1969, an estimated 200,000 to 240,000 harbour seals were killed in British Columbia for pelts and bounties. This may have caused a population bottleneck that reduced that amount of genetic diversity in the harbour seal population in British Columbia. Since their protection in 1970, harbour seals have been increasing at rates as high as 12.5% per year, although rates may have slowed in recent years. In the last 25 years, the number of harbour seals in British Columbia has increased from 9,000 to approximately 135,000 (Olesiuk *et al.* 1990, 1995).

The increase in the number of harbour seals in British Columbia could be due to immigration of seals from Alaska and Washington or an increase in the reproductive

rates of harbour seals in British Columbia. If the increase was due to immigration from surrounding areas and the population is structured, harbour seals in B.C. should have haplotypes similar to those from populations to the north and south. If an increase in reproductive rates caused the increase in population size, the B.C. population should contain haplotypes that are not found in the Alaska and Washington populations, assuming low migration rates.

Male harbour seals have an average life span of 22 years and become sexually mature between 3 and 6 years of age. Females can live up to 27 years and give birth to one pup annually starting at the age of 3 or 4 years. Fecundity rates increase from 80 to 97% as the females get older (Bigg 1969).

Harbour seals exhibit strong site fidelity and limited movements of less than 200 km (Olesiuk *et al.* 1995, Thompson *et al.* 1989, Cottrell 1995, Pitcher and McAllister 1981). Translocation experiments have further shown that animals transported across land from Nanaimo, B.C., on the east coast of Vancouver Island, to Bamfield, B.C., on the west coast of Vancouver Island, could find their way back to Nanaimo, a distance of more than 270 km, within 2 to 10 days (Olesiuk *et al.* 1995).

Questions regarding movements and population structuring of natural populations can be addressed using DNA analysis. PCR (polymerase chain reaction) and DNA sequencing have recently been used to identify genetic variation and analyze phylogeographic structuring in natural populations. Many of these studies use mitochondrial DNA (mtDNA) sequence analysis because mtDNA evolves rapidly, and universal primers are available (Kocher *et al.* 1989, Palumbi *et al.* 1991). Other studies have used biparentally inherited nuclear markers such as EPICs (exon primed intron crossing) or microsatellites to study population structuring and levels of genetic variation (Palumbi and Baker 1994, Paetkau *et al.* 1995, Paetkau and Strobeck 1994,

Roy *et al.* 1994, Taylor *et al.* 1994). Only a few studies have combined nuclear and mtDNA analysis (Palumbi and Baker 1994, Gottelli *et al.* 1994, and Roy *et al.* 1994, Degnan 1993).

Both nuclear and mitochondrial markers can be used to study population structure, migration rates and address concerns regarding possible population bottlenecks. MtDNA provides important information on maternal lineages, but it only represents a single locus due to the lack of intermolecular recombination. It also evolves rapidly and therefore often responds to changes in population structure faster than many nuclear markers. Nuclear DNA provides information on multiple independent genealogies with different modes of evolution. By combining mtDNA and nuclear markers, such as microsatellites, one can address questions regarding phylogeographic structuring and movements of males and females in the population. For example, if only microsatellites or mtDNA were used one could not detect differences in migration rates between males and females. Migration rates of males and females are different in some mammalian species (Karl *et al.* 1992, Palumbi and Baker 1994, Degnan 1994). For this reason, it is important to combine both nuclear and mtDNA to see if harbour seals also show sex specific differences in migration rates.

This study uses both mtDNA sequence data and microsatellite analysis to analyze the geographic distribution of different maternal lineages and phylogeographic structuring of harbour seal populations in British Columbia and parts of Alaska. A secondary goal is to assess the amount of genetic diversity present in the harbour seals in British Columbia with respect to the putative population bottleneck. Attempts are also made to determine migration rates and divergence times between different populations.

The thesis is divided into three sections. Chapter 2 presents the mitochondrial DNA analyses of harbour seals from British Columbia and southeast Alaska. Results provide insights into the colonization of the Pacific by harbour seals, the geographic distribution of maternal lineages, the haplotypic diversity, and the migration rates of females. Chapter 3 examines results of the microsatellite analysis in terms of population structuring, heterozygosity, Hardy-Weinberg equilibrium, and migration rates. Finally, Chapter 4 combines the mtDNA and microsatellite analyses and compares the phylogeographic structuring, migration rate, and divergence time estimates, and discusses possible influences of glaciation on population structuring.

Chapter 2 Mitochondrial DNA

2.1 Introduction

Mitochondrial DNA (mtDNA) is a double stranded, closed circular molecule. It is maternally inherited in almost all organisms and reportedly does not exhibit intermolecular recombination (Wilson *et al.* 1985). MtDNA is useful for molecular analysis because it is made up of 37 genes that evolve 5 to 10 times faster than most nuclear genes (Brown *et al.* 1982). In mammals, the D-loop is a non-transcribed region about 1,600 bp in length that evolves three to five times faster than the other portions of the mitochondrial genome (Aquadro and Greenberg 1983, Vigilant *et al.* 1989, 1991, Horai and Hayasaka 1990). In addition, the different genes within the mitochondrial genome evolve at different rates and therefore different genes can be used in specific analyses. The more slowly evolving genes are often used for phylogenetic analysis while the more rapidly evolving regions tend to be used for population studies (Árnason and Gullberg 1996, Baker *et al.* 1993, Stevens *et al.* 1989).

Previous studies of mtDNA have shown that populations are often partitioned into phylogeographic units based on geographic distance or the presence of topographical boundaries between populations (Avise *et al.* 1987). Phylogeographic structuring may also be caused by behavioural differences. Genetic differentiation without spatial separation may result from a secondary recolonization of an area, such as might occur after the retreat of a glacier.

Separate genetic stocks have been identified for a number of different species in the eastern Pacific. For example, Steller sea lions have a continuous distribution in

the eastern Pacific, yet mitochondrial control region sequence data show that the Steller sea lion population can be separated into an eastern and western stock (Bickham *et al.* 1995). A similar separation is seen in humpback whales which can be divided into a Californian and a Hawaiian population based on mtDNA (Baker *et al.* 1990). Similarly, the sea cucumber (*Cucumaria pseudocurata*) can be separated into a northern population comprising Alaska and the Queen Charlotte Islands, and a southern population consisting of Vancouver Island south to California (A. Arndt, Simon Fraser University, pers. comm.). In addition, several salmon species also exhibit a north south split in their population structure (Taylor *et al.* 1996, Taylor *et al.* 1994,Varnavsakya and Beacham 1992, Wilson *et al.* 1987, Okazaki 1984).

MtDNA has previously been used to study geographic variation, speciation, gene flow and population structure (Ferris *et al.* 1983, Carr *et al.* 1987, Cann *et al.* 1987). Recently, RFLP (restriction fragment length polymorphism) and sequence analyses have been used to study humpback whales (Baker *et al.* 1990), striped dolphins (Rosel *et al.* 1994), bottlenose dolphins (Garcia-Martinez *et al.* 1995), California sea lions (Maldonado *et al.* 1995), and red wolves (Wayne and Jenks 1991).

The following attempts to determine if geographic differences are present in harbour seals from British Columbia by analyzing a portion of the hypervariable D-loop. It also considers migration rates, and divergence times, and examines patterns of colonization of the eastern Pacific by harbour seals.

2.2 Materials and Methods

2.2.1 Sample Collection

Whole blood or tissue samples were collected from 128 harbour seals along the coast from B.C. to southeast Alaska (Figure 2.1, Appendix 1). Blood samples were collected from abandoned harbour seal pups which were part of a rehabilitation program at the Vancouver Aquarium. Tissue samples (liver, skin, muscle and intestine) were obtained from dead harbour seals that had washed up on the beaches, and were stored in dimethylsulphoxide (DMSO) at room temperature (Amos and Hoelzel 1991). Blood samples were frozen at -80°C.

2.2.2 DNA Extraction

2.2.2.1 Blood

To lyse the blood cells, three microlitres of whole blood was added to 1 mL of sterile double distilled water and put at room temperature for 20 minutes. The tube was then centrifuged at 13,000 rpm for 3 minutes. All but 30 µL of the supernatant was removed. One hundred and fifty microlitres of 5% w/v Chelex was added to the pellet and incubated at 56°C for 15 to 30 minutes. The sample was vortexed for 10 seconds and boiled for 8 minutes (Walsh *et al.* 1991). The tube was vortexed for an additional 10 seconds and the DNA was stored at -80°C until required.

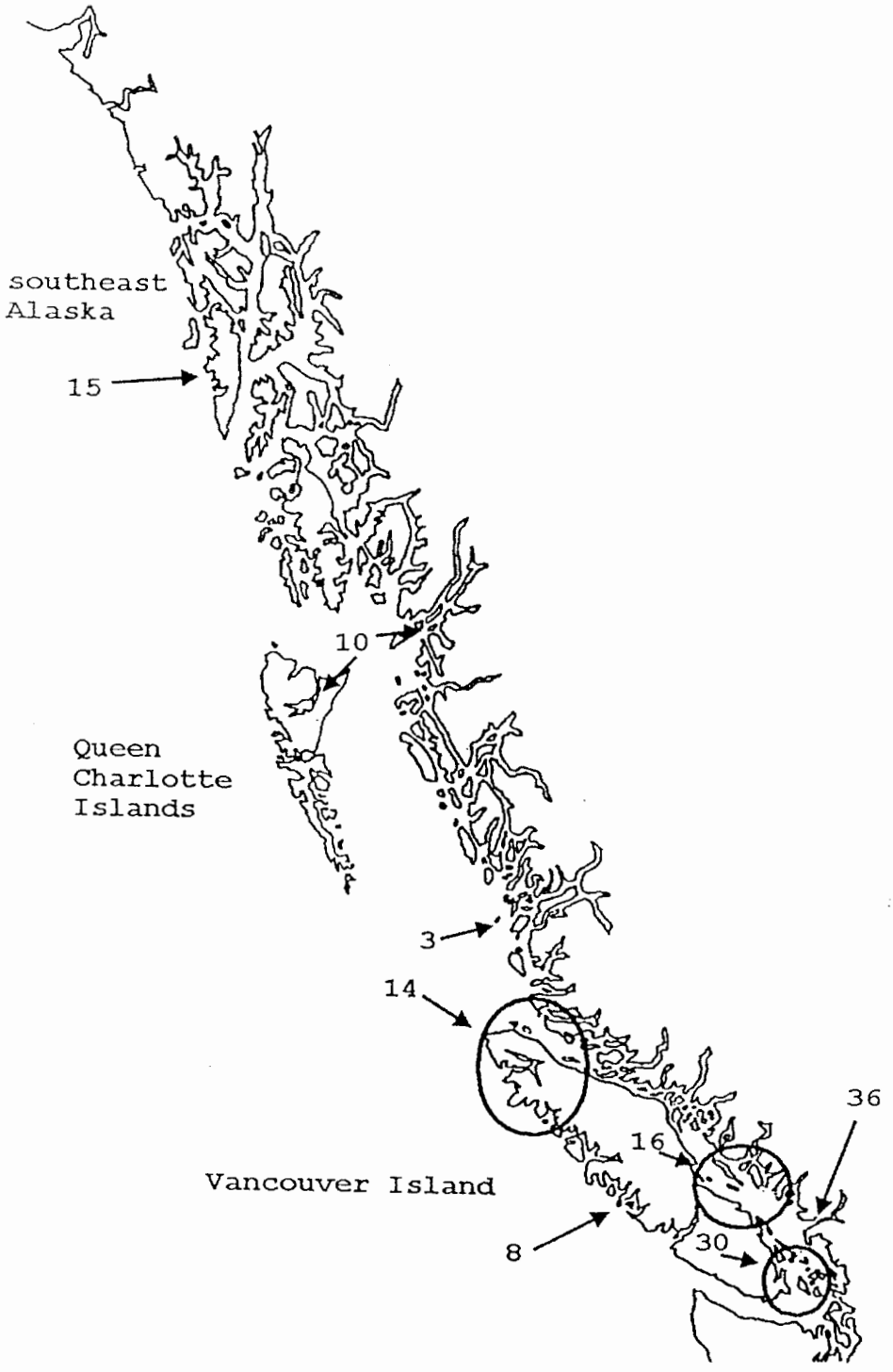

Figure 2.1 Map of sampling sites for mtDNA analysis. Numbers indicate the sample size at that location.

2.2.2.2 Tissue

Approximately 200 ng of tissue was ground in a 1.5 mL microcentrifuge tube containing 300 µL of protease buffer (0.1 M Tris pH 8, 0.05 M disodium EDTA, 0.2 M NaCl, 1% SDS) and 200 µg/mL protease K. An additional 200 µL of protease buffer with enzyme was added and the sample was incubated at 65°C for 1 to 4 hours or until no large tissue particles were visible. DNA was extracted with one volume of phenol, followed by two phenol-chloroform isoamyl (25:24:1) and two chloroform isoamyl (24:1) extractions and an ethanol precipitation (Emmons *et al.* 1979). The dried DNA pellet was rehydrated in 200 µL of sterile double distilled water.

2.2.3 PCR and Sequencing

Two PCR primers (WKT115 5'-ATGACCCTGAAGAA(G/A)GAACCAG-3' and WKT283 5'-TACACTGGTCTTGTAAACC-3') were obtained from W. Kelly Thomas (University of Missouri, Kansas City). They amplify a 520 bp product containing a portion of the tRNA threonine and proline and part of the control region. PCR reactions were performed in a 25 µL reaction volume containing 100 µM each dNTP, 2.5 µM $MgCl_2$, 1x reaction buffer (10x buffer = 200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl (pH 8.8), 0.1% Tween 20), 0.2 U Ultratherm polymerase (BioCan Scientific) and 4 pmol of each primer. Amplification consisted of one cycle at 95°C for 60 s, 52°C for 60 s and 72°C extension for 90 s, 25 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 30 s and one final cycle at 94°C for 30 s, 52°C for 30 s and 72°C for 5 minutes. Two microlitres of the PCR product were electrophoresed on a 1% agarose gel and stained with ethidium bromide to visualize the PCR product.

Five microlitres of the PCR product (approximately 10-20 ng/μL) were treated with shrimp alkaline phosphatase and Exonuclease I, and sequenced using the PCR sequencing kit from USB Amersham and 2 pmol of primer WKT115 or WKT283. Sequencing reactions were run on a 4.5% denaturing acrylamide gel.

2.2.4 Sequence Analysis and Phylogenies

Several different analyses were conducted to examine the phylogeographic relationship among the 128 harbour seals sampled. First, sequences were manually aligned using ESEE (Cabot and Beckenbach 1989) and SeqApp (Gilbert 1992). Individuals with identical sequences were removed and assigned to a shared haplotype (indicated by a single upper case letter). Pairwise distances were calculated using both Kimura 2-parameter model and gamma distance (a = 0.5) estimates in MEGA (Kumar *et al.* 1993) and neighbor joining trees were constructed. Insertion/deletion events were used as an additional character state whenever possible. Maximum likelihood and maximum parsimony analyses were also conducted using Phylip (Felsenstein 1989) and PAUP (Swofford 1991), respectively. Maximum parsimony analysis had poor resolving power at terminal nodes due to the large number of taxa and relatively few informative sites and large number of equally parsimonious trees (>800). Due to the difficulty in differentiating between haplotypes, a minimum spanning tree (Rohlf 1993) was constructed to resolve the relationship between the different haplotypes. A minimum spanning tree places the OTUs at the nodes in addition to terminal branches. Using the minimum spanning tree one can see more clearly the relationship between the different animals and between the different groups. Published sequences for harbour seal (*Phoca vitulina vitulina*) and grey seal (*Halichoerus grypus*) (Árnason and Johnsson 1992, Árnason *et al.* 1995) were used for outgroups analyses.

2.2.5 Regional Patterns of Geographic Subdivision

Analysis of Molecular Variance (AMOVA) was used to determine if significant geographic partitioning was present in the harbour seal population. AMOVA uses F-statistic analogs, designated as Φ statistics, to analyze the correlation between haplotype distances at various levels of organization and random haplotype distribution. Random permutation of sequences among the populations was used to determine the significance of the groupings. There are three different Φ-statistics as defined by Excoffier *et al.* (1992): Φ_{ST}, Φ_{CT}, and Φ_{SC}. Φ_{ST} is the F_{ST} analog and is the correlation between random haplotypes within a population relative to that of the entire population of that species. It specifically measures the proportion of genetic variation within populations. Φ_{CT} is the correlation of random haplotypes within a group of populations relative to that of the entire species and measures the amount of variance among the groups. The last Φ-statistic is Φ_{SC}. It measures the proportion of variation among populations within a region and is the correlation of random haplotypes within a population relative to that within a regional grouping of populations (Excoffier *et al.* 1992). This last Φ-statistic is more comparable to F_{ST} since the traditional F_{ST} does not use a hierarchical approach. Several different groupings of populations were examined based on geographic distribution and those suggested by phylogenetic analysis. In addition to Φ_{ST}, Hudson's F_{ST} analog was also determined using a program developed by S.R. Palumbi (Palumbi pers. comm., Hudson *et al.* 1992).

The amount of gene flow between different regions was estimated as $N_e m_f$ (the number of female migrants per generation). It was determined from:

$$F_{ST} = \frac{1}{(1 + 2N_e m_f)}$$

where N_e is the effective population size and m_f is the proportion of migrating females (Wright 1951, Slatkin 1987, 1993, Palumbi and Baker 1994).

2.3 Mitochondrial DNA Results

2.3.1 Haplotypic Diversity

A 475 bp fragment spanning positions 16,254 to 16,774 of the published harbour seal mitochondrial genome (Árnason and Johnsson 1992) was sequenced from 128 harbour seals from British Columbia and southeast Alaska. Within this region, 60 variable sites (Figure 2.2) defined a total of 72 mitochondrial DNA haplotypes (Figure 2.3). Only 14 of these haplotypes were shared between two or more individuals and the remaining 58 were unique (Table 2.1). Twelve of the 14 shared haplotypes (A-N) were found only on Vancouver Island and the adjacent mainland (hereafter referred to as southern B.C.). The other two (C and D) were also found in seals sampled in northern B.C./Alaska. The most frequent haplotype (A) represented 18% of all the individuals analyzed while the next most frequent haplotypes (B, D and N) were found in 4.7% of the harbour seals.

	haplotype													
area	A	B	C	D	E	F	G	H	I	J	K	L	M	N
sBC	23	6	4	5	3	3	4	3	2	2	2	2	2	6
nBC/AK	0	0	1	1	0	0	0	0	0	0	0	0	0	0
total	23	6	5	6	3	3	4	3	2	2	2	2	2	6

Table 2.1 Distribution of the 14 shared haplotypes between 128 harbour seals sampled from southern B.C. (sBC) and northern BC/southeast Alaska (nBC/AK). An additional 58 unique haplotypes were identified.

Figure 2.2 D-loop sequence from *Phoca vitulina richardsi* (individual QC4). Numbers refer to position relative to published harbour seal sequence (Árnason and Johnsson 1992)showing variable sites (*) and insertion/deletion events (†).

```
16,289      ACTCTCCCTAAGACTCAAGGAAGAGGTAAACAACCCCACCACCAGCACCC                16,338
             tRNA^Thr---> ||                        **  *

16,339      AAAGCTGACATTCTAATTAAACTATTCCCTGACGCCCGCCCAATCCCCCT                16,388
                    <---tRNA^Pro      *     || *              *

16,389      TTCACTCCTCAATTCATATAATAATATCACCTTACTGTGCTATCACAGTA                16,438
                control region    *** *

16,439      TTCACGCACACTGGCCTATGTACTTCGTGCATTGCATGTCCCCCCCCC-ATCC             16,488
               *     †**   *      *** *      **   *****  * †   *

16,489      TCGGACCCCCTATGTATATCGTGCATTAATGGTTTGCCCCATGCATATAA                16,538
              * * *                       *       *

16,539      GCATGTACATAGAGTGGTTGATTTTACATATATGGCATACGGTTGTAACA                16,588
                 *    ** *      *         **       ****       *

16,589      CCAAGTTCTAAAGCATAATTACCTGTCATGAACGCATTTCACCTAGTCCA                16,638
                 *             *     * *   *          *  *

16,639      CGAGCCTTAATCACCATGCCTCGGGAAATCAGCAACCCTTGTGAAACGTG                16,688
            *                      *                    **

16,689      TACCTAGATCTCGCTCCGGGCCCATAACATGTGGGGGTTTCTATACTGGA                16,738
                 *             *         *

16,739      ACTATACCTGGCATCTGGTTCTT                                           16,761
```

Variable sites

```
          1111111111111 1111111111111111 11111111111111111111111111111111
          6666666666666 6666666666666666 66666666666666666666666666666666
          3333344444444 4444444444444444 44445555555555555555666666666677
          2226781111444 4456666777777888 89991244555667778890111233688901
          5694281235289 3930124126789034 81468649029897890847359929145488

QC4       CAATGTAATTAACTCCTTGGCTCCCCC-CGCCTCTAGGAATACGGATTGCAATCGACAGT
V8        ............................A...................AT...T......
V2        ............................A..C.....G.........AT.......G..
J         ..............C.........T.......................T..........
B         ..................A..............................T..........
CH5       ..................A.....................G..A....T.G........
SS1       ..................A..............................T........A.
F         ..................A......T.......................T.........C
Se2       ..................AT.............................T.........C
WVan1     ..................A..............................T.........C
TC3       ....................C.........T.................T..........
QC2       ................C..................................T.....G....
QC3       ................C..................................T..........
D         ...................................................T..........
FI1       ...................................................T....T.T...
C1        .....C.....-.............T.............C....G..............
CS3       .....C.....-.......A.....T.............C....G..............
FI2       ...C.C.....-...................T.........C....G..............
SE3       .....C.....-............T..C..T.........C....G.C............
C         .....C.....-............T.....T.........C....G.C............
Sid1      .....C.....-............T.....T.............G.C..G.........
JB3       .....C.....-............T.....T.................C..G.........
QC1       .....C.....-...................T.........C....G.C............
PR1       ..T........-...................T.........C....G.C............
J1        .....C.....-...................T.........C....G.C.T..C.......
TC4       ........................T......T.........C....................
N3        ...........................................G.........T.....G....
CH7       ....................T...................G.........T..........
N         .....C....G-........T......C...........G....A....T..C.......
A         .....C....G-........T...................G....A....T..C.......
SS9       .....C....G-........T..............A..G....A....T..C.......
PH1       AC...C....G-........T...................G....A....T..C.......
G         .....C....G-.C......T.....T............G.........TG.C.......
N2        .....C....G-........T......C...........G....A....TG.C.......
E         .....C....G-........T...................G....A....TG.C.......
PtH2      .....C....G-.......AT......C...........G....AG...T..........
L         .....C....G-........T...................G....AG...TG.C.......
I         .....C....G-........T...................G....AG...T..C.......
SS10      .....C....G-........T............T......C...A....T..C.......
CS2       .....C....G-........T...............................T..C.......
JB1       .....CG....-...T..A.T.T.T.........C.....C..A.GC.AT..C.......
R1        .....CG....-...T..A.T.T.T.........C.....C..A.GC..T..C.......
SQ1       .....CG....-......A.T.T.T.........C.....C..A.GC.AT..C.......
V9        .....CG....-......A...T.T..............C..A.GC.A...C.......
V5        .....CG....-...T..A.TCT.T..C............C..A..C.AT..C.......
TC2       ............T...........T..............................T..C.......
CH8       ..........G.........T...................G.........T..C.......
BC1       ...................................A..G...A.....T..........
CH6       ....................T..............A..G...A.....T..........
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```
H       ..................AT..............................T.........
SS11    ................................T..................T.........
K       .....CG....-...T..A.T.T.T.........C.....C..AAGC.AT..C.......
WR3     .....CG....-......A.T.T.T...............C..AAGC.AT..C.......
M       .....C.....-............T.....T......A..C....G.C............
WR4     .....C....G-.....CA.T..................G.........T..C.......
CS9     ...................A..............................T..C.......
QB1     .....C....G-......A.TC.................G....A....T..C.......
SaI2    ..............................A........G.........AT..........
Sel     .....C....G-.......AT..................G....AG...TG.C.......
N1      .....C....G-......A.T................A.G....A...AT..C.......
QC5     .....C...C.-..................T.........C....G......C.......
PR3     .....C.....-............T.....T....G....C...AG.C............
BC3     .....C....G-........T.....T............G.........TG.C.......
SE1     .....C..C..-..................T.........C..A.G.C............
SE2     ....A.....................................................TG..........
SE4     ....A.....................................................T...........
SE5     .....C.....-..................T..............G.C............
SE6     .....C.....-..................T.........C..A.G.C............
SE7     .....C.....-........T....T....T.........C....G..............
SE8     .....C.....-..............................C..A.G.C............
SE9     .....C.....-................T.T..............G.C............
CS10    ...................T..................G....A....T..C.......
Pvv     .....C.G...-..T......C.............GA.....TAA....T...........
```

Figure 2.3 Aligned control region sequences from 128 harbour seals showing variable sites and insertion deletion events (-). Numbered sites refer to published sequence for *Phoca vitulina vitulina* (Pvv) (Árnason and Johnsson 1992). Refer to Figure 2.2 for distribution of variable sites.

Two insertion-deletion events were present in the mtDNA sequence. The first insertion or deletion (between positions 16,447 and 16,448) was present in only eight haplotypes while the second (between positions 16,484 and 16,485) was found in 60% of the haplotypes (Figure 2.3).

2.3.2 Sequence Divergence and Haplotype Relationships

Gamma and Kimura distances between haplotypes ranged from 0.026% to 5%. In general, haplotypes within the same geographic area were more similar to each other than to those haplotypes from other geographic areas, with one notable exception (Figure 2.4 and 2.5). A group from southern Vancouver Island (group 3) contains seven haplotypes and forms a clade of its own that is separated from the other seals in southern B.C. by a large number of nucleotide substitutions (minimum of eight nucleotide substitutions).

The neighbor joining trees produced using both gamma and Kimura distances were similar. They suggest three primary divisions: one comprising northern B.C. and southeast Alaska (group 2), another comprising southern B.C. (group 1 and 1a) and a group from southern Vancouver Island (group 3) (Fig. 2.4 and 2.6). A group is defined as the largest set of haplotypes from similar geographic locations that are consistently placed together using various phylogenetic approaches. Group 1a in the neighbour-joining tree shown in figure 2.4 appears as a subgroup of group 1, however, the parsimony tree (not shown), places them as two separate groups. Bootstrap values show that these groupings are strongly supported at the base of each group, but resolution of terminal branching order is poor. The four groups are consistently reproduced using different phylogenetic approaches including maximum likelihood and maximum parsimony.

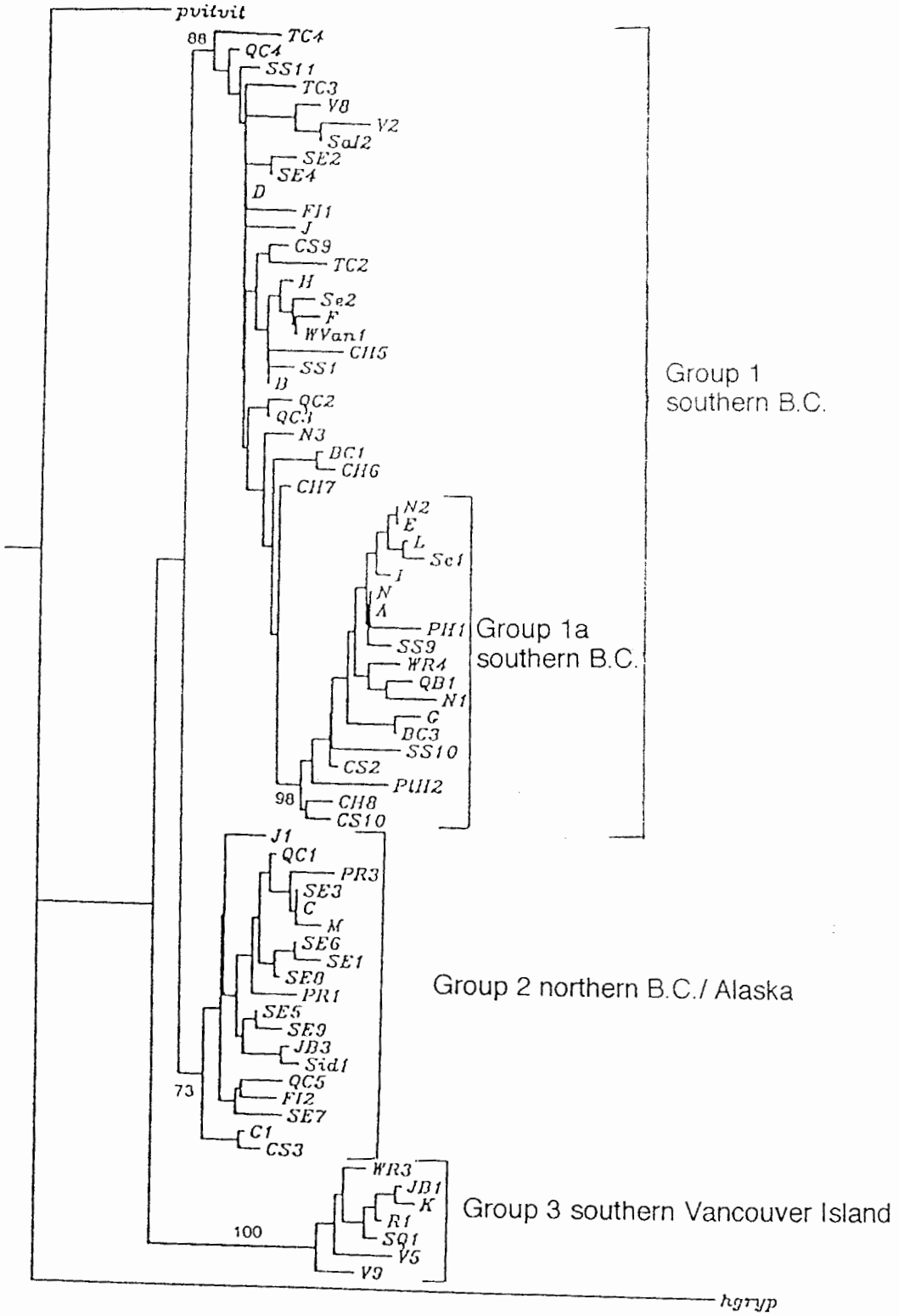

Figure 2.4 Neighbour joining tree based on Jukes-Cantor distances constructed from a 475 base pair region of the D-loop from 128 harbour seals from B.C. and southeast Alaska. Bootstrap values are indicated at the branch points of the major groups. Published sequences from an eastern Atlantic harbour seal (pvit) and grey seal (hgryp) are used for outgroups (Árnason and Johnsson 1992, Árnason *et al.* 1995). Refer to Appendix 1 for abbreviations of haplotypes.

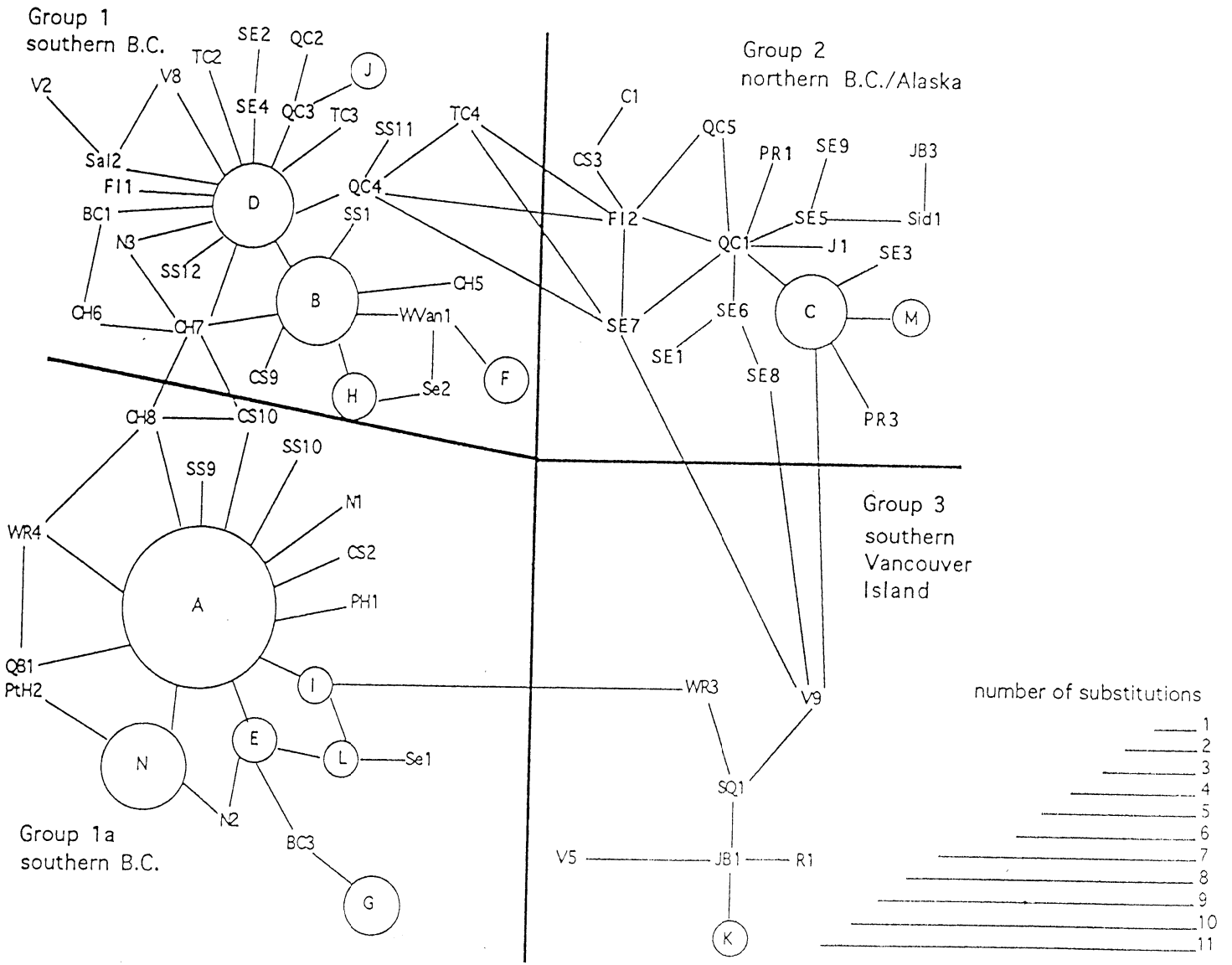

Figure 2.5 Minimum spanning tree based on pairwise nucleotide differences between mitochondrial haplotypes. Samples from northern BC/Alaska are shown in bold. The number of nucleotide substitutions is indicated by the length of the branch connecting the haplotypes. The presence of a circle indicates a shared haplotype and the larger the area of the circle, the more seals share that haplotype.

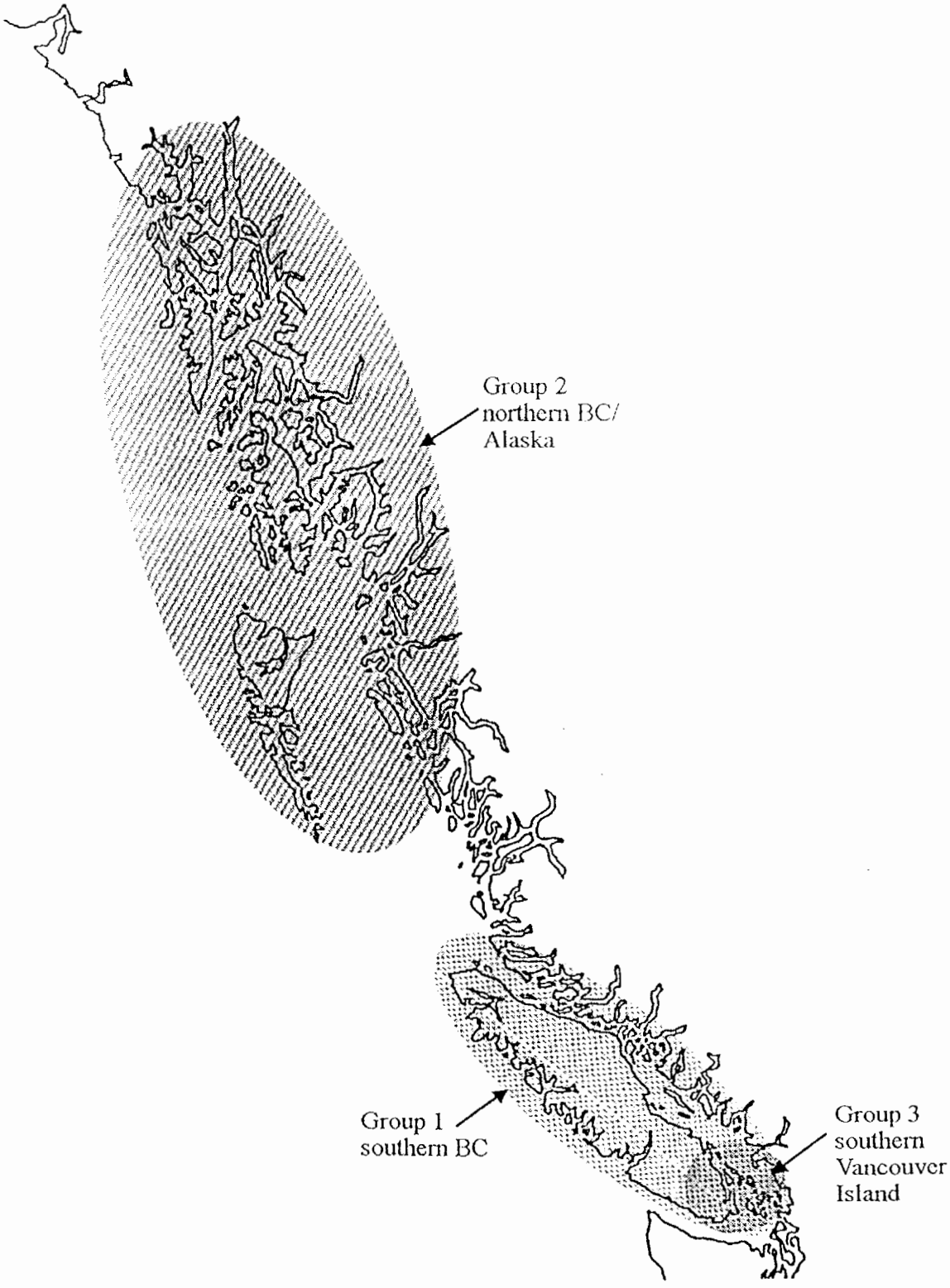

Figure 2.6 Geographic distribution of groups in southern BC (sBC), southern Vancouver Island (sVI) and northern BC/Alaska (nBC/AK) based on mtDNA.

The minimum spanning tree shows the division between these four groups more clearly (Fig. 2.5). The minimum spanning tree also clearly differentiates between groups 1 and 1a. Group 1 contains seals mainly from southern B.C., but also contains six unique haplotypes and one shared haplotype that are found in seals from northern B.C. and Alaska. Furthermore, the haplotypes in group 1 form an articulating network in which several alternative pathways exist between some animals. For example, from haplotype B to Se2, two possible paths through haplotype H or WVan1 exist. Group 1a contains seals from southern B.C. only. Most haplotypes have radiated from a central shared haplotype, haplotype A. This group also contains three other shared haplotypes, N, E, and I, which are linked by single mutation events to A. The minimum number of substitutions between haplotypes in group 1 and either haplotype CH8 or CS10 is three. An additional two nucleotide substitutions are required to get to the main cluster of haplotypes in group 1. Group 2 contains animals mostly from northern B.C. and Alaska, but also contains five haplotypes from southern B.C. including haplotype C which for the most part comprises seals from southern British Columbia. Unlike groups 1 and 1a from southern B.C., group 2 has three central haplotypes, QC1, FI2 and C. Seals from group 2 are most similar to those found in group 1 with only six nucleotide substitutions separating the two groups. Group 3 represents southern B.C. with seven haplotypes which are distantly removed from all other groups (eight substitutions to group 2 and ten to group 1a).

2.3.3 Analysis of Molecular Variance

Analysis of molecular variance (AMOVA) was used to compare the divisions suggested by the minimum spanning tree and geographic location. The division of harbour seals into populations which consistently show high levels of among group variation were those suggested by the minimum spanning tree. Based on geographic

locations, these four groups were divided into three populations comprising southern B.C. (Vancouver Island and the adjacent mainland), northern B.C./Alaska and southern Vancouver Island. This resulted in 62% of the among population variation and 38% within population variation. The Φ_{ST} values were significant for the different geographic regions, with values ranging between 0.44 and 0.86. The net effective migration rate ($N_e m_f$) or the number of seals moving between all the southern B.C. groups and northern B.C./southeast Alaska is approximately 0.637 females/generation. A value of 0.595 was also calculated according to Hudson *et al.* (1992) and is similar to the Φ_{ST} (0.662). The effective migration rate of 0.34 females/generation between the two populations is slightly higher than that from the Φ_{ST} (0.26 females/generation).

2.3.4 Divergence Times

Divergence time between the harbour seal and grey seal is estimated at 2-2.5 MYA based on Cytochrome b data (Árnason *et al.* 1995). The amount of sequence divergence is 11.87% between harbour seals and grey seals and 4.51% between Pacific and Atlantic harbour seals. If harbour seals and grey seals diverged 2.5 MYA, then the mutation rate is approximately 2.4%/MY. If this mutation rate is used to estimate the time of separation between Pacific and Atlantic harbour seals, the divergence time is 1 MYA. This is consistent with the findings of Árnason *et al.* (1995), but half of the Stanley *et al.* (1996) estimate of 2-3 MYA. Estimated time of separation is 0.67 MYA between the southern Vancouver Island group of harbour seals and the other two groups and 0.38 MYA between the southern B.C. and northern populations.

2.4 Discussion

Sixty variable sites in the hypervariable region of the mitochondrial control region define 72 haplotypes in harbour seals from British Columbia and southeast Alaska. Phylogenetic analysis of 128 seals from British Columbia and southeast Alaska support the presence of three main ancestral maternal lineages (southern Vancouver Island, southern B.C. and northern BC/Alaska). The geographic discreteness of these lineages suggests the possibility that the multiple unique haplotypes that are present today radiated from two closely related maternal lineages that arose thousands of years ago.

Average pairwise nucleotide differences within the eastern Pacific harbour seals (average of 2.6% ± 0.29%) is comparable to that found in other studies of harbour seals (M. LaMont, Moss Landing, pers. comm.; R. Westlake, southwest Fisheries Centre, pers. comm.), California sea lions (4.4%) (Maldonado *et al.* 1995) and humpback whales (3.0%) (Baker *et al.* 1993), but considerably higher than the 1.11% reported for bottlenose dolphins (Rosel *et al.* 1994). Interestingly, the level of divergence found by Stanley *et al.* (1996) were much lower (1.19% ± 0.65%) in the Pacific harbour seals and average levels of sequence divergence between the Atlantic and Pacific harbour seals (3.28% ± 0.384%) were more similar to those found in this study in the Pacific. These lower amounts of sequence divergence in the study by Stanley *et al.* (1996) were partially due to a larger number of shared haplotypes and fewer variable sites.

The two main maternal lineages that represent seals in southern British Columbia may be explained by a number of different scenarios. For example, they may have arisen as the result of separation by glaciation and subsequent

recolonization by seals from three separate refugia given that parts of the northwestern Graham Island, western Vancouver Island and the outer coast of Washington were unglaciated during the last glaciation (Clague 1989). A second, and more probable explanation, is that two maternal lineages colonized southern B.C. around the same time while the third lineage (group 3) probably represents an earlier colonization by a small number of harbour seals that migrated south during the initial colonization of the Pacific by harbour seals from the Atlantic. This conjecture is supported by the position of group 3 as a basal group to the other eastern Pacific harbour seals. Subsequent glaciations, may have restricted the small number of these seals that survived to southern Vancouver Island. Furthermore, the first invasion occurred around the same time as the walruses invaded the Atlantic from the Pacific (Cronin *et al.* 1994) and the second just about 70,000 years before sea urchins last colonized the Atlantic from the Pacific (Palumbi and Wilson 1990).

Published harbour seal sequences from Stanley *et al.* (1996) showed that the animals from California and Washington grouped with those from southern B.C., while seals from Japan formed a group with those animals from northern B.C. and Alaska (Fig. 2.7a). The group from southern Vancouver Island (group 3) remained as a distinct group and is most closely related to animals from Japan (n17 and n21). This further supports the idea that group 3, which is the furthest removed from the other groups, is most likely the result of an early colonization by seals from the Atlantic (about 670,000 years ago). Results from the combined analysis of the four harbour seal species presented in Stanley *et al.* (1996) and those from this study further support the separation of the southern Vancouver Island group from the other two Pacific groups (Fig. 2.7b). Subsequent glaciations may have reduced this group to a small number of animals in southern Vancouver Island and Japan. Recolonization of Japan, Alaska and northern Vancouver Island could have occurred during a second

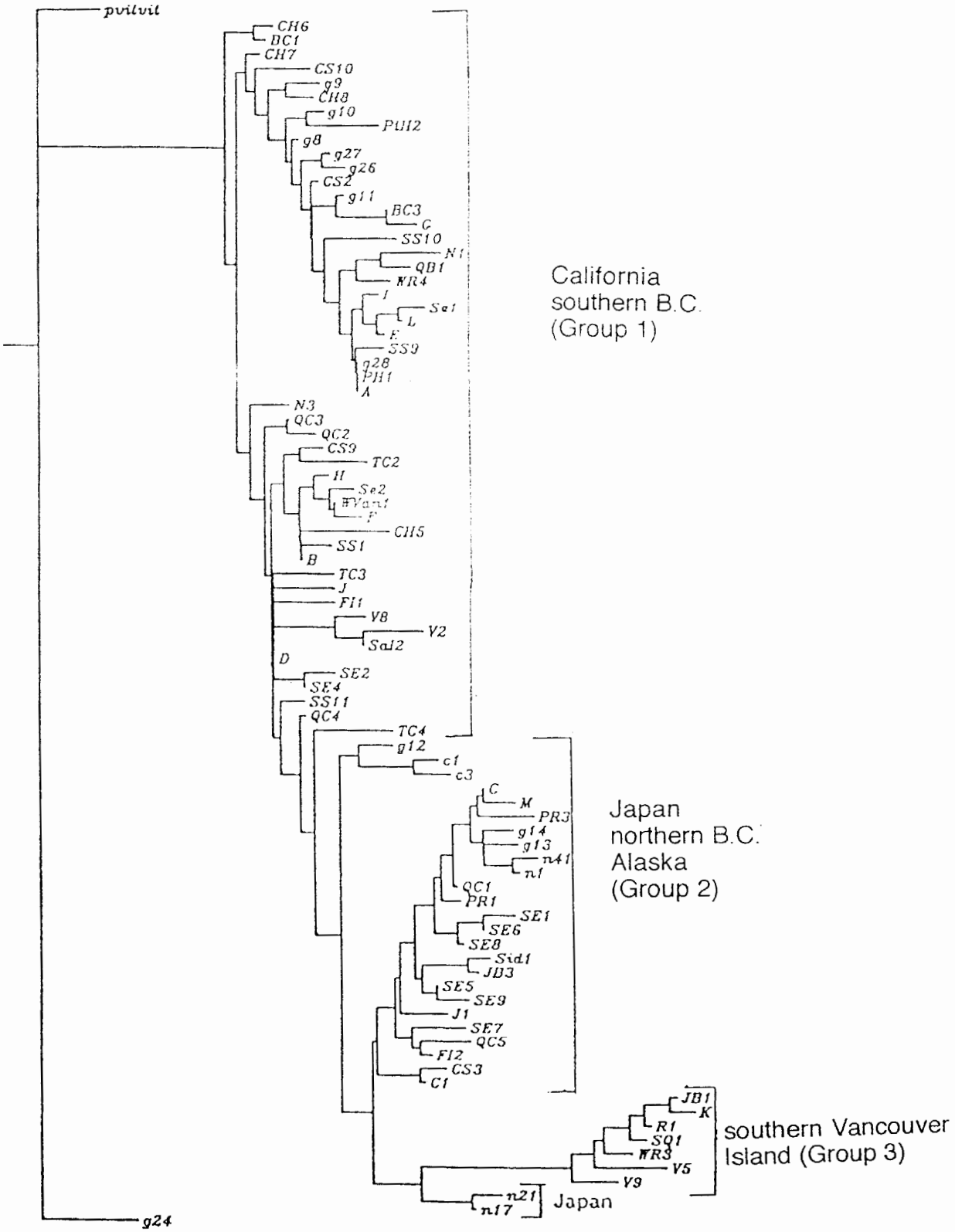

Figure 2.7a Neighbour joining tree based on 394 bp region of the D-loop from this study and Stanley *et al.* (1996) showing the relationship between the eastern Pacific harbour seals. Two eastern Atlantic harbour seals (pvitvit and g24) are used as outgroups (Árnason *et al.* 1995 and Stanley *et al.* 1996).

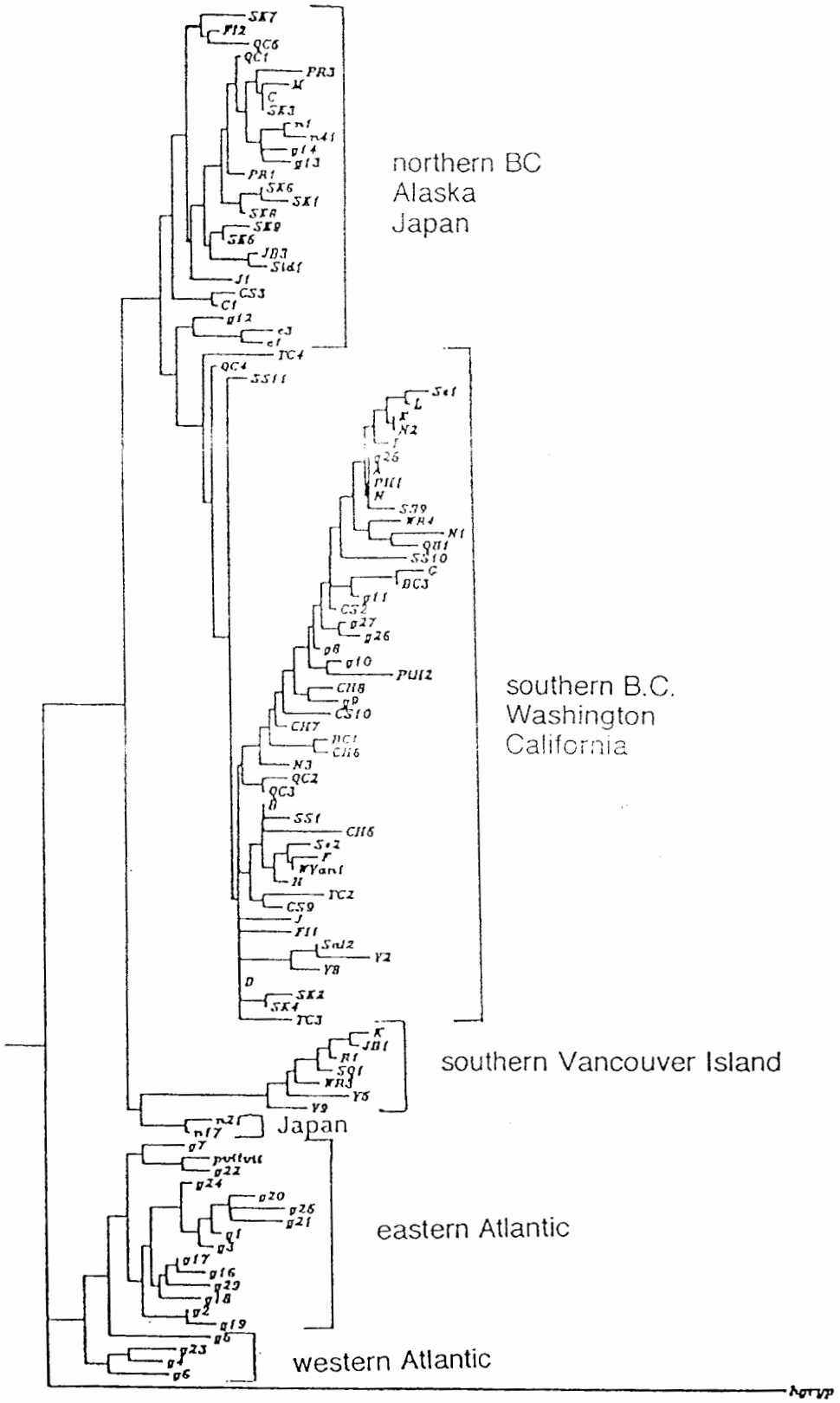

Figure 2.7b Neighbour joining tree based on 394 bp region of the D-loop from this study and Stanley *et al.* (1996) showing the relationship between Pacific and Atlantic harbour seals. Grey seals sequence (hgryp) is used for outgroup analysis (Árnason *et al.* 1995).

invasion of harbour seals into the Pacific (about 380,000 years ago) from the Atlantic Ocean through the Arctic Ocean.

The best grouping of harbour seals, as indicated by the large among group variation, was the one containing all three populations as opposed to three groups each with a single population. Assuming the one group and three population model, migration rate estimates of 0.3 females per generation may not be accurate because 58 of the 72 haplotypes were unique. It is also difficult to determine population partitioning since few haplotypes were shared. Those that were shared often came from seals from southern B.C. due to the larger number of samples obtained from that area. Stanley *et al.* (1996) found that harbour seal populations from the Pacific Ocean were not dramatically different and population divisions in the Atlantic were not precise. Including the harbour seal sequences from Stanley *et al.* (1996) resulted in higher Φ_{ST} values and a large reduction in the among group variation. This is probably the result of the larger proportion of shared haplotypes found by Stanley *et al.* (1996).

The grouping of southern B.C. animals with California harbour seals from the study by Stanley *et al.* (1996) suggests that the southern B.C. harbour seals shared a common ancestor more recently with California harbour seals than any of the other groups despite the closer geographic distance to northern British Columbia. Migration rates between California, southern B.C./Puget Sound, northern B.C./Alaska and Japan suggest that seal migration between California and southern B.C./Puget Sound occurs more frequently than between southern B.C./Puget Sound and northern British Columbia. Similarly, migration from northern B.C./Alaska to Japan is higher than to any other area (Table 2.2).

	California	sBC/ Puget Sd.	nBC/Alaska	Japan
California		2.80	0.51	0.29
sBC/Puget Sd.	0.151		0.99	0.73
nBC/Alaska	0.497	0.336		3.36
Japan	0.629	0.405	0.130	

Table 2.2 Migration rates between California, southern B.C./ Puget Sound (sBC/ Puget Sd.), northern B.C./Alaska (nBC/Alaska) and Japan are shown above diagonals and Φ_{ST} values are below diagonals.

Preliminary mtDNA sequence data of 103 harbour seals sampled in Alaska failed to show any geographic partitioning (Westlake pers. comm.); however, mtDNA analysis of harbour seals from California, Oregon and Washington did show geographic differentiation (LaMont pers. comm., Bickham and Patton 1994). LaMont found that some of the harbour seals from Puget Sound form a group separate from seals found in California, Oregon and the outer coast of Washington. However, LaMont also found that harbour seals from Puget Sound clustered with harbour seals from the other areas to the south. Bickham and Patton (1994) found that haplotypes were shared between seals from Puget Sound and Neah Bay and between seals from the outer coast of Washington and Oregon, but not between the two areas. LaMont, Westlake (pers. comm.) and Bickham and Patton (1994) have found a large number of unique haplotypes and few shared haplotypes. This also contradicts Stanley *et al.* (1996) who found twelve shared haplotypes and four unique haplotypes in harbour seals from California, Washington, Alaska and Japan.

Results of the mtDNA analyses suggest that the Pacific Ocean was colonized twice. One maternal lineage invaded the Pacific approximately 670,000 years ago and is now restricted to southern Vancouver Island, Puget Sound and Japan. A

second invasion occurred approximately 380,000 years ago. This group of animals appears to have colonized Japan and Alaska and a small group of females moved south from northern B.C. and Alaska to colonize southern B.C. and the southern portion of their range.

Chapter 3 Microsatellite Analysis

3.1 Microsatellite Introduction

While mtDNA evolves rapidly and is useful for determining maternal information, new techniques such as microsatellite analysis are more powerful, require less time and provide both paternal and maternal information. Microsatellites consist of short tandem repeats usually 1-6 bp in length such as $(CA)_n$ or $(ATT)_n$ (Beckman and Weber 1992). They are found approximately every 10 kb in the eukaryotic genome and are often highly polymorphic (Tautz 1989, Stallings *et al.* 1991). Polymorphism arises through variation in the number of repeat units present possibly due to slipped strand mispairing (Schlötterer and Tautz 1992). Mutation rates are quite high in microsatellites and generally range from 10^{-2} to 10^{-5} per generation (Dallas *et al.* 1995).

It is necessary to understand a little bit about how microsatellites mutate in order to interpret the data correctly. Comparisons of observed and simulated allele frequency distributions suggest that microsatellites mutate by the stepwise mutation model (Shriver *et al.* 1993, Valdes *et al.* 1993) which states that mutations result in alleles that differ by one repeat unit from the previous allelic state. Another mutation model, the infinite alleles model, states that when an allele mutates it is not dependent on its previous allelic state and any mutation erases all memory of prior allele size. New alleles arise predominantly through intra-allelic polymerase slippage during DNA replication (Schlötterer and Tautz 1992) under either the stepwise mutation or the infinite alleles model. In general, a continuous distribution of size classes is observed as predicted by the stepwise mutation model, but the distribution of the allele size classes corresponds to the infinite alleles model. Furthermore, the type of the

microsatellite repeat (e.g. dinucleotide or pentanucleotide) also seems to affect the mutational process. Shriver *et al.* (1993) used computer simulations to estimate the expected number, size, heterozygosity and frequency distribution of alleles under different stepwise mutation rates. While resulting heterozygosity agreed with data from natural populations, the average number of alleles was larger in the simulation. The microsatellites were divided into three groups based on the size of the repeat: microsatellites (1 - 2 bp repeats), microsatellites (3 - 5 bp repeats, which they termed short tandem repeats (STR)) and minisatellites (15 - 70 bp repeats). They found that the number of alleles, size range and modality of STR corresponds to the stepwise mutation model best, followed by the microsatellites while the infinite allele model was a better fit for minisatellites. With microsatellites, the size of the new allele depends on the size of the previous state (Valdes *et al.* 1993). More recent research shows that microsatellites probably mutate by the two-phase stepwise model (DiRienzo *et al.* 1994) which represents a slight variation on the stepwise mutation model where most mutations involve a change of one repeat unit, but some mutations result in a change of two repeat units (DiRienzo *et al.* 1994).

Regardless of the debate about how the microsatellite alleles mutate, there are several obvious advantages to using microsatellites. For example, many independent loci can be analyzed and alleles are easily scored using PCR and gel electrophoresis (Hughes and Queller 1993). Microsatellites have many uses including genetic mapping, linkage analysis, paternity testing and genetic structuring (Weber and May 1989, Paetkau and Strobeck 1994, 1995, Bowcock *et al.* 1994, Roy *et al.* 1994). For example, Amos *et al.* (1993) used highly variable minisatellites to study pod structure of pilot whales, and found that mature males remained with the pod into which they were born, yet sired none of the offspring in that pod. Microsatellites have also been used to compare the amount of variation between species. For example, the Ethiopian wolf was found to have 30-40% of the heterozygosity present in domestic dogs

(Gottelli *et al.* 1994). In addition, Gottelli *et al.* (1994) used microsatellites to detect a hybridization event between a male domestic dog and a female Ethiopian wolf. Several studies have recently used microsatellites to analyze population subdivision in mammals (Bowcock *et al.* 1994; Edwards *et al.* 1992; Dallas *et al.* 1995; Paetkau and Strobeck 1994; Paetkau *et al.* 1995; Roy *et al.* 1994; Gottelli *et al.* 1994; Taylor *et al.* 1994; Goodman pers. comm.).

The following uses seven microsatellite loci to determine the extent of genetic differentiation, levels of heterozygosity and rates of movement between populations of harbour seals in British Columbia and Alaska. Unlike the mtDNA, microsatellite analysis will provide information on total population structure and is not restricted to maternal lineages.

3.2 Microsatellites Materials and Methods

3.2.1 Isolation and Cloning of 200-600 bp Fragments

Approximately 100 µg of total genomic DNA were digested using Sau3A and electrophoresed on a 1.5% low melting point agarose gel. Fragments between 200 and 600 base pairs were isolated from the low melting point agarose gel. The sample was incubated at 65°C until the agarose was melted. One volume of TE saturated phenol (pH 8) was added and the tube was placed on dry ice for 5 minutes followed by an incubation at 37°C for 5 minutes. The freeze thaw process was repeated twice, inverting the tube periodically (Thuring *et al.* 1975). The sample was then centrifuged at 10K rpm for 10 minutes at 4°C. Following ethanol precipitation, the fragments were ligated into BamH1 cut pUC18, transformed into *E. coli* DH5α and incubated at 37°C for 18 hours on NZY plates (1% w/v NZ amine, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, 0.1% $MgCl_2$, 40 µg/mL Xgal, 160 µg/mL IPTG) containing 50 µg/mL ampicillin.

3.2.2 Detection of Positives

3.2.2.1 Biotin Labeled $(AC)_{12}$ Probe

Microsatellites were detected using a biotin labeled [$(AC)_{12}$] probe (University Core DNA Services, Calgary, AB) and BLUgene kit from Gibco BRL as follows. After transferring the colonies to the hybond N^+ nylon membrane (Amersham), the membrane was treated with a 0.5 M NaOH: 1.5 M NaCl for 5 minutes, followed by 1 M Tris pH 8 for 5 minutes, and 0.1 M Tris (pH 7.5): 2X SSC (1x SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.5) for 5 minutes (Beckenbach *et al.* 1992). The membrane

was UV cross-linked and incubated in 25 mM Tris buffered saline (TBS) (pH 7.4) containing 200 µg/mL protease K for 1 hour at 37°C. The filter was rinsed in TBS followed by a 2X SSC wash and prehybridized in 50% formamide, 5x SSC, 5x Denhardt's (1x Denhardt's = 0.02% BSA, 0.02% polyvinylpyrolidine, 0.02% Ficoll) and 25 mM sodium phosphate (pH 6.5) solution at 42°C for 2 hours. The filter was removed and placed in hybridization solution (45% formamide, 5x SSC, 1x Denhardt's and 20 mM sodium phosphate (pH 6.5)) and 100 ng/mL of biotin labeled probe was added. After hybridizing overnight at 42°C, the filters were washed as follows: 1) two washes in 250 mL of 2x SSC: 0.1 % w/v SDS for 3 minutes at room temperature, 2) two washes in 0.2x SSC: 0.1 % w/v SDS for 3 minutes at room temperature, 3) two washes in 0.16 x SSC: 0.1% w/v SDS for 10 minutes at 50°C, and 4) a rinse in 2x SSC at room temperature. The filters were rinsed with buffer 1 [0.1 M Tris HCl (pH 7.5), 0.15 M NaCl] for 1 minute and then incubated in buffer 2 (3% w/v BSA in buffer 1) for one hour at 65°C. Buffer 2 was removed and replaced with buffer 1 (7 mL/ 100 cm^2) containing 1 µL Streptavidin-Alkaline Phosphatase (SA-AP) per millilitre of buffer 1. The filters were incubated in the SA-AP solution for 10 minutes with gentle agitation and washed twice in buffer 1 (20-40 times the amount used in the previous step) for 15 minutes. Finally the filters were washed for 10 minutes in buffer 3 [0.1 M Tris HCl (pH 9.5), 0.1 M NaCl and 50 mM $MgCl_2$]. Detection of positives was obtained by incubating the filters in buffer 3 (7.5 mL/100 cm^2) containing 33 µL NBT(nitroblue tetrazolium) and 25 µL BCIP (5-bromo-4-chloro-3-indolylphosphate) in a plastic bag in the dark for 20 minutes or until the positive colonies started to appear as blue spots on the membrane.

3.2.2.2 P^{32} Detection Using $(CAC)_{10}$ and $(AAT)_{10}$

Detection was also performed using P^{32} labeled oligonucleotides. After transforming the plasmids and incubating the plates overnight, colonies were

transferred to a hybond N^+ nylon membrane (Amersham). Once transferred to the nylon membrane, the bacteria colonies were lysed and the DNA was fixed to the membrane by placing the membranes in 100 mL of 0.5 M NaOH: 1.5 M NaCl for 2 minutes followed by a wash in 50 mL of 1 M Tris: 1.5 M NaCl. Finally the membranes were placed in 3x SSC for 2 minutes (Beckenbach *et al.* 1992). The filters were UV cross-linked and prehybridized at 40°C in 5x SSC, 0.3% SDS (10-20 mL/100 cm^2) for 30 minutes. Probes were labeled with γP^{32}-ATP using the exchange reaction as described by Tabor (1994). The filters were hybridized with P^{32} labeled [$(CAC)_{10}$ and $(AAT)_{10}$] overnight at 40°C. After hybridizing the filters, they were washed in 3x SSC and 0.2% SDS three times. The membranes were dried and exposed to X-ray film (Kodak X-Omat full speed blue) for up to one week.

3.2.3 Isolation of Positive Colonies

Each positive colony was transferred to a 5 mL sterile tube of LB (1% NZ amine, 0.5% yeast extract, 0.5% NaCl, 0.1% $MgCl_2$, 50 µg/mL ampicillin) and incubated at 37°C overnight. One and a half millilitres of the overnight culture was centrifuged for 30 seconds at 10,000 rpm in a microcentrifuge tube. The supernatant was removed and 100 µL of lysis buffer (1 mM EDTA, 15% w/v sucrose, 0.2 mg/mL pancreatic RNase, 0.1 mg/mL BSA and 2 mg/mL lysozyme) was added to the pellet and agitated for 5 minutes. The lysed sample was placed in boiling water for 60 seconds and on ice for an additional 60 seconds. The sample was centrifuged for 20 minutes at 13,000 rpm to pellet the proteins (modified from Holmes and Quigley 1981). The supernatant was removed and put in clean, sterile tube. To confirm the presence of an insert, 5 µL of the isolated plasmid was digested with EcoRI and HindIII and electrophoresed on an agarose gel. Clones were sequenced using dideoxy termination method (Sanger *et al.* 1977) and sequenase (USB). The procedure was

modified by adding 1 μL DMSO to the template and primer, boiling for five minutes and snap cooling on a dry ice ethanol bath. An additional 0.5 μL DMSO were added to the labeling reaction (T. Snutch, University of British Columbia, pers. comm.).

3.2.4 Designing primers

Primers were designed using Oligo 4 (Rychlik 1992) minimizing self complementarity and primer dimer formation (Table 3.1). Primer pairs were designed to have similar annealing temperatures. Primers were only designed for microsatellites with eight or more perfect repeats. At the onset of this study, no known microsatellite primer sets were available for pinnipeds, so a genomic library was constructed to screen for microsatellites. After some microsatellites were found through the screening of the library, primer sets from other studies became available. Additional microsatellite primers were obtained from S. Goodman (University of Cambridge) and R. Slade (University of Queensland). The primer names are made up of three components: the first two letters indicate person who designed the primer, Pv refers to species for which primers were designed (*Phoca vitulina*) and the number refers to the loci with the exception of primer BG which is named for its location in the beta globulin gene. For example, TBPv1 is the first primer designed by myself (TB) from harbour seals (Pv).

Locus	primer sequences
TBPv1	*TBPv1F- 5' ATAAAGAGGACACAGTTCAA 3'
	TBPv1R- 5' ATCACAGTTGTCAATATGAA 3'
TBPv2	*TBPv2F- 5' CTCTCCCATCCTCATATTAA 3'
	TBPv2R- 5' GTACTACCCAATATAGAGAC 3'
SGPv9	*SGPv9A- 5' CTGATCCTTGTGAATCCCAGC 3'
	SGPv9B- 5' TAGTGTTTGGAAATGAGTTGGC 3'
SGPv10	*SGPv10A- 5' TTCACTTAGCATAATTCCCTC 3'
	SGPv10B- 5' TCATGAATTGGTATTAGACAAAG 3'
SGPv11	SGPv11A- 5' CAGAGTAAGCACCCAAGGAGCAG 3'
	*SGPv11B- 5' GTGCTGGTGAATTAGCCCATTATAAG 3'
SGPv16	*SGPv16A- 5' AGCTAGTGTTAATGATGGTGTG 3'
	SGPv16B- 5' TCTGAGAGATTCAGAGTAACCTTC 3'
BG	beta5- 5' AATTAGTATGATGCTGGGCTGTC 3'
	*beta6- 5' AATTGGGCATGTGATGTGATGAG 3'

Table 3.1 Microsatellite primer sequences. Asterisk indicated the primer which was end-labeled. SGPv loci are from *P. v. vitulina* (Simon Goodman) and BG locus from *M. leonina* (Rob Slade).

3.2.5 Samples

The microsatellite analysis used the 128 samples from the mtDNA analysis plus an additional 94 tissue samples from Alaska for a total of 222 (Fig. 3.1). The latter samples were collected between 1975-1978 and 1993-1995 by the Alaska Department of Fish and Game (J. Lewis pers. comm.).

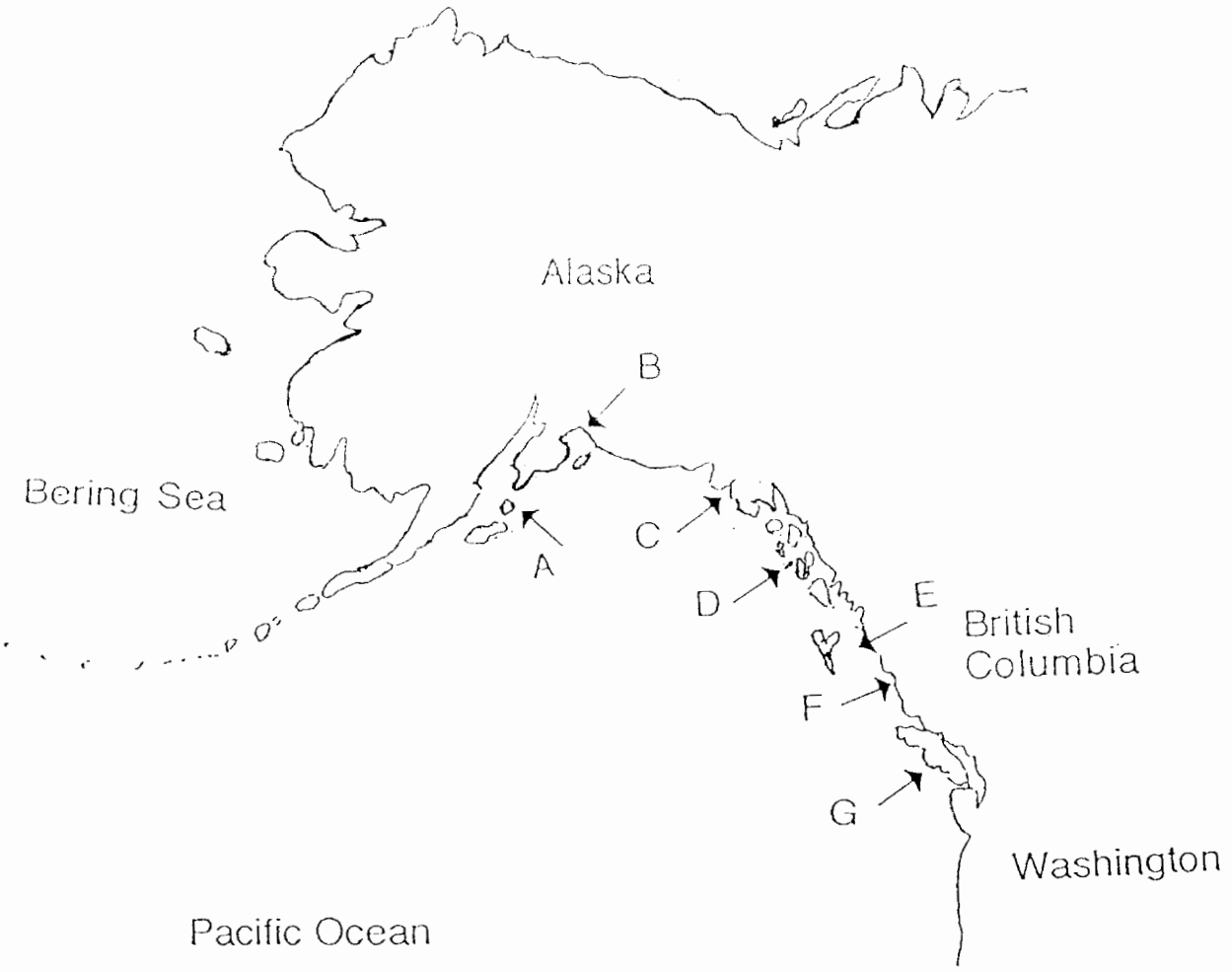

Figure 3.1 Map of the eastern Pacific showing where harbour seal samples were collected for microsatellite analysis. Areas include Kodiak Island (A, n=29), Prince William Sound and Copper River Delta (B, n=30), Icy Bay (C, n=15), southeast Alaska (D, n=30), northern B.C. (E, n=10), Bella Bella (F, n=3), and Vancouver Island and the adjacent mainland (G, n=105).

3.2.6 Amplifying Microsatellites Using PCR

3.2.6.1 End-labeling Primer

One of the PCR primers (Table 3.1) was end labeled using [γ P^{32}]-ATP. First the primer was dephosphorylated at the 5' end using shrimp alkaline phosphatase (SAP) (10x reaction buffer = 200 mM Tris-HCl (pH 8.0), 100 mM $MgCl_2$). Eight units of SAP was used to dephosphorylate 400 pmol of primer. The reaction was placed at 37°C for 15-60 minutes. The enzyme was heat inactivated by placing it at 80°C for 15 minutes. Fifteen units of T4 kinase was used to end label 90 pmol of primer with 2 μCi of γ P^{32}-ATP. The tube was placed at 37°C for 15-30 minutes to incorporate the P^{32} and transferred to 80°C for 10 to 15 minutes to inactivate the kinase.

3.2.6.2 PCR Amplification of Microsatellite Loci

PCR amplification was performed in a 10 μL cocktail consisting of approximately 100 ng DNA template, 2-4 pmol each primer, 200 μM dNTP, 0.5 mM $MgCl_2$, 1x PCR buffer (10x buffer = 200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl (pH 8.8), 0.1% Tween 20) (BioCan), 0.2 units Ultratherm polymerase (BioCan Scientific), and varying concentrations of DMSO (Table 3.2) using GTC Genetic Thermocycler (GL Applied Research Inc.). Amplification consisted of an initial 2 minute denaturation at 94°C followed by 7 cycles of denaturation at 94°C for 60 s, annealing at lower annealing temperature (Table 3.2) for 60 s and extension at 72°C for 60 s. An additional 25 cycles consisting of denaturation at 89°C for 40 s, annealing at a higher annealing temperature (Table 3.2) for 40 s and extension at 72°C for 40 s.

Locus	annealing temperatures	percent DMSO
TBPv2	48°C - 48°C	0
SGPv9	53°C - 55°C	10
SGPv10	57°C - 59°C	3
SGPv11	58°C - 60°C	10
BG	58°C - 60°C	10

Table 3.2 PCR reaction conditions for microsatellite loci. The first annealing temperature is the lower annealing temperature (7 cycles) and the second value is the higher annealing temperature (25 cycles).

3.2.7 Separation of Microsatellite Alleles

Approximately two microlitres of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol FS, 20 mM EDTA) was added to each PCR reaction. The sample was heat denatured for 90 seconds and cooled on ice. Five microlitres of the denatured PCR product was loaded onto a 6% denaturing TBE acrylamide gel, and electrophoresed at 45W for 2-4 hours depending on the size of the PCR product. For example, a 200 bp product required 3 hours of electrophoresis to sufficiently separate the alleles while a 250 bp product required 3 1/2 hours (Glenn 1995). The gels were dried on 3MM Whatman filter paper and exposed overnight to X-ray film (Kodak X-Omat full speed blue).

3.2.8 Scoring Alleles

The alleles were scored according to their size, and assigned numbers starting at 1 for the largest band (Figure 3.2). Exact sizes of the alleles were determined using a sequencing reaction as a size ladder. Samples from some animals were amplified

and the resulting PCR product run on each gel as markers to ensure consistent scoring of the alleles. The average heterozygosity, allele frequencies and genotype frequencies were calculated. In addition, F-statistics, distances and tests for Hardy-Weinberg equilibrium and allele homogeneity were determined.

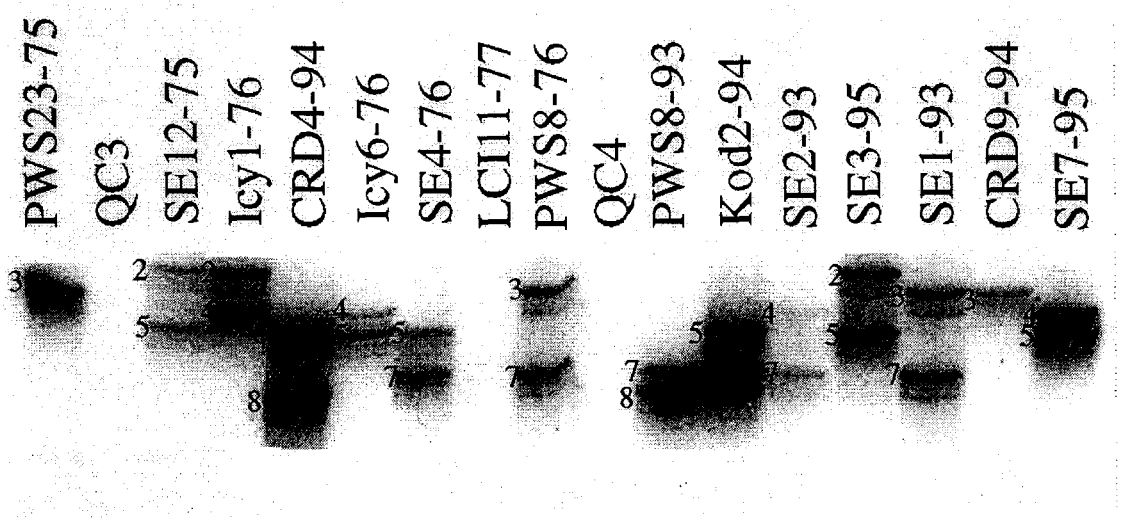

Figure 3.2 Microsatellite gel showing alleles variation at TBPv2 locus. Numbers next to the bands refer to allele number.

3.2.9 Statistical Analysis

A G-test was performed at each locus for all pairwise comparisons to test for differences in allele distribution between the different geographic areas (Zar 1984). The null hypothesis was that the two populations have homogenous allele distributions. With microsatellites, a large number of alleles are often present, resulting in low values of some allele classes. These small values can lead in turn to

erroneously rejecting the null hypothesis. However, the final subpopulation differences that were detected met all the assumptions of the goodness-of-fit test. The fixation index (F_{ST}) and inbreeding coefficient (F_{IS}) were also calculated according to Hartl and Clark (1980).

Heterozygosity and probability of identity were calculated as follows:

Observed heterozygosity

$$H_{obs} = \sum \frac{p_{ij}}{n}$$

Expected heterozygosity

$$H_{\exp} = 1 - \frac{\left(n\sum p_i^2 - 1\right)}{n-1}$$

and probability of identity

$$I = 1 - \left[\sum p_i^4 + \sum\sum \left(2p_i p_j\right)^2\right]$$

where p_i and p_j are the frequency of the i^{th} and j^{th} allele, p_{ij} is the number of heterozygotes for alleles i and j, and n is the number of animals (Nei and Roychoudhury 1974).

Deviations from Hardy-Weinberg equilibrium at each locus in each geographic location were examined using Fisher's exact test (Guo and Thompson 1992). Delta mu distances were calculated using the program microsat (Goldstein *et al.* 1995b).

F_{ST}, G_{ST}, and R_{ST} were also determined. F_{ST} was used to determine the population subdivision and assumed an infinite island model of population substructure i.e. an individual has equal probability of migrating to any of the demes within a population. From the F_{ST} values, one can obtain estimates of migration:

$$F_{ST} = \frac{1}{1 + 4N_e m_e}$$

where N_e is the population size and m is the proportion of individuals migrating each generation (Wright 1951). One problem with F_{ST} and many other statistics used in population genetics is that they are based on the infinite alleles model and mutation rates that are much lower than those found in microsatellite data. In addition, as discussed earlier it is believed that microsatellites do not evolve according to the infinite alleles model. Slatkin (1995) developed R_{ST} which is based on the two-phase model to address these concerns. R_{ST} was calculated using a program developed by S. Goodman (pers. comm.) according to:

$$R_{ST} = \frac{\bar{S} - S_W}{\bar{S}}$$

where $\bar{S}$ is twice the variance in allele size over d populations and S_W is twice the average within population variance in allele size. Migration rates, M_R, based on R_{ST} were also calculated from the average R_{ST} according to Slatkin (1995):

$$M_R = \left(\frac{d-1}{4d}\right)\left(\frac{1}{R_{ST}-1}\right)$$

where d is the number of populations sampled.

Migration rates were also calculated using the average frequency of private alleles (Slatkin 1985, Slatkin and Barton 1989) which is relatively insensitive to mutation rate.

3.3 Microsatellite Results

Eleven microsatellites were found by screening of a genomic library. Of those, only two dinucleotide repeats were of sufficient length (> 8 base pairs) to make primers. It is possible that the shorter repeats for which no primers were made may represent a small allele at that locus. Since only two suitable loci were found, additional microsatellite primer sets were obtained from S. Goodman (SGPv9, SGPv10, SGPv11 and SGPv16) and R. Slade (BG).

3.3.1 Allele Frequency Distribution

The number and frequency of alleles at each of the five loci differed between southern B.C. and northern B.C./Alaska (Fig. 3.3 and Table 3.3). Allele frequency distribution was highly variable between loci, with most loci having at least one rare allele (Table 3.3). Compared to the northern B.C./Alaska population, the southern B.C. population had the highest frequency of the smallest allele (allele 7) of the BG locus and the lowest frequency of the largest allele (allele 1) (Figure 3.3a). Locus SGPv10 had five alleles in the southern population while the two smallest alleles were absent from the northern population (Figure 3.3c). Locus SGPv11 had a larger number of alleles in the northern population (seven alleles) compared to southern B.C. (six alleles) (Figure 3.3d). The SGPv9 locus had four alleles in both populations although the allele frequency varied dramatically between the two populations (Figure 3.3b). The most frequent allele (allele 3) in the northern population was found in only a small number of harbour seals in the southern population. Finally, locus TBPv2 had twelve alleles in the southern B.C. group and only ten in the northern animals. The

distribution and frequency of the alleles was also quite different between the two populations (Figure 3.3e).

		population				population	
Locus	Allele	sBC	nBC/AK	Locus	Allele	sBC	nBC/AK
TBPv2	1	0.043	0.074	SGPv9	1	0.427	0.387
	2	0.117	0.064		2	0.440	0.347
	3	0.122	0.167		3	0.011	0.073
	4	0.234	0.211		4	0.287	0.194
	5	0.043	0.118				
	6	0.101	0.191				
	7	0.202	0.064	SGPv10	1	0.012	0.030
	8	0.011	0.044		2	0.367	0.371
	9	0.069	0.010		3	0.604	0.598
	10	0.005	0.000		4	0.006	0.000
	11	0.021	0.059		5	0.012	0.000
	12	0.032	0.000				
BG	1	0.033	0.063	SGPv11	1	0.105	0.121
	2	0.179	0.278		2	0.042	0.121
	3	0.271	0.233		3	0.447	0.328
	4	0.217	0.188		4	0.321	0.318
	5	0.120	0.119		5	0.037	0.056
	6	0.038	0.068		6	0.047	0.051
	7	0.140	0.051		7	0.000	0.005

Table 3.3 Observed allele frequency distributions for five polymorphic microsatellite loci (also see Figure 3.3).

3.3.2 Geographic Differences

Microsatellite data from the 222 seals sampled in British Columbia and Alaska were analyzed using seven microsatellite loci. Five of the seven microsatellites were polymorphic having four to twelve alleles per locus and an average heterozygosity of 47%. This is not unusual given the high mutation rates of microsatellites. Homogeneity of allele distribution was tested using a G test (Zar 1984). Pairwise comparisons between adjacent geographic locations were made for each locus and

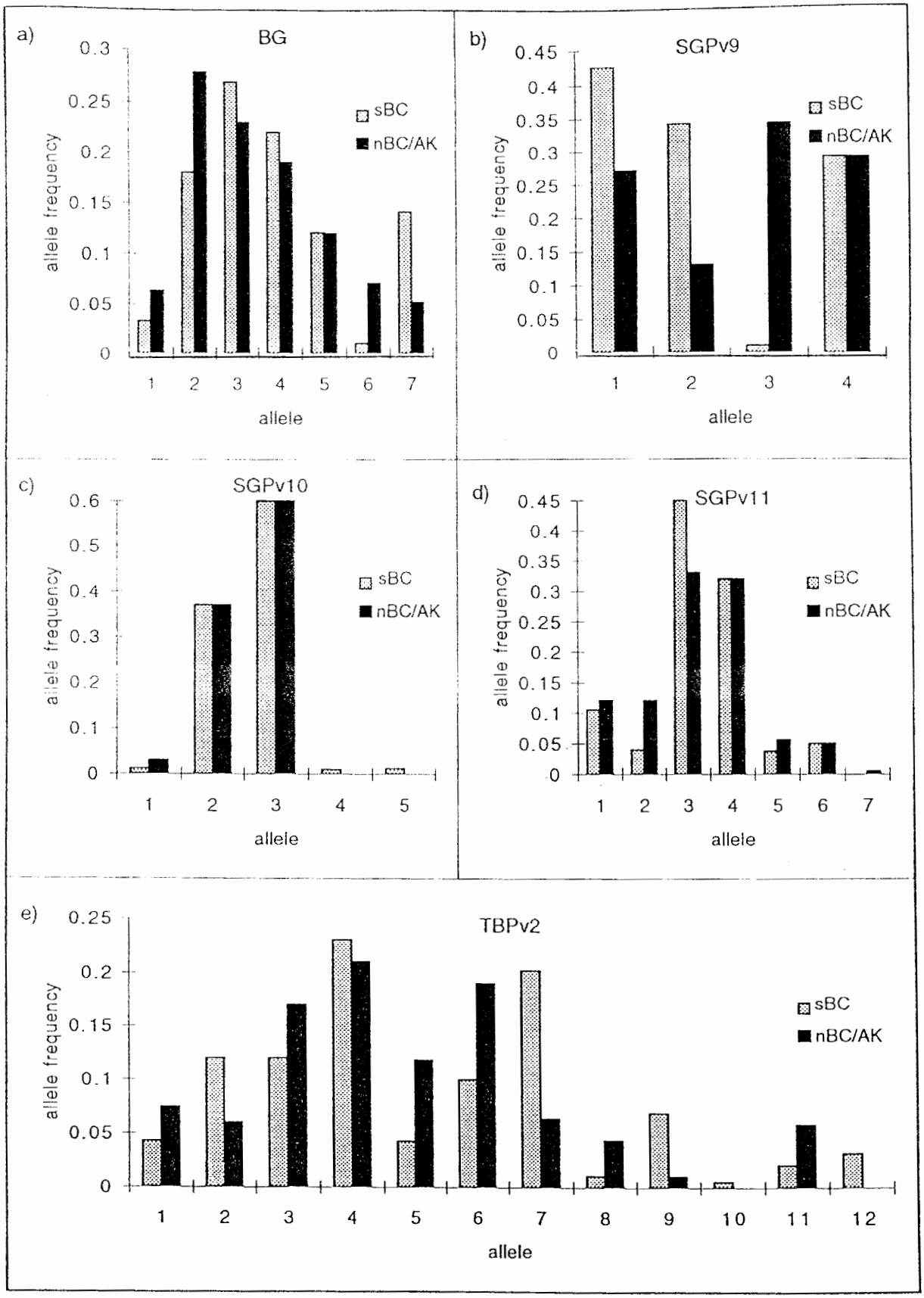

Figure 3.3 Microsatellite allele distribution for the five polymorphic loci in southern British Columbia (sBC) and northern British Columbia and Alaska (nBC/AK). Refer to Table 3.3 for exact values.

the values were summed across all loci. The only significant difference in allele frequency was between the southern B.C. population (Vancouver Island and the adjacent mainland) and northern B.C./Alaska.

Analysis of four microsatellite loci (BG, TBPv2, SGPv9 and SGPv11) showed significant differences between southern B.C. and northern B.C./Alaska (P values of 0.01, < 0.001, <0.001, and 0.03 respectively). Two of the loci (SGPv10 and SGPv11) further subdivided the northern B.C./Alaska group. The SGPv11 locus divides the harbour seal populations into three significantly different groups: 1) Kodiak Island, AK 2) Prince William Sound to northern B.C. and 3) southern B.C. ($P < 0.001$).

There were no significant differences in the SGPv10 locus when the eastern Pacific was divided into three regions: southern B.C., northern B.C./southeast Alaska and Icy Bay/Prince William Sound/Kodiak Island ($P = 0.056$). However, there was a significant difference when southern B.C. was excluded from the analysis ($P = 0.005$). The only significant difference in allele distribution when all five polymorphic loci were combined was between southern B.C. and northern B.C./Alaska ($P < 0.001$).

3.3.3 Heterozygosity

The observed heterozygosity ranged from 44% to 81% for the five polymorphic microsatellite loci, while the expected heterozygosity within each population varied between 51% and 87% with an average of 66% and 72.5%, respectively. The southern B.C. population had a slightly lower overall heterozygosity compared to northern B.C./Alaska. The total observed heterozygosity was lower than the overall expected heterozygosity (Table 3.4).

	H_{obs}		H_{exp}		prob. of identity		no. of alleles	
locus	sBC	nBC/AK	sBC	nBC/AK	sBC	nBC/AK	sBC	nBC/AK
TBPv1	0.00	0.00	0.00	0.00	1	1	1	1
TBPv2	0.75	0.81	0.86	0.87	0.148	0.074	12	10
SGPv9	0.66	0.79	0.67	0.70	0.238	0.138	4	4
SGPv10	0.52	0.44	0.51	0.51	0.347	0.347	5	3
SGPv11	0.53	0.60	0.69	0.76	0.154	0.097	6	7
SGPv16	0.00	0.00	0.00	0.00	1	1	1	1
BG	0.79	0.73	0.82	0.82	0.063	0.064	7	7
overall	0.46	0.48	0.51	0.52	1×10^{-4}	2×10^{-5}	5.1	4.7

Table 3.4 Observed and expected heterozygosity (H_{obs} and H_{exp}), probability of identity and number of alleles between southern B.C. (sBC) and northern BC/Alaska (nBC/AK) for all seven microsatellite loci.

3.3.4 Hardy-Weinberg Equilibrium

The proportion of microsatellite genotypes observed in the two areas was compared with Hardy-Weinberg proportions (HWP) using Fisher's exact test (Guo and Thompson 1992). Highly significant departures ($P < 0.01$) from HWP were found in three out of ten comparisons (Table 3.5). The P value is lower (0.005) because the tests are not independent of each other (Zar 1984). The two loci that did not exhibit HWP (SGPv10 and SGPv11) were not used to calculate F_{ST}, Nm or genetic distances.

locus	area	significance (P)	standard error
SGPv10	nBC/AK	0.042	0.00146
	sBC	0.00005**	0.00005
SGPv11	nBC/AK	0.00094**	0.00021
	sBC	0.00029**	0.00012
BG	nBC/AK	0.163	0.00299
	sBC	0.097	0.00236
SGPv9	nBC/AK	0.014	0.00095
	sBC	0.752	0.002
TBPv2	nBC/AK	0.012	0.00088
	sBC	0.120	0.00243

Table 3.5 CMC test for Hardy Weinberg proportions (HWP) show highly significant departures (**) from HWP ($P < 0.005$).

3.3.5 Genetic Distances

Delta mu distances and Nei's standard distances were also calculated for the microsatellite loci that showed no evidence of null alleles (Goldstein *et al.* 1995b, Nei 1978) (Table 3.6). The standard errors calculated for the combined loci were high. Delta mu is based on average square distances and dependent on time, not on population size which makes it ideal for estimating time of separation between populations. In addition, delta mu is based on the stepwise mutation model unlike Nei's standard distance which is based on the infinite alleles model. Both calculations showed that the two populations (nBC/AK and sBC) are separated by a small genetic distance.

	sBC	nBC	PWS	SE AK	Kodiak	Icy Bay
sBC		-0.015	0.035	-0.012	0.040	0.026
nBC	0.089		-0.027	-0.045	-0.009	-0.045
PWS	0.133	0.091		-0.051	0.007	0.004
SE AK	0.143	0.166	0.027		-0.022	-0.028
Kodiak	0.069	0.068	0.085	0.144		0.011
Icy Bay	0.089	0.101	0.108	0.185	0.093	

Table 3.6 Pairwise delta mu (below diagonal) and Nei's (above diagonal) distances for southern B.C. (sBC), northern B.C. (nBC), Prince William Sound (PWS), southeast Alaska (SE AK), Kodiak Island area (Kodiak) and Icy Bay.

3.3.6 Probability of Identity

Another measure of genetic diversity is the probability of identity. This is the probability that two individuals drawn at random from the same population have identical genotypes at all loci as shown in Table 3.4. The probability of two harbour seals from southern B.C. having the same genotype is 1 in 10,000 and in northern B.C./Alaska 1 in 50,000 seals at the seven loci examined.

3.3.7 F Statistics

The inbreeding coefficient, F_{IS}, and fixation index, F_{ST}, were calculated according to Weir and Cockerham (1989). The F_{IS} values were near zero indicating random breeding was occurring. Overall F_{ST} values were small and a migration rate ($N_e m_e$) of 3 seals/generation was calculated. Migration rates based on private alleles method (7.3 seals/generation) and R_{ST} (104 seals/generation) were higher.

3.4 Microsatellite Discussion

Analysis of the 222 harbour seals sampled in British Columbia and Alaska using seven microsatellite loci revealed distinct populations of harbour seals. Five microsatellites were polymorphic in eastern Pacific harbour seals and had 4-12 alleles per locus with an average heterozygosity of 66% which is not unusual given the high mutation rates of microsatellites. Low levels of genetic divergence between southern B.C. and northern B.C./Alaska populations is evident by the different major allele classes at the same locus (Fig. 3.3) and the lack of homogenous allele distributions.

Deviations from Hardy-Weinberg proportions (HWP) were observed at several microsatellite loci. This may be due to either non-random mating or, more likely, the presence of null alleles since the inbreeding coefficient values (F_{IS}) are near zero. Null alleles are ones that fail to amplify usually due to a mutation in the unique flanking sequence where the PCR primer anneals (Chakraborty *et al.* 1992). The presence of null alleles can sometimes be detected by heterozygosity deficiencies or by the absence of certain microsatellite alleles in different populations. One way to detect null alleles is by designing new primers. However it has been shown that selecting a primer that is farther away from the microsatellite repeat does not decrease or increase the probability of detecting null alleles. A second way to detect null alleles is to lower the annealing temperature during PCR amplification. However this often results in secondary non-specific binding. The best way to detect null alleles is through pedigree analysis, but this option was not available. The deviations from HWP observed in two of the five loci were probably due to the presence of null alleles as evident by a heterozygote deficit. Null alleles have been detected in other animals such as coyotes, grey wolves (Roy *et al.* 1994), deer (Pemberton *et al.* 1995), bears

(Paetkau and Strobeck 1995), mice (Dallas *et al.* 1995) and minke whales (vanPijlen *et al.* 1995), although at a much lower rate.

Null alleles may be present in the SGPv11 locus and probably represent an ancestral null allele since both populations do not exhibit HWP and show heterozygote deficiencies. Locus SGPv10 also has a large number of rare alleles (three of the five alleles) in the southern B.C. population.

In addition to using allelic variation as a measure of genetic diversity, probability of identity was also examined. The probability of identity for the seven loci examined is 1×10^{-4} for southern B.C. and 2×10^{-5} for northern B.C./Alaska. This is comparable to values found for black bear populations (4.6×10^{-2} to 2×10^{-5} Paetkau and Strobeck 1994).

Migration rate estimates obtained using the various methods showed considerable variation. This is probably due to the assumptions made by the various statistics. Similar discrepancies in migration rate estimates were found in other studies (Goodman pers. comm., Allen *et al.* 1995). Both R_{ST} and F_{ST} take the mutation rate into consideration, but Wright's F_{ST} does not take the mutational history into account and assumes that mutations always result in new alleles. Slatkin's R_{ST} is less biased as it includes back-mutations of microsatellites. However, the Nm estimates from R_{ST} are considerably higher than those from F_{ST} (104 seals/generation compared to 3 seals/generation). A third model, Slatkin's private alleles model, uses the extent to which interchange prevents novel alleles from reaching a high frequency in one population to derive migration rate estimates. This last method results in a migration rate similar to that obtained from F_{ST} and is in better agreement with the differences in allele frequency that were observed. The best explanation for the differences in

migration rates is that the microsatellite loci used in this study do not meet one of the underlying assumptions of Slatkin's R_{ST} model and may not be valid for these loci.

Comparison of eastern Pacific harbour seals with two similar studies conducted on the western Atlantic harbour seals (*P. v. concolour*) and the eastern Atlantic harbour seals (*P. v. vitulina*) reveal interesting differences. The western Atlantic population on Sable Island appears to have gone through an extreme bottleneck severely reducing its genetic variation. In fact the amount of genetic diversity is so low that analysis of eight microsatellite loci, including TBPv2, revealed that no more than one to three alleles were present (Coltman *et al.* 1996 and unpublished data). Levels of heterozygosity were extremely low ranging from 5% to 47%. This was not the case with the eastern Atlantic harbour seal. Analysis using thirteen microsatellite loci, including five used in this study, showed the presence of six distinct populations (Goodman unpublished data).

Comparison between *P. v. vitulina* and *P. v. richardsi* showed that *P. v. richardsi* has higher levels of heterozygosity. This higher level of genetic diversity agrees with RAPD and DNA fingerprinting analysis (Kappe *et al.* 1995). In addition, two loci (SGPv9 and SGPv10) display a higher number of alleles in the eastern Pacific subspecies than in the eastern Atlantic subspecies of harbour seal.

In the eastern Atlantic, microsatellite analysis shows distinct harbour seal populations only where the geographic distribution is disjunct. Furthermore, the overall population density of harbour seals in the eastern Atlantic is lower than that in the eastern Pacific due to an outbreak of phocid distemper in the late 1980's that killed 50% of the European harbour seals (Heide-Jørgensen *et al.* 1992). Amplified alleles in the eastern Pacific harbour seals were also of a different size range (Table 3.7). Comparison of microsatellite alleles between different species has revealed that they

are often of different size ranges depending on the species (Coltman *et al.* 1996, Schlötterer *et al.* 1991, FitzSimmons *et al.* 1995, Roy *et al.* 1994).

		P. v. richardsi			*P. v. vitulina*		
Locus	Repeat type	Allele size	no. of alleles	no. of repeats	Allele size	no. of alleles	no. of repeats
TBPv1	AC	184	1	14	n/a	n/a	n/a
TBPv2	AC	240-264	12	19-30	n/a	n/a	n/a
SGPv9	GT	162-168	4	13-16	164	1	14
SGPv10	GT	138-146	5	12-16	132-136	2	9 and 11
SGPv11	AC	162-174	7	18-24	152-166	7	13-20
SGPv16	AC	127	1	17	127	1	17
BG	GGAAA	282-312	7	8-14	277-307	7	7-13

Table 3.7 Comparison of allele size, total number of alleles and number of repeats in microsatellite loci in the eastern Pacific (*P. v. richardsi*) and eastern Atlantic (*P. v. vitulina*) harbour seals.

Movements of harbour seals in B.C. during the breeding season may result in gene flow along the Strait of Georgia and the west coast of Vancouver Island. Numbers of harbour seals in southern B.C. are currently quite high and the home ranges of harbour seals probably overlap which may contribute to the gene flow and lack of population structuring. In the past two decades, some harbour seal populations in western Alaska have declined and while their levels appear to be starting to stabilize, their current levels are far below what they were twenty years ago. Nevertheless, the new smaller populations may not have been isolated long enough from the eastern populations to show any significant phylogeographic partitioning. The division of northern B.C./Alaska into smaller populations by some of the microsatellite loci, may be due to genetic drift in these new isolated groups.

Two refugia are thought to have existed in British Columbia during the last glaciation: one on the Queen Charlotte Islands and a second in the Brooks Peninsula on northern Vancouver Island (Clague 1989). The climate along the B.C. coast during the last glaciation was similar to present day western Alaska and was capable of supporting harbour seals (Clague 1989). The last glaciation likely caused a separation in the distribution of harbour seals and prevented gene flow between southern B.C. and northern B.C. allowing genetic drift and mutation to affect allele distribution in the two areas.

Approximate estimates of divergence between southern B.C. and northern B.C./Alaska populations obtained using delta mu (Goldstein *et al.* 1995a) estimate the time of separation around 9,000 years ago. Genetic differentiation in the eastern Atlantic harbour seal populations occurred earlier (13,780 to 32,500 years ago) (Goodman pers. comm.). During the last glaciation, most of the Pacific coast was covered by ice and a large piece of the Cordilleran ice sheet extended out into the Pacific over the Queen Charlotte Islands. The glaciers retreated earlier (13,000 years ago) in northern B.C. and Alaska compared to Vancouver Island (10,000 years ago). This means that for 3,000 years, when Vancouver Island was covered by ice, there was a topographical barrier present between the two present day populations. Since the retreat of the glacier, the allele frequencies in the two populations have become separated by genetic drift and the non-migratory nature of harbour seals has prevented a large genetic exchange between the two populations.

While minimal population structure was detected using microsatellites, the allele frequency distribution between northern B.C./Alaska and southern B.C. suggests that the eastern Pacific harbour seal populations are not entirely panmictic. The last glaciation probably resulted in a discontinuity in the distribution of harbour seals. However nuclear markers have a larger effective population size than mtDNA and the

period of isolation may not have been long enough for sufficient drift and mutation to occur. In addition, the large number of harbour seals in the eastern Pacific results in overlapping home ranges and may add to gene flow. These factors combined may be sufficient to prevent detection of high levels of geographic differentiation that may be present. Alternatively, the number of loci and variation in the microsatellites used may have been insufficient to allow detection of population structuring.

Chapter 4 Comparison of Mitochondrial and Nuclear DNA

Both mtDNA and nuclear DNA support the presence of two separate populations or stocks in the eastern Pacific, southern B.C. (Vancouver Island and the adjacent mainland) and northern B.C./Alaska (Fig. 4.1). The mtDNA shows that southern B.C. contains three ancient maternal lineages, one of which appears to have been restricted to a few animals in southern Vancouver Island. However, these three separate lineages in southern B.C. were probably not detected with the microsatellite analysis because of recombination of the microsatellite alleles. Nevertheless, four of five microsatellite loci show significant differences between southern B.C. and northern B.C./Alaska.

Figure 4.1 Distribution of the two harbour seal populations in British Columbia and Alaska based on mtDNA and microsatellite analysis. Area with question marks indicates insufficient samples were collected in that area.

The southern and northern populations appear to have been separated for 0.38 MYA based on the mitochondrial DNA. A similar estimate is difficult to obtain from microsatellite data due to computational difficulties and an associated large variance. However, the microsatellite data suggest an approximate divergence time of 9,000 years between the southern B.C. and northern B.C./Alaska populations.

It appears that harbour seals from the Atlantic invaded the Pacific twice. The first invasion occurred 670,000 years ago and resulted in the colonization of British Columbia and other regions of the north Pacific. Glaciation or some other large scale event severely reduced this group of harbour seals and all that is left is a small group on southern Vancouver Island and in Japan. Following the reopening of the Arctic, a second invasion of harbour seals from the Atlantic recolonized the north Pacific (380,000 years ago). This group first colonized Alaska and Japan and two maternal lineages moved south to colonize southern B.C. This was followed by a second movement of seals from southern B.C. to Washington, Oregon and California. Further glaciation 14,000 to 18,000 years ago probably resulted in some mixing of these groups when animals moved to the refugia on the Brooks Range (western Vancouver Island), Queen Charlotte Island and south of Washington state (Clague 1989). The two populations have remained separated since the last glaciation which ended 10-11,000 years ago in the eastern Pacific (Fig. 4.2).

The dividing line between the northern and southern populations in B.C. occurs somewhere north of Vancouver Island and south of the Queen Charlotte Islands for both the mtDNA and microsatellites. More samples from the Bella Bella region are required to more accurately determine where this division occurs (Fig 4.1). A similar north-south split in populations is observed in sea cucumbers (*C. pseudocurata*) (A. Arndt pers. comm.), chinook salmon (Wilson *et al.* 1987), chum salmon (*O. keta*) (Taylor *et al.* 1994), pink salmon (*O. gorbuscha*) (Varnavsakya and Beacham 1992),

steelhead and rainbow trout (*O. mykiss*) (Okazaki 1984) and sockeye and kokanee salmon (*O. nerka*) (Taylor *et al.* 1996).

Figure 4.2 Position of Cordilleran ice sheet 14,000 (left) and 18,000 (right) years ago (from Fulton 1989).

The two populations found in BC and Alaska have different pupping times (Fig. 1.3). Harbour seals in northern B.C. and Alaska give birth in May and June while those from Vancouver Island give birth in July and August. The possibility that differences in pupping times are the result of genetic isolation raises two questions: Is the difference in pupping time the result of genetic separation or did the difference in pupping time maintain the distinction of the two populations?

LaMont (pers. comm.) found that some Puget Sound harbour seals were genetically distinct from those on the outer coast of Washington, Oregon and California. However, the relationship of those haplotypes from Washington to California to those in my study is not yet known. Analyses of the control sequences from seals in Washington and California published by Stanley *et al.* (1996) suggests that the pupping times do not correspond to genetically distinct populations. Harbour seals from California grouped with those from Vancouver Island as did those from Puget Sound. However, some Puget Sound harbour seals from LaMont (pers. comm.) are distinct from the outer coast of Washington, Oregon and California. Furthermore, the position of the harbour seals from California, as a side branch on the minimum spanning tree (not shown) with the group containing seals only from southern B.C. (group 1a), suggests that a gradient may exist along the coast even though no clearly differentiated maternal lineages are present.

Populations based on pelage pattern differences are not supported by genetic data. Pelage differences suggest that the Queen Charlotte Islands and Glacier Bay are two separate populations, while all the loci examined here indicate that they are one population.

The presence of two separate harbour seal populations in British Columbia may represent separate colonization events and subsequent mixing of the southern B.C.

maternal lineages as a result of the last glaciation 13,000 years ago. This is evident by the multiple maternal lineages present in southern B.C. and the lack of further population division detected by microsatellite loci.

Migration rates for microsatellites (3.0 seals per generation) were higher than those from mtDNA data (0.3 females/generation). The higher migration rate implied by the microsatellite data could be the consequence of the different mutation rates of microsatellites compared to mitochondrial DNA (Scribner *et al.* 1994). On the other hand, the difference may mean that males migrate more than females. Higher rates of male migration are common in many marine species (Palumbi and Baker 1994, Bowen *et al.* 1992, Karl *et al.* 1992). For example, Palumbi and Baker (1994) combined nuclear and mtDNA analysis to determine that male humpback whales were migrating between different areas and that females were exhibiting site fidelity to the summer feeding and winter breeding grounds. Female green sea turtles also show strong homing ability to their natal rookery, yet genetic exchange occurs between rookeries due to male mediated gene flow (Bowen *et al.* 1992, Karl *et al.* 1992).

Satellite telemetry data from a small number of harbour seals has yet to show a significant difference between the movements of males and females. However, it is unlikely that migration rates as low as 0.3 to 3.0 seals per generation could be estimated from telemetry studies unless a large number of animals were monitored over a long period of time.

Migration rate between northern B.C./Alaska and southern B.C. is 0.3 females/generation. This low migration rate should result in strong phylogeographic partitioning for the mtDNA (Slatkin 1987). One reason for the lack of strong partitioning may be due to the last glaciation which ended approximately 11,000 years ago. This last glaciation may have caused a mixing of the pre-glacial populations

resulting in the present day population. Another reason may be the large number of unique haplotypes, such as those found in humpback whales (Baker *et al.* 1993). A combination of glaciation, large population numbers, continuous distribution and male biased migration may prevent the detection of other distinct genetic populations of harbour seals in the eastern Pacific.

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Appendix 1 List of abbreviations and locations of harbour seals used for mtDNA sequencing. If haplotype is given it refers to shared haplotype.

Seal ID	haplotype	Sex	Location
V1	B	M	Victoria, B.C.
V2		F	Victoria, B.C.
V3	A	F	Victoria, B.C.
V4	D	F	Victoria, B.C.
V5		M	Victoria, B.C.
V6	E	F	Victoria, B.C.
V7	G	M	Victoria, B.C.
V8		M	Victoria, B.C.
V9		F	Victoria, B.C.
V11	B	M	Victoria, B.C.
V13	C	F	Victoria, B.C.
V14	A	F	Victoria, B.C.
TB1	A	F	Victoria, B.C.
S1	C	M	Sooke, B.C.
SS1	I	M	Salt Spring Island, B.C.
SS2	B	M	Salt Spring Island, B.C.
SS3	E	F	Salt Spring Island, B.C.
SS4	B	M	Salt Spring Island, B.C.
SS5	G	F	Salt Spring Island, B.C.
SS6	E	F	Salt Spring Island, B.C.
SS7	C	M	Salt Spring Island, B.C.
SS8	I	M	Salt Spring Island, B.C.
SS9		F	Salt Spring Island, B.C.
SS10		M	Salt Spring Island, B.C.
SS11		M	Salt Spring Island, B.C.
SS12	A	F	Salt Spring Island, B.C.
SS13	A	F	Salt Spring Island, B.C.
SS15	G	F	Salt Spring Island, B.C.
Sid1		M	Sidney, B.C.
WVan1		F	Vancouver, B.C.
WVan2	B	F	Vancouver, B.C.
WVan3	J	M	Vancouver, B.C.
R1		M	Vancouver, B.C.
R2	N	M	Vancouver, B.C.
R3	H	F	Vancouver, B.C.
NV1	A	M	Vancouver, B.C.
NV2	A	M	Vancouver, B.C.
NV3	N	F	Vancouver, B.C.
NV4	A	M	Vancouver, B.C.
WR1	D	F	Vancouver, B.C.
WR2	G	M	Vancouver, B.C.
WR3		F	Vancouver, B.C.
WR4		M	Vancouver, B.C.
BB1	I	F	Vancouver, B.C.
BB2	D	F	Vancouver, B.C.
DC1	A	M	Vancouver, B.C.
JB1		F	Vancouver, B.C.
JB2	A	F	Vancouver, B.C.

JB3		M	Vancouver, B.C.
JB4	H	F	Vancouver, B.C.
Bl1	F	F	Vancouver, B.C.
Bl2	F	F	Vancouver, B.C.
Gl1	A	F	Vancouver, B.C.
Gl2	A	M	Vancouver, B.C.
Gl3	N	F	Vancouver, B.C.
PM1	B	F	Vancouver, B.C.
PM2	D	M	Vancouver, B.C.
Il1	A	M	Vancouver, B.C.
Il2	A	F	Vancouver, B.C.
Bol1	A	M	Vancouver, B.C.
Bol2	A	M	Vancouver, B.C.
Bol3	A	F	Vancouver, B.C.
Van1	A	F	Vancouver, B.C.
SQ1		M	Squamish, B.C.
Se1		M	Sechelt, B.C.
Se2		F	Sechelt, B.C.
Gib1	A	M	Gibsons, B.C.
PH1		M	Pender Harbour, B.C.
N1		M	Nanaimo, B.C.
N2		F	Nanaimo, B.C.
N3		F	Nanaimo, B.C.
N4	A	F	Nanaimo, B.C.
N5	K	F	Nanaimo, B.C.
N6	K	M	Nanaimo, B.C.
Sl1	L	M	Nanaimo, B.C.
Sl2	A	M	Nanaimo, B.C.
QB1		F	Qualicum Beach, B.C.
C1		M	Comox, B.C.
C2	A	F	Comox, B.C.
C3	H	M	Comox, B.C.
Sal2		F	Comox, B.C.
CR1	M	F	Campbell River, B.C.
TC2		M	Telegraph Cove, B.C.
TC3		F	Telegraph Cove, B.C.
TC4		F	Telegraph Cove, B.C.
PtH1	D	M	Port Hardy, B.C.
PtH2		F	Port Hardy, B.C.
CH1	N	F	Coal Harbour, B.C.
CH2	J	M	Coal Harbour, B.C.
CH3	F	F	Coal Harbour, B.C.
CH4	N	F	Coal Harbour, B.C.
CH5		M	Coal Harbour, B.C.
CH6		?	Coal Harbour, B.C.
CH7		?	Coal Harbour, B.C.
CH8		?	Coal Harbour, B.C.
CS1		M	Clayoquot Sound, B.C.
CS2		M	Clayoquot Sound, B.C.
CS3		M	Clayoquot Sound, B.C.
CS4	M	M	Clayoquot Sound, B.C.
CS5	C	?	Clayoquot Sound, B.C.

CS8	A	?	Clayoquot Sound, B.C.
CS9		?	Clayoquot Sound, B.C.
CS10		M	Clayoquot Sound, B.C.
BC1		M	Bella Coola, B.C.
BC2	D	M	Bella Coola, B.C.
BC3		M	Bella Coola, B.C.
QC1		M	Queen Charlotte Islands, B.C.
QC2		F	Queen Charlotte Islands, B.C.
QC3		?	Queen Charlotte Islands, B.C.
QC4		?	Queen Charlotte Islands, B.C.
QC5		M	Queen Charlotte Islands, B.C.
PR1		F	Prince Rupert, B.C.
PR2	C	M	Prince Rupert, B.C.
PR3		M	Prince Rupert, B.C.
FI1		?	Forrester Island, AK
FI2		M	Forrester Island, AK
J1		M	Juneau, AK
SE1		M	Vixen Island, AK
SE2		F	Vixen Island, AK
SE3		M	Vixen Island, AK
SE4		M	Vixen Island, AK
SE5		F	Vixen Island, AK
SE6		F	Vixen Island, AK
SE7		M	Vixen Island, AK
SE8		M	Vixen Island, AK
SE9		F	Vixen Island, AK