

# Assessment of stock structure among gray whales utilizing feeding grounds in the Eastern North Pacific

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## ABSTRACT

Although the majority of Eastern North Pacific (ENP) gray whales spend their summers feeding in the Bering, Beaufort, and Chukchi Seas, a small number of individuals, referred to as the Pacific Coast Feeding Group (PCFG), feed in waters between northern California and southeastern Alaska during summer and fall. Many individuals identified within this southern feeding area demonstrate intra- and inter-seasonal fidelity to the region, suggesting that structure could be present among ENP gray whales utilizing different areas for feeding. Little is known, however, about patterns of site fidelity of individuals feeding in northern waters. We utilized samples collected from individual gray whales within both southern (n=100) and northern (n=106) feeding areas to assess possible stock structure using both mtDNA control region sequences and 8 microsatellite markers. Significant mtDNA differentiation was found when the subset of samples representing individuals (n=71) sighted over two or more years within the seasonal range of the PCFG were compared to the combined set of samples collected from the northern feeding area(s) ( $F_{ST}=0.01$ ,  $p=0.005$ ; Fisher's exact test,  $p=0.008$ ) as well as when the PCFG samples were compared to only those samples which were collected off Chukotka, Russia (n=71,  $F_{ST}=0.01$ ,  $p=0.012$ ; Fisher's exact test,  $p=0.030$ ). No significant differences were found for any of the comparisons utilizing microsatellites. These results indicate that structure is present among gray whales utilizing different feeding areas and suggest that matrilineal fidelity plays a role in creating such structure. The lack of differentiation detected using nuclear markers ( $\chi^2$  test,  $p=0.636$ , PCFG versus northern;  $p=0.753$ , PCFG versus Chukotka) suggests that individuals from different feeding areas may interbreed. These results are important in evaluating the management of the ENP gray whale population, especially in light of the Makah Tribe's proposal to resume whaling in an area of the Washington coast utilized by both feeding and migrating whales. Although the proposed hunt is designed to target whales migrating to/from the northern feeding grounds, the possibility of taking a PCFG whale cannot be eliminated. Increasing our understanding of recruitment into this group is needed to assess potential impacts of a hunt.

## INTRODUCTION

The current distribution of gray whales is limited to the eastern and western margins of the North Pacific (Rice & Wolman, 1971), where a small western population (~130 individuals, Cooke *et al.*, 2008) and a much larger eastern population (~19,000 individuals based on surveys in 2006/2007, Laake *et al.*, 2009) are recognized. Much of what is known about the western population is derived from photo-identification and genetic studies of individuals on the population's primary feeding ground, which is located in the coastal waters of northeastern Sakhalin Island, Russia (Weller *et al.*, 1999; Weller *et al.*, 2008; 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 in years 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). Genetic comparisons of samples collected from gray whales feeding off Sakhalin 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).

Gray whales in the ENP population feed in waters between California and the Bering, Beaufort, and Chukchi Seas during summer and fall. Most of the population then migrates south along the coast of North America to overwinter in the lagoons and coastal waters of Baja Mexico. Three primary calving lagoons are utilized, with some females known to make repeated returns to specific lagoons (Jones, 1990). Genetic studies have demonstrated small but significant mtDNA differentiation between females (mothers with calves) utilizing two of the primary calving lagoons and females sampled in other areas (Goerlitz *et al.*, 2003). An additional study, utilizing both mtDNA and microsatellites with samples collected from all three of the primary calving lagoons, also identified small but significant departure from panmixia between two of the lagoons using nuclear data, although no significant differences were identified using mtDNA (Alter *et al.*, 2009).

Sub-structuring within the feeding range of the eastern population could also be present. Although little is known about fidelity of gray whales feeding north of the Aleutians, a small number of individuals, referred to as the Pacific Coast Feeding Group (PCFG; IWC, 2010), are known to show fidelity to more southern feeding grounds located in the coastal waters between northern California and southeastern Alaska (Gilmore, 1960; Pike, 1962; Hatler & Darling 1974; Darling, 1984; Calambokidis *et al.*, 2002, 2010). Within these waters, photo-identification research, which commenced in the early 1970s, has identified some whales that demonstrate consistent return to specific areas within this larger region, although movements between areas within the region also occur regularly (Hatler & Darling, 1974; Darling, 1984; Calambokidis *et al.*, 2002, 2010). In addition, photographic evidence has shown that some whales considered part of the PCFG move at least as far north as Kodiak Island, Alaska (Calambokidis *et al.*, 2010, Gosho *et al.*, 2011). Recent estimates of the annual abundance of the PCFG suggest that at most a few hundred individuals utilize this feeding area (Calambokidis *et al.*, 2010).

Satellite tagging studies of 18 whales off the coast of Oregon and California have provided additional information on the movements of individual whales considered part of the PCFG (Mate *et al.*, 2010). Although the duration of tag attachment differed between individuals, movement patterns of the tagged animals were variable, with some individuals remaining in a relatively small area within the larger PCFG seasonal range and others traveling more widely. Only two of the eighteen whales moved north of Washington while tagged; one of these animals traveled at least as far north as southeastern Alaska (Mate *et al.*, 2010). All six of the individuals whose tags continued to transmit through the southbound migration utilized the wintering area within and adjacent to Laguna Ojo de Liebre. Although this lagoon is by far the most heavily used of the three major wintering lagoons, these results raised the possibility that PCFG whales may demonstrate philopatry to this particular wintering area (Mate *et al.*, 2010).

Concern for the PCFG of gray whales has stemmed in part from recent interest in the resumption of whaling by the Makah Tribe in northwest Washington, an area used by migrating whales as well as by whales considered part of the PCFG. The current proposal by the Makah Tribe includes time/area restrictions which will limit the hunt to between 1 December and 31 May and will not allow hunting in the Strait of Juan de Fuca

east of Cape Flattery. The Makah Tribe also proposes to compare photographs of any whales harvested in the hunt to a photo-identification catalogue of known PCFG whales and to suspend the hunt for the year if the number of known PCFG gray whales struck is equal to the annual allowable bycatch level calculated for the PCFG (Makah Tribal Council, 2011). These restrictions are designed to reduce the probability of killing a PCFG whale and to focus the hunt on whales migrating to/from feeding areas north of the PCFG. Nevertheless, it is impossible to ensure that no PCFG whales would be killed. Evaluating whether such kills would, over time, have the potential to deplete the PCFG requires an understanding of how individuals are recruited into the group. If recruitment into the area is exclusively driven by calves learning the location of feeding grounds from their mothers (i.e., internally), then a PCFG individual that is removed would not be replaced by immigration. However, if recruitment is largely external, such that some whales stop to feed during the migration and then return to the PCFG area as their primary feeding destination in subsequent years, then it is likely that any takes from the PCFG would be offset by immigration into the group by whales that in previous years fed in northern areas.

Understanding recruitment into the PCFG is relevant to management under the Marine Mammal Protection Act (MMPA). The goal of the MMPA is to maintain population stocks as functioning elements of their ecosystem. The National Marine Fisheries Service considers stocks to be demographically independent units, such that the population dynamics of the affected group is more a consequence of births and deaths within the group (internal dynamics) rather than of immigration or emigration (external dynamics). Thus, the exchange of individuals between population stocks is not great enough to prevent the depletion of one of the populations as a result of increased mortality or lower birth rates (NMFS, 2005).

Previous genetic studies of the PCFG whales have focused on evaluating patterns of recruitment. Initial work utilizing a simulation-based approach indicated that if the PCFG originated from a single recent colonization event in the past 40 to 100 years, with no subsequent external recruitment into the group, detectable mtDNA genetic differentiation would be generated (Ramakrishnan & Taylor, 2000). Subsequent empirical analysis, however, failed to detect such a signal when comparing 16 samples collected from known PCFG whales utilizing Clayoquot Sound, British Columbia, with samples (n=41) collected from individuals presumably feeding in more northern areas (Steeves *et al.*, 2001). Additional genetic analysis utilizing an extended set of samples (n=45) collected from whales within the range of the PCFG indicated that the level of genetic diversity and the number of mtDNA haplotypes identified were inconsistent with measures, based on simulations, which would be expected if recruitment into the group were exclusively internal (Ramakrishnan *et al.*, 2001). However, both simulation-based studies focused on evaluating only the hypothesis of founding by a single and recent colonization event and did not evaluate alternative scenarios, such as limited dispersal of whales from other areas into the PCFG, which could have implications for management (Ramakrishnan and Taylor 2000, Ramakrishnan *et al.*, 2001). More recently, Frasier *et al.* (In press) have shown significant levels of mtDNA differentiation when comparing samples collected from 40 individuals considered part of the PCFG with published data generated from 104 samples collected from ENP gray whales, most of which stranded along the migratory route (LeDuc *et al.*, 2002). These results suggest that matrilineally directed fidelity may play a role in use of this area and led the authors to support recognition of the PCFG as a distinct management unit.

The lack of available samples collected from gray whales feeding in northern areas has limited previous genetic studies from directly addressing the potential for demographic independence among whales utilizing different feeding regions within the ENP. Here we use samples collected from various locations north of the Aleutians as well as samples collected from within the seasonal range of the PCFG. A high proportion of the samples collected north of the Aleutians were collected from individuals harvested off Chukotka, Russia, where between 111 and 134 whales per year have been taken during aboriginal whaling over the last decade (IWC, 2010). We also increased the number of samples collected from whales within the seasonal PCFG range and, for those samples linked to photographed individuals, were able to further refine our representation of the PCFG by incorporating sighting histories of known individuals in some comparisons.

The primary goal of this study was to evaluate whether multiple demographically independent units of gray whales exist on feeding grounds, with a special focus on comparing PCFG whales with whales utilizing

northern feeding areas. Although other scenarios are possible, here we test three hypotheses using data from both mitochondrial and nuclear markers (n=8 microsatellite loci):

1. No population structure (e.g., panmixia) is present among gray whales utilizing feeding areas in the ENP; individuals move between feeding areas and exhibit random mating. This hypothesis would be supported by a finding of no nuclear or mitochondrial differentiation between samples collected in northern versus southern feeding areas.
2. Utilization of feeding areas is influenced by internal recruitment, with calves following their mothers to feeding grounds and returning in subsequent years. Mating is random with respect to feeding ground affiliation. This hypothesis would be supported by a finding of significant differences in mtDNA haplotype frequencies when comparing samples collected on northern versus southern feeding grounds, but no significant differences are expected in microsatellite allele frequencies between groups of samples from specific geographic areas (i.e., "strata").
3. Utilization of feeding areas is influenced by matrilineal fidelity and mating is not random with respect to feeding ground affiliation. This hypothesis would be supported by a finding of significant differences in both mtDNA haplotype and microsatellite allele frequencies.

Support for the second hypothesis would indicate that groups of individuals feeding in northern and southern areas are demographically independent but not reproductively isolated, while support for the third hypothesis would provide support for both demographic independence and reproductive isolation.

## METHODS

### Sample Collection

A total of 277 samples were processed for this study. The majority of samples (n=185, including all samples collected between Northern California and British Columbia, Canada) were collected as biopsies from free-ranging individuals, with the remainder collected from individuals taken as part of the subsistence whaling (n= 75 samples from Chukotka) or from stranded individuals (n=17). Collection locations ranged from northern California to Barrow, Alaska and Chukotka, Russia (Figure 1).

For each of the biopsy samples collected, efforts were made to obtain a photograph of the biopsied whale. For whales biopsied between northern California and British Columbia, Canada, photographs were compared to photo-identification catalogues maintained by Cascadia Research Collective. This approach allowed sighting histories of individual individuals to be linked to samples and utilized (as described below) in the stratification of samples for comparisons.

Figure 1 shows that most of the PCFG samples utilized in this study came from the southern portion of the PCFG range. Although the original design of the study was to have both a Russian and a Barrow, Alaska strata, the sample size for the latter (n=14) was insufficient to characterize genetic frequencies from that area. We were therefore unable to directly address hypotheses about whether there are multiple demographically independent feeding units to the north of the Aleutian Islands.

### Laboratory Processing

*DNA extraction, PCR Amplification and Sequencing* – DNA was extracted from samples using standard protocols. The 5' end of the hyper-variable mtDNA control region was amplified from extracted genomic DNA, using the polymerase chain reaction (PCR) and then sequenced using standard techniques (Saiki *et al.*, 1988; Palumbi *et al.*, 1991). DNA was amplified using a 25 ul reaction of 1ul DNA, 18.25 ul of water, 2.5 ul of buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 ul of 10 mM dNTP], 0.75 ul of each 10 μM primer, and 0.25 ul of Taq DNA polymerase. The PCR cycling profile consisted of 90°C for 2 min, followed by 35 cycles of 94°C for 50 sec, an annealing temperature of 60°C for 50 sec, and 72°C for 1 min, then a final extension of 72°C for 5 min. A 523 base pair region of the 5' end of the mtDNA control region was amplified using primers B (5'-TACCAAATGTATGAAACCTCAG - 3'; Rosel *et al.*, 1995) and TRO (5'- CCTCCCTAAGACTCAAGG-3'; developed at SWFSC). Both strands of the amplified DNA product were sequenced independently as mutual controls on the Applied Biosystems Inc. (ABI) model 3730 sequencer. All sequences were aligned using Sequencher v4.8

software (Gene Codes Corp., 2000). If discrepancies were found within the replication the sample was re-sequenced from extracted DNA. If the discrepancy was still not resolved, DNA was re-extracted from tissue and the sample was resequenced until the haplotype was confirmed. For a small number of samples (n=4), the mtDNA sequence contained an ambiguous base call which could not be resolved; these samples were excluded from the mtDNA analysis. In addition, if a sample was identified as having a mtDNA haplotype that was not found among any of the other samples, mtDNA amplification and sequencing was replicated to confirm the haplotype identity.

*Nuclear DNA processing* – Eight microsatellite loci isolated from other cetacean species were used to genotype the samples (Table 1). Extracted DNA was amplified using a 25 µl reaction of 1 µl of DNA, 18 µl of MilliQ water (Millipore, Bedford, MA), 2.5 µl of 10x PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, and 15 mM MgCl<sub>2</sub>), 1.5 µl of 10 mM dNTP, 0.75 µl of each 10 µM primer, and 0.5 units of Taq DNA polymerase. The PCR cycling profile included 90 °C for 2.5 min, followed by 35 cycles of 94 °C for 45 sec, 1 min at the optimal annealing temperature (Table 1), and 72 °C for 1.5 min, then a final extension of 72 °C for 5 min. PCR products were assessed electrophoretically. Genotype data was generated on ABI's 3730 genetic analyzer and analyzed with ABI's Genemapper (version 4.0) software.

*Sex determination* - Samples were genetically sexed by amplification and Real-Time PCR (MX3000p, Stratagene Inc) of the zinc finger (ZFX and ZFY) genes. Sex was determined by the amplification pattern: males had two products and females had one (Morin *et al.*, 2005).

*Quality Control* – Quality control and sample tracking procedures, as detailed in Morin *et al.* 2010, were implemented for all laboratory processing by incorporating control samples (negative and positive) into all amplifications. In addition, a set of samples were randomly chosen to act as replicates for error tracking and error rate estimation. For these samples (“random replicates”), which represented ≥10% of all samples processed, the mtDNA sequence, sex, and microsatellite genotype were re-generated from DNA for each sample.

#### Analysis

*Stratification of Samples* – Two stratification hypotheses were tested in the analysis. The “Northern versus Southern” hypothesis assumed that individuals utilize each of these general regions in a relatively uniform manner such that sampling location within each stratum does not matter. The stratification used for the Northern-versus-Southern hypothesis included all samples described above (Figure 1). Those samples which were collected north of the Aleutian Island Chain were included in the “North” stratum, while all samples collected between northern California and southeastern Alaska (i.e. from within the described range of the PCFG) were included in the “South” stratum (Figure 1).

The second hypothesis is referred to as the “Fine-scale Feeding Aggregation” hypothesis. This hypothesis considers that there may be multiple feeding aggregations north of the Aleutians and hence sampling location within each stratum does matter. The only fine-scale area that was sampled adequately to capture genetic frequencies in the “North” stratum included the individuals hunted off Chukotka (Figure 2). The “Fine-scale Feeding Aggregation” hypothesis also used more stringent criteria than location and season to define individuals assigned to the PCFG stratum. The rationale for more stringent criteria is that photo-identification studies have indicated that whales utilizing the PCFG's seasonal range fall into two categories: 1) whales that return frequently and account for the majority of sightings, and 2) apparent stragglers from the migration that are sighted in only one year (Calambokidis *et al.*, 2010). The criteria for assigning samples to the PCFG stratum were intended to make this stratum representative of the first category of whales. Inclusion in the PCFG stratum for the “Fine-scale Feeding Aggregation” hypothesis relied on two criteria: 1) the sample was linked to a photographed animal with high or medium confidence, and 2) the photographed animal had been sighted two or more years within the season (June – November) and area representative of the PCFG.

*Data Review* – To avoid including duplicate samples, the Excel Microsatellite Toolkit (Park, 2001) was used to identify samples with identical genotypes, indicating that they may have been collected from the same animal. These sample pairs were then checked to see if they also shared the same mtDNA haplotype and sex, and, when possible, photo-identification records were used to confirm the genetic match. For all samples which shared identical mtDNA haplotypes, sexes, and genotypes, one sample from each pair was removed.

*Genotyping Error Rate* – For all plates of samples used to generate microsatellite genotypes, a random subset of samples, representing >10% of the samples on each plate, were assigned as replicates. Replicate and original genotypes were compared, and a per-allele error rate was calculated by determining the number of discrepant allele calls divided by the total number of allele calls compared across all loci.

*Genetic Diversity* – For the mtDNA, haplotypic diversity ( $h$ ) and nucleotide diversity ( $\pi$ ) were calculated using Arlequin 3.5.1.2 (Excoffier *et al.*, 2005). For the microsatellite data, the number of alleles per locus and observed and expected heterozygosities were calculated using custom R-code (eiaGenetics, available upon request<sup>1</sup>). Fstat (Goudet 1995) was used to calculate allelic richness for each stratum. 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. All tests were run for the combined dataset as well as for each stratum, and a sequential Bonferroni correction was applied across all tests for each stratum.

*Genetic Structure* – Pairwise estimates of genetic divergence were calculated using both  $F_{ST}$  and  $\Phi_{ST}$  (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. 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 microsatellite data,  $F_{ST}$  (Weir & Cockerham, 1984), Jost's D (Jost, 2008), and a  $\chi^2$  test were used to assess genetic differentiation. These tests were implemented using custom code (eiaGenetics<sup>1</sup>) written in the statistical program language R (R Core Development Team, 2009). Statistical significance was determined from 10,000 permutations of each data set.

## RESULTS

*Data Review* - Fifteen samples (including  $n = 11$  samples collected from stranded whales) amplified at  $\leq 5$  microsatellite loci and were removed from the analysis. The remaining samples were genotyped for at least seven of the eight microsatellite loci. Fifty-six samples had microsatellite genotypes, mtDNA haplotypes, and sexes which matched at least one other sample in the dataset; these samples were removed from further analysis. No movements of animals between regions representing different strata were identified based on genetic matches (i.e., all samples sharing identical genetic profiles were part of the same stratum).

*Genotyping Error Rate* – Based on the samples randomly chosen for replication, a per-allele error rate of 0.16% was detected for the microsatellite data.

*Genetic Diversity* – Thirty-nine mtDNA haplotypes defined by 37 variable sites were identified from the 202 gray whale samples representing unique individuals (Table 2). Haplotype diversity ( $h$ ) was high in all four strata (“Northern v. Southern” and “Fine-scale Feeding Aggregations”) defined for the analysis (0.945 - 0.953). Nucleotide diversity ( $\pi$ ) was also similar among the four defined strata (1.4 - 1.6%).

The frequency of each haplotype in the defined strata (including Barrow) is shown in Table 3. For the “Fine-scale Feeding Aggregations” strata, eighteen haplotypes were shared between Chukotka and the PCFG, with nine haplotypes found only in Chukotka and five haplotypes found only in the PCFG. For both Chukotka and the PCFG, many haplotypes were found in only one individual ( $n=12$  haplotypes in Chukotka,  $n = 8$  haplotypes in the PCFG).

The median-joining network shows the relationship among mtDNA haplotypes and their frequency in each stratum (Figure 4). MtDNA haplotypes from both Chukotka and the PCFG are dispersed throughout the network, and no phylogeographic pattern is apparent.

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A summary of microsatellite diversity for each locus is shown in Table 4. None of the tests for HWE were significant after the correction for multiple tests was applied. Significant linkage disequilibrium was found for only one pair of loci (EV14t and Gt023t) in the PCFG strata. No significant LD was found for these two loci in any of the other strata or for the combined dataset, so these loci were retained for the analysis.

Measures of genetic diversity for each stratum after averaging across loci are shown in Table 5. As in the comparisons of mtDNA diversity, nuclear diversity was similar across all strata.

*Sex Ratio* – A female bias was present among the samples, ranging from 1.3 – 1.5 females per male in each stratum (Table 6). This female bias is similar to that (1.47 females per male) described in Frasier *et al.* (In press) but contrasts with earlier studies (Steeves *et al.*, 2001; Ramakrishnan *et al.*, 2001). The male bias (1.7 males per female) described in Steeves *et al.* 2001 was based on a small sample size (n=16 samples). When the gender determination method utilized here was applied to the sample set used in the Ramakrishnan *et al.* 2001 study, only a slight male bias was identified (1.25 males/female). These results contrast with those presented in Ramakrishnan *et al.* (1.8 males/female) and indicates that an issue with the gender determination assay used at that time was responsible for falsely identifying some samples as males.

*Genetic Structure* – The results of the mtDNA comparisons are shown in Table 7. Low but statistically significant differences were detected when the PCFG stratum was compared with the North stratum ( $\Phi_{ST} = 0.030$ ,  $p = 0.0118$ ;  $F_{ST} = 0.010$ ,  $p = 0.0052$ ; Fisher's exact test  $p = 0.0080$ ) and with the Chukotka stratum ( $\Phi_{ST} = 0.020$ ,  $p =$ ;  $F_{ST} = 0.012$ ,  $p = 0.0295$ ; Fisher's exact test  $p = 0.0304$ ). The  $F_{ST}$  comparisons for mtDNA were also significant when the North and South strata were compared ( $F_{ST} = 0.007$ ,  $p = 0.0272$ ), although none of the other mtDNA comparisons involving the South stratum demonstrated significant differences. None of the comparisons across strata utilizing the microsatellite data were significant (Table 8), providing no evidence of nuclear structure among feeding areas.

## DISCUSSION

The results presented here are consistent with the second hypothesis that was evaluated, indicating that utilization of at least some feeding areas is influenced by internal recruitment (e.g., matrilineal fidelity), but that individuals from different feeding grounds interbreed. The extent of differentiation, while significant, was low and was detected only in the mtDNA comparisons. Diversity within the PCFG strata was high and similar to that found among strata in the north.

The low level of mtDNA differentiation between strata, as well as the high diversity found in the PCFG, could be a reflection of relatively recent colonization (or re-colonization following depletion of the population by commercial whaling) of the PCFG area. If recruitment into the PCFG is driven exclusively by the return of individuals which followed their mothers to the area as calves, then over time those mtDNA haplotypes originally found only in males or non-reproducing females would be removed via genetic drift, while haplotypes found in females and their returning offspring would build to higher frequencies. By this process, genetic differences would develop between the PCFG and other feeding aggregations, and, given its small size, the PCFG would be expected to maintain low haplotypic diversity. However, if colonization of the PCFG area occurred relatively recently, strong mtDNA differences between the PCFG and individuals feeding further north may not have had time to develop, and the number and distribution of haplotypes in the PCFG may not yet have been affected by genetic drift.

The low level of mtDNA differentiation and high diversity is also consistent with a scenario in which the population structuring is largely driven by matrilineal fidelity (perhaps over longer time scales) but in which some low-level external recruitment also occurs. Some degree of external recruitment would slow the accumulation of genetic differences between the PCFG and northern individuals. As well, external recruits would likely carry haplotypes not previously found among PCFG individuals and would increase the number and diversity of haplotypes found.

These two explanations are not exclusive, and it is plausible that some combination of these scenarios (recent colonization and/or low-level external recruitment) may be occurring. The origin of the PCFG is unknown, and use of the area may date back to the "Little Ice Age" [ca. 1450-1850] when access to the Bering Sea

feeding areas would have been limited by heavy ice and some whales may have started to use the PCFG range. Gray whales have been recorded feeding in the southern portion of the PCFG range as early as 1926, when a single gray whale, which was reported to have been feeding with four other whales, was taken by the Trinidad whaling station off the entrance to the Crescent City Harbor in July (Howell & Huey, 1930). The repeated return of individual whales to the area was first documented starting in the 1970s (Hatler & Darling, 1974; Darling, 1984). Photo-identification studies have identified some individuals that have consistently returned to the PCFG seasonal range over time, including some known reproductive females and their calves (Calambokidis *et al.*, 2010). However, “new” whales continue to appear annually and many are resighted in subsequent years (Calambokidis *et al.*, 2010). These new individuals may be internal recruits that were not sighted as calves, but could also be external recruits that return to the area following a successful feeding season. Even if some low-level external recruitment to the area is occurring, however, the differences in mtDNA haplotype frequencies observed in our comparisons indicate that matrilineal fidelity to the area does occur and is important in influencing population structure on the feeding grounds utilized by ENP gray whales.

Conception in gray whales is thought to primarily occur during a three week period between late November and 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) estimate that the median (peak) sighting dates for the southbound migration are 12 December for Unimak Pass, Alaska, suggesting that many gray whales would be north of the PCFG seasonal range during the first mating period. In addition, of the eight individuals which had retained their satellite tags when they started the southbound migration, four (two males and two females) remained on the PCFG feeding ground after mid-December, with two staying until mid-January or later (Mate *et al.*, 2010). These findings raise the possibility that some segregation in breeding could occur based on feeding ground affiliation. However, while the results of the mtDNA comparisons indicate that matrilineal fidelity is generating structure among feeding areas utilized by ENP gray whales, the lack of differentiation found in the nuclear comparisons supports mixing of individuals from different feeding areas while breeding.

The genetic signal of matrilineal fidelity in the PCFG is less marked than that seen among gray whales feeding off Sakhalin Island in the western North Pacific (WNP). Although significant differences in  $F_{ST}$  and  $\Phi_{ST}$  were observed in the mtDNA comparisons between the PCFG and the northern strata, the magnitude of differentiation is lower than that seen in the WNP versus ENP comparisons ( $F_{ST}=0.068$ ,  $p\leq 0.001$ ; Lang *et al.*, 2010). In addition, a pattern of matrilineal fidelity to the area is also reflected in the distribution of haplotypes among individuals in the western population, such that two haplotypes are found in very high frequencies (representing 36% and 31% of all sampled individuals, Lang *et al.*, 2010). 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), and examination of the haplotypes carried by individuals revealed that 16 of the 23 known reproductive females (between 1995 and 2007, Weller *et al.*, 2008) share one of these two common haplotypes (Lang, 2010). In the PCFG stratum, however, the three highest frequency haplotypes are found in only 10 to 13% of sampled individuals, which is consistent with more recent colonization of the PCFG area by a relatively large number of founders. In addition, genetic differentiation based on microsatellite allele frequencies was observed between the Sakhalin and ENP strata ( $F_{ST} = 0.009$ ,  $p\leq 0.001$ ; Exact test,  $p\leq 0.001$ ), indicating that, unlike what has been suggested in the PCFG, some degree of reproductive isolation also occurs between these groups.

The results presented here are consistent with those presented in Frasier *et al.* (In press), which also found evidence of maternally driven structure when comparing samples collected from PCFG whales with samples from LeDuc *et al.* 2002, which were collected primarily from animals which stranded along the migratory route. The samples utilized in the Frasier *et al.* (In press) study were all collected from Clayoquot Sound, British Columbia. In contrast, the majority of samples representing the PCFG in this study were collected from animals in the waters off northern California, Oregon, and Washington, with only 11 samples collected from waters off British Columbia. Although some whales are known to move throughout the range of the PCFG, sightings of most whales are concentrated within subareas of the range (Calambokidis *et al.* 2010). This pattern is illustrated in Figure 7 of Calambokidis *et al.* (2010), which shows the distribution of latitudes of sightings for whales with 6 or more sightings after 1 June from 1998-2008. The patterns evident in this figure



reveal that individual gray whales do not utilize the range of the PCFG randomly and indicate that, while there is likely overlap among the individuals sampled in Frasier *et al.* (In press) and the current study, neither represents random sampling across the range of the PCFG. To date, the photographs and/or genetic identities of sampled whales in the Frasier *et al.* (In press) study have not been compared with those used in the current study. In the future, such comparisons, along with the collection of additional samples from whales in the northern portion of the PCFG range, would be valuable in allowing sampling effort to be more evenly distributed throughout the range of the PCFG.

As aforementioned, the results presented here are consistent with the hypothesis that utilization of at least some feeding areas by ENP gray whales is influenced by internal recruitment. Within the PCFG, these findings are concordant with photo-identification records demonstrating site fidelity of individuals, including some known reproductive females and their calves, to the seasonal range (Calambokidis *et al.*, 2010). However, interpretation of the results is complicated by our lack of understanding of the potential for structuring within the northern feeding ground(s). If there is no structure on the feeding grounds north of the Aleutians, then the northern strata (both “north” and “Chukotka”) can be considered representative of the genetic diversity of whales feeding throughout the northern feeding area. As such, the mtDNA differences observed here would be driven by fidelity of individuals to the PCFG seasonal range. However, if structuring is present among northern feeding areas, then the differences demonstrated here may be influenced by fidelity of individuals in either or both areas (Chukotka and PCFG). The collection of additional samples from northern feeding areas would be valuable in further elucidating the mechanisms creating the observed differences and in evaluating whether structuring is present among whales utilizing the northern feeding grounds.

Although the lack of nuclear differentiation found in our study indicates that gray whales from different feeding regions may be interbreeding, the significant differences in mtDNA haplotype frequencies that were identified in the study suggest that groups of gray whales utilizing different (northern versus southern) feeding regions are demographically independent. A similar pattern has been observed among humpback whales in the North Atlantic, where four feeding regions are present (Katona & Beard, 1990; Stevick *et al.*, 2006). Within feeding regions, individuals demonstrate intra- and inter-seasonal site fidelity, with only low levels of interchange between regions (Stevick *et al.*, 2006). Although most of the whales from these four feeding regions share a common mating ground in the West Indies (Katona & Beard, 1990; Clapham *et al.*, 1993; Palsbøll *et al.*, 1997; Stevick *et al.*, 1998), individuals utilizing the Gulf of Maine have been classified as a separate feeding stock, based on matrilineally-derived fidelity of individuals to this area and the assumption that, should this subpopulation be extirpated, repopulation by whales using adjacent areas would not occur on a management timescale (Waring *et al.*, 2000). It has been suggested that the timeframe for management should be, at most, decadal in scope (i.e., <100 years; Clapham *et al.*, 2008).

*Future Work* - The low level of differentiation identified, as well as the high diversity found in the PCFG strata, may indicate relatively recent colonization of the PCFG but is also consistent with a scenario in which some low-level external recruitment into the PCFG may occur. Relatedness analysis, in which microsatellite genotypes are used to identify putative parent-offspring pairs, would provide insight into the proportion of internal versus external recruitment that is occurring. Such analysis would require genotyping additional microsatellite loci for sampled individuals and would benefit from the collection of additional samples from individuals within the PCFG.

As part of previous work exploring genetic differentiation between gray whales in the eastern and western North Pacific (Lang *et al.*, 2010), the genetic profiles of samples collected from individuals on the Sakhalin feeding ground (n=142) were compared to those generated from samples collected in the eastern North Pacific (n=136). Two individuals that were sampled off Sakhalin had matching genders, genotypes (n=13 loci), and mtDNA haplotypes to two individuals sampled off central California in 1995 (Lang, 2010). Although subject to caveats, these genetic matches may have represented movements of gray whales between the eastern and western North Pacific. Given that additional gray whale samples from feeding grounds in the ENP have been processed as part of this study, an expanded genetic comparison of all processed samples is currently underway to look for additional matches between the eastern and western populations.

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Figure 1. Map of sample collection locations showing the “Northern versus Southern” stratification

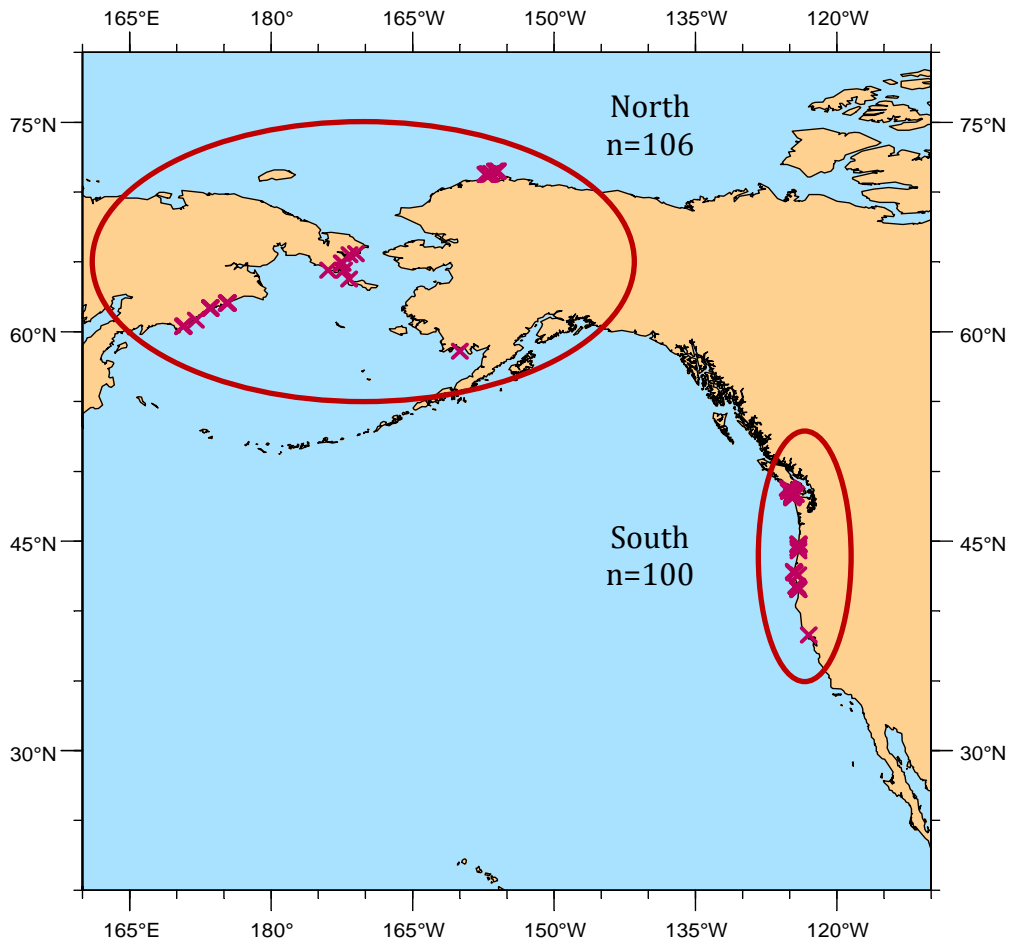


Figure 2. Map of sample collection locations showing the “Fine-scale Feeding Aggregations” stratification

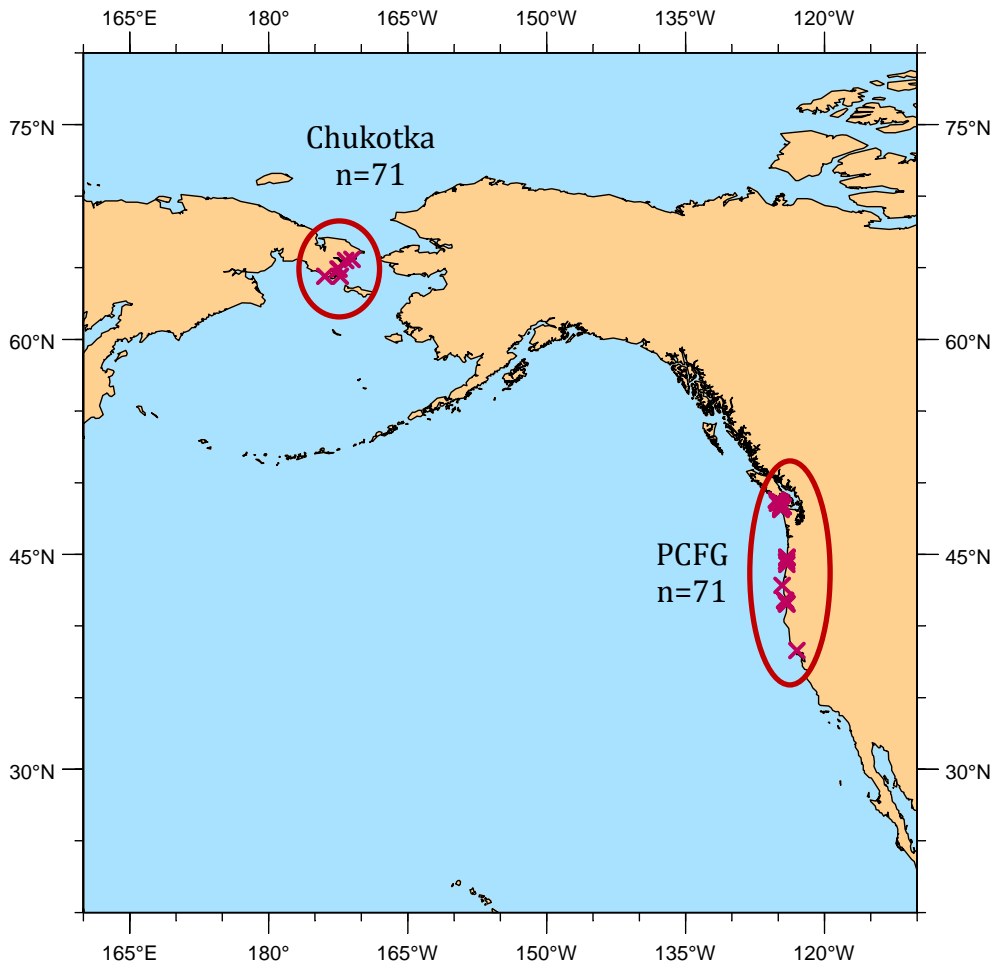




Table 1. Microsatellite loci used in the study. Includes the species for which primers were initially designed, size of repeats, annealing temperature ( $T_a$ ), and reference listing primer sequences.<sup>2</sup>

| Locus    | Source Species                | Repeat    |            | Reference                     |
|----------|-------------------------------|-----------|------------|-------------------------------|
|          |                               | Size (bp) | $T_a$ (°C) |                               |
| EV14t    | <i>Megaptera novaeangliae</i> | 2         | 55         | Valsecchi and Amos 1996       |
| EV94t    | <i>Megaptera novaeangliae</i> | 2         | 52         | Valsecchi and Amos 1996       |
| Gata028t | <i>Megaptera novaeangliae</i> | 4         | 54         | Palsboll <i>et al.</i> , 1997 |
| Gata417t | <i>Megaptera novaeangliae</i> | 4         | 54         | Palsboll <i>et al.</i> , 1997 |
| Gt023t   | <i>Megaptera novaeangliae</i> | 2         | 54         | Palsboll <i>et al.</i> , 1997 |
| RW31t    | <i>Eubalaena glacialis</i>    | 2         | 54         | Waldick <i>et al.</i> , 1999  |
| SW13t    | <i>Physeter macrocephalus</i> | 2         | 55         | Richard <i>et al.</i> , 1996  |
| SW19t    | <i>Physeter macrocephalus</i> | 2         | 55         | Richard <i>et al.</i> , 1996  |

<sup>2</sup> For all primers, the sequence has been modified from the original design by placing the sequence GTTTCTT on the 5' end of the reverse primer (Brownstein *et al.*, 1996)

Table 2. Sequence statistics for gray whale mitochondrial DNA control region sequences for the strata used in the population structure analysis

| Strata                            |          | No. of Samples | No. of Haplotypes | Gene Diversity (h)    | Nucleotide Diversity ( $\pi$ ) |
|-----------------------------------|----------|----------------|-------------------|-----------------------|--------------------------------|
| All                               |          | 202            | 39                | 0.955 ( $\pm 0.004$ ) | 0.0151 ( $\pm 0.008$ )         |
| "Northern v. Southern"            | North    | 103            | 32                | 0.952 ( $\pm 0.008$ ) | 0.0141 ( $\pm 0.007$ )         |
|                                   | South    | 99             | 29                | 0.953 ( $\pm 0.007$ ) | 0.0160 ( $\pm 0.008$ )         |
| "Fine-scale Feeding Aggregations" | PCFG     | 71             | 23                | 0.945 ( $\pm 0.010$ ) | 0.0148 ( $\pm 0.008$ )         |
|                                   | Chukotka | 69             | 27                | 0.953 ( $\pm 0.011$ ) | 0.0142 ( $\pm 0.007$ )         |

Table 3. The number of samples with each mtDNA haplotype for each stratum.

| MtDNA<br>Haplotype<br>ID | "Northern v.<br>Southern" |                 | "Fine-scale Feeding Aggregations" |                  |                  |
|--------------------------|---------------------------|-----------------|-----------------------------------|------------------|------------------|
|                          | North<br>(n=103)          | South<br>(n=99) | Chukotka<br>(n=69)                | PCFG 2<br>(n=71) | Barrow<br>(n=14) |
| 1                        | 10                        | 7               | 8                                 | 7                | 2                |
| 2                        | 3                         | 7               | 2                                 | 4                | 0                |
| 3                        | 14                        | 4               | 9                                 | 1                | 1                |
| 4                        | 5                         | 9               | 4                                 | 6                | 0                |
| 5                        | 1                         | 2               | 1                                 | 1                | 0                |
| 7                        | 7                         | 8               | 4                                 | 6                | 0                |
| 8                        | 1                         | 3               | 1                                 | 2                | 0                |
| 9                        | 1                         | 0               | 1                                 | 0                | 0                |
| 11                       | 3                         | 3               | 2                                 | 3                | 1                |
| 12                       | 5                         | 4               | 4                                 | 3                | 1                |
| 13                       | 5                         | 10              | 3                                 | 9                | 0                |
| 14                       | 1                         | 9               | 1                                 | 7                | 0                |
| 15                       | 3                         | 0               | 0                                 | 0                | 2                |
| 16                       | 1                         | 0               | 0                                 | 0                | 1                |
| 17                       | 1                         | 0               | 0                                 | 0                | 0                |
| 18                       | 3                         | 2               | 3                                 | 2                | 0                |
| 20                       | 6                         | 4               | 1                                 | 2                | 2                |
| 21                       | 2                         | 3               | 1                                 | 3                | 1                |
| 22                       | 1                         | 0               | 1                                 | 0                | 0                |
| 23                       | 5                         | 1               | 4                                 | 0                | 0                |
| 24                       | 2                         | 3               | 2                                 | 3                | 0                |
| 25                       | 6                         | 2               | 4                                 | 1                | 0                |
| 26                       | 2                         | 1               | 1                                 | 0                | 1                |
| 27                       | 0                         | 4               | 0                                 | 4                | 0                |
| 28                       | 2                         | 2               | 2                                 | 2                | 0                |
| 29                       | 2                         | 1               | 2                                 | 0                | 0                |
| 30                       | 0                         | 1               | 0                                 | 1                | 0                |
| 31                       | 1                         | 0               | 1                                 | 0                | 0                |
| 33                       | 5                         | 3               | 4                                 | 1                | 0                |
| 35                       | 1                         | 0               | 0                                 | 0                | 1                |
| 36                       | 1                         | 1               | 0                                 | 1                | 1                |
| 38                       | 1                         | 0               | 1                                 | 0                | 0                |
| 39                       | 0                         | 1               | 0                                 | 0                | 0                |
| 42                       | 1                         | 0               | 1                                 | 0                | 0                |
| 43                       | 1                         | 0               | 1                                 | 0                | 0                |
| 44                       | 0                         | 1               | 0                                 | 0                | 0                |
| 45                       | 0                         | 1               | 0                                 | 0                | 0                |
| 46                       | 0                         | 1               | 0                                 | 1                | 0                |
| 47                       | 0                         | 1               | 0                                 | 1                | 0                |

Figure 4. Median joining network

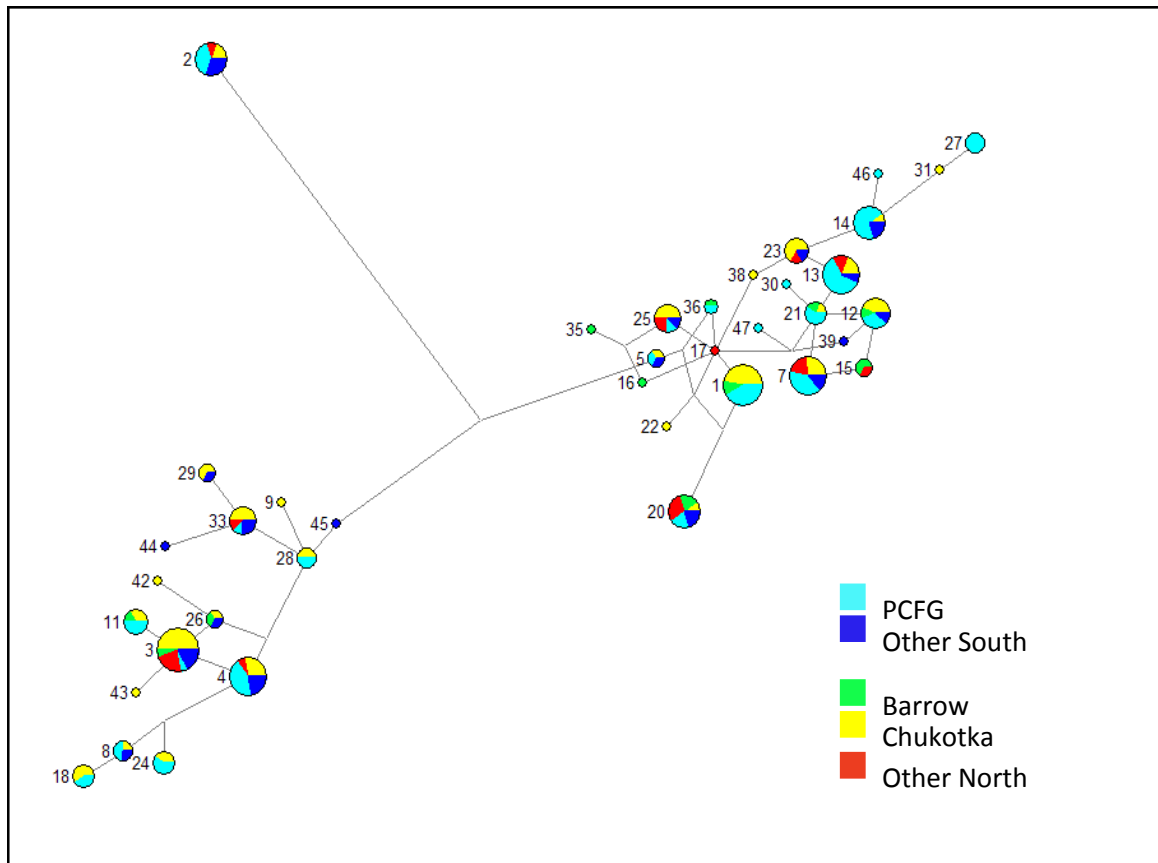


Table 4. Characteristics of the microsatellite loci utilized in the study.

| Locus    | Number of alleles | Number of missing genotypes | He    | Ho    | HWE (prob) |
|----------|-------------------|-----------------------------|-------|-------|------------|
| EV14t    | 10                | 0                           | 0.829 | 0.850 | 0.533      |
| EV94t    | 11                | 1                           | 0.790 | 0.766 | 0.065      |
| Gata028t | 7                 | 0                           | 0.766 | 0.777 | 0.656      |
| GATA417t | 6                 | 1                           | 0.715 | 0.737 | 0.690      |
| Gt023t   | 8                 | 0                           | 0.730 | 0.714 | 0.220      |
| RW31t    | 10                | 0                           | 0.830 | 0.782 | 0.017      |
| SW13t    | 7                 | 0                           | 0.603 | 0.612 | 0.775      |
| SW19t    | 10                | 1                           | 0.709 | 0.707 | 0.213      |

Table 5. Gene diversity for the nuclear DNA data set, including the mean number of alleles, mean observed heterozygosity, and mean allelic richness.

| Strata                            | No. of Samples | Mean number of alleles | Mean Ho | Mean allelic richness |      |
|-----------------------------------|----------------|------------------------|---------|-----------------------|------|
| "Northern v. Southern"            | North          | 106                    | 8.25    | 0.728 ( $\pm 0.068$ ) | 8.18 |
|                                   | South          | 100                    | 8.38    | 0.758 ( $\pm 0.088$ ) | 8.36 |
| "Fine-scale Feeding Aggregations" | PCFG           | 71                     | 7.38    | 0.752 ( $\pm 0.085$ ) | 7.37 |
|                                   | Chukotka       | 71                     | 7.88    | 0.737 ( $\pm 0.095$ ) | 7.86 |

Table 6. The sex ratio for each strata.

| Strata                           | No. of Females | No. of Males | Ratio |     |
|----------------------------------|----------------|--------------|-------|-----|
| Overall                          | 117            | 85           | 1.4   |     |
| "Northern v. Southern"           | North          | 61           | 42    | 1.5 |
|                                  | South          | 56           | 43    | 1.3 |
| "Fine-scale Feeding Aggregation" | PCFG           | 42           | 29    | 1.5 |
|                                  | Chukotka       | 41           | 28    | 1.5 |

Table 7. Results of MtDNA comparisons across strata. Significant p-values are shown in bold.

| Pairwise Comparison         | $\phi_{st}$ | p-value       | $F_{st}$ | p-value       | Fisher exact test p-value |
|-----------------------------|-------------|---------------|----------|---------------|---------------------------|
| North (103) v. South (99)   | 0.006       | 0.1295        | 0.007    | <b>0.0272</b> | 0.0693                    |
| North (103) v. PCFG (71)    | 0.020       | <b>0.0232</b> | 0.012    | <b>0.0052</b> | <b>0.0080</b>             |
| Chukotka (69) v. South (99) | 0.011       | 0.0872        | 0.005    | 0.0932        | 0.2234                    |
| Chukotka (69) v. PCFG (71)  | 0.030       | <b>0.0118</b> | 0.010    | <b>0.0295</b> | <b>0.0304</b>             |

Table 8. Results of nuclear comparisons across strata

| Pairwise Comparison          | $F_{st}$ | p-value | Jost's D | p-value | $X^2$ p-value |
|------------------------------|----------|---------|----------|---------|---------------|
| North (106) v. South (100)   | -0.002   | 0.9740  | -0.003   | 0.9491  | 0.9331        |
| North (106) v. PCFG (71)     | -0.002   | 0.8362  | -0.001   | 0.8032  | 0.7532        |
| Chukotka (71) v. South (100) | -0.002   | 0.9520  | -0.003   | 0.9021  | 0.9021        |
| Chukotka (71) v. PCFG (71)   | -0.001   | 0.7303  | 0.000    | 0.6813  | 0.6364        |