Small-scale population structure of eastern North Pacific harbour porpoises (*Phocoena phocoena*) indicated by molecular genetic analyses

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ABSTRACT

Concern about the conservation and management of harbour porpoise (*Phocoena phocoena*) populations, which have experienced relatively large incidental fishery kills in localised areas throughout their range, has prompted research to better understand their population structure. Both mitochondrial and nuclear (microsatellites) DNA were used to examine the intra-specific structure of harbour porpoise inhabiting the eastern North Pacific Ocean. Null hypotheses of panmixia were tested after mitochondrial DNA (mtDNA) control region sequence data (402 base pairs; $n = 249$) and allele frequency data (9 polymorphic loci; $n = 194$) were sub-divided into geographic strata defined *a priori*. Strata were based on sampling discontinuities and not discontinuities in population distribution. The mtDNA and nuclear gene data revealed statistically significant genetic differentiation between most strata ($\alpha = 0.05$) suggesting demographic independence of fairly small sub-units within the population. Since harbour porpoises are essentially continuously distributed in the eastern North Pacific, this degree of genetic differentiation was unexpected and needs to be considered in developing a sound management plan to protect them.

KEYWORDS: HARBOUR PORPOISE; CONSERVATION; GENETICS; NORTH PACIFIC; DISTRIBUTION; STOCK IDENTITY

INTRODUCTION

Concern over whether harbour porpoise (*Phocoena phocoena*) populations can sustain the level of observed incidental fisheries mortality has prompted research on their intra-specific population structure. Knowledge of this structure is necessary to properly assess the impact of fisheries mortalities by defining the appropriate regions for which to estimate animal abundance and incidental mortality. Recently published molecular genetic studies of harbour porpoises inhabiting the northwest Atlantic (Rosel *et al.*, 1999), the North and Baltic Seas (Tiedemann *et al.*, 1996; Wang and Berggren, 1997), West Greenland (Andersen *et al.*, 1997) and the seas around the UK (Walton, 1997) detected significant genetic differentiation between sampled strata. The detection of genetic differences between the geographically adjacent sampled areas in each study is quite remarkable, because harbour porpoises appear to be essentially continuously distributed and there are no apparent barriers to movement throughout the habitats they occupy.

Throughout their range, harbour porpoises are vulnerable to coastal gillnet fishing and the eastern North Pacific Ocean population focused on in this study is no exception (Gaskin, 1984; Perrin *et al.*, 1994). Harbour porpoises are locally abundant throughout their distribution, which in the eastern North Pacific extends from Point Conception, California around the North Pacific rim to the northern islands of Japan and as far north as Barrow, Alaska (Leatherwood *et al.*, 1983; Jefferson *et al.*, 1993). Their mortality has been well documented in coastal gillnet fisheries for halibut in California (Hanan *et al.*, 1993; Julian and Beeson, 1998) and in gillnet fisheries for salmon that operate near Spike Rock, Washington and in Puget Sound, Washington (Gearin *et al.*, 1994; 2000; Pierce *et al.*, 1996). However, the incidental take of harbour porpoises in gillnet fisheries operating around Vancouver Island, British Columbia and throughout Alaska is not well known because observer coverage is either lacking or less than 5% of the fishery (Barlow *et al.*, 1995a; Small and DeMaster, 1995). The common characteristic of these gillnet fisheries is that the fishing effort is generally intensive and localised, and if they operate in areas where harbour porpoises occur, the incidental take may be large.

The existing national management plan for the harbour porpoise in the eastern North Pacific Ocean recognises seven management units or stocks: (1) central California; (2) northern California; (3) Oregon/Washington coastal; (4) Washington inland waterways; (5) Southeast Alaska; (6) Gulf of Alaska; and (7) Bering Sea (Barlow *et al.*, 1995a; 1997; 1998; Hill *et al.*, 1996; Hill and DeMaster, 1998; Forney *et al.*, 1999). These management units cover fairly large geographic areas, and because fisheries mortality has been locally high within several of the units, there is concern about whether their scale is biologically appropriate and whether the boundaries are in the right place. Originally, these stocks were designated based on knowledge of the distribution of animals as well as analyses of mitochondrial DNA (mtDNA) control region sequences (Rosel *et al.*, 1995) and contaminant concentrations (Calambokidis and Barlow, 1991). Both of the studies concluded that there was evidence of limited dispersal between the strata represented in their study but that there was probably additional, finer structure within the populations (i.e. areas not yet sampled but inhabited by harbour porpoise). Management goals specified in the US Marine Mammal Protection Act of 1972 and its subsequent amendments for marine mammal populations occupying US territorial waters include, among others, maintaining populations as functional elements of their ecosystem. This has been interpreted to mean that a species' historical distribution and range should be maintained

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(Barlow *et al.*, 1995b). Due to the limited understanding of population structure, whether the current management plan for the eastern North Pacific harbour porpoise population is adequate to meet these goals is uncertain.

This paper extends the existing knowledge by using molecular genetic techniques on a dataset that includes samples collected from additional areas that were not represented in previous studies. Both mitochondrial and nuclear gene markers were used to measure genetic differentiation between strata defined *a priori*. These markers quantify different aspects of gene flow, because they evolve at different rates and the mtDNA marker is maternally inherited, whereas, the nuclear markers are bi-parentally inherited. The maternal mode of inheritance for mtDNA means that the effective population size is approximately a quarter that of nuclear markers, which will result in more rapid differentiation of population sub-units, primarily due to genetic drift, when gene flow is limited (i.e. negligible movement of breeding females). Furthermore, the evolutionary rate of mtDNA makes it useful for reconstructing phylogeographic relationships, and these relationships were examined before performing intra-specific structure analyses to see whether an evolutionary process played an overall role in the population's structure. Analyses of both mitochondrial and nuclear DNA markers reveal patterns of gene flow, which can be used to infer movement and dispersal patterns of the breeding portion of the population studied, and thus provide evidence of intra-specific structure.

MATERIALS AND METHODS

Samples

Samples used in this study were collected along the west coast of the USA and Canada between 1984 and 1998 from animals incidentally taken in fisheries, found stranded on the beach or biopsied at sea (Fig. 1). The samples collected were predominantly skin tissue (92% skin; 8% muscle or internal organ tissue) preserved in a 20% dimethylsulphoxide solution saturated with NaCl (Amos and Hoelzel, 1991; Amos, 1997). All samples are stored in the Southwest Fisheries Science Center's Genetic Tissue Archive (contact author SJC for information).

DNA extraction

Standard molecular protocols were used to extract genomic DNA (Saiki *et al.*, 1988; Palumbi *et al.*, 1991). Extractions of DNA with a CTAB (cetyltrimethylammonium bromide) protocol (Winnepenninckx *et al.*, 1993) were successful for most samples, but when DNA yield was initially low, a phenol-chloroform technique was used for a second extraction (Sambrook *et al.*, 1989). Prior to amplification, the concentration of DNA extracted was determined spectrophotometrically and the purity assessed electrophoretically.

mtDNA amplification and sequencing

The 402 base pair region of the 5′ end of the hypervariable control region of the mtDNA gene was amplified using the polymerase chain reaction (PCR). The following primers were used: L15812 (5'-cctccctaagactcaagg-3') (Southwest Fisheries Science Center Laboratory, unpublished); or L15926 (5'acaccagtcttgtaaacc3'); and H16498 (5'cctgaagtaagaaccagatg3') (Rosel *et al.*, 1994), which are named according to their position in the mtDNA sequence of the fin whale (Árnason *et al.*, 1991). Standard protocols for the PCR were used with 50 µl reactions containing 1 µl (approximately 10-100ng) of genomic DNA, 37.75 µl MilliQ water, 5 µl of buffer (10mM Tris-HC1 (pH 8.3), 50mM KC1, 1.5mM $MgC1_2$), 3 µl of 10mM dNTP, 0.25 µl of *Taq* DNA polymerase, and 1.5 µl of 1 µM of each primer for the amplification. The PCR cycling was done on the *Perkin Elmer* 9600 thermocycler at 90°C for 2.5 min for the initial denaturation, followed by 35 cycles of 94°C for 45 sec, 48°C for 1 min and 72°C for 1.5 min, and a final extension at 72°C for 5 min (Saiki *et al.*, 1988). PCR products were cleaned using purification columns (QIAquick 250®;Qiagen).

Cycle sequencing was done using a profile of 25 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4.0 min on the *Perkin Elmer* 9600 thermocycler. 12 µl sequencing reactions were used containing 3 µl of cleaned PCR product, 3 µl of primer (L15812 and H16498), 2 µl of PRISM® dRhodamine dye terminators (PE Applied Biosystems, Inc.) and 4µl MilliQ water. Both strands of the amplified DNA product of each specimen were sequenced independently as mutual controls using standard protocols on the Applied Biosystems Inc. (ABI) model 373 and 377 automated sequencers with most samples run on the ABI 377. All sequences were aligned by eye using SEQED, version 1.0.3 software (Applied Biosystems Inc., 1992).

Nuclear DNA processing

Nine dinucleotide primers were optimised for harbour porpoise: DlrFCB3, DlrFCB6 (Buchanan *et al.*, 1996), EV1, EV14, EV94, EV104 (Valsecchi and Amos, 1996), SL1026 (L. Garrison, Southwest Fisheries Science Center; pers. comm.), 415/416 and 417/418 (Andersen *et al.*, 1997). Extracted DNA was amplified using the PCR in 25 µl reactions containing 1 µl (approximately 10-100ng) genomic DNA, 18.25 µl water, 2.5 µl of buffer (same as sequencing buffer), 0.75 µl of each primer, 1.5 µl 10mM dNTP and 0.25 µl *Taq* DNA polymerase. All forward primers were labelled with a fluorescent dye. The thermal cycling profile for each locus was an initial 3 min at 97°C, followed by 35 amplification cycles of 30 sec at 90°C, 1 min at the specified annealing temperature for each primer (list follows) and 1 min at 72°C, and a final 5 min period at 72°C ensured extension of the PCR products. The optimal annealing temperature for each primer was 55°C for DlrFCB3, DlrFCB6, EV94 and SL1026, 49°C for EV1 and EV14, 48°C for EV104, and 45°C for 415/416 and 417/418. Size and purity of the amplicon was assessed electrophoretically. Successful amplifications were loaded onto an ABI 377 automatic sequencer for sizing with a commercial internal lane standard (ROX350®; PE Applied Biosystems Inc.). Allele fragment size was determined against a size standard using ABI's GENESCAN, version 3.1 software. The size of the allele is the number of repeat units × 2 plus the size of the flanking region for each base pair, and the size of each allelic pair for each loci constituted the raw data for analyses.

The primers listed above were selected from 22 that were optimised for use on harbour porpoises. For the 14 primers that were optimised, these were screened by plotting the sized alleles to ensure that dinucleotide repeats were amplified for all samples in the dataset and by testing each for the presence of non-amplifying loci, or so-called 'null' alleles. This additional screening was important because none of the primers were developed on the study species.

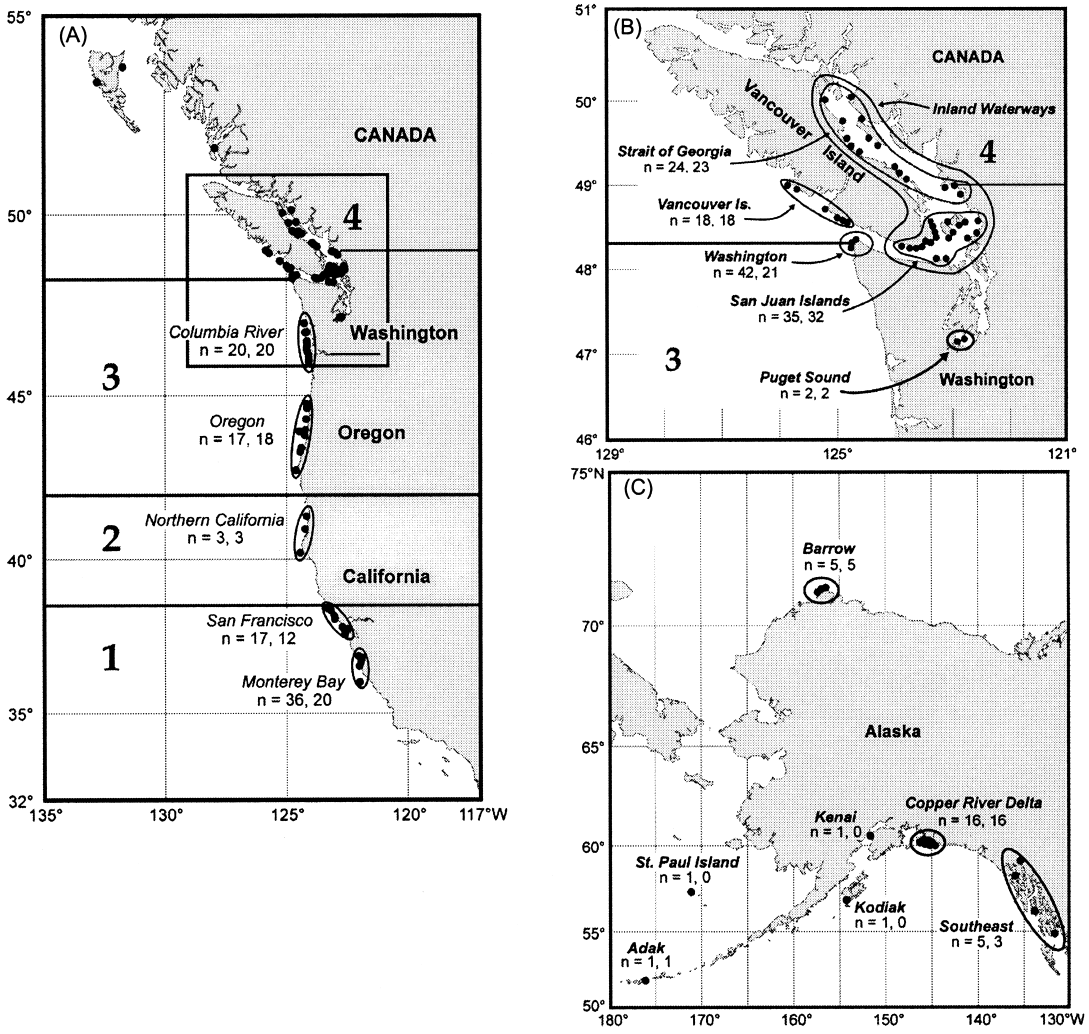

Fig. 1 Geographic locations of harbour porpoise specimens used in the molecular genetics study of population structure are plotted on three maps: (A) the California, Oregon and Washington coastal area; (B) the northern coast of Washington and the inland waterways of Washington and British Columbia, Canada; and (C) Alaska. The region outlined in (A) is expanded in (B) to show the detail of sample collection within that area. The large numbers and bold horizontal lines identify the existing management units and their boundaries: (1) central California; (2) northern California; (3) Oregon/Washington coastal; and (4) Washington inland waterways. Circles around groups of specimens identify each sampling stratum used in the genetic population structure analyses (see Methods for description). The name and sample size for each molecular marker (i.e. n = mtDNA sequence, nuclear DNA microsatellite) is listed next to each stratum. Discrepancies between the sample sizes of the mitochondrial and nuclear DNA datasets listed for a particular locale were due either to the inclusion of control region sequences for the Monterey Bay, California and northern Washington strata used in Rosel *et al.* (1995) for which microsatellite data were not available, or to low yield of DNA extracted from tissue samples of decomposed stranded animals that precluded sizing microsatellite loci.

Five primers were excluded from the dataset, because mutations in the alleles of one or more samples were detected. Null alleles were tested by adjusting annealing temperatures during PCR, because if null alleles are present, a heterozygotic state may appear as a homozygotic one under these conditions (Pemberton *et al.*, 1995; Jarne and Lagoda, 1996). The presence of null alleles was not detected in the nine primers selected for use in this study.

To further screen this dataset prior to analyses, individual relatedness was estimated using *Relatedness*, version 5.0.6 (Goodnight, 2000) and tested for evidence of linkage disequilibrium and deviations from Hardy-Weinberg equilibrium using *Arlequin*, version 2.0 (Schneider *et al.*, 2000). The estimated coefficients of relatedness (e.g. parent-offspring, full siblings) were calculated and used to identify duplicate individuals and first-order relatives in the dataset (Queller and Goodnight, 1989). Two parent-offspring pairs were identified and the offspring of each were removed from the dataset prior to analyses. No evidence of linkage disequilibrium was detected. However, in the tests of Hardy-Weinberg equilibrium, 2 of 72 comparisons (9 loci $\times$ 8 strata) were rejected. These results were not considered indicative of further sub-division within the strata in question, because the observed heterozygosity and allelic diversity was high for both, and furthermore, when making 72 separate tests, 5% would likely be rejected by chance alone.

Genetic variation analyses

For each sampling strata, the genetic variation of the control region was characterised by the number of unique haplotypes present and by estimates of haplotypic and nucleotide diversity (Nei and Tajima, 1981; Nei, 1987). Observed and expected heterozygosity for each locus processed was calculated using the procedure incorporated in Arlequin, version 2.0 (Schneider *et al.*, 2000).

Phylogeographic analyses

Prior to testing hypotheses of intra-specific structure, the control region sequences were examined for evidence of a phylogeographic (i.e. evolutionary) signal. In these analyses, no *a priori* assumptions about intra-specific structure are required, but inferences about phylogeographic patterns are made demonstrating concordance between related haplotypes and their sampling locations. The phylogeographic analyses included control region sequences from an additional 23 specimens from strata not used in the intra-specific structure analyses. For these strata, there were too few specimens to include with the other, more robustly sampled strata. The additional specimens included specimens collected off northern California ($n = 3$); Puget Sound, Washington ($n = 2$); northern British Columbia ($n = 4$); and several, geographically distant locations around Alaska ($n = 14$; Fig. 1). Only unique haplotypes were used and a minimum spanning network was generated with MINSPNET (Excoffier and Smouse, 1994) to construct the phylogeographic relationships. For reference, three haplotypes from Atlantic Ocean harbour porpoise and haplotypes for two sister species (*P. sinus* and *P. spinipinnis*) were included in the dataset. There were two dominant clades apparent in the optimal network, but there was no geographic concordance apparent in the network. Therefore, these results are not presented but are available from the authors.

Genetic population structure analyses

Conventional analyses designed to detect intra-specific structure are based on *a priori* stratification of the samples using non-genetic criteria (e.g. a distributional hiatus or geographic barriers). Therefore, the data were further analysed with *a priori* stratifications that sub-divided the dataset based on the current management scheme or sampling discontinuities. Different schemes were tested, because results from these analyses are fundamentally dependent on decisions about stratification.

The first *a priori* stratification of the dataset recognised the current management units: central California, Oregon/Washington coastal, Washington; and Washington inland waterways (Barlow *et al.*, 1995a; 1997; 1998). The existing northern California management unit was not represented in the analyses, since only three samples were collected from that area although it is known to have high densities of harbour porpoise (Forney, 1999).

The second *a priori* stratification was defined based on geographic sampling discontinuities and resulted in eight fairly fine-scale strata: Monterey Bay, California; San Francisco Bay and Russian River, California; central and southern Oregon; Columbia River, Oregon; northern Washington (Spike Rock); western shore of Vancouver Island, British Columbia; inland waterways of Washington and British Columbia; and Copper River Delta, Alaska (Fig. 1). These strata will be subsequently referred to as: Monterey Bay, San Francisco, Oregon, Columbia River, Washington, Vancouver Island, Inland waterways and Copper River Delta, respectively. The Inland waterways stratum covered a larger geographic range than any other strata in the study, and was therefore split into two: (1) the Strait of Georgia, Canada; and (2) the area south of the Strait of Georgia primarily around the San Juan Islands, Washington and southern tip of Vancouver Island. The null hypothesis of panmixia was tested for these two sub-strata, they were statistically distinguishable (see Results), and therefore, the second *a priori* stratification was modified to include sub-division of the Inland waterways. Thus, nine sampling strata were used in the fine-scale intra-specific analyses and the shorthand names used to reference the strata were: Monterey Bay, San Francisco, Oregon, Columbia River, Washington, Vancouver Island, San Juan Islands, Strait of Georgia and Copper River Delta. The Copper River Delta stratum was included as a 'reference' stratum (Fig. 1c). At the very least, genetic differences were expected between this geographically distant stratum and all others to the south.

Using the mtDNA data, the null hypothesis of panmixia was tested for intra-specific structure using both χ^2 and Φ_{ST}, because each statistic characterises a unique aspect of genetic differentiation. The χ^2 statistic detects differences in haplotype frequencies between strata and makes no assumptions about the evolution or relatedness of haplotypes (Rolf and Bentzen, 1989). On the other hand, Φ_{ST} detects differences in the relatedness of haplotypes between strata. That is, statistically significant Φ_{ST} values mean that haplotypes within a stratum are more closely related (i.e. have a smaller genetic distance or are more genetically homogenous) to each other than to those found in other strata. This statistic uses genetic distance to quantify relatedness and the number of homologous nucleotide differences between two individuals was used as the measure of genetic distance. Φ_{ST} is analogous to the more familiar F-statistic but is modified for pairwise comparisons of genetic distance data and tests significance with a non-parametric permutation method in an analysis of variance framework (AMOVA; Excoffier *et al.*, 1992).

Using the microsatellite data, the same *a priori* stratifications of data established for analyses of the mtDNA marker were tested. F_{ST} (Wright's fixation index; Wright, 1965; Cockerham and Weir, 1993) was the test statistic used in an AMOVA for the microsatellite data (Excoffier *et al.*, 1992). For analyses of both the mtDNA and microsatellite data, AMOVA was used as implemented in Arlequin, version 2.0 (Schneider *et al.*, 2000). For all intra-specific structure analyses, the null hypothesis was panmixia.

The Bonferonni multiple-test correction factor has become fairly routinely applied to results of genetic population structure analyses for cetacean species. However, the application of any correction factor needs to be carefully considered (see Rothman, 1990; Perneger, 1998; Bender and Lange, 1999). For example, one assumption of correction factors is that all null hypotheses are true simultaneously. When applied to genetic population structure analyses, the correction factor is routinely applied to all comparisons regardless of whether they are biologically plausible. In this study, the essentially linear coastal distribution of harbour porpoise means animals most likely move through adjacent (i.e. neighbouring) strata as they move along the coast, and therefore, only comparisons of adjacent strata would likely be biologically plausible, and the only comparisons that would likely be simultaneously true would be those with the Washington stratum, which has three neighbours: Columbia River, Vancouver Island and San Juan Islands. An additional consideration when applying correction factors is that they effectively reduce the critical value (α), or Type I error rate, but at the expense of the Type II error rate. In conservation management applications, reducing the Type I error rate means that one is more willing to commit an under-protection error (i.e. incorrectly pooling strata) than an over-protection error (i.e. incorrectly sub-dividing strata). Because the results of the analyses will likely be applied to management of the harbour porpoise, and the acceptance of

particular Type I and Type II error rates has implications for resource management (Dizon *et al.*, 1995; Taylor *et al.*, 1997), a correction factor was not applied to the analyses and the results were interpreted with $\alpha = 0.05$.

RESULTS

Genetic variation

Analysis of mtDNA data

There were 74 unique haplotypes identified with 88 variable sites among the 249 control region sequences in the dataset (Fig. 2). The overall nucleotide diversity was 0.014 and haplotypic diversity was 0.876. For each sampling strata, nucleotide diversity ranged from 0.0056-0.0243 and haplotypic diversity ranged from 0.377-0.956 (Table 1). Similarly, the smaller dataset used for the intra-specific structure analyses ($n = 225$: 115 males; 77 females; 33 unknown) had an estimated overall nucleotide diversity of 0.018 and haplotypic diversity of 0.876, and 63 of the 74 unique haplotypes identified in the complete dataset were represented (Table 2). As mentioned in the methods, 23 specimens were excluded from the intra-specific structure analyses, because there were too few specimens/locale to adequately represent separate strata that were geographically distant (Fig. 1).

Analysis of nuclear DNA data

All nine microsatellite loci used were polymorphic and the number of alleles per locus ranged from six for DlrFCB-6 to 24 for EV-1. There were 194 specimens in the dataset, of which 180 specimens (107 males; 73 females) were used in the analyses of intra-specific structure. The average observed heterozygosity was > 0.7 for each locus except 415/416 and 417/418, which had average observed heterozygosities of 0.689 and 0.509, respectively (Table 3).

Genetic population structure

Analysis of mtDNA data

For the first *a priori* stratification of data, which was the currently recognised management units: central California, Oregon/Washington coastal and Washington inland waterways, the overall Φ_{ST} was not statistically significant ($\Phi_{ST} = 0.014$, $P = 0.061$). Of the pairwise comparisons using Φ_{ST}, the null hypothesis was marginally rejected for only the central California versus Washington inland waterways comparison ($\Phi_{ST} = 0.034$, $P = 0.046$). However, statistically significant evidence was found of genetic differentiation in χ^2 for comparisons of the central California stratum with: (1) the Oregon/Washington coastal stratum (χ^2 $P < 0.001$); and with (2) Washington inland waterways stratum (χ^2 $P < 0.001$) (Table 4).

When the second *a priori* stratification of data was tested (i.e. Monterey Bay, San Francisco, Oregon, Columbia River, Washington, Vancouver Island, San Juan Islands, Strait of Georgia and Copper River Delta), more evidence of genetic differentiation was found. The overall Φ_{ST} was statistically significant ($\Phi_{ST} = 0.062$, $P < 0.0001$), and of the nearest neighbour comparisons considered most relevant to the question of intra-specific structure in this population, six of the nine were statistically significant using either χ^2 or Φ_{ST}. The comparisons of Columbia River to Washington, Washington to San Juan Islands and Vancouver Island to San Juan Islands were not statistically significant for either χ^2 or Φ_{ST} (Table 5).

Analysis of nuclear DNA data

When the current management units: central California, Oregon/Washington coastal and Washington inland waterways, were tested as population strata using the nuclear markers, the overall F_{ST} was not statistically significant ($F_{ST} = 0.0025$). However, the comparison of central California and Washington inland waterways strata was statistically significant ($F_{ST} = 0.0087$, $P = 0.020$) (Table 6).

When these data were analysed using our second *a priori* stratification (i.e. Monterey Bay, San Francisco, Oregon, Columbia River, Washington, Vancouver Island, San Juan Islands, Strait of Georgia and Copper River Delta), the overall F_{ST} was not statistically significant ($F_{ST} = 0.0075$). Furthermore, none of the nearest neighbour strata comparisons were statistically significant. It was expected that evidence of reproductive isolation for the Copper River Delta stratum would be found because it is geographically distant from all other strata. Significant genetic differences were detected in comparisons of the Copper River Delta to most strata, but it was not significantly distinguishable from the San Francisco, San Juan Islands and Strait of Georgia strata (Table 7). Similarly, significant differences were detected between comparisons of Monterey Bay and the strata most geographically distant to it. The Monterey Bay stratum is the one nearest the southern edge of the range of harbour porpoise in the eastern North Pacific, and therefore might be expected to be genetically distinguishable, if dispersal of breeding males and females is limited within the range.

DISCUSSION

The analyses of the mtDNA control region and nuclear DNA genetic markers of harbour porpoise provide evidence of a genetically sub-divided population organised into surprisingly small geographic units. Although results of analyses of the more broadly drawn first *a priori* stratification of data (i.e. the existing management units) provided evidence of intra-specific genetic distinctness, analyses of the final, most finely drawn *a priori* strata by and large demonstrated that each stratum was likely an isolated unit. Contrasting the results of these two analyses suggest that the current management units are likely composed of sub-units with unique genetic characteristics. In other words, the current management boundaries are drawn too broadly.

This conclusion was based on results from all of these analyses. The lack of statistical significance for the overall AMOVA results for both mtDNA and nuclear markers suggest that the within strata genetic variation is too great to be able to detect between strata differences, thus suggesting that structure exists within the current management units. The results from the second, more finely stratified dataset, were interpreted by considering detection of any significant difference in any genetic measurement between strata to be indicative of genetic differentiation (Table 8). Statistically significant genetic differentiation was detected with the mtDNA marker using either χ^2 or Φ_{ST} for six of our nine nearest neighbour comparisons. At least from a demographic perspective, these results indicate that there has been essentially no dispersal of breeding females between strata. The three comparisons that showed no evidence of genetic differentiation were Columbia River to Washington, Washington to San Juan Islands and San Juan Islands to Vancouver Island. These comparisons were all neighbours of each other, and possible explanations for the lack of evidence for genetic differentiation may be low statistical power or poorly defined strata that do not appropriately

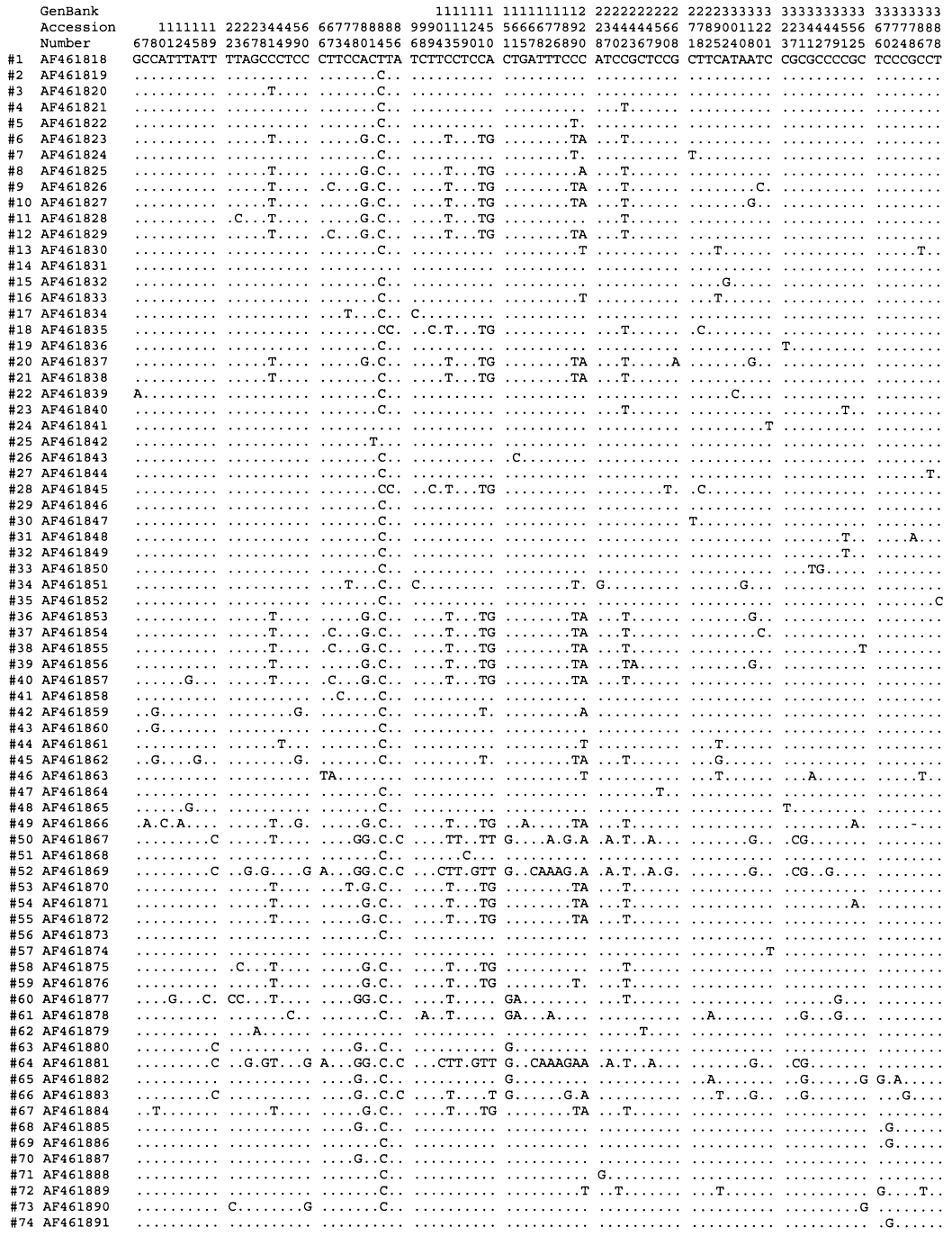

Fig. 2 The variable sites in 74 unique haplotypes identified for eastern North Pacific Ocean harbour porpoise.

Table 1

Sample size, number of haplotypes, haplotypic diversity and nucleotide diversity for each stratum.

Location	Sample size	No. of haplotypes	Haplotypic diversity	Nucleotide diversity
Monterey Bay, California	36	13	0.890	0.0153
San Francisco Bay, California	17	12	0.956	0.0162
Oregon (southern/central)	17	8	0.728	0.0083
Columbia River, Oregon	20	12	0.910	0.0243
Washington (Spike Rock)	42	14	0.892	0.0136
Inland waterways, Washington and British Columbia:				
San Juan Islands (and inland Strait of Juan de Fuca)	35	15	0.924	0.0186
Strait of Georgia, British Columbia	24	5	0.377	0.0061
Vancouver Island, British Columbia (western shore)	18	10	0.810	0.0127
Copper River Delta, Alaska	16	10	0.892	0.0056

Table 2
Frequencies for each unique mitochondrial DNA haplotype identified by sampling strata used in the intra-specific structure analyses.

Haplotype no.	Monterey Bay, CA	San Francisco, CA	Oregon (southern/central)	Columbia River, OR	Washington (Spike Rock)	Vancouver I., BC	San Juan Is, WA	Strait of Georgia, BC	Copper River Delta, AK	Frequency
1	0	0	0	0	0	0	0	0	1	1
2	8	3	9	5	10	8	7	19	5	74
3	0	0	0	0	0	0	0	0	3	3
4	0	0	0	0	3	0	0	0	1	4
5	0	0	0	2	1	0	2	0	1	6
6	5	2	0	1	6	0	3	0	0	17
7	0	2	0	0	0	0	0	0	0	2
8	1	1	0	0	3	0	0	0	0	5
9	1	2	0	0	0	0	0	0	0	3
10	0	1	0	0	1	0	1	0	0	3
11	0	1	1	4	2	1	4	1	0	14
12	4	0	0	0	0	0	0	0	0	4
13	3	0	1	0	3	2	4	0	0	13
14	7	0	0	0	0	0	0	0	0	7
15	1	1	1	1	0	0	0	0	0	4
16	0	0	0	0	0	0	2	1	0	3
17	0	0	0	1	2	1	1	0	0	5
18	0	0	0	1	2	0	0	0	0	3
19	0	0	1	0	1	0	0	0	0	2
20	0	0	0	0	0	0	0	2	0	2
21	0	0	2	0	0	0	1	0	0	3
22	0	0	0	0	0	0	2	0	0	2
23	0	0	0	0	0	0	4	0	0	4
24	0	0	0	0	2	0	0	0	0	2
25	1	0	0	0	0	0	0	0	0	1
26	0	0	0	0	0	0	0	1	0	1
27	1	0	0	0	0	0	0	0	0	1
28	1	0	0	0	0	0	0	0	0	1
29	0	0	0	0	0	0	0	0	1	1
30	0	0	0	0	0	0	0	0	1	1
31	0	0	0	0	0	0	0	0	1	1
32	0	0	0	0	0	0	0	0	0	0
33	0	0	0	1	0	0	0	0	0	1
34	0	0	1	0	0	0	0	0	0	1
35	0	0	0	0	0	0	0	0	0	0
36	0	0	1	0	0	0	0	0	0	1
37	0	0	0	0	0	0	0	0	0	0
38	1	0	0	0	0	0	0	0	0	1
39	1	0	0	0	0	0	0	0	0	1
40	1	0	0	0	0	0	0	0	0	1
41	0	0	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0	0	1	1
44	0	0	0	0	0	0	0	0	0	0
45	0	0	0	1	0	0	0	0	0	1
46	0	0	0	0	0	0	0	0	0	0
47	0	0	0	0	0	0	1	0	0	1
48	0	0	0	0	0	0	1	0	0	1
49	0	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	1	0	0	0	1
51	0	0	0	0	0	1	0	0	0	1
52	0	0	0	0	0	1	0	0	0	1
53	0	0	0	1	0	0	0	0	0	1
54	0	0	0	1	0	0	0	0	0	1
55	0	0	0	0	0	0	1	0	0	1
56	0	0	0	0	0	0	1	0	0	1
57	0	0	0	1	0	0	0	0	0	1
58	0	1	0	0	0	0	0	0	0	1
59	0	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0	0
61	0	0	0	0	0	0	0	0	1	1
62	0	1	0	0	0	0	0	0	0	1
63	0	1	0	0	0	0	0	0	0	1
64	0	1	0	0	0	0	0	0	0	1
65	0	0	0	0	0	1	0	0	0	1
66	0	0	0	0	0	1	0	0	0	1
67	0	0	0	0	0	1	0	0	0	1
68	0	1	0	0	0	0	0	0	0	0
69	0	0	0	0	1	0	0	0	0	1
70	0	0	0	0	1	0	0	0	0	1
71	0	0	0	0	1	0	0	0	0	1
72	0	0	0	0	1	0	0	0	0	1
73	0	0	0	0	1	0	0	0	0	1
74	0	0	0	0	1	0	0	0	0	1
Total	36	17	17	20	42	18	35	24	16	225

Table 3

Genetic diversity of the nuclear DNA dataset: the number of alleles, and the expected and observed heterozygosity of the microsatellite dataset for each locus by stratum.

Locus	No. alleles	Monterey Bay, CA (*n*=20)		San Francisco Bay, CA (*n*=12)		Oregon (south/central) (*n*=18)		Columbia River, OR (*n*=20)		Vancouver Island, BC (*n*=18)		Washington (Spike Rock) (*n*=21)		San Juan Islands, WA (*n*=32)		Strait of Georgia, BC (*n*=23)		Copper River Delta, AK (*n*=16)	
		H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O
DlrFCB-3	19	0.897	0.900	0.964	0.833	0.941	0.833	0.914	0.800	0.935	0.889	0.920	0.952	0.919	0.812	0.921	0.826	0.946	0.938
DlrFCB-6	6	0.788	0.850	0.862	0.917	0.776	0.833	0.796	0.800	0.752	0.667	0.816	0.762	0.772	0.844	0.751	0.783	0.758	0.812
EV-1	24	0.931	0.900	0.913	0.833	0.903	0.833	0.913	0.950	0.905	0.833	0.943	0.857	0.911	0.844	0.860	0.826	0.833	0.812
EV-14	8	0.622	0.600	0.750	0.833	0.611	0.667	0.833	0.950	0.763	0.833	0.700	0.714	0.743	0.688	0.735	0.739	0.744	0.750
EV-94	8	0.792	0.600	0.808	0.917	0.802	0.667	0.782	0.850	0.765	0.722	0.715	0.857	0.730	0.812	0.693	0.739	0.822	0.938
EV-104	13	0.873	0.850	0.793	0.917	0.849	0.722	0.883	1.000	0.859	0.889	0.908	0.857	0.905	0.906	0.908	0.870	0.879	0.812
SL10-26	16	0.865	0.750	0.840	0.833	0.790	0.889	0.838	0.900	0.914	0.889	0.915	0.857	0.891	0.938	0.857	0.913	0.897	0.812
415/416	7	0.740	0.850	0.627	0.667	0.697	0.778	0.660	0.600	0.808	0.611	0.653	0.714	0.653	0.812	0.638	0.609	0.605	0.562
417/418	7	0.654	0.550	0.475	0.417	0.527	0.500	0.533	0.450	0.522	0.500	0.645	0.428	0.496	0.531	0.501	0.391	0.613	0.812

Table 4

Results of intra-specific structure analyses using the currently recognised management units using the mitochondrial DNA control region sequences. In the upper diagonal are the Monte Carlo *P*-values for the comparison of test strata using χ^2, and in the lower diagonal are the Φ_{ST} test statistics with the corresponding *P*-value written in parentheses underneath. All text in cells with *P*-values $\leq$ 0.05 are printed in **bold** text and *P*-values between 0.05 and 0.10 are printed in *italics*.

	1. Central California (*n*=53)	2. Oregon/ Washington coastal (*n*=79)	3. Washington inland waterways (*n*=35)
1. Central California	-	**<0.001**	**<0.001**
2. Oregon/Washington coastal	*0.019 (0.063)*	-	**0.050**
3. Washington inland waterways	**0.034 (0.046)**	-0.002 (0.429)	-

reflect population distribution. Additional samples will be needed to resolve population structure in this region. Using the nuclear DNA markers, significant differences were detected only when the more distant strata were compared. These results are more difficult to interpret, because the effective population size is four times greater than that for mtDNA, but they suggest that breeding males move greater distances than breeding females within the study area. If this were true, even stronger evidence of genetic differentiation would be expected between strata using only females in the mtDNA dataset. However, the sample sizes for all *a priori* strata were too small to perform meaningful analyses using females only, because the dataset was approximately 60% male.

The rationale for combining evidence from the two markers: mtDNA and nuclear DNA, and from both statistics used to analyse the mtDNA data: χ^2 and Φ_{ST}, is that this approach is analogous to combining evidence from several disparate datasets, for example, morphological, contaminant and genetic studies. This type of approach has been applied in studies of intra-specific structure as a means to make an inference about animal movement patterns based on a preponderance of evidence (Dizon *et al.*, 1992). In such cases, you would not necessarily expect or demand each contrast to be significant for each criterion, but a significant finding in any marker provides relevant information. In this study, each genetic marker and statistic provides a different measure of genetic distinctness. The mtDNA marker provides information about the relative movement of breeding females, because it is maternally inherited, and the two statistics used provide information: (1) about the relative frequencies of haplotypes, which are expected to be different due to the complicated interplay of dispersal (albeit low) and genetic drift and would be detected by χ^2; and (2) about the relatedness, or evolution, of haplotypes, which change due to drift and mutation and would be detected by Φ_{ST} when there is essentially no gene flow between groups. On the other hand, nuclear markers are bi-parentally inherited, and therefore, provide information about the relative movements of both breeding males and females. When statistically significant differences are detected, they are interpreted as evidence that there is essentially no dispersal of males and females and that the strata are likely reproductively isolated.

The primary advantage of analysing molecular genetic data for evidence of population structure is that the data directly reflect gene flow and not transient, non-breeding interchange. The disadvantage is that the statistical power of the tests used to detect differences is inherently low (Dizon *et al.*, 1995; Taylor *et al*., 1997), and results of several studies (e.g. Slatkin, 1985; 1987; Slatkin and Barton, 1989; Hudson *et al.*, 1992) indicate that only near zero dispersal rates are reliably detected (i.e. approximately one migrant/generation). Therefore, when statistically significant differences in genetic variation are found between hypothesised population sub-units, the sub-units should be recognised as demographically distinct and managed separately. Even demographically insignificant amounts of dispersal will eliminate detectable genetic differences (Mills and Allendorf, 1996). While the interpretation of statistical significance is that there is effectively no movement of animals between strata compared (i.e. the dispersal rate was only a couple of animals per generation), the interpretation of negative results (i.e. no statistically significant differences) remains problematic. If an analysis fails to reject the null hypothesis of panmixia, the effective dispersal rate between population sub-units may still be several percent per year (e.g. 1-3%). At such low dispersal rates, population sub-units may be sufficiently isolated to warrant independent management, because the movement of animals between sub-units would be unlikely to compensate for anthropogenic mortality that exceeds the dispersal rate in at least one sub-unit (Taylor, 1997).

Estimating statistical power for genetic analyses is not straightforward and requires simulation modelling (Taylor *et al*., 1997). However, the role of statistical power in these

Table 5

Results of the fine-scale intra-specific structure analyses using Φ_{ST} and χ^2 for the mitochondrial DNA dataset. In the upper diagonal are the Monte Carlo *P*-values for the comparison of test strata using χ^2, and in the lower diagonal are the Φ_{ST} test statistics with the corresponding *P*-value written in parentheses underneath. All text in cells with *P*-values $\leq$ 0.05 are printed in **bold** text and *P*-values between 0.05 and 0.10 are printed in *italics*. Comparisons of nearest neighbours are on the off diagonal in bordered cells.

	1. Monterey Bay, CA	2. San Francisco, CA	3. Oregon (south/central)	4. Columbia River, OR	5. Washington (Spike Rock)	6. Vancouver Island, BC	7. San Juan Islands, WA	8. Strait of Georgia, BC	9. Copper River Delta, AK
	n=36	*n*=17	*n*=17	*n*=20	*n*=42	*n*=18	*n*=35	*n*=24	*n*=16
1. Monterey Bay, CA	-	**0.027**	**0.012**	**0.001**	**0.001**	**0.012**	**0.001**	**0.000**	**0.004**
2. San Francisco, CA	-0.016 (0.545)	-	*0.066*	0.258	*0.089*	*0.062*	**0.033**	**0.000**	*0.077*
3. Oregon (south\central)	**0.110 (0.023)**	**0.109 (0.036)**	-	0.158	0.118	0.806	*0.084*	*0.055*	*0.083*
4. Columbia River, OR	0.021 (0.154)	0.0006 (0.365)	**0.073 (0.043)**	-	0.164	0.176	0.259	**0.000**	0.129
5. Washington (Spike Rock)	0.005 (0.254)	-0.002 (0.372)	*0.067 (0.054)*	0.001 (0.334)	-	0.226	*0.055*	**0.000**	**0.048**
6. Vancouver I., BC	**0.132 (0.007)**	**0.126 (0.016)**	-0.012 (0.542)	**0.077 (0.018)**	**0.096 (0.013)**	-	0.144	**0.026**	*0.099*
7. San Juan Islands, WA	*0.033 (0.065)*	0.020 (0.156)	0.013 (0.224)	0.002 (0.352)	0.006 (0.249)	0.018 (0.158)	-	**0.001**	**0.012**
8. Strait of Georgia, BC	**0.157 (0.004)**	**0.170 (0.009)**	-0.027 (0.688)	**0.117 (0.006)**	**0.110 (0.010)**	-0.0002 (0.340)	**0.044 (0.049)**	-	**0.000**
9. Copper River Delta, AK	**0.189 (0.003)**	**0.211 (0.002)**	-0.002 (0.357)	**0.136 (0.002)**	**0.148 (0.003)**	0.019 (0.151)	**0.071 (0.016)**	-0.005 (0.442)	-

Table 6

Results for comparisons of the currently recognised management units using nuclear DNA. In the lower diagonal of this table are the F_{ST} test statistics with the corresponding *P*-value written in parentheses underneath. All text in cells with *P*-values $\leq$ 0.05 are printed in bold text.

	1. Central California (*n*=32)	2. Oregon/ Washington coastal (*n*=59)	3. Washington inland waterways (*n*=32)
1. Central California	-		
2. Oregon/Washington coastal	0.0012 (0.296)	-	
3. Washington inland waterways	**0.0087 (0.020)**	0.0003 (0.398)	-

analyses can be partially illustrated by comparing the number of statistically significant comparisons to sample size by stratum. The expectation is that the number of significant comparisons would increase with increasing sample size. In general, this is what is observed, but there are three notable exceptions: Monterey Bay, Strait of Georgia and Copper River Delta (Table 9). Both Monterey Bay and Copper River Delta were statistically distinguishable from all other strata in at least one analysis, even though Monterey Bay had the second largest mtDNA sample size and Copper River Delta had the smallest sample size (Table 8). In part, the genetic distinctness of these strata may be due to their location within the study area. Monterey Bay is at the southern extreme of the study area, while Copper River Delta is at the northern extreme. The Strait of Georgia stratum was statistically distinguishable in at least one analysis from all other strata except Oregon. The uniqueness of this stratum is likely due to the low haplotypic diversity observed (Table 1). Although a lack of statistical power due to relatively small sample sizes may help explain some of the inconsistencies in the results (i.e. not all comparisons were statistically significant when the nearest neighbour comparisons were significant), the discrepancies observed in the resolution of genetic population structure, particularly for the Oregon, Columbia River, Washington and San Juan Islands strata, likely have multiple explanations. For example, the comparisons of *a priori* strata, which were defined by sampling discontinuities, may not reflect the distribution of the population. Additional sampling in nearby areas together with a better understanding of regional distribution and seasonal movement patterns of the population will be needed to determine the influence of the *a priori* stratification in analyses. Additionally, even though χ^2 had been demonstrated to be a more powerful statistic than Φ_{ST} (Hudson *et al.*, 1992; Taylor and Chivers, 2000), this statistic did not appear to perform particularly well in the analyses (Table 9). Briefly, it is noted that sample size is not the only determinant of statistical power but that the number of unique haplotypes and observed haplotypic diversity also play a role. The performance of χ^2 observed most likely indicates that the sample sizes were likely too small for the relatively high haplotypic diversity/stratum (Table 1).

One of the goals of this study was to apply the results to management of the eastern North Pacific harbour porpoise population. However, achieving that goal remains elusive, because the samples were collected from discrete locales within the population's range, which do not necessarily correspond to the regional distribution of the population. In fact, some areas inhabited by large numbers of harbour porpoise were essentially un-sampled (e.g. northern California; Fig. 1a). The samples used were collected opportunistically in areas where people actively participate in stranding networks and where fishery observer programmes operate, and thus resulted in discrete geographic sampling. The discreteness of sampled areas ultimately dictated the *a priori* stratification of data for

Table 7

Results of the intra-specific population structure analyses using the nuclear DNA data. In the lower diagonal are the F_{ST} test statistics with the corresponding *P*-value written in parentheses underneath. All text in cells with *P*-values $\leq$ 0.05 are printed in bold text and *P*-values between 0.05 and 0.10 are printed in *italics*. Results for comparisons of nearest neighbor strata are in bordered cells.

	1. Monterey Bay, CA	2. San Francisco, CA	3. Oregon (south/central)	4. Columbia River, OR	5. Washington (Spike Rock)	6. Vancouver Island, BC	7. San Juan Islands, WA	8. Strait of Georgia, BC	9. Copper River Delta, AK
	n=20	*n*=12	*n*=18	*n*=20	*n*=21	*n*=18	*n*=32	*n*=23	*n*=16
1. Monterey Bay, CA	-								
2. San Francisco, CA	0.0030 (0.296)	-							
3. Oregon (south/central)	-0.0068 (0.884)	-0.0058 (0.761)	-						
4. Columbia River, OR	*0.0094* *(0.064)*	-0.0010 (0.504)	0.0001 (0.436)	-					
5. Washington (Spike Rock)	**0.0157** **(0.008)**	0.0072 (0.156)	**0.0142** **(0.019)**	*0.0084* *(0.075)*	-				
6. Vancouver I., BC	*0.0083* *(0.078)*	0.0077 (0.148)	-0.0004 (0.467)	-0.0010 (0.536)	*0.0081* *(0.084)*	-			
7. San Juan Islands, WA	**0.0108** **(0.021)**	0.0072 (0.138)	*0.0067* *(0.094)*	0.0002 (0.437)	0.0028 (0.243)	0.0055 (0.127)	-		
8. Strait of Georgia, BC	**0.0189** **(0.001)**	**0.0193** **(0.012)**	*0.0089* *(0.079)*	*0.0085* *(0.069)*	**0.0159** **(0.008)**	*0.0080* *(0.089)*	0.0040 (0.174)	-	
9. Copper River Delta, AK	**0.0111** **(0.048)**	*0.0126* *(0.070)*	**0.0140** **(0.026)**	**0.0129** **(0.037)**	**0.0107** **(0.050)**	**0.0180** **(0.005)**	0.0050 (0.181)	0.0059 (0.159)	-

Table 8

Combined results from all intra-specific structure analyses using mitochondrial (mtDNA) and nuclear DNA markers are presented in this table. The symbol for each test statistic: χ^2 and Φ_{ST} for mtDNA and F_{ST} for the nuclear DNA markers is printed in the cells for comparisons with $P \leq 0.05$. A "♦" denotes comparisons of sampling strata with no evidence of genetic differentiation. Nearest neighbour strata comparisons are the bordered cells.

	1. Monterey Bay, CA	2. San Francisco, CA	3. Oregon (south/central)	4. Columbia River, OR	5. Washington (Spike Rock)	6. Vancouver Island, BC	7. San Juan Islands, WA	8. Strait of Georgia, BC	9. Copper River Delta, AK
mtDNA	*n* = 36	*n* = 17	*n* = 17	*n* = 20	*n* = 42	*n* = 18	*n* = 35	*n* = 24	*n* = 16
Nuclear DNA	*n* = 20	*n* = 12	*n* = 18	*n* = 20	*n* = 21	*n* = 18	*n* = 32	*n* = 23	*n* = 16
1. Monterey Bay, CA	-								
2. San Francisco, CA	χ^2	-							
3. Oregon (south/central)	χ^2 Φ_{ST}	Φ_{ST}	-						
4. Columbia River, OR	χ^2	♦	Φ_{ST}	-					
5. Washington (Spike Rock)	χ^2 F_{ST}	♦	F_{ST}	♦	-				
6. Vancouver I., BC	χ^2 Φ_{ST}	Φ_{ST}	♦	Φ_{ST}	Φ_{ST}	-			
7. San Juan Islands, WA	χ^2 F_{ST}	χ^2	♦	♦	♦	♦	-		
8. Strait of Georgia, BC	χ^2 Φ_{ST} F_{ST}	χ^2 Φ_{ST} F_{ST}	♦	χ^2 Φ_{ST}	χ^2 Φ_{ST} F_{ST}	χ^2	χ^2 Φ_{ST}	-	
9. Copper River Delta, AK	χ^2 Φ_{ST} F_{ST}	Φ_{ST}	F_{ST}	Φ_{ST} F_{ST}	χ^2 Φ_{ST}	F_{ST}	χ^2 Φ_{ST}	χ^2	-

analyses, and thus, limits the conclusions regarding the true underlying intra-specific structure of the population and precludes precise placement of population sub-unit boundaries. However, dependence on opportunistic sampling means that it will be quite some time before there are sufficiently more samples to analyse and to refine existing knowledge.

Detecting genetic differences between neighbouring strata in these analyses was striking because harbour porpoises appear to be essentially continuously distributed in the

Table 9

Sample size and number of statistically significant ($P \leq 0.05$) comparisons detected by each statistic by stratum. The sampling strata are listed in order of decreasing sample size. The maximum number of significant comparisons possible was 24 or 8 per statistic.

Location	Sample size (mtDNA/ nuclear)	χ^2	Φ_{ST}	F_{ST}	Total no. significant comparisons
Washington (Spike Rock)	42/21	3	3	3	9
Monterey Bay, CA	36/20	8	4	4	16
Inland waterways, WA (Strait of Juan de Fuca and Juan Is)	35/32	4	2	1	7
Strait of Georgia, BC	24/23	7	5	3	15
Columbia River, OR	20/20	2	4	1	7
Vancouver I., BC (western shore)	18/18	2	4	1	7
San Francisco Bay, CA	17/12	3	4	1	8
Oregon (southern/central)	17/18	1	3	2	6
Copper River Delta, AK	16/16	4	5	4	13

eastern North Pacific Ocean and have high haplotypic diversity. Failure to find significant differences between all neighbouring strata in a single analysis should not be considered contradictory because the low statistical power of genetic analyses makes them sensitive to insufficient sample sizes. It is acknowledged that there are no obvious geographic barriers to restrict movement of animals in this population, and that knowledge about habitat preferences, movement patterns and seasonal distributions in the eastern North Pacific harbour porpoise is limited, but required, before sub-unit boundaries can be identified. However, the results of this study indicate that this population is likely highly stratified and that smaller management units would better preserve the population's intra-specific structure.

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