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Genetic analysis of stock structure and movements of gray whales in the eastern and western North Pacific

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ABSTRACT

Previous studies have documented genetic differentiation between gray whales in the eastern and western North Pacific on the basis of both mtDNA haplotype and microsatellite allele frequencies. In these studies, the eastern North Pacific (ENP) population of gray whales has been represented by a sample set comprised primarily of samples from animals that stranded along the migratory route. Recent studies assessing population substructuring of gray whales within the ENP have resulted in the collection and analysis of additional samples from ENP gray whales feeding north of the Aleutian Islands (n=106 sampled individuals). Here we update previous assessments of genetic differentiation between ENP and western North Pacific (WNP) gray whales using these additional ENP samples and samples collected from whales (n=142 individuals) feeding off the northeastern coast of Sakhalin Island, Russia. In addition, comparison of the mtDNA haplotype, sex, and genotypes (8 to 13 loci) of all analyzed samples (n=380) was used to identify samples with identical genetic profiles, and these genetic matches were used to infer movements of individuals between areas. Consistent with the results of previous studies, significant levels of differentiation were found between WNP and ENP gray whales using both mitochondrial (e.g., Sakhalin versus Chukotka, $F_{ST} = 0.082$, $p < 0.0001$; $\Phi_{ST} = 0.037$, $p < 0.001$) and nuclear (e.g., Sakhalin versus Chukotka, $F_{ST} = 0.010$, $p = 0.001$; $F_{ST}' = 0.037$, $p = 0.001$) markers (n=8 loci). Seven pairs of samples shared identical genetic profiles, including one match between an animal sampled off the coast of San Diego and an animal taken in the Chukotka hunt, four matches between animals biopsied on the Sakhalin feeding ground and animals biopsied off southeastern Kamchatka, and two matches between animals biopsied on the Sakhalin feeding ground and animals biopsied off the coast of southern California. While the significant levels of genetic differentiation support demographic independence and a degree of reproductive isolation between whales feeding in the WNP and the ENP, the putative movements detected here, in combination with information derived from photo-identification comparisons and telemetry studies, suggest that some of the animals summering off Sakhalin overwinter in the ENP in at least some years. Given that recent records document gray whales in Japanese waters during winter and spring, these results suggest that population structure in gray whales may be more complex than previously believed, such that not all of the animals which feed off Sakhalin share a common wintering ground, or that some animals may switch between wintering grounds. Thus, the number of gray whales remaining in the WNP year-round may be lower than previously thought, highlighting the need for additional studies focusing on identifying migratory routes and wintering ground(s) used by gray whales in the WNP.

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INTRODUCTION

Like many species of baleen whales, gray whales (*Eschrichtius robustus*) exhibit seasonal movements between high- latitude summer feeding grounds and low- latitude wintering areas. The current distribution of this species is limited to the eastern and western margins of the North Pacific (Rice & Wolman 1971), where two populations are recognized. Although both populations were greatly depleted by commercial whaling, the population in the eastern North Pacific (ENP) currently numbers ~19,000 animals, (based on surveys in 2006/2007; Laake *et al.*, 2009). Most animals in the ENP population feed in the waters of the Bering, Beaufort, and Chukchi Seas during summer and early fall and then migrate south to the lagoons and coastal waters off Baja California, Mexico to spend the winter months. However, a small number of animals (~200, Calambokidis *et al.*, 2010), referred to as the Pacific Coast Feeding Group (PCFG) of gray whales (IWC, 2010), demonstrate consistent return to more southern waters between northern California and southeastern Alaska during the summer feeding season (Darling, 1984; Calambokidis *et al.*, 2002, 2010). Recent genetic studies have demonstrated significant mtDNA differentiation between the PCFG and ENP gray whales feeding in areas north of the Aleutians (Lang *et al.*, 2011), as well as between the PCFG and a sample set comprised primarily of whales which stranded along the migratory route in the ENP (Frasier *et al.*, 2011). No significant differentiation in nuclear markers was identified, however, suggesting that PCFG whales may interbreed with animals feeding north of the Aleutians (Lang *et al.*, 2011).

The population of whales in the western North Pacific (WNP) was reduced to much lower numbers than its eastern counterpart during commercial whaling. This population has been estimated to contain only about 130 animals of age one or older (Cooke *et al.*, 2008) and is currently listed as Critically Endangered by the IUCN (Weller *et al.*, 2002; Baillie *et al.*, 2004). Much of what is known about this population is derived from combined photo-identification and genetic studies of individuals on the population's primary feeding ground in the coastal waters of northeastern Sakhalin Island, Russia (Weller *et al.*, 1999; Weller *et al.*, 2008a; LeDuc *et al.*, 2002; Lang *et al.*, 2010). Photo-identification studies have documented seasonal site fidelity and annual return of individuals to this feeding area (Weller *et al.*, 1999). Reproductive females are known to utilize the Sakhalin feeding ground year after year when they are accompanied by calves as well as when they are pregnant or resting, and the return of many individuals first identified as calves accompanying their mothers has been documented (Weller *et al.*, 2009). Some of the whales feeding in the coastal waters off Sakhalin are also known to utilize feeding areas slightly offshore, as well as off the southern and eastern coast of Kamchatka (Tyurneva *et al.*, 2010; Burdin *et al.*, 2011). The whales identified off Kamchatka include some mother-calf pairs also identified on the Sakhalin feeding ground (Tyurneva *et al.*, 2010). However, not all of the whales photographed off Kamchatka have been identified off Sakhalin, and their population affiliation is unknown.

Genetic samples have been collected from 83 % (n=142) of the whales identified on the Sakhalin feeding ground between 1995 and 2007 (Lang, 2010). Comparison of this sample set with samples collected from whales in the eastern North Pacific (ENP) have supported recognition of the two populations as distinct, with differentiation in both mtDNA haplotype and microsatellite allele frequencies (LeDuc *et al.*, 2002; Lang *et al.*, 2010). Assessment of the paternity of animals first identified as calves on the Sakhalin feeding ground between 1995 and 2007 (n = 57 sampled mother-calf pairs) resulted in the assignment of putative fathers for 46-53% of the calves, supporting interbreeding among animals that feed off Sakhalin but also raising questions about the identity of the unassigned fathers (Lang, 2010).

Little is known about the location of migratory routes and wintering ground(s) currently used by the whales that feed off Sakhalin. The coastal waters of southeastern Russia, the Korean Peninsula, and Japan are thought to have been used as migratory corridors historically, and some evidence exists suggesting that the coastal waters of southern China may have been used as wintering grounds (reviewed in Weller *et al.*, 2002). Aside from sightings of whales in feeding areas, the majority of recent records of gray whales in the WNP are of sightings, strandings, and entrapments in the coastal waters of Japan (see details in Kato *et al.*, 2010). Although little is known about the identity of most of the whales recorded off Japan, photographs of one animal which was entrapped off the Pacific coast of Japan in January 2007 were matched to an animal first

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photographed as a calf on the Sakhalin feeding ground in 2006, providing the first known link between the Sakhalin feeding ground and a migratory route in the western North Pacific (Weller *et al.*, 2008b).

In October 2010, a team of scientists from Russia and the United States deployed a satellite tag on a gray whale off the coast of Sakhalin Island¹. The tagged individual (“Flex”) was a 13 year-old male that had been first identified as a calf off Sakhalin in 1997 and subsequently demonstrated repeated return to the Sakhalin feeding ground. “Flex” was tracked for ~4 months, during which time he traveled from the feeding ground off Sakhalin Island to the western coast of the U.S.^{1, 2, 3}. Subsequent comparison of photographs of “Flex” collected off the coast of Sakhalin with photographs collected by Cascadia Research Collective in the Pacific Northwest revealed that Flex had previously been photographed off the coast of Vancouver Island in April 2008 (Weller *et al.*, 2011). The satellite track and photographic record of “Flex” have since raised questions about the potential for movements of gray whales between the eastern and western North Pacific.

Prior analyses of genetic differentiation between ENP and WNP gray whales have been based on an ENP sample set in which the majority of samples were collected from animals which stranded along the migratory route (LeDuc *et al.*, 2002; Lang *et al.*, 2010). As part of recent efforts to better understand the potential for substructure in ENP gray whales (Lang *et al.*, 2011), additional samples were collected and analyzed from gray whales utilizing feeding grounds north of the Aleutians. This sample set provides the opportunity to update previous assessments of genetic differentiation between eastern and western gray whales and will allow direct comparisons to be made between animals utilizing feeding areas in the ENP and in the WNP. The expanded data set will also be used to identify samples with identical genetic profiles, which may provide information on movements of animals both within and between the eastern and western North Pacific.

METHODS

Sample Collection

The collection location for all samples utilized in the study is shown in Figure 1. Within the WNP, samples were collected between 1995 and 2007 via biopsy-darting of 142 individual whales on the feeding ground off Sakhalin Island, Russia. All except one of these samples are linked to a photographically identified animal, and this sample set represents 83% of all animals (n=169) identified on the Sakhalin feeding ground through 2007. Additional samples were collected via biopsy darting of whales between Kamenistaya Bay and Asacha Bay on the southeastern coast of Kamchatka, Russia during the summer months of 2004 (n=3 samples) and 2010 (n=12 samples).

Within the ENP, samples were collected from 228 individuals. Table 1 shows the year of sample collection as well as the collection method, with samples subdivided into those collected from animals south of the Aleutians (“CA->AK”) and those collected on the northern feeding ground(s) (“N of Aleutians”).

Lab Processing:

Details on protocols for extraction, sequencing, molecular sexing, and genotyping of the samples collected off Sakhalin Island and samples collected in the ENP between California and the Aleutians are provided in Lang *et al.* 2010. These samples were genotyped at 13 loci, including D17t, EV14t, EV37, EV94t, Gata028, Gata098, Gata417, Gt023, RW31, RW48, SW10t, SW13t, and SW19t.

Protocols used for generating data for the samples collected north of the Aleutians and those collected off of the coast of southeastern Kamchatka are described in Lang *et al.* 2011. These samples have been genotyped at 8 loci, including EV14t, EV94t, Gata028, Gt023, Gata417, RW31, SW13t, and SW19t. Genotypes for the additional five loci included in Lang *et al.* 2010 are currently being generated. Analysis of genetic diversity

¹<http://www.iucn.org/wgwap/?6614/International-scientists-track-endangered-whale-to-discover-breeding-grounds>¹

²<http://mmi.oregonstate.edu/Sakhalin2010>

³http://www.sevin.ru/menues1/index_rus.html?..ExpeditionsRAS/Gray_whale/Gray_whale.html

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and differentiation for nuclear markers used only the eight microsatellite loci which have been generated for all samples, although the calculation of probabilities associated with identifying genetic matches utilized the 13-loci dataset when available.

Analysis

Sample Stratification

Samples were separated into strata for analysis based on the geographic location of sample collection. Within the WNP, samples were subdivided into two strata. All samples collected from animals off the northeastern coast of Sakhalin Island were included in the Sakhalin stratum; the composition of this stratum is identical to that utilized in Lang *et al.*, 2011. Samples collected from whales off the southeastern coast of Kamchatka were included in a southeastern Kamchatka stratum.

Within the ENP, samples were first classified into two broad-scale strata, with all samples from feeding grounds north of the Aleutians included in the “north of Aleutians” stratum and all samples from animals obtained along the migratory route between southern California and southeastern Alaska included in the “CA->AK” strata. The “north of the Aleutians” stratum is identical in composition to the “North” stratum utilized in Lang *et al.*, 2011. The “CA->AK” stratum is similar in composition to the stratum used to represent ENP gray whales in previous comparisons (LeDuc *et al.*, 2002; Lang *et al.*, 2010; Frasier *et al.*, 2011); however, all samples which were collected from animals north of the Aleutians were removed and were retained in the “north of the Aleutians” stratum. Although all samples included in the “CA->AK” stratum were obtained from regions utilized as part of the migratory route in the ENP, the region between northern California and southeastern Alaska is also used as a feeding ground by the PCFG whales. While none of the samples included in the “CA->AK” stratum were known to be from PCFG whales (i.e., none of these samples were included in the “South” or “PCFG” strata utilized in Lang *et al.*, 2011), it is possible that some PCFG whales could be included in this stratum.

Little is known about whether or not additional substructuring occurs among whales feeding in different areas within the larger “north of the Aleutians” feeding ground. To avoid inadvertently using a stratum that may contain unrecognized structure, samples collected north of the Aleutians were further subdivided into the “Chukotka” and “Barrow” strata. These strata are the same as those used in Lang *et al.*, 2011, and additional details on the rationale for this stratification system are included in that manuscript.

The Microsoft EXCEL program MS_TOOLKIT (Park, 2001) was used to identify samples with genotypes that matched at all loci. The mtDNA haplotype and sex of animals with identical genotypes were used to confirm all identified matches. For those samples that shared identical genetic profiles and were collected within the same region (i.e., were included in the same stratum), one of each pair of matching samples was removed prior to analysis.

Genetic Diversity

For the mtDNA, haplotypic diversity (h) and nucleotide diversity (π) were calculated using Arlequin 3.5.1.2 (Excoffier *et al.*, 2005). For the 8-loci microsatellite dataset, the number of alleles per locus and observed and expected heterozygosities were calculated using custom R-code (eiaGenetics, available upon request¹). Deviations from Hardy-Weinberg equilibrium (HWE) were assessed for each microsatellite locus using Genepop (version 4.0.11, Rousset 2008). Both the probability test (Guo & Thompson, 1992) and the test for heterozygote deficiency (Rousset and Raymond 1995) were conducted using the program defaults for the Markov chain parameters (10,000 dememorization steps, 20 batches, 5000 iterations/batch). Genepop was also used to test for linkage disequilibrium (LD) for each pair of loci.

Genetic Structure

Pairwise estimates of genetic divergence were calculated using both F_{ST} and Φ_{ST} (based on pairwise differences between sequences as the measure of genetic distance) for the mtDNA data as implemented in Arlequin 3.5.1.2 (Excoffier *et al.*, 2005). Statistical significance was assessed using 20,000 permutations.

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Fisher's exact test (Raymond & Rousset, 1995) was also used to test for mtDNA differentiation between strata using 100,000 replications to test for significance.

For the 8-loci microsatellite dataset, F_{ST} (Weir & Cockerham, 1984), normalized F_{ST} , Jost's D (Jost, 2008), and a χ^2 test were used to assess genetic differentiation. These tests were implemented using custom code (eiaGenetics⁴) written in the statistical program language R (R Core Development Team, 2009). Statistical significance was determined from 10,000 permutations of each data set.

Movements

As aforementioned, the EXCEL add-in MS_TOOLKIT (Park, 2001) was used to identify samples with genotypes that matched at all loci, and the mtDNA haplotype and sex of animals with identical genotypes were used to confirm all identified matches. Although the majority of the genetic matches detected were collected from animals within the same region, several were collected from animals in different areas and may represent movements of individuals. The program GENEAP (Wilberg & Dreher, 2004) was used to calculate the probability of identity using the microsatellite genotypes. The probability of identity (PID) is defined as the probability that two individuals drawn randomly from the dataset will have the same genotype at multiple loci. This statistic was initially calculated under the assumption of Hardy-Weinberg equilibrium (PID_{HW} , Paetkau & Strobeck, 1994). However, such estimates may be biased in the presence of population structure. Calculations assuming the presence of full siblings within the dataset (PID_{SIB}) are considered more conservative (Waits *et al.* 2001) and were calculated using the formula of Evett & Weir (1998).

The match probability (*e.g.*, individual probability of identity) is defined as the probability that given the genotype of one individual, a second individual will have the same genotype. For the genetic matches between samples collected in different areas, the match probability was calculated for all identified pairs of duplicate genotypes both under the assumption of Hardy-Weinberg equilibrium and assuming that the two individuals were full siblings (Woods *et al.*, 2009).

RESULTS

Population Structure –

Forty mtDNA haplotypes defined by 38 variable sites were identified among the 377 gray whale samples for which mtDNA sequences were produced (Table 2). Haplotype diversity (h) was high in all of the ENP strata ($h = 0.952-0.967$) but was reduced in the WNP strata ($h=0.77$, Sakhalin; $h=0.80$, southeastern Kamchatka). Nucleotide diversity (π) was also similar across all strata, although slightly higher in the WNP strata (1.8-1.9%) than in the ENP strata (1.2 – 1.6%).

The number of individuals with each haplotype in each stratum is shown in Table 3, with data for the PCFG stratum taken from Lang *et al.*, 2011. Within the Sakhalin stratum, two haplotypes were found in very high frequencies, with 36% of sampled animals having haplotype 1 and 31% having haplotype 2. Within the ENP strata, no haplotypes were found in frequencies greater than 14%. The three highest frequency haplotypes in the PCFG stratum were found in 10% and 13% of individuals.

Within the Sakhalin stratum, the number of known mother-calf pairs with each haplotype, relative to the total number of animals with each haplotype, is shown in Figure 2. Of the 51 animals with haplotype 1, 59% were part of a known mother-calf pair, while 66% of the individuals with haplotype 2 ($n=44$) were animals from a known mother-calf pair. With one exception, all haplotypes that were identified in more than two sampled animals in the Sakhalin stratum are composed of at least one known mother-calf pair.

The median-joining network shows the relationship among mtDNA haplotypes and their frequency in each

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stratum (Figure 3). MtDNA haplotypes identified among animals feeding off Sakhalin are dispersed throughout the network, and no phylogeographic pattern is apparent.

Measures of microsatellite diversity for each stratum after averaging across the eight loci common to both datasets are shown in Table 4. Nuclear diversity was similar across all strata. None of the tests for HWE were significant after the correction for multiple tests was applied. Significant linkage disequilibrium was found for two pairs of loci (one in the “Sakhalin” stratum and one in the “north of the Aleutians” stratum) after the correction for multiple tests was applied. No significant LD was found for these two loci pairs in any of the other strata, so these loci were retained for the analysis.

The results of the mtDNA comparisons are shown in Table 5. All comparisons between ENP strata and the Sakhalin stratum were highly significant ($p < 0.001$). No significant differentiation was found when the CA->AK stratum was compared to either Chukotka ($F_{ST} = 0.010$, $p = 0.0883$; $\Phi_{ST} < 0.001$, $p = 0.5009$; χ^2 $p = 0.5466$) or the combined set of all samples collected north of the Aleutians ($F_{ST} = 0.005$, $p = 0.1488$; $\Phi_{ST} = 0.001$, $p = 0.3477$; χ^2 $p = 0.4049$).

Similar results were found in the microsatellite comparisons (Table 6). With one exception (Sakhalin versus all samples collected north of the Aleutians, Jost's $D = 0.0004$, $p = 0.1169$), all other comparisons between the ENP strata and the Sakhalin stratum were highly significant ($p < 0.001$ to $p = 0.002$). No significant differentiation was identified when strata within the ENP were compared.

Genetic Matches

Of the 380 samples genotyped, seven pairs of samples were identified that were collected in different areas and that shared identical microsatellite genotypes, mtDNA haplotypes, and sexes. These genetic matches included four matches between samples collected from the Sakhalin feeding ground and samples collected from southeastern Kamchatka, one match between an animal sampled off San Diego, California and an animal killed in the Chukotka hunt in Russia, and two matches between animals sampled off Sakhalin and animals sampled off the coast of southern California (Figure 1). The average probability of identity based on allele frequencies of all sampled animals was 1.35×10^{-8} (PID_{HW}) and 7.50×10^{-4} (PID_{SIB}) for the 8-loci dataset.

The individual match probabilities are shown in Table 7. For the 8-loci dataset, the individual match probabilities ranged from 6.74×10^{-4} to 1.24×10^{-3} (P_{SIB}) and 9.10×10^{-10} to 1.16×10^{-8} (P_{HW}). There were no samples that mismatched at only 1 allele but two pairs of samples were identified that mismatched at only 2 alleles and had identical mtDNA haplotypes and sexes. However, both pairs were part of the Sakhalin dataset analyzed in Lang *et al.* 2010, and when the 13-loci genotypes were compared the two pairs differed at 6 and 7 loci. Genotypes for the additional five loci are currently being generated for the Lang *et al.* 2011 dataset.

Although the 8-loci match probabilities assuming HW equilibrium were relatively low, the more conservative P_{SIB} measures are high, suggesting probabilities as great as 1 in 1000 that two individuals could share the same genotype by chance. Although P_{SIB} values are considered overly conservative in most cases (Rewe *et al.*, 2011), the genetic matches based on the 8-loci dataset should be considered only preliminary evidence of movements until the matches can be corroborated with the addition of more loci. However, although no photographs exist for the animal(s) included in the San Diego-Chukotka match, photographs were collected of the animals biopsied off Kamchatka. Comparison of these photographs to the Sakhalin photo-identification catalogue maintained by the joint Russia-U.S. research program verified that in all four cases the genetic matches represented samples collected from the same animal.

The two sets of matching samples identified between Sakhalin and southern California were genotyped at 13 loci. For both pairs, the genotypes were heterozygous for 10 of the 13 loci genotyped, and all other samples in the 13-loci dataset had genotypes that mismatched at five ($n=1$ pair) or more loci. Precautions, including replication of genetic profiles after re-extraction of the DNA from the tissue, were taken to ensure that the matching genotypes were not an artifact of lab error (detailed in Lang, 2010). The power of the microsatellite panel used to discriminate between individuals was high when the 13-loci dataset was analyzed ($PID_{HW} =$

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8.65×10^{-14} ; $PID_{SIB} = 1.04 \times 10^{-5}$) and comparable to that used in other studies utilizing genetic tagging to infer movements of individuals between areas (*e.g.*, $PID_{Dave} = 1.51 \times 10^{-7}$, Palsboll *et al.* 1997; $PID_{sib} = 2.8-3.11 \times 10^{-5}$, Pomilla and Rosenbaum 2005;). The more conservative estimates of the match probabilities (P_{SIB}) calculated from the gray whale microsatellite data suggest that the probability of finding two different animals with these identical genotypes is 1/50,000 (for the match between females) and 1/100,000 (for the match between males). The less conservative measures (P_{HW}) suggest that the probability is almost infinitesimally small. It is likely that the true probability lies somewhere between these two estimates, given that there is evidence for population structure in our data but it is unlikely that many full siblings were represented. Based on these considerations, the probability that the two sets of matching genotypes identified between the eastern and western North Pacific are an artifact of laboratory errors or a lack of resolution in the markers used is small.

Both of the biopsies from the ENP were collected during the same expedition. Sample #3947 was collected on 20 March 1995, and sample #3950 on 23 March 1995. Both samples were taken from whales in the Santa Barbara Channel off southern California, and the timing and location indicates that the samples were likely collected from animals which would have been migrating north after overwintering in the ENP. Although some video was taken during this sampling trip, it proved to be of too poor resolution to be useful in photographic comparisons. Sample #12186 was collected from an animal on the Sakhalin feeding ground on 14 August 1998. Photo-identification records link this whale to an animal that was first identified in that area on 19 August 1995. This male was sighted off Sakhalin during ten of the 12 seasons covered by the study. Sample #50728 was collected from a whale on the Sakhalin feeding ground on 27 August 2004. Photo-identification was used to link this animal to a whale first identified in that area in 1999. This female was then sighted off Sakhalin in all subsequent years of the study. Extensive photo and video documentation exists for both of these animals.

DISCUSSION

Population Structure –

The genetic signal of matrilineal fidelity among the whales sampled off Sakhalin is apparent both in the measures of mtDNA differentiation and in the distribution of haplotypes among individuals. Similar to the results of previous comparisons of samples collected in the WNP with samples obtained primarily from whales on migratory routes in the ENP (LeDuc *et al.*, 2002; Lang *et al.*, 2010), mtDNA comparisons between the Sakhalin stratum and strata comprised of animals feeding north of the Aleutians were highly significant. The magnitude of mtDNA differentiation between the Sakhalin stratum and the ENP strata (WNP v. North, $F_{ST}=0.086$, $p<0.0001$; Fisher's exact test, $p<0.0001$; WNP v. Chukotka, $F_{ST}=0.082$, $p<0.0001$, Fisher's exact test, $p<0.0001$) is greater than that seen in comparisons between whales utilizing different feeding grounds within the ENP (PCFG v. North, $F_{ST}=0.01$, $p=0.005$; Fisher's exact test, $p=0.008$; PCFG v. Chukotka, $F_{ST}=0.01$, $p=0.012$; Fisher's exact test, $p=0.030$; Lang *et al.*, 2011).

As has been previously described (LeDuc *et al.*, 2002; Lang *et al.*, 2010), the distribution of haplotypes among sampled individuals in the Sakhalin stratum is highly skewed, with two haplotypes found in very high frequencies, representing 36% and 31% of all animals sampled from that area. Given the maternal inheritance of mtDNA, this pattern would be expected if utilization of this area was driven in large part by the continued return over time of a small number of females and their offspring (and eventually their offspring's offspring). Examination of the haplotypes carried by sampled individuals revealed that 16 of the 23 known reproductive females identified between 1995 and 2007 (Weller *et al.*, 2008a) share one of these two common haplotypes (Lang, 2010), and known mother-calf pairs comprise a large proportion of animals with the two common haplotypes. Within any of the strata representing the ENP, there were no haplotypes found in frequencies greater than 14% of sampled individuals. This was also true of the PCFG, in which the three highest frequency haplotypes were found in 10 and 13% of sampled animals (Lang *et al.*, 2011).

Similar to results of previous comparisons of samples collected in the WNP with those obtained primarily from animals along the migratory route in the ENP (based on $n=13$ loci, Lang *et al.*, 2010), significant levels of

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nuclear differentiation were also found between animals feeding north of the Aleutians and those feeding off of Sakhalin. Although highly statistically significant, the magnitude of nuclear differentiation was relatively low, which has led to speculation that some limited degree of dispersal or gene flow could be occurring between the WNP and ENP populations (LeDuc *et al.*, 2002; Lang *et al.*, 2010; Lang, 2010). However, the significant differences identified in both the mtDNA and the microsatellite comparisons indicate that the group of animals feeding off Sakhalin is not only demographically independent from animals feeding in the eastern North Pacific, but also that a degree of reproductive isolation is occurring. This differs from what has been reported thus far within the ENP (Lang *et al.*, 2011), where comparisons of microsatellite differentiation have suggested that animals feeding in different areas of the ENP may interbreed.

One limitation of this analysis is that all of the WNP samples were collected from animals on feeding grounds. A preliminary mtDNA analysis of six samples collected from gray whales (five females and one of unknown sex) which were stranded or entrapped in Japanese coastal waters found high haplotype ($h=0.933$) and nucleotide ($\pi=1.85\%$) diversity (Kanda *et al.*, 2010). No significant differentiation was found when these Japanese samples were compared to their samples ($n=7$) collected from whales taken in the hunt off Chukotka, although the small sample sizes used in the comparison limited the conclusions that could be drawn (Kanda *et al.*, 2010). One of the whales included in the analysis was the animal that was entrapped off the Pacific coast of Japan in January 2007 and that had previously been identified off Sakhalin. This animal has a haplotype (Haplotype 2 or B) which is common among animals sampled off Sakhalin. Although one of the other samples analyzed had a haplotype (Haplotype 1 or A) that is found in high frequencies among animals sampled off Sakhalin, the other four animals had haplotypes which have been identified in only one or two animals sampled off Sakhalin.

Movements –

Within the WNP, comparison of the genetic profiles of sampled animals indicates that four of the ten whales biopsied off southeastern Kamchatka were also sampled while on the Sakhalin feeding ground. These four genetic matches, which were confirmed photographically, include samples collected from two animals (one male and one female) first identified as calves on the Sakhalin feeding ground in 2007, indicating that these animals would have been ~3 years old when they were sampled off Kamchatka in 2010. The mother of one of these calves is also the mother of one of the animals first identified as a calf off Sakhalin and later photographed in the ENP (Weller *et al.*, 2011). The other two samples were collected from males first identified as non-calves off Sakhalin in 1994 and 1995. One of these males has also been photographed in the ENP (Weller *et al.*, 2011). The remaining six samples could not be matched to the genetic profiles of whales sampled off Sakhalin. These samples may represent animals that do not utilize the Sakhalin feeding ground, or they could be from animals known to feed off Sakhalin but from which no genetic sample has been collected. These results are consistent with patterns identified in photo-identification comparisons between southeastern Kamchatka and Sakhalin, in which 61 of the 116 animals identified off Kamchatka between 2004 and 2009 had also been sighted on the Sakhalin feeding ground (Tyurneva *et al.*, 2010). Photo-identification work has also documented the use of the southeastern Kamchatka area by young whales (Tyurneva *et al.*, 2010; Burdin *et al.*, 2011).

The detection of two pairs of matching samples between the ENP and WNP was more surprising. If these genetic matches do represent movements of individuals, they suggest that these animals, both of which are known to demonstrate fidelity to the western Pacific during the feeding season, have overwintered in the eastern Pacific for at least one season. Although the results presented here, as well as those from previous studies (LeDuc *et al.*, 2002; Lang *et al.*, 2010), have confirmed that the eastern and western gray whale populations are genetically differentiated, the relatively low level of differentiation observed at nuclear markers suggested that some degree of dispersal of eastern animals onto the western feeding ground may occur (Lang *et al.* 2010, Lang, 2010). In the past, this dispersal was hypothesized to have been mediated largely by males, as supported by observations that the majority of haplotypes found in only one or two individuals are represented only by males (LeDuc *et al.*, 2002; Lang *et al.*, 2010) as well as by the lower levels of differentiation found when only males were compared between ENP and WNP strata (Lang, 2010). However, the putative movements associated with these genetic matches suggest that both males and females

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may travel between these two areas. Other factors, such as age and oceanographic conditions, may also influence any movements.

In addition, the female associated with sample #50728 is a known reproductive female and was identified (both behaviorally and genetically) as the mother of three calves between 2003 and 2007. Although two of her calves were not assigned a putative father among the sampled western animals in the paternity analysis, her 2007 calf was assigned a putative father that has been sighted regularly on the Sakhalin feeding ground (Lang, 2010). The paternity analysis assigned the male associated with sample #12186 as the putative father of a calf born in 2007 (Lang, 2010). This calf was one of the two animals first identified as calves off of Sakhalin which were genetically and photographically matched to the southeastern coast of Kamchatka in 2010. This evidence suggests that both of the whales sampled off Sakhalin interbred at least one time with other animals which feed in the WNP.

Even if all 130 of the animals which feed off Sakhalin were to have visited the eastern Pacific during the study period, it would seem unlikely that one of the western animals would have been sampled given the size of the eastern population and the relatively low proportion of animals sampled in the eastern Pacific. If samples obtained from stranded or harvested animals in the ENP prior to 1995 ($n=24$) are excluded, samples were obtained from only 203 individuals in the ENP during the period of time (*i.e.*, in 1995 or later) in which an animal identified on the western feeding ground could have feasibly been sampled (Table 1). Only 48 of these samples were collected via biopsy-darting, while the rest were collected from animals which were stranded, hunted, or taken in fishing gear. The probability of sampling one of ~ 130 Sakhalin animals among an estimated 19,000 eastern gray whales is approximately $1/150$; assuming the two events are independent, the probability of capturing two Sakhalin animals is approximately $1/22500$. These probabilities would be even lower if only a small number of the animals identified in the WNP travel to the ENP.

However, if the genotype matches do represent dispersal of whales between Sakhalin and the ENP, the short time span over which the two samples were collected in the ENP raises questions about the independence of the two events. One possible explanation is that some segregation takes place on the migratory route relative to the feeding location. Southbound migration timing has been correlated with feeding ground origin in North Atlantic humpback whales (Stevick *et al.*, 2004), which utilize two different feeding areas but a common breeding area in the West Indies. Animals that fed in the Gulf of Maine and eastern Canada had earlier mean sighting dates in the West Indies than did animals known to feed in Greenland, Iceland, and Norway, suggesting that the migration from feeding area to breeding area might be segregated. Although stratification of the gray whale migration relative to age, sex, and reproductive status is known to occur (Rice & Wolman 1971), no additional mechanisms for segregation have been identified.

Weller *et al.* (2011) presents the results of a comparison between the Sakhalin photo-identification catalogue and a catalogue of whales photographed in the Pacific Northwest that is maintained by Cascadia Research Collective. The results of this photographic comparison were similar in several regards to the findings from the genetic comparison. Six animals that were photographed off Sakhalin were also identified in the Pacific Northwest during months (late April and early May, when field efforts in this area are relatively low) when the animals would have been migrating north. Three of the animals were photographed on one day, while the other three were photographed on another day, further suggesting that some segregation with respect to feeding ground origin may occur on the northbound migration. The combined findings indicate that, despite the low probability of sampling or photographing one of ~ 130 Sakhalin animals while the majority of the 19,000 whales comprising the eastern population are migrating, as many as eight animals have been either photographed or sampled despite relatively low field effort. These results suggest that the potential for photographing or sampling a Sakhalin whale, at least while on the northbound migration, is higher than would be expected based on the assumption of random intermixing of animals with eastern versus western summer destinations.

While estimates of genetic differentiation support demographic independence and a degree of reproductive isolation between whales feeding in the WNP and the ENP, information from tagging¹, photo-identification comparisons (Weller *et al.*, 2011), and genetic comparisons suggest that some of the animals summering off

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Sakhalin overwinter in the ENP in at least some years. Conception in gray whales is thought to primarily occur during a three week period from late November to early December (Nov 27 – Dec 13), although if no conception occurs during this first period, a second estrus may occur about 40 days later when whales are on or near their wintering grounds (Rice & Wolman, 1971). Rugh *et al.* (2001) estimated that the median (peak) sighting date for the southbound migration in the ENP is 12 December for Unimak Pass, Alaska, suggesting that many animals from the ENP are north of the Aleutians during the first mating period. With the exception of the 13 year-old male (“Flex”) that was tagged off Sakhalin this past fall and remained off northeastern Sakhalin until early December⁵, little is known about the current migratory timing of and route(s) used by any whales traveling between Sakhalin and the ENP. However, it is plausible that animals making this journey would be relatively far west during the first mating period, suggesting a mechanism by which some degree of reproductive isolation could develop between animals feeding off Sakhalin and those feeding in eastern areas even if they shared a common wintering destination.

Recent records of gray whales off Japan, however, indicate that some whales remain in the WNP during winter months. Since 1955, there have been 19 reports of gray whales in Japanese waters, most of which (n=13) occurred in 1990 or later (Kato *et al.*, 2010). These reports span the months of November through August, although the majority (n=11) were recorded between March and May, when animals would likely be migrating north. At least one of the whales, an approximately one year old female that was entrapped off the coast of Honshu in January 2007, is known to have also visited Sakhalin with her mother the summer prior to her entrapment (Weller *et al.*, 2008b). Although it is not known what proportion of the other gray whales reported in Japanese waters were also animals that visited Sakhalin, this link indicates that not all of the gray whales feeding off Sakhalin show fidelity to wintering destinations in the ENP.

These observations suggest that population structure in gray whales may be more complex than previously thought, such that animals utilizing the Sakhalin feeding ground may not all share a common wintering ground. Photo-identification and genetic studies of humpback whales in the North Pacific have revealed similar, albeit more complicated, patterns (Calambokidis *et al.*, 2008; Baker & Steel, 2010). Significant differences in mtDNA haplotype frequencies (overall $F_{ST} = 0.179$) have been used to define 7 different “eco-stocks” among the feeding grounds. Differences in both mitochondrial and nuclear DNA have also been used to delineate five reproductive units or “breeding stocks” on the wintering grounds (overall $F'_{ST} = 0.034$ for nuclear comparisons, Baker & Steel, 2010). However, some feeding grounds (e.g., Kodiak) are comprised of individuals from different breeding stocks (Calambokidis *et al.*, 2008).

Conclusion

Although questions remain about the movements of Sakhalin gray whales when they are not on the feeding range, the significant mtDNA and nuclear genetic differences between animals utilizing the Sakhalin feeding ground and those summering in the ENP support the continued recognition of the Sakhalin animals as a distinct unit. Additional satellite tagging of Sakhalin gray whales, along with continued collection and analysis of photo-identification and genetic data, especially from Japan, is needed to address these questions. If some proportion of the animals that feed off Sakhalin overwinter in the ENP, then the number of animals remaining in the WNP year-round may be smaller than previously estimated, and the impact of potential threats to this group of animals may be greater than predicted. As such, learning more about migratory routes and wintering grounds in the WNP should be a priority of future work.

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⁵ <http://mmi.oregonstate.edu/Sakhalin2010>

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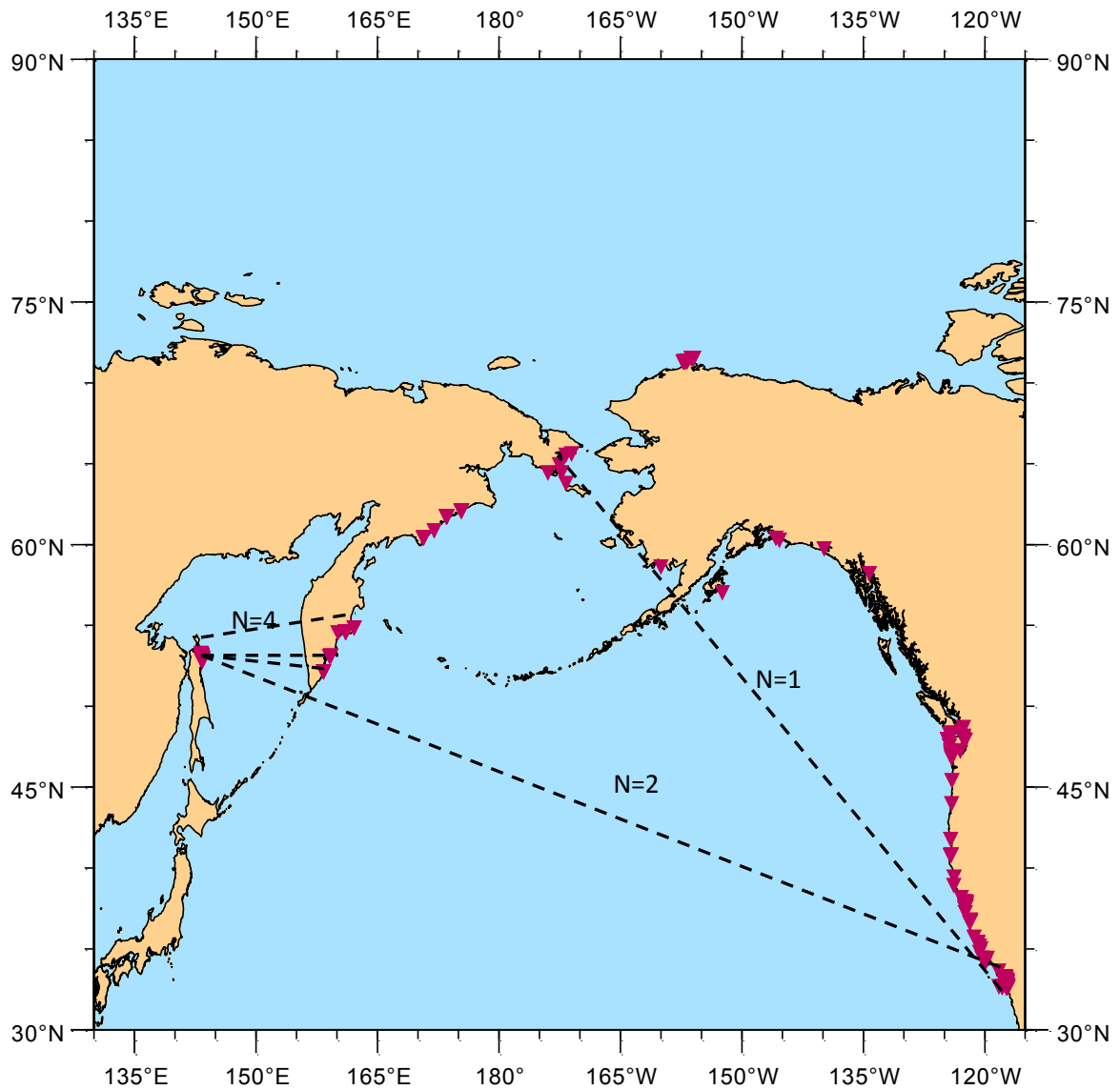
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Figure 1. Map showing collection locations for samples analyzed in the study. Lines link recapture locations of genetic matches but do not denote movement tracks of animals. Text above lines indicates the number of matching genotypes between areas.



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Table 1. List of genetic samples used in the study which were collected from gray whales in the eastern North Pacific. The year of sample collection, source of tissue, and designated strata are shown

YEAR	Biopsy		Strand		Fishery	Harvest	Total
	CA->AK	N of Aleutians	CA->AK	N of Aleutians	CA->AK	N of Aleutians	
?						1	1
1979			1				1
1990			1				1
1992			5				5
1993			2				2
1994			2			12	14
1995	6						6
1996			1				1
1997			4	1			5
1998			13		1		14
1999			13	1	1		15
2000			41	1			42
2001	2		1	1		25	29
2002			3				3
2003	2		3			13	18
2004			8	1		11	20
2005	3		1	1	1	9	15
2006	6		1				7
2010		29					29
Total	19	29	100	6	3	71	228

Table 2. Genetic diversity estimates for each strata based on mtDNA control region sequences (523 bp in length). For haplotype diversity and nucleotide diversity, standard deviations are included in parentheses.

Region	Strata	No. of Samples	No. of Haplotypes	Haplotype Diversity (h)	Nucleotide Diversity (π)
ENP	North of Aleutians	103	32	0.952 (+/- 0.008)	0.0141 (+/- 0.007)
	Chukotka	69	27	0.953 (+/- 0.011)	0.0142 (+/- 0.007)
	Barrow	14	11	0.967 (+/- 0.037)	0.0123 (+/- 0.007)
	CA->AK	122	34	0.956 (+/- 0.006)	0.0162 (+/- 0.008)
WNP	Sakhalin	142	22	0.770 (+/- 0.025)	0.0182 (+/- 0.009)
	SE Kamchatka	10	5	0.800 (+/- 0.100)	0.0192 (+/- 0.011)
All		377†	40	0.914 (+/- 0.008)	0.0177 (+/- 0.009)

† “North of the Aleutians” includes samples from both Chukotka and Barrow

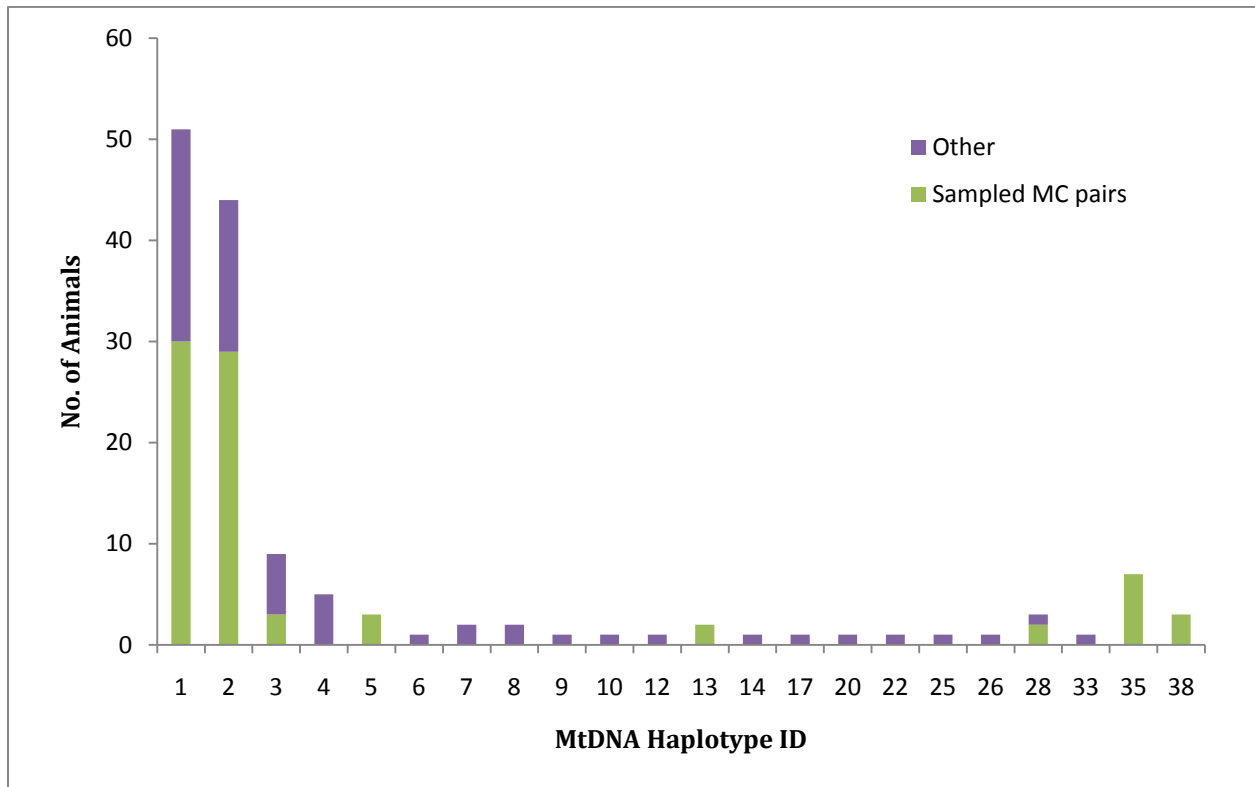
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Table 3. The number of individuals with each haplotype in each stratum. Data for the PCFG stratum, which is taken from Lang *et al.*, 2011, is included for comparative purposes.

MtDNA Haplotype	Strata					
	Barrow	Chukotka	CA->AK	SE Kamchatka	Sakhalin	PCFG
1	2	8	13	3	51	7
2		2	11	4	44	4
3	1	9	9		9	1
4		4	6		5	6
5		1	4		3	1
6					1	
7		4	8		2	6
8		1	1		2	2
9		1			1	
10				1	1	
11	1	2	4			3
12	1	4	5		1	3
13		3	6		2	9
14		1	5		1	7
15	2					
16	1		2			
17			1		1	
18		3	7			2
19			1			
20	2	1	7		1	2
21	1	1	3	1		3
22		1	3		1	
23		4	1			
24		2	5			3
25		4	2	1	1	1
26	1	1	1		1	
27			2			4
28		2	2		3	2
29		2	1			
30			3			1
31		1	1			
32			1			
33		4	1		1	1
34			1			
35	1		1		7	
36	1		2			1
38		1			3	
41			1			
42		1	1			
43		1				
46						1
47						1
Total	14	69	122	10	142	71

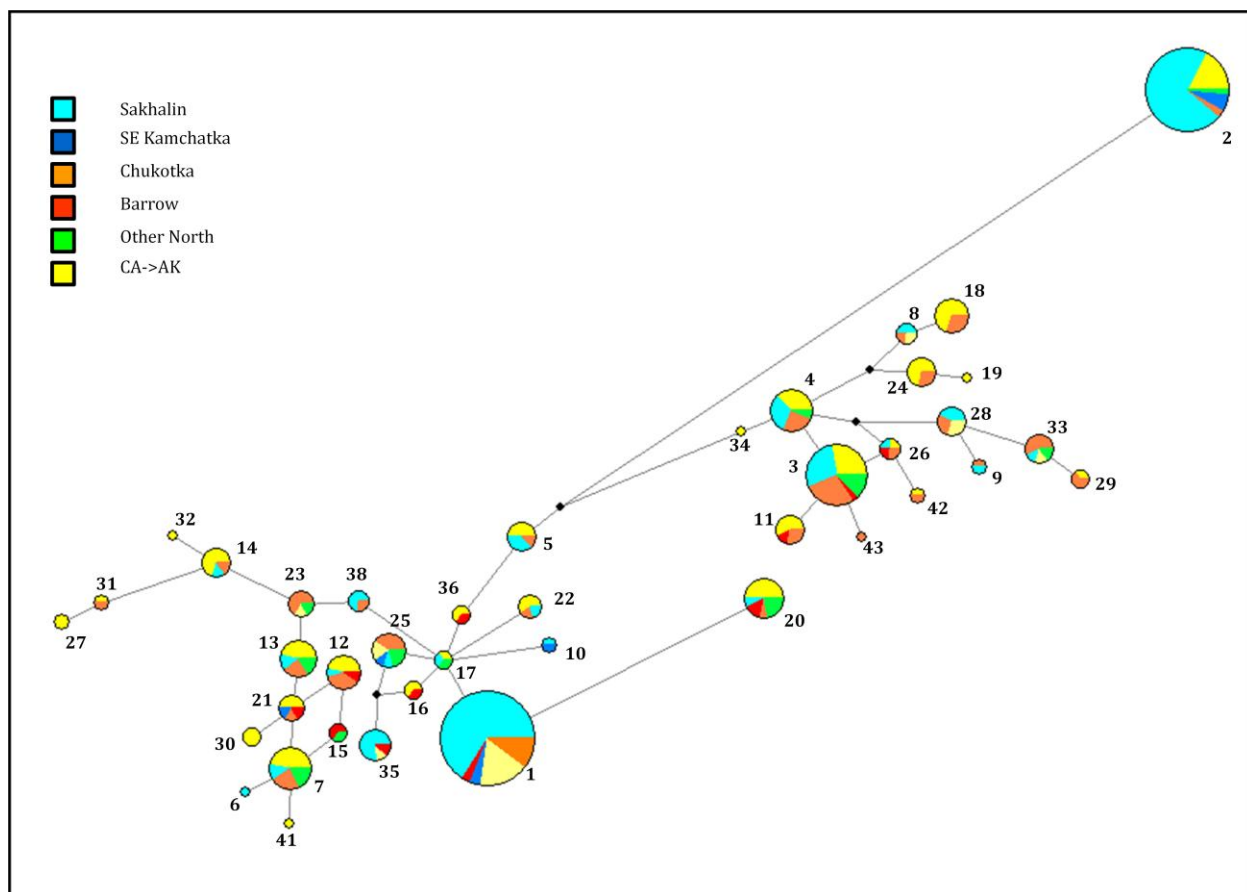
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Figure 2. Graph showing the distribution of mtDNA haplotypes in the Sakhalin stratum. Known mother-calf pairs with each haplotype are represented in green.



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Figure 3. Median joining network for the mtDNA haplotypes.



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Table 4. Genetic diversity measures for the nuclear DNA data set based on 8 microsatellite loci, including the mean number of alleles, and mean expected and observed heterozygosity.

Region	Strata	No. of Samples	Mean No. of alleles	Mean He	Mean Ho
ENP	North of Aleutians	106	8.25	0.746	0.728
	Chukotka	71	7.88	0.748	0.737
	Barrow	14	5.00	0.723	0.705
	CA->AK	122	8.88	0.746	0.747
WNP	Sakhalin	142	7.75	0.712	0.724
	SE Kamchatka	10	5.13	0.730	0.713
All		380†	9.38	0.739	0.737

† “North of the Aleutians” includes samples from both Chukotka and Barrow

Table 5. Results of MtDNA comparisons across strata, with sample sizes shown in parentheses. Significant p-values are highlighted in bold.

Pairwise Comparison	Φ_{ST}	p-value	F_{ST}	p-value	χ^2 p-value
Sakhalin (142) v. Chukotka (69)	0.150	<0.0001	0.082	<0.0001	<0.0001
Sakhalin (142) v. North of Aleutians (103)	0.152	<0.0001	0.086	<0.0001	<0.0001
Sakhalin (142) v. CA->AK (122)	0.100	<0.0001	0.065	<0.0001	<0.0001
CA->AK (122) v. Chukotka (69)	0.010	0.0883	<0.001	0.5009	0.5466
CA->AK (122) v. North of Aleutians (103)	0.005	0.1488	0.001	0.3477	0.4049

Table 6. Results of microsatellite comparisons (n=8 loci) across strata, with sample sizes shown in parentheses. Significant p-values are highlighted in bold.

Pairwise Comparison	F_{ST}	p-value	F'_{ST}	p-value	Jost's D	p-value	χ^2 p-value
Sakhalin (142) v. Chukotka (71)	0.010	0.0010	0.037	0.0010	0.008	0.0020	0.0010
Sakhalin (142) v. N of Aleutians (106)	0.010	0.0010	0.037	0.0010	0.000	0.1169	0.0010
Sakhalin (142) v. CA->AK (122)	0.008	0.0010	0.028	0.0010	0.018	0.0010	0.0010
CA->AK (122) v. Chukotka (71)	-0.001	0.7053	-0.004	0.7123	-0.001	0.7542	0.8951
CA->AK (122) v. N of Aleutians (106)	-0.001	0.8362	-0.005	0.8492	-0.001	0.8661	0.9820

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Table 7. Match probabilities, as calculated in GENECAAP, for pairs of identical genotypes identified in the gray whale microsatellite data. PHW refers to probabilities calculated assuming Hardy-Weinberg equilibrium, while PSIB refers to probabilities calculated assuming that the two individuals are full siblings. Information on the location and date the sample was collected, the mtDNA haplotype, sex, and number of microsatellite loci compared is included. All samples were collected via biopsy of live whales except where noted.

Labid	Location Sampled	Date Sampled	MtDNA Hap	Sex	No. of Loci Compared	Sib Prob	HW Prob																																																																											
15164	Sakhalin Island, Russia	8/22/1999	1	M	8	9.07×10^{-4}	2.21×10^{-09}																																																																											
100791	SE Kamchataka, Russia	7/10/2010	1	M				72878	Sakhalin Island, Russia	7/27/2007	2	F	8	1.12×10^{-3}	2.21×10^{-08}	100792	SE Kamchataka, Russia	8/26/2010	2	F	72884	Sakhalin Island, Russia	8/19/2007	2	M	8	1.06×10^{-3}	9.39×10^{-09}	100765	SE Kamchataka, Russia	6/18/2010	2	M	19050	Sakhalin Island, Russia	8/10/2000	2	M	8	1.24×10^{-3}	1.16×10^{-08}	100790	SE Kamchataka, Russia	7/10/2010	2	M	100735	Chukotka, Russia	Summer 2001*	12	M	8	6.74×10^{-4}	9.10×10^{-10}	18838	San Diego, CA	1/21/2001	12	M	3950	Santa Barbara Channel, CA	3/23/1995	2	M	13	2.10×10^{-5}	1.87×10^{-13}	12186	Sakhalin Island, Russia	8/14/1998	2	M	3947	Santa Barbara Channel, CA	3/20/1995	35	F	13	1.06×10^{-5}	5.43×10^{-15}	50728	Sakhalin Island, Russia
72878	Sakhalin Island, Russia	7/27/2007	2	F	8	1.12×10^{-3}	2.21×10^{-08}																																																																											
100792	SE Kamchataka, Russia	8/26/2010	2	F				72884	Sakhalin Island, Russia	8/19/2007	2	M	8	1.06×10^{-3}	9.39×10^{-09}	100765	SE Kamchataka, Russia	6/18/2010	2	M	19050	Sakhalin Island, Russia	8/10/2000	2	M	8	1.24×10^{-3}	1.16×10^{-08}	100790	SE Kamchataka, Russia	7/10/2010	2	M	100735	Chukotka, Russia	Summer 2001*	12	M	8	6.74×10^{-4}	9.10×10^{-10}	18838	San Diego, CA	1/21/2001	12	M	3950	Santa Barbara Channel, CA	3/23/1995	2	M	13	2.10×10^{-5}	1.87×10^{-13}	12186	Sakhalin Island, Russia	8/14/1998	2	M	3947	Santa Barbara Channel, CA	3/20/1995	35	F	13	1.06×10^{-5}	5.43×10^{-15}	50728	Sakhalin Island, Russia	8/27/2004	35	F										
72884	Sakhalin Island, Russia	8/19/2007	2	M	8	1.06×10^{-3}	9.39×10^{-09}																																																																											
100765	SE Kamchataka, Russia	6/18/2010	2	M				19050	Sakhalin Island, Russia	8/10/2000	2	M	8	1.24×10^{-3}	1.16×10^{-08}	100790	SE Kamchataka, Russia	7/10/2010	2	M	100735	Chukotka, Russia	Summer 2001*	12	M	8	6.74×10^{-4}	9.10×10^{-10}	18838	San Diego, CA	1/21/2001	12	M	3950	Santa Barbara Channel, CA	3/23/1995	2	M	13	2.10×10^{-5}	1.87×10^{-13}	12186	Sakhalin Island, Russia	8/14/1998	2	M	3947	Santa Barbara Channel, CA	3/20/1995	35	F	13	1.06×10^{-5}	5.43×10^{-15}	50728	Sakhalin Island, Russia	8/27/2004	35	F																							
19050	Sakhalin Island, Russia	8/10/2000	2	M	8	1.24×10^{-3}	1.16×10^{-08}																																																																											
100790	SE Kamchataka, Russia	7/10/2010	2	M				100735	Chukotka, Russia	Summer 2001*	12	M	8	6.74×10^{-4}	9.10×10^{-10}	18838	San Diego, CA	1/21/2001	12	M	3950	Santa Barbara Channel, CA	3/23/1995	2	M	13	2.10×10^{-5}	1.87×10^{-13}	12186	Sakhalin Island, Russia	8/14/1998	2	M	3947	Santa Barbara Channel, CA	3/20/1995	35	F	13	1.06×10^{-5}	5.43×10^{-15}	50728	Sakhalin Island, Russia	8/27/2004	35	F																																				
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*This sample was collected from a harvested whale.