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Population structure of nuclear and mitochondrial DNA variation among humpback whales in the North Pacific

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Abstract

The population structure of variation in a nuclear actin intron and the control region of mitochondrial DNA is described for humpback whales from eight regions in the North Pacific Ocean: central California, Baja Peninsula, nearshore Mexico (Bahia Banderas), offshore Mexico (Socorro Island), southeastern Alaska, central Alaska (Prince Williams Sound), Hawaii and Japan (Ogasawara Islands). Primary mtDNA haplotypes and intron alleles were identified using selected restriction fragment length polymorphisms of target sequences amplified by the polymerase chain reaction (PCR–RFLP). There was little evidence of heterogeneity in the frequencies of mtDNA haplotypes or actin intron alleles due to the year or sex composition of the sample. However, frequencies of four mtDNA haplotypes showed marked regional differences in their distributions (Φ_{ST} = 0.277; P < 0.001; n = 205 individuals) while the two alleles showed significant, but less marked, regional differences (Φ_{ST} = 0.033; P < 0.013; n = 400 chromosomes). An hierarchical analysis of variance in frequencies of haplotypes and alleles supported the grouping of six regions into a central and eastern stock with further partitioning of variance among regions within stocks for haplotypes but not for alleles. Based on available genetic and demographic evidence, the southeastern Alaska and central California feeding grounds were selected for additional analyses of nuclear differentiation using allelic variation at four microsatellite loci. All four loci showed significant differences in allele frequencies (overall F_{ST} = 0.043; P < 0.001; average n = 139 chromosomes per locus), indicating at least partial reproductive isolation between the two regions as well as the segregation of mtDNA lineages. Although the two feeding grounds were not panmictic for nuclear or mitochondrial loci, estimates of long-term migration rates suggested that male-mediated gene flow was several-fold greater than female gene flow. These results include and extend the range and sample size of previously published work, providing additional evidence for the significance of genetic management units within oceanic populations of humpback whales.

Keywords: control region, intron, microsatellite, management, stocks, gene flow

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Introduction

For species with sex-biased dispersal or philopatry, the population structure of genetic variation can be different for the maternally inherited mitochondrial genome and the biparentally inherited nuclear genome (e.g. Karl *et al*. 1992). The contrasting structure of this genetic variation can, in turn, reveal the complexity of mating systems and one-way or return dispersal in species where demographic evidence is difficult to obtain. For endangered or commercially exploited species, this complexity must be considered when developing strategies for protecting habitat or in setting quotas for hunting or incidental mortality (Bowen & Avise 1995). For species without complex patterns of sex-biased dispersal or philopatry, the two unlinked genomes provide independent evidence for defining genetic management units or evolutionarily significant units (Moritz 1994). Although mtDNA can be particularly powerful for describing historical demographic processes (Avise 1994), some management strategies recognize, explicitly or implicitly, reproductive isolation as a requirement for defining population subdivisions (e.g. the International Whaling Commission, Donovan 1991; and the US Endangered Species Act, Waples 1991).

Humpback whales in the North Pacific Ocean provide an example of a population with a complex seasonal and geographical structure (Baker 1985). These whales feed during spring, summer and autumn in temperate and near-polar water along the rim of the North Pacific. During winter months, they migrate to near-tropical waters to mate and give birth. Although hunting has reduced and may have altered the historical range of humpback whales, primary winter concentrations are now found in three areas (NMFS 1991): the Pacific coast and offshore islands of Mexico; the main or leeward islands of Hawaii; and the Ogasawara (Bonin) and Ryukyu Islands near Japan. The timing of migratory arrival in the wintering grounds is stratified by age–sex class (Nishiwaki 1959; Medrano *et al*. 1994) and the migratory cycle of individual whales may be influenced by other factors, including reproductive status (Brown *et al*. 1995).

Observations of naturally marked individuals indicate strong fidelity to specific feeding grounds and consistent migratory return between these and some winter mating and calving grounds (e.g. Darling & McSweeney 1985; Baker *et al*. 1986). Whales found along the coast of Alaska are generally thought to winter near Hawaii while those found along the coast of California, Oregon and Washington are thought to winter primarily in the coastal waters of the Mexican Pacific (Calambokidis *et al*. 1996). Movement between Mexico and Hawaii and between Hawaii and the Ogasawara Islands has been documented (Darling & McSweeney 1985; Baker *et al*. 1986; Darling & Mori 1993), but the relative or absolute rate of this interchange is unknown. Although similarities in the song of humpback whales from Mexico, Hawaii and Japan suggest acoustic contact between whales migrating to these wintering grounds (Winn *et al*. 1981; Payne & Guinee 1983; Helweg *et al*. 1990), the degree of contact required to exchange songs is unknown.

The distribution of mitochondrial (mt) DNA haplotypes shows a strong influence of maternally directed fidelity to migratory destinations, probably as a result of experience during a calf's first year of life (Baker *et al*. 1990; Baker *et al*. 1994). A complete segregation of maternal lineages is found between the Californian and southeastern Alaskan feeding grounds, confirming results from photographic comparisons. Differences between the Hawaiian and Mexican wintering grounds are also significant (Medrano-Gonzalez *et al*. 1995), although less marked, as are differences between the nearshore Mexican wintering grounds (the Baja Peninsula and Bahia Banderas) and the offshore Revillagigedo Islands (Socorro Island). A strong migratory connection between southeastern Alaska and Hawaii is indicated by a similarity in haplotype frequencies in these two regions. However, frequencies on the Mexican wintering grounds are intermediate between those of the southeastern Alaska and California feeding grounds.

The analysis of variation in nuclear markers has shown less distinct patterns of geographical structure in North Pacific humpback whales, as a result of either preferential dispersal of males between breeding grounds or the larger effective population size of nuclear genes (Palumbi & Baker 1996). DNA fingerprints showed differentiation between the North Pacific and North Atlantic populations and a clinal pattern of variation within the North Pacific but the latter effect was not significant (Baker *et al*. 1993). Variation in sequences of an intron from the nuclear actin gene defined two distinct clades of alleles differing in frequencies between oceanic populations but not between two regions (California and Hawaii) in the North Pacific (Palumbi & Baker 1994). However, in both studies of nuclear variation, sample sizes have been small and the geographical range of sampling has been less extensive than for mtDNA.

A similar pattern of reduced, or less consistent, geographical structure in nuclear variation is reported in other oceanic populations of humpback whales based on the analysis of microsatellite loci. Feeding grounds in the central and eastern North Atlantic differ significantly in frequencies of mtDNA haplotypes (Palsboll *et al*. 1995) but not microsatellite alleles, suggesting oceanic panmixis as a result of interbreeding on a common wintering ground (Larsen *et al*. 1996). Feeding grounds in the western and central North Atlantic, however, show significant differences in allele frequencies at several microsatellite loci, suggesting that individuals from these regions do not interbreed freely despite the absence of geographical

barriers (Valsecchi *et al*. 1997). On a global scale, microsatellite variation provides inconsistent estimates of genetic distances within and between oceanic populations (Valsecchi *et al*. 1997), perhaps as a result of inappropriate assumptions about models and rates of mutations at these loci.

Here, we describe the population structure of variation in both mtDNA and the intron of the nuclear actin gene among humpback whales from eight seasonal habitats in the North Pacific. We used previously published sequences of the mitochondrial control region and the actin intron to select restriction sites that reflected the phylogenetic relationship of common haplotypes and alleles (Amato & Gatesy 1994). Our assays of genetic variation include a larger geographical range and number of samples than previously reported, allowing us to test the question of reproductive isolation between components of this populations and to evaluate potential heterogeneity due to the sex composition or annual collection of regional samples. Based on these results, the southeastern Alaska and central California feeding grounds were selected for additional analyses of reproductive isolation using allelic variation at four microsatellite loci. These results include and extend the range and sample size of previously published work, providing additional evidence for the complexity of male and female gene flow and the significance of genetic management units within oceanic populations of humpback whales.

Materials and methods

Sample collection, DNA extraction and sex identification

Samples for genetic analysis were available from 205 individual humpback whales representing eight regional habitats within the North Pacific, including three feeding grounds and five wintering grounds (Fig. 1; Table 1). In most cases, skin tissue was collected from free-ranging whales using a biopsy dart described in detail by Lambertsen (1987), with slight modifications (Weinrich *et al*. 1991; Baker *et al*. 1994). In the Ogasawara Islands, skin tissue was collected as it sloughed naturally from whales during activities such as aerial behaviour or competitive interactions (Baker *et al*. 1991; Clapham *et al*. 1993). Tissue samples were stored in 70% ethanol or a solution of 5 M NaCl and 20% DMSO while in the field and placed at – 80 °C for long-term storage. Total cellular DNA was extracted from skin tissue as described previously (Baker *et al*. 1990, 1991). Molecular genetic identification of the sex of humpback whales followed the Southern hybridization method described by Baker *et al*. (1991) and the PCR-based method described by Palsboll *et al*. (1992).

PCR and RFLP of mtDNA and actin intron

Published sequences of mitochondrial DNA control region from 91 humpback whales (Baker *et al*. 1993; Medrano-Gonzalez *et al*. 1995) were inspected for restriction enzyme sites that could distinguish the most common haplotypes or maternal lineages in the North Pacific Ocean. As described previously, 15 variable sites in the first 317 bp of the control region defined nine haplotypes among these 91 whales. Phylogenetic reconstructions showed that the nine haplotypes form two distantly related clades. The 'CD' clade included two closely related types, 'F1' and 'F2', only one of which was common. The 'AE' clade included seven closely related haplotypes, only three of which were common.

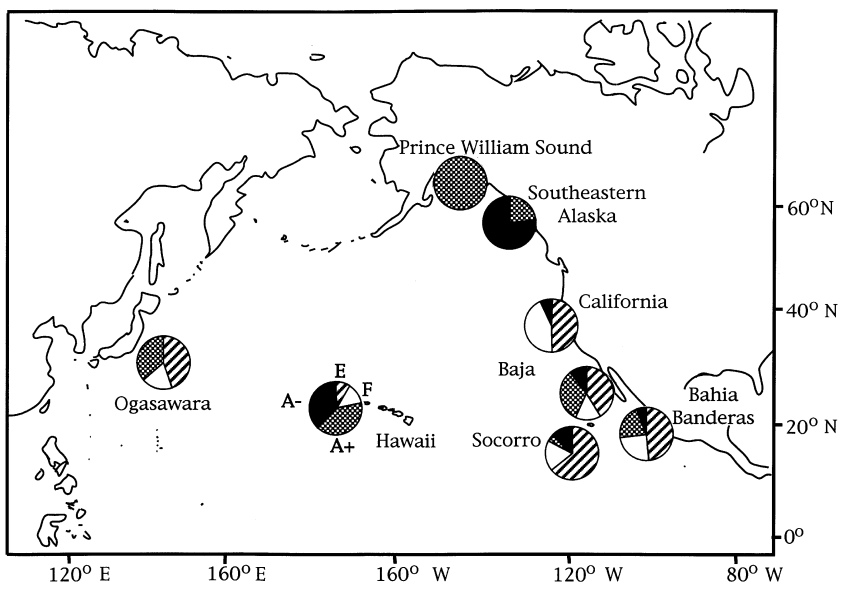

Fig. 1 The sampled feeding and wintering grounds of humpback whales in the North Pacific and the frequencies of the four mtDNA haplotypes (A+, A–, E and F frequencies shaded as noted in the Hawaiian diagram).

Table 1 Summary of tests for heterogeneity in mtDNA haploptypes and nuclear intron alleles due to the year and sex composition of regional samples of humpback whales in the North Pacific

				Yearly sampling		Sex composition	
Region	Habitat	*n* = mtDNA	*n* = alleles	Years	Heterogeneity mtDNA/allele	males: heterogeneity females: mtDNA/allele	
Bahia Banderas	Wintering	21	42	90–92	ns/ns	10:9	ns/ns
Baja Peninsula	Wintering	21	42	91	na/na	18:3**	ns/ns
Socorro Island	Wintering	23	46	91, 92	ns/*	16:5*	ns/ns
California	Feeding	57	108	88, 91	ns/ns	29:20	ns/ns
Southeastern Alaska	Feeding	39	76	87, 88	ns/ns	19:15	*/ns
Prince William Sound	Feeding	6	12	94	na/na	2:4	ns/ns
Hawaii	Wintering	27	54	89, 92	ns/ns	17:8	ns/ns
Ogasawara Islands	Wintering	11	20	91	na/na	10:0**	na/na
Total		205	400			121:64**	

*, $P < 0.05$; **, $P < 0.01$; ns, not significant at the 0.05 level of probability; na, not applicable.

Within the amplified fragment of the control region, three restriction enzymes distinguished the two clades and the three common haplotypes within the 'AE' clade (Fig. 2). The 'AE' clade can be distinguished from the 'CD' clade by the loss of a *Nco*I site at position 136 and a *Dra*I site at position 274. Following the previous convention, individuals of the 'CD' clade in the North Pacific were referred to as 'F' types. Within the 'AE' clade, the 'E*' and 'A*' types were distinguished by a *Dra*I site at position 270. Within the 'A*' types, the 'A–' and 'A+' haplotypes were distinguished by a *Sau*96I site at position 028. The 'A' and 'E' haplotypes identified previously by RFLP analysis of the whole mtDNA genome (Baker *et al*. 1990; Baker *et al*. 1994) were consistent with this PCR–RFLP assay. An uncommon haplotype, referred to previously as the 'AE' type, shared the loss of the *Nco*I site with the 'CD' clade but could be recognized by the presence of the *Dra*I site at position 270 and 274 and by a *Sau*96I fragment pattern characteristic of the 'E*' types. The 'AE' type individuals were grouped with the 'A+' types for statistical analysis. The uncommon 'H' and 'G' types, identified previously by genomic RFLP analysis, were not distinguishable from the 'E*' types by the PCR–RFLP assay.

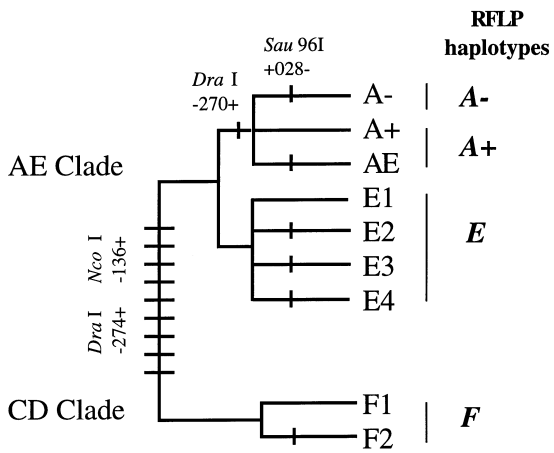

Fig. 2 The position of nucleotide substitutions resulting in a loss or gain of a restriction site defining the four common haplotypes within the amplified fragment of the humpback whale mtDNA control region (adapted from Medrano-Gonzalez *et al*. 1995). The orientation of the loss (–) or gain (+) is shown adjacent to the nucleotide postion for each site.

Where the haplotype identity of whales could not be inferred from previously published genomic RFLP or control region sequences, a 550 bp fragment of the mtDNA control region was amplified using standard protocols (Saiki *et al*. 1988; Palumbi 1995) and the oligonucleotide primers, light-strand tPro-whale (5'-TCACCCAAAGCTGRARTTCTA-3') and heavy-strand Dlp5 (5'- CCATCGWGATGTCTTATTTAAGRGGAA-3'). The tPro-whale primer used here bracketed the Dlp10 primer used previously (Baker *et al*. 1993; Medrano-Gonzalez *et al*. 1995), extending the fragment in the 5' direction to include the *Sau*96I site that distinguished the A– and A+ haplotypes. Following amplification, 5–10 μL of the double-stranded (ds) DNA product was digested with *Nco*I, *Dra*I and *Sau*96I following the manufacturer's recommendations and electrophoresed through a 1.6% agarose/TBE gel to separate restriction fragments by size.

A similar procedure was used for the analysis of variation in the nuclear actin intron. As described previously (Palumbi & Baker 1994), a phylogenetic reconstruction of sequence variation in the actin intron of humpback whales showed that alleles formed two distinct clades, referred to as 'A' and 'B'. A nucleotide substitution at position 1344 in the reference sequence (see Fig. 2, Palumbi & Baker 1994) results in the loss of a *Kpn*I site

distinguishing the two clades. Using this diagnostic restriction site, homozygotes and heterozygotes for the A and B clades could be assayed by digestion with *Kpn*I followed by electrophoresis of the restriction fragments through a 1.6% agarose/TBE gel (see Fig. 5, Palumbi & Baker 1994). To increase the efficiency of PCR reactions, two primers (Act1162, 5'-TGTCATAGTGGCGAACAAGAC-3' and Act1385, 5'-CTTGTGAACTGATTACAGTCC-3') were designed to amplify a short fragment (265 bp including primers) of the actin intron bracketing the *Kpn*I site in the humpback whale (courtesy of C. Conway).

Microsatellite loci

Following the comprehensive analysis of actin intron alleles and mtDNA haplotypes, regional samples from southeastern Alaska and central California were chosen for screening with four dinucleotide, microsatellite loci previously determined to be polymorphic in humpback whales (415/416 and 464/465, Schlotterer *et al*. 1991; EV14 and EV104, Valsecchi *et al*. 1997). These four loci were chosen because they were found to have a small number of alleles and low levels of heterozygosity, reducing the probability that assumptions for traditional mutation/drift models were violated (see below).

The 5' end of one primer for each locus was modified to incorporate a fluorescent dye (6-FAM or HEX) to allow visualization of alleles on an ABI 373 autosequencer. All PCR reactions followed standard protocols, as described above, and published recommendations for thermal-cycle profiles. In some cases, the 6-FAM- and HEX-labelled products were loaded together in a single lane (duplexed). The output of the ABI 373 autosequencer was interpreted using computer programs GENESCAN and GENOTYPER (Applied Biosystems Division/Perkin-Elmer). The size of each allele (in bp) was measured using the GENESCAN-350 size standard run in each lane. Repeated runs of the same individuals gave size estimates differing by less than 0.5 bp, indicating that the expected minimum difference of 2 bp between alleles could be consistently resolved. This is within the range expected for gel-to-gel precision with a single ABI 373 autosequencer (Ghosh *et al*. 1997).

Statistical analysis

The hierarchical structure of mtDNA and nuclear intron variation among regions was tested using the analysis of molecular variance model (AMOVA) of Excoffier *et al*. (1992). This procedure calculated standard variance components and an array of haplotypic correlation measures, referred to as Φ statistics, for each level of population. The significance of the observed variance components and Φ statistics was tested using a random permutation procedure available in the computer program AMOVA (version 1.55, courtesy of L. Excoffier).

Information on the molecular distances between mtDNA haplotypes and nuclear intron alleles was not used in the analysis of population subdivisions (the 'no molecular information' option in AMOVA, version 1.55). The inclusion of molecular distance would have no effect on the analysis of the actin intron because only two alleles were resolved, and little effect on the mtDNA haplotypes because three of these differed by only one or two nucleotides. Instead, interhaplotypic distances were assumed to be equal and the analysis was essentially a multivariate analysis of variance. Thus, in the nonhierarchical model, the Φ_{ST} of the AMOVA is equivalent to Weir & Cockerham's (1984) F_{ST} statistic (theta, see below). Differences in frequencies of haplotypes and alleles were also tested using contingency tables and a randomized Chi-squared test of independence (Roff & Bentzen 1989). These results are reported only where they differ from the AMOVA or where the AMOVA was considered inappropriate for the analysis, e.g. for tests of heterogeneity within geographical region. For both the AMOVA and the test of independence, the significance of observed values was compared against the null distribution of the respective test statistic generated by 1000 simulations. Multiple pairwise comparisons of haplotype and allele frequencies were tested for statistical significance using the sequential Bonferroni adjustment (Rice 1989).

Differences in allele frequencies between selected regional samples of microsatellite loci were tested with an exact procedure (Raymond & Rousset 1995a) available in the program GENPOP (Raymond & Rousset 1995b). The degree of population subdivision in microsatellite loci was quantified using Weir & Cockerham's (1984) F_{ST} statistic (theta), and the 95% confidence interval of the overall F_{ST} estimate for microsatellite and intron loci was calculated using Weir's (1996) bootstrap procedure as implemented in GENPOP. The frequencies of nuclear intron and microsatellite genotypes were tested for departure from Hardy–Weinberg equilibrium using the exact procedure (Louis & Dempster 1987; Guo & Thompson 1992) available in the computer program GENPOP.

An infinite allele model was assumed in the analysis of population structure for mtDNA and both of the nuclear systems, microsatellites and the actin intron. Under the island model of populations (Wright 1951; Takahata & Palumbi 1985), combined male and female gene flow was estimated using the approximation $Nm_e = ((1-\Phi_{ST})/4(\Phi_{ST})$, or the F_{ST} analogue, and female migration was estimated using the approximation $Nm_f = (1-\Phi_{ST}))/2(\Phi_{ST})$. Alternate estimators of population subdivision and gene flow based on stepwise mutation models (e.g. Slatkin 1995) were not used because the low level of allelic variation in the microsatellite loci chosen for this analysis did not suggest high rates of mutation.

Results

Heterogeneity within regions

The mtDNA haplotype of 205 whales from eight regions was identified by a combination of diagnostic PCR–RFLP, whole genomic RFLP (Baker *et al*. 1990; Baker *et al*. 1994) and direct sequencing of the control region (Baker *et al*. 1993; Medrano-Gonzalez *et al*. 1995). The actin intron genotype was identified for 200 of these whales (400 chromosomes) and the sex was identified for 185. Each regional sample was tested for heterogeneity in the frequencies of mtDNA haplotypes and actin intron alleles due to the year and sex composition of the sample (Table 1). Only two of the 24 pairwise tests were significant at $P < 0.05$ (unadjusted for multiple comparisons): a greater frequency of mtDNA A– haplotypes was found among males in southeastern Alaska ($\chi^2 = 4.45$, $P = 0.033$); and, a greater frequency of the nuclear actin B allele was found in the 1992 sample from Socorro Island ($\chi^2 = 5.17$; $P = 0.023$).

The genotype frequencies of the overall sample did not differ significantly from expected under the assumptions of the Hardy–Weinberg equilibrium (Table 2). A significant, although marginal ($P = 0.05$, unadjusted for multiple comparisons), deviation from Hardy–Weinberg equilibrium due to an excess of homozygotes was found for the actin allele in Baja California. The sex ratio of the overall sample was significantly biased towards males (121 males to 64 females, $\chi^2 = 17.6$, $P < 0.01$) as were regional samples from the Baja Peninsula (as noted by Baker *et al*. 1994), Socorro Island (as noted by Medrano *et al*. 1994) and the Ogasawara Islands. The most extreme male bias was found in the Ogasawara Islands where all samples were collected from sloughed skin.

Regional differences

An initial analysis of differences in frequencies of mtDNA haplotype and nuclear actin alleles was conducted using only regional geographical divisions (Table 2). This non-hierarchical model imposed no *a priori* assumptions about the structure of humpback whale populations and thus ignored evidence of known relationships between habitats connected by seasonal migration (see below).

There were marked differences in the frequencies of the four mtDNA PCR–RFLP haplotypes among the eight regional samples (Table 3). The overall Φ_{ST} value calculated

Table 2 Frequencies of mtDNA haplotypes (n = number of individuals) and actin intron alleles (n = chromosomes, i.e. 2 × number of individuals) among humpback whales in regions of the North Pacific and the probability that the intron genotypes are in Hardy–Weinberg (H-W) equilibrium

	mtDNA haplotypes					Intron alleles		
	n	E	F	A+	A–	n	B	H– W (P <)
Bahia Banderas	21	0.49	0.24	0.24	0.05	42	0.81	1.00
Baja Peninsula	21	0.43	0.14	0.33	0.10	42	0.64	0.05
Socorro Island	23	0.65	0.17	0.04	0.13	46	0.74	1.00
California	57	0.49	0.44	0.07	0	108	0.70	0.51
Southeastern Alaska	39	0	0	0.23	0.77	76	0.51	1.00
Prince Williams Sound	6	0	0	1	0	12	0.67	1.00
Hawaii	27	0.07	0.15	0.41	0.37	54	0.54	0.26
Ogasawara Islands	11	0.45	0.18	0.36	0	20	0.75	0.48
Total	205	0.34	0.21	0.23	0.22	400	0.66	0.35

Table 3 The Φ_{ST} values (below the diagonal) for comparisons of mtDNA haplotype frequencies between regional samples of humpback whales in the North Pacific and the probability (above the diagonal) of a greater value by chance based on 1000 permutations of the data matrix (unadjusted for multiple comparisons). Values of Φ_{ST} with permutation probabilities < 0.05 are shown in bold

	BB	BP	SI	CA	SEA	PWS	HI	OG
Bahia Banderas	–	0.837	0.224	0.187	0.000*	0.000*	0.002*	0.814
Baja Peninsula	–0.032	–	0.084	0.013	0.000*	0.008	0.017	0.999
Socorro Island	0.019	0.060	–	0.053	0.000*	0.000*	0.000*	0.121
California	0.026	**0.097**	0.066	–	0.000*	0.000*	0.000*	0.075
Southeastern Alaska	**0.447**	**0.396**	**0.501**	**0.514**	–	0.000*	0.002*	0.000*
Prince William Sound	**0.404**	**0.321**	**0.591**	**0.534**	0.635	–	0.021	0.024
Hawaii	**0.150**	**0.095**	**0.274**	**0.280**	**0.158**	**0.254**	–	0.023
Ogasawara Island	–0.057	–0.065	0.062	0.071	**0.480**	**0.364**	**0.124**	–

* Significant at $P < 0.05$ after adjustment for multiple comparison with the sequential Bonferroni test (Rice 1989).

in the nonhierarchical AMOVA model showed that 27.7% of the variance in haplotype frequencies was explained by the eight regions ($P < 0.001$). Pairwise comparisons between regions (as reflected by the Φ_{ST} values) explained from –5.7 to 59.1% of the variance in frequencies (Table 4), with negative values suggesting that regional divisions explained less variance than random permutations of the pooled samples (Excoffier 1995). Most of these pairwise comparisons were significant at the 0.05 level of probability after adjustment for multiple comparisons.

As described previously (Baker *et al*. 1990, 1994), the greatest differences in mtDNA haplotype frequencies were found among the three feeding grounds (Table 2). The California feeding ground was dominated by E and F types while southeastern Alaska was dominated by A– and A+ types. The small number of individuals from Prince William Sound were all A+ types. Pairwise differences among the three feeding grounds were all significant. Differences among the wintering grounds were less marked. As reported previously based on an analysis of haplotype sequences (Medrano-Gonzalez *et al*. 1995), the two coastal wintering grounds in the Mexican Pacific were dominated by E, F and A+ types. Socorro Island showed a greater frequency of E and A– types but did not differ significantly from the coastal Mexican wintering grounds (although a previous analysis of sequence variation in mtDNA haplotypes showed a weak but significant difference for coastal and offshore regions, Medrano-Gonzalez *et al*. 1995). The Ogasawara Islands showed a surprising similarity to coastal Mexico and only a slight, but nonsignificant, difference with Socorro Island. Only Hawaii differed significantly from all other wintering grounds, being dominated by A+ and A– types.

Results of the comparisons of feeding grounds and wintering grounds were complex. Southeastern Alaska and Prince William Sound were most similar to Hawaii, as indicated by relatively lower Φ_{ST} values, but differed significantly from all wintering regions in haplotype frequencies. California was most similar to the Bahia Banderas and Ogasawara wintering regions but differed significantly from the Baja Peninsula as well as Hawaii in haplotype frequencies.

Regional differences in the nuclear actin alleles were less marked than for mtDNA haplotypes, as reported previously based on a smaller sample of whales from Hawaii and California (Palumbi & Baker 1994). Unlike the previous analysis of Palumbi & Baker (1994), however, the larger size and geographical range of the samples revealed some significant differences in the frequency of the two alleles. The Φ_{ST} of the nonhierarchical AMOVA model explained 3.31% of the variance in allele frequencies among the eight regions ($P < 0.013$, confirmed by the GENPOP exact test of differentiation) and between –05.5 and 15.4% of the variance in pairwise comparisons between regions (Table 4). Φ_{ST} values with permutation probabilities less than 0.05 (unadjusted) were found in comparisons of two central regions, Hawaii and southeastern Alaska, to three eastern regions, Bahia Banderas, Socorro Island, and California. Only the comparison of southeastern Alaska to Bahia Banderas was significant after adjustment for the 28 multiple comparisons.

Stock structure

To test the significance of the hypothesized 'stock structure' in the North Pacific, we conducted analyses of mtDNA and nuclear actin alleles using a hierarchical AMOVA model. Based on previous demographic and genetic evidence, the two Alaskan feeding regions and the Hawaiian wintering region were considered to form a 'central' stock or subpopulation. The Californian feeding region and the two coastal Mexican wintering regions were considered to form an 'eastern' stock. Socorro and the Ogasawara Islands were not included in either stock division because the primary feeding regions for whales from these wintering regions are unknown.

Table 4 The Φ_{ST} values (below the diagonal) for comparisons of intron allele frequencies between regional samples of humpback whales in the North Pacific and the probability (above the diagonal) of a greater value by chance based on 1000 permutations of the data matrix (unadjusted for multiple comparisons). Values of Φ_{ST} with permutation probabilities $P < 0.05$ are shown in bold

	BB	BP	SI	CA	SEA	PWS	HI	OG
Bahia Banderas	–	0.055	0.287	0.219	0.000*	0.431	0.008	0.741
Baja Peninsula	0.045	–	0.377	0.429	0.110	0.999	0.417	0.570
Socorro Island	–0.009	–0.001	–	0.684	0.019	0.469	0.026	0.999
California	0.012	–0.008	–0.012	–	0.012	0.999	0.056	0.770
Southeastern Alaska	**0.154**	0.015	**0.085**	**0.064**	–	0.362	0.865	0.076
Prince William Sound	0.004	–0.055	–0.041	–0.045	–0.002	–	0.325	0.403
Hawaii	**0.134**	0.002	**0.065**	0.046	–0.015	–0.018	–	0.135
Ogasawara Island	–0.027	–0.011	–0.037	–0.025	0.078	–0.053	0.057	–

* Significant at $P < 0.05$ after adjustment for multiple comparison with the sequential Bonferroni test (Rice 1989).

The hierarchical AMOVA of stock structure explained 38.0% of the variance in the distribution of mtDNA haplotypes and 6.0% of the variance in actin alleles. The majority of explained variance in mtDNA distributions (27.8%; $P < 0.001$) was due to the two putative stock divisions but a significant proportion was also due to the regional habitats within stocks (10.2%; $P < 0.001$). All of the explained variance in the actin allele was due to the two stock divisions (6.1%; $P < 0.001$) with only a slight negative value (–0.1%) due to regions within stocks.

Microsatellite differentiation

Differentiation of nuclear variation between the southeastern Alaska and central California feeding grounds was further tested using four microsatellite loci. These regional populations were selected because comparisons of individual identification photographs (see the Introduction), as well as frequencies of mtDNA haplotypes, indicate very low levels of demographic interchange. This reduced the probability of sampling individuals engaged in temporary migratory interchange rather than effective migration, a potential bias when sampling from the more complex congregations on wintering grounds.

The results of the four microsatellite loci were consistent with those of the actin intron in showing significant differences in allele frequencies between the two regions (Table 5). The four loci ranged in heterozygosity from a low of 0.04 (415/416, southeastern Alaska) to a high of 0.78 (464/465; southeastern Alaska). The estimates of F_{ST} ranged from 0.010 to 0.133, overlapping the value of 0.064 for the actin allele. The overall average F_{ST} for the five nuclear loci was 0.048 with a 95% bootstrap confidence

Table 5 The allele frequencies, observed (H_O) (and expected, H_E) heterozygosity, sample size (number of chromosomes) and significance of genetic differentiation (F_{ST}) for humpback whales from the California and southeastern Alaska feeding grounds at four microsatellite loci

Locus*	Allele size (bp)	California	Southeastern Alaska
415/416			
$F_{ST} = 0.060$	227	0.122	0.018
$P = 0.041$	229	0.878	0.982
		$H_O = 0.24$ ($H_E = 0.22$) $n = 74$	$H_O = 0.04$ ($H_E = 0.04$) $n = 56$
464/465			
$F_{ST} = 0.010$	137	0.014	0.000
$P = 0.018$	139	0.514	0.500
	141	0.014	0.141
	143	0.403	0.297
	149	0.055	0.031
	151	0.000	0.031
		$H_O = 0.56$ ($H_E = 0.58$) $n = 72$	$H_O = 0.78$ ($H_E = 0.65$) $n = 64$
EV14			
$F_{ST} = 0.017$	129	0.000	0.033
$P = 0.014$	131	0.553	0.650
	133	0.160	0.250
	135	0.042	0.000
	137	0.106	0.050
	139	0.128	0.017
	141	0.011	0.000
		$H_O = 0.62$ ($H_E = 0.65$) $n = 94$	$H_O = 0.63$ ($H_E = 0.52$) $n = 60$
EV104			
$F_{ST} = 0.133$	147	0.069	0.151
$P < 0.001$	149	0.917	0.651
	151	0.014	0.197
		$H_O = 0.11$ ($H_E = 0.16$) $n = 72$	$H_O = 0.61$ ($H_E = 0.52$) $n = 66$
Average per locus			
$F_{ST} = 0.048$			
$P < 0.001$		$H_O = 0.38$ ($H_E = 0.40$) $n = 78$	$H_O = 0.51$ ($H_E = 0.43$) $n = 61$

*F_{ST} statistic (Weir & Cockerham 1984) and the exact probability (Raymond & Rousset 1995a) of the observed differences in allele frequencies at each loci were calculated with the program GENPOP (Raymond & Rousset 1995b).

intervals of 0.016–0.101. There was no evidence for heterogeneity in the frequencies of microsatellite alleles due to the year and sex composition in each regional sample except for the 464/465 locus where a significant difference ($P = 0.007$) was found between years in California (due to fewer 139 bp alleles and more 143 bp alleles in the 1989 sample). All of the loci were in Hardy–Weinberg equilibrium for each of the two populations.

The combined nuclear and mtDNA data allowed estimation of the relative magnitude of gene flow attributable exclusively to females check Nm (Nm_f) and that attributable to males and females together (Nm_e). For southeastern Alaska and central California, this estimate was 0.5 females per generation (Nm_f) using the value $\Phi_{ST} = 0.51$ for the mtDNA genome, and five males and females per generation (Nm_e) using the overall value $F_{ST} = 0.048$ for the five nuclear loci.

Discussion

Maternal fidelity and reproductive isolation

The genetic structure of the North Pacific population of humpback whales showed significant partitioning of both nuclear and mtDNA variation into regional and stock components. This confirmed the strong influence of maternally directed fidelity to migratory destinations suggested previously (Baker *et al*. 1990, 1994) and indicated at least partial reproductive isolation between some components of the oceanic population. These genetic differences paralleled the complex demographic structure of this population as described previously from observation of migratory movement by naturally marked individuals (Darling & McSweeney 1985; Baker *et al*. 1986; Darling & Mori 1993; Urban *et al*. 1994; Calambokidis *et al*. 1996; Darling *et al*. 1996).

Although the relationship between some seasonal habitats of the North Pacific cannot yet be determined, differences in both nuclear and mtDNA variation supported recognition of at least two stocks or subpopulations. A central stock includes at least the southeastern and central Alaskan feeding grounds and the Hawaiian wintering grounds. An eastern or 'American' stock includes the feeding grounds of coastal California (and perhaps Oregon and Washington) and wintering grounds of coastal Mexico. Regional differences within each stock in frequencies of mtDNA haplotypes but not nuclear alleles suggested an additional influence of maternally directed segregation among feeding grounds for whales sharing a common wintering ground. This was most obvious in the comparison of southeastern Alaska and Prince William Sound, two regions known to be connected to Hawaii by seasonal migration. These regions were distinguished by difference in haplotype frequencies, although the sample size for Prince William Sound was small and requires confirmation. Reasons for the differences in frequencies of mtDNA haplotypes between feeding and wintering regions of the same stock were less obvious. California and Mexico, for example, are known to be connected by seasonal migration but differed significantly in haplotype frequencies.

'Missing' stock components

The most plausible explanation for this heterogeneity in haplotype frequencies is the migration of whales from unsampled feeding grounds to the Hawaiian and Mexican wintering grounds. An obvious example of this problem is the unknown feeding habitats of whales wintering near the Ogasawara Islands and Socorro Island. Additional evidence of a 'missing' stock component comes from a comparison of abundance estimates from wintering grounds and feeding grounds within the eastern and central stocks. Estimates of abundance on the wintering grounds are typically several-fold larger than those from known feeding regions. In the American stock, for example, abundance in the central California regions was estimated to be 582 in 1991–92 (Calambokidis *et al*. 1993), while the coastal and offshore Mexican wintering grounds were estimated to be 2200–2800 in 1994 (Urban *et al*. 1994). In the central stock, abundance in southeastern Alaska was estimated to be 504–590 in 1986 (Baker *et al*. 1992), while Hawaii was estimated to be 1400–2000 in 1981–84 (Darling & Morowitz 1986; Baker & Herman 1987). It seems likely that this discrepancy is due to whales from, as yet, unsampled feeding grounds congregating on these wintering grounds.

Information on the pre-exploitation feeding range of humpback whales points to some probable locations for one or more of these missing stock components. Records of Russian and Japanese pelagic whaling fleets from 1952–65 show the greatest number of humpbacks around the Aleutian Islands. During this period, more than 4700 humpbacks were killed in this region (Rice 1978). The current status of humpback whales along the Aleutians is largely unknown although aerial surveys report substantial numbers around Kodiak Island (Brueggeman *et al*. 1987).

The similarity of haplotype frequencies in the Ogasawara Islands of Japan and the coastal wintering grounds of Mexico is puzzling considering that both regional samples differ from the geographically intermediate wintering grounds of Hawaii. A larger sample size from the Ogasawara Islands is needed to confirm this pattern. It may also be necessary to use a biopsy dart, rather than relying on sloughed skin, to collect a more representative sample of the sexes on this wintering ground. However, the genetic evidence of an association, current or historical, between the Ogasawara Islands and the American coast is consistent with available demographic

evidence. Although discovery marking showed a connection between the Ryukyu Islands, south of Japan, and the southern Bering Sea near the Aleutian Islands (Nishiwaki 1967; Ohsumi & Masaki. 1975), the only known migratory connection between the Ogasawara Islands and a feeding ground is to the coast of British Colombia based on individual identification photographs (Darling *et al*. 1996).

Genetic management units

The finding of significant differences between a central and an eastern stock of humpback whales in both mitochondrial and nuclear DNA loci fulfils the criterion of reproductive isolation implicit in some management schemes, including the US Endangered Species Act and the Revised Management Procedure of the International Whaling Commission (Donovan 1991; Waples 1991; Dizon *et al*. 1992). Reproductive isolation within components of these stocks (southeastern Alaska and California) was confirmed by a significant difference in the allele frequencies at four microsatellite loci, in addition to the actin intron. These findings also conform to Moritz's (1994) recommendation that 'genetic management units' should be recognized by significant differences in frequencies of mtDNA and, when possible, nuclear alleles. Further segregation of mtDNA haplotypes on feeding grounds within stocks is evidence for significant demographic units that may share a common nuclear gene pool but retain unique maternal traditions of migration and habitat use.

The presence of shared mtDNA haplotypes among stocks, however, falls short of the criterion of reciprocal monophyly suggested by Moritz (1994) and others (Vogler & Desalle 1994), as evidence for the distinction of 'evolutionary significant units'. In addition, the comparison of mtDNA haplotype and intron allele frequencies confirmed previous observations that population differentiation in humpback whales is less marked for nuclear loci than for maternal lineages (Palumbi & Baker 1994). This difference was most obvious in the hierarchical analysis of haplotypes and alleles. Here, there was no partitionable variance for the allele frequencies among regions within stocks, suggesting that mating on a common wintering ground was sufficient to maintain panmixis among individuals migrating to different feeding grounds. Between stocks, differences in intron allele frequencies were significant but considerably weaker than for mtDNA haplotypes. This pattern and magnitude of differentiation was confirmed for the two feeding grounds using the four microsatellite loci.

Male- and female-mediated gene flow

There are several possible reasons for the reduced magnitude of nuclear differentiation compared to mtDNA in populations of humpback whales (Palumbi & Baker 1996), including: male-biased gene flow; the larger effective population size of nuclear genes; intralocus sampling variance; and balancing polymorphism operating on nuclear loci. The comparison of gene flow estimates from mtDNA, the actin intron and the four microsatellite loci provides some evidence to discount the latter three factors. Given that the gene flow estimate Nm_f corrects for haploid, but not maternal, inheritance the expected difference in the nuclear and mtDNA estimates should be twofold in the absence of sex-biased gene flow. Instead, the observed differences between Nm_f and Nm_e were found to be approximately 10-fold in magnitude. Intralocus sampling variance was relatively low, considering the expectations for this parameter (Nei 1987), and estimates of gene flow from the 95% confidence intervals of the nuclear loci (Nm_e = 2.2–15.4) did not overlap the value of Nm_f = 0.5, even after the twofold adjustment for maternal inheritance. Finally, balancing polymorphism would be improbable at all nuclear loci given the general assumption that most microsatellite loci are not under selection (Jarne & Lagoda 1996) and the observation of weak but significant differentiation in each of the five loci.

Although it is difficult to exclude the actions of these other factors with certainty, the hypothesis of male-biased gene flow is consistent with the known breeding pattern of most mammals (Greenwood 1983). However, the nature of such gene flow in a migratory marine species can be complex and need not require permanent male-biased dispersal. For example, Karl *et al*. (1992) suggested two possible mechanisms to explain greater male-mediated gene flow in the migratory green sea turtle *Chelonia mydas*: (i) reduced philopatry by males; or, (ii) mating along migratory routes of otherwise separate populations. Recent analyses of mtDNA, single-copy nuclear and microsatellite markers in Australian green sea turtles discount the hypothesis of reduced male phylopatry in this population and support, instead, the hypothesis of mating during migration from feeding to courtship areas (FitzSimmons *et al*. 1997a, b).

Similar to the green sea turtle, male-mediated gene flow in the North Pacific humpback whales could be due to occasional mating during migration between otherwise discrete populations. There is little known about the migratory routes of humpback whales in the North Pacific but some overlap in these paths seems probable given known connections between migratory destinations. Baja California, for example, could be a region of overlap for animals migrating to primary wintering grounds further to the south or west. The excess of homozygotes for the actin intron in the sample from Baja suggests a Wahlund effect due to mixing of genetically distinct groups. Alternatively, there could be greater interchange between wintering grounds by one or both sexes

and some form of assortative mating to maintain the nuclear differentiation observed between the southeastern Alaska and central California feeding grounds. Finally, it is possible that the majority of mating takes place on or near the feeding grounds during the late autumn and early winter (Straley 1990) rather than on arrival in tropical waters. This could result in the differentiation of nuclear genes despite opportunities for intermingling during the remainder of the migratory cycle. However, this hypothesis conflicts with observations of behaviour assumed to be related to competition for mating in the wintering grounds (Tyack & Whitehead 1983; Baker & Herman 1984).

Although a comprehensive description of the North Pacific humpback whale will require additional samples from 'missing' components of the population, our results confirm the power of a comparative genetic and demographic approach using both nuclear and mitochondrial markers (Karl *et al*. 1992; Palumbi & Baker 1994). Additional insight into the population dynamics of long-lived, social vertebrates can be gained by further integration of fine-scale genetic analyses with parallel detailed studies of mating systems, social organization and behavioural displays (e.g. Packer *et al*. 1991; Morin *et al*. 1994). In humpback whales, the combined survey of nuclear and mtDNA markers, the documentation of migratory movement by naturally marked individuals, and the analysis of annual and regional changes in the winter song could describe the dynamics of population change at three levels of interest to evolutionary biologists: genetic, demographic and cultural.

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The authors all share an interest in the geographical or genetic structure of humpback whale populations. This work was motivated, in part, by the need to define demographic or genetic units of management for evaluating rates of recovery or probabilities of extinctions in formerly and currently exploited populations of whales.
