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ABSTRACT

Within the North Pacific, gray whales (*Eschrichtius robustus*) are recognized as distinct eastern and western populations. Although both populations were severely reduced by whaling, the eastern population is generally considered to have recovered while the western population has remained highly depleted. Previous studies have documented genetic differentiation between the two populations on the basis of mtDNA haplotype frequencies. Since mtDNA represents only maternal inheritance patterns, the present study used bi-parentally inherited microsatellite markers (n=13) to measure differentiation between populations as well as to compare levels of nuclear genetic diversity retained in each. Mean levels of genetic diversity, as measured by the microsatellites, were similar between the eastern and western populations, indicating that the western population has retained relatively high levels of nuclear genetic diversity despite its small size. Comparison of microsatellite allele frequencies confirmed that eastern and western populations are genetically distinct. Although highly statistically significant, the level of differentiation between the two populations is relatively low, and sex-specific analyses suggest that some amount of male-biased dispersal may occur between populations. While these results suggest some movements between the eastern and western populations may take place, the maintenance of genetic differences between the two populations supports their recognition as separate eastern and western populations. Future efforts should focus on elucidating the nature and extent of any dispersal which is occurring in order to better understand factors potentially influencing the recovery of the small western population.

INTRODUCTION

Although gray whales (*Eschrichtius robustus*) once inhabited the North Atlantic Ocean (Mead and Mitchell 1984), the current distribution of the species is limited to the eastern and western margins of the North Pacific (Rice and Wolman 1971). Within this region, gray whales are recognized as having distinct eastern and western populations. Eastern gray whales winter in the lagoons and adjacent waters of Baja California, Mexico and then migrate north along the west coast of North America to feed in the Bering and Chukchi Seas during summer (Rice and Wolman 1971), with a small number of animals remaining in more southern waters between northern California and southeastern Alaska during summer months (Darling 1984, Calambokidis *et al.* 2002). For western gray whales, the primary feeding ground is in the coastal waters off northeastern Sakhalin Island, Russia (Weller *et al.* 1999, 2002). The location of the wintering ground(s) for

this population remains unknown, but limited information from sightings, strandings, and catches shows that some animals winter in the coastal waters of southern China (Wang 1984, Henderson 1990, Zhu 1998).

Both gray whale populations were greatly reduced by intensive commercial whaling during parts of the 19th and 20th centuries, but the two populations have exhibited different trajectories in abundance following exploitation. Commercial whaling for eastern gray whales ceased in 1936 (Brownell and Swartz 2006), and the population's size has increased since that time (Rugh *et al.* 2005). Eastern gray whales were removed from the U.S. List of Endangered and Threatened Wildlife and Plants in 1994, and recent abundance estimates indicate that the population contains approximately 22,000 animals (Punt and Wade 2010). In the western population, however, hunting continued through at least 1966 (Brownell and Chun 1977). This population was reduced to a much smaller size than the eastern populations and was considered by some to be extinct as recently as the 1970s (Bowen 1974). Today western gray whales exist only as a small remnant population. Recent population assessment utilizing a Bayesian individually-based stage-structure model and photo-identification data collected between 1994 and 2007 projected a median non-calf population size of 130 individuals in 2008, assuming current demographic and population trends continue (Cooke *et al.* 2008). This population was listed as Critically Endangered by the IUCN in 2000 (Weller *et al.* 2002, Baillie *et al.* 2004), and its continued survival is jeopardized by problems associated with small population size (reviewed in Clapham *et al.* 1999), as well as by a wide range of potential anthropogenic threats, including the rapid expansion of oil and gas development on its summer feeding ground off Sakhalin Island, Russia (Weller *et al.* 2002, Reeves *et al.* 2005, IISG 2006) and mortality due to net entrapment while on the migratory route off Japan (Brownell *et al*. 2007, Weller *et al.* 2008b).

Concern for the conservation status of the western population led to the initiation of a joint Russia-U.S. research program in 1995. This program is based on the summer feeding ground off Sakhalin Island, Russia and has incorporated both photo-identification studies and biopsy sampling (Weller *et al.* 1999, 2002). Photo-identification research has shown that most whales demonstrate high rates of annual return and pronounced seasonal site fidelity to the Sakhalin feeding ground (Weller *et al.* 1999, 2002). The majority (83% of identified whales (n=169) have also been genetically sampled, allowing a male bias (58% males) to be documented among sampled individuals (Weller *et al.* 2002, 2008). This male bias is particularly pronounced in individuals first identified as calves, of which 66% are males (Weller *et al.* 2008).

Biopsy samples collected between 1995 and 1999 have been used to show that the eastern and western populations are genetically distinct based on mitochondrial DNA (mtDNA) haplotype frequencies (LeDuc *et al.* 2002). This study found that western gray whales have retained a relatively high number of mtDNA haplotypes for such a small population. Genetic differentiation between the eastern and western populations was based on differences in the frequency distributions of haplotypes within each population. While haplotypes were apportioned relatively evenly among the eastern gray whale samples, the haplotype distribution found within the western gray whale samples was highly skewed, with two haplotypes found in very high frequencies and the remaining haplotypes identified in only one or two individuals (LeDuc *et al.* 2002).

The work presented here used thirteen microsatellite markers to further examine population structure of gray whales. Unlike mtDNA, which is maternally inherited and provides information about historic gene flow of females only, microsatellites are nuclear bi-parentally inherited markers and reflect gene flow of both males and females. The primary goal of this study was to examine genetic differentiation between eastern and western populations using microsatellites, as well as to assess factors which might contribute to that differentiation. Secondarily, levels of nuclear genetic diversity were compared between the two populations to determine if substantial genetic variability has been lost in the much smaller western population and could thus be affecting its ability to recover. Finally, since additional western gray whale samples have been collected since the LeDuc *et al.* (2002) study, further analysis of population structure and genetic diversity using mtDNA was also conducted.

MATERIALS AND METHODS

Sample collection and DNA extraction

One hundred forty-two western gray whale samples were collected between 1995 and 2007 via biopsy darting of free-ranging whales on the population's feeding ground off Sakhalin Island, Russia. All except for one of the western gray whale samples are linked to a photographically identified animal, and this sample set represents 83.4% of all animals (n=169) identified on the western feeding ground through 2007. One hundred thirty-seven eastern gray whale samples obtained from the archive at the Southwest Fisheries Science Center were used for comparison to the western population. These samples were taken primarily from stranded animals (n=105), with some samples obtained from directed subsistence takes (n=12), fisheries bycatch (n=3), and biopsies (n=17) from free ranging whales. Collection locations ranged from southern California north to the Chukotka Peninsula in Russia.

DNA had been previously extracted for 120 of the eastern gray whale samples and 45 of the 142 western gray whale samples (those collected between 1995 and 1999) for use in an earlier study (LeDuc *et al.* 2002). For the remaining samples, whole genomic DNA was extracted using either the QIAGEN DNeasy™ tissue kit or the Corbett Robotics X-tractor Gene robot with the recommended protocols.

Molecular sexing and mtDNA control region sequencing

For those samples (n=114) not analyzed in the prior study by LeDuc *et al.* (2002), molecular sexing and mtDNA control region sequencing were conducted. For all of the eastern gray whale samples as well as the western gray whale samples which were collected prior to the 2006 season (n=94), a polymerase chain reaction (PCR) was used to determine sex utilizing primers described in Fain and Lemay (1995) and following the methods described in Gilson *et al.* (1998). For western gray whale samples collected in 2006 and 2007 (n=20), the protocol described in Morin *et al.* (2005) was used to determine the sex of individuals.

PCR was used to amplify a 523-base-pair fragment from the mtDNA control region using the primers 5'-TACCAAATGTATGAAACCTCAG-3' (H00034, Rosel *et al.* 1995) and 5'-CCTCCCTAAGACTCAAGGAAG-3' (L15812, Escorza-Trevino *et al.* 2005). Amplification products were cleaned through purification columns (QIAquick, Qiagen) and then sequenced using standard protocols with ABI-PRISM® Dye-DeoxyTerminator Big Dye™ v3.1 (Applied Biosystems) and the same primers. Following ethanol precipitation, sequenced products were run on an ABI 3100 or ABI3130 capillary sequencer. Consensus sequences for both strands were generated using ABI SEQSCAPE v2.5 software.

Microsatellite genotyping

Thirteen microsatellite loci isolated from other cetacean species were used to genotype the samples (*Table 1*). Reactions were performed in 25-uL volumes containing approximately 100 ng of genomic DNA and 2.5 uL of 2.0 mM $MgCl_2$ buffer, 1.5 uL of 10 mM dNTPs, 0.75 uL of each primer (at 10uM concentrations, with the forward primer of each pair fluorescently labeled), and 0.25 uL Taq. The thermal cycling profile included an initial hot start of 94°C for 2 min followed by 35 cycles of 94°C for 45 s, 1 min at the annealing temperature (see *Table 1*), and 1.5 min at 72°C, with a final 5-min extension at 72°C. Amplified products were mixed with a size standard and loaded onto an ABI 3100 or ABI 3130 sequencer. Sizing and binning of allele fragments using ABI GENESCAN and GENOTYPER analysis software were automated and relied on the use of internal lane standards, with subsequent manual evaluation of all labeled peaks.

Microsatellite scoring errors and identification of replicate samples

Prior to inclusion in this study, photo-identification data collected during biopsy sampling was used to identify and remove any duplicate samples (*i.e.,* samples taken from the same individual) from the western population sample set. Genotypic data were used to search for duplicates within the eastern gray whale sample set using MS Excel Toolkit v3.1 (Park 2001); one duplicate was identified and removed prior to

analyses, leaving a total of 136 eastern gray whale samples. Microsatellite data were also examined for signs of large-allele dropout and null alleles using MICRO-CHECKER v2.2.1 (van Oosterhout *et al.* 2004).

Genetic variability within populations

ARLEQUIN v3.01 (Excoffier *et al.* 2005) was used with the mitochondrial control region data to calculate standard indices of genetic variation (nucleotide diversity, π, and haplotype diversity, h; Nei 1987) for each population. Genetic diversity at the nuclear level was characterized by generating the number of alleles, observed heterozygosity, and expected heterozygosity for each microsatellite locus in each population using ARLEQUIN. Within each sample set, a Markov-chain approximation of an exact test, as implemented in GENEPOP v3.4 (Raymond and Rousset 1995a), was used to test for departures from Hardy Weinberg expectations and for linkage disequilibrium between all pairs of loci.

Bottleneck analyses

Populations which have undergone recent bottlenecks are expected to exhibit genetic signatures characteristic of a reduction in effective population size (Cornuet and Luikart 1996, Luikart and Cornuet 1998, Luikart et al. 1998, Garza and Williamson 2001). One such signature is a transient excess of heterozygosity (H_e) relative to that expected in a population of constant size, which results from the rapid loss of rare alleles contributing little to overall heterozygosity (Cornuet and Luikart 1996). Here we utilized the program BOTTLENECK v1.2 (Piry *et al.* 1999) to determine if the gray whale microsatellite data demonstrated evidence of population bottlenecks. As recommended (Piry *et al.* 1999), a two-phase model assuming 95% single-step mutations and 5% multiple-step mutations was employed, with the variance among multiple steps set to 12. The distribution of gene diversity at equilibrium was estimated using a coalescent process with 10,000 simulations, and a one-tailed Wilcoxon test was used to determine if an excess of heterozygosity, relative to that expected in populations at equilibrium, was present (Cornuet and Luikart 1996).

The loss of rare alleles during a population bottleneck may also result in gaps in the size distribution of microsatellite alleles. This can be measured as the mean ratio (M) of the number of alleles to the allele size range across all loci (Garza and Williamson 2001); bottlenecked populations demonstrate reduced M values. Here we used ARLEQUIN to calculate M for both gray whale populations and then compared our values to those reported for reduced and stable populations by Garza and Williamson (2001).

Genetic differentiation among populations:

Two approaches were used to assess the degree of genetic differentiation between the two sampling regions. In the first approach, samples were divided *a priori* into populations based on the geographic location in which they were collected. The extent of genetic differentiation between populations was then examined using both mtDNA sequences and microsatellite data. For mtDNA data, an analysis of molecular variance (AMOVA, Weir and Cockerham 1984, Excoffier *et al.* 1992) was used to generate frequency-based (F_{ST}) estimates of differentiation using the program ARLEQUIN (20,000 permutations were used to test for significance). For microsatellite loci, genetic differentiation was examined using an AMOVA (ARLEQUIN) and allelic frequencies (with 20,000 permutations to test for significance) to generate F_{ST} values (Weir and Cockerham 1984). Modified exact tests based on genotype counts, as implemented in GENEPOP v3.4 (Raymond and Rousset 1995b), were also utilized to measure levels of differentiation. Significance was tested using 10,000 permutations. Since the western gray whale sample set included 57 mother-calf pairings, analyses of genetic differentiation were repeated after removal of the sample representing the calf in each pair, in order to avoid biasing the results by including known first-degree relatives.

As an alternative to *a priori* stratification of samples by geographic location, population structure was also explored using a Bayesian model-based clustering approach (STRUCTURE v2.2, Pritchard *et al.* 2000) with the microsatellite data. STRUCTURE assumes that within a set of samples there are K populations, each of which is characterized by allele frequencies at each locus. The program then divides all samples into K genetically distinct clusters by assigning individuals to putative populations such that Hardy-

Weinberg and linkage disequilibrium are minimized within each group. Five independent runs of K=1-5 were performed with a burn-in period of 50,000 iterations followed by 100,000 Markov-chain Monte Carlo repetitions, using a model based on admixture with correlated allele frequencies (Falush *et al.* 2003). After averaging across runs, the log probability of the data given K (Ln P(X|K) was used as the criterion to infer the number of clusters (K) most compatible with the our data.

Detection of sex-biased dispersal

The potential for sex-biased dispersal between populations was investigated using the microsatellite data with the methods described by Goudet *et al.* (2002) and implemented in FSTAT v2.9 (Goudet 2001). Since the signal of sex-biased dispersal disappears with mating (Goudet *et al.* 2002), animals first sampled as calves in the western population were omitted prior to analysis. This program generates a number of statistics aimed at identifying patterns of sex-biased dispersal. The statistics utilized here were 1) F_{st}, the proportion of genetic variation among populations; 2) the mean corrected assignment index (mAIc) and 3) the variance around the assignment index (vAIc) (Favre *et al.* 1997, Mossman and Waser 1999). The p values were estimated using 10,000 randomizations, and a one-tailed test was utilized based on the expectation that, as in most mammals, dispersal is biased toward males. F_{st} and mAIc are expected to be higher in the more philopatric sex, while vAIc should be lower (Goudet *et al.* 2002).

To further explore the potential for sex-biased dispersal between populations, sex-specific estimates of genetic differentiation were generated using the methods outlined above with both the mtDNA and microsatellite data. In addition, values of cluster membership (Q) produced by the STRUCTURE model assuming K=2 clusters were compared between males and females.

RESULTS

Genetic diversity

Forty haplotypes defined by 39 variable sites were identified from the 278 gray whale samples. Thirty-five haplotypes were found among the eastern gray whale samples, while 22 haplotypes were found in the western gray whale sample set. Seventeen haplotypes were shared between the two populations. The frequency of haplotypes in each population is shown in *Table 2*. When all samples were combined, nucleotide diversity (π) was 0.018 (SD=0.0092), while haplotypic diversity (h) was 0.89 (SD=0.012). When subdivided by population, nucleotide diversity was relatively similar in both populations (π=0.016 ±0.0081SD, eastern population; π=0.018 ±0.0093SD, western population), while measures of haplotype diversity were higher in the eastern (h=0.95 ±0.006SD) than the western (h=0.77±0.025SD) population (*Table 3*). Sex-specific diversity measures indicated that although haplotypic diversity was similar between the male (h=0.96) and female (h=0.95) subsets of the eastern population, lower levels of haplotype diversity were found among the western female subset (h=0.77) when compared to the western male subset (h=0.83).

No signal of large-allele dropout or null alleles was identified by MICROCHECKER for any locus in either of the two populations. No deviation from Hardy-Weinberg Equilibrium was detected in either population after controlling for the False Discovery Rate (FDR, Benjamini and Hochberg 1995). After correcting for the FDR, only one loci combination was found to be in significant linkage disequilibrium in the eastern population. However, significant linkage disequilibrium was detected for eight loci combinations in the western population. Given that the same loci pairs were not in disequilibrium in both populations, it is unlikely that this result was derived from physical linkage. Linkage disequilibrium can result from inclusion of related individuals within a sample set. Therefore, known relatives were removed and the tests were rerun on the remaining genotypes. Six loci combinations remained out of linkage disequilibrium after controlling for the FDR.

After averaging across loci, measures of microsatellite diversity were higher in the eastern population (H_o=0.74, H_e=0.74, K=9.8) than in the western population (H_o=0.71; H_e=0.70, A=8.8); however, these differences were relatively small (*Table 4*). A total of 18 private alleles were observed in the eastern population, while only 5 private alleles were found in the western population.

Statistical analysis of the microsatellite allele frequency data using the program BOTTLENECK did not detect evidence of a recent (2-4N_e generations) bottleneck in either population. Under the model utilized, heterozygosity excess was not observed in the eastern (Wilcoxon test, *P*=0.989) or the western population (Wilcoxon test, *P*=0.999). In addition, the calculated *M* values (0.823±0.15and 0.808±0.17) in the eastern and western populations, respectively) were more consistent with those described for stable populations and were considerably higher than the upper bound (0.70) that Garza and Williamson (2001) derived for reduced populations.

Genetic differentiation among populations

Significant genetic structuring between eastern and western populations on the basis of both mtDNA haplotypes and microsatellite allele frequencies was observed (*Table 5*). Similar results were also observed for the microsatellite data when genetic differentiation was assessed using the exact test; the overall results were significant ($p \leq 0.001$), with 11 of the 13 loci showing significant differences when analyzed independently (data not shown). These comparisons remained significant ($P \leq 0.001$) after known relatives (n = 57 calves which had sampled mothers) were removed from the analysis (*Table 5*); however, only three of the thirteen loci showed significant differences when analyzed independently.

STRUCTURE analyses (Pritchard *et al.* 2000, Falush *et al.* 2003) supported the presence of two populations (P ~1.0), with a clear increase in the log-likelihood of the data for K = 2 when compared to that for other numbers of clusters (*Table 6*). The probability that the data contained only one cluster was < 0.001, suggesting that eastern and western populations are not panmictic. When Q values, which represent the proportion of each individual's genotype that can be attributed to each of the clusters, were used to assign individuals into clusters, 80% (n = 109 of 136) of animals sampled in the east were grouped into the same cluster while 65% (n = 92 of 142) of animals sampled in the west were grouped into a cluster (*Figure 1*). However, average source population Q values were relatively low for both populations; they averaged 0.69 (± 0.209SD) for animals sampled in the east and 0.60 (± 0.296SD) for animals sampled in the west.

Sex-specific comparisons

Sex-specific estimates of differentiation were much more marked among females than among males. Using mtDNA haplotype frequencies (*Table 5*), both the male and female comparisons were highly significant, although the F_{st} value estimated for females (F_{st} = 0.078) was more than twice as high as that estimated for males (F_{st} = 0.033). Interestingly, while the male-specific comparisons remained significant (P = 0.029) in the microsatellite exact test, F_{st} estimates based on microsatellite allele frequencies suggested no significant differences between eastern and western males. The sex-biased dispersal tests in FSTAT also supported greater philopatry among females when compared to males. While difference in males and females were not significant for the mean assignment index (*P* = 0.365) or the variance in the mean assignment index (*P* = 0.9262), females demonstrated significantly higher F_{st} values (*P* = 0.0176).

Results of the STRUCTURE analysis provided further evidence that male-biased dispersal may be occurring. After removing animals first identified as calves, average Q values were similar between eastern males (Q_{EM} = 0.70 ± 0.211SD) and females (Q_{EF} = 0.67 ± 0.208SD, *P* = 0.26, t-test); 80% and 87% of males and females were assigned to their source population. In contrast, average Q values were lower for western males (Q_{WM} = 0.47 ± 0.339SD) than for western females (Q_{WF} = 0.63 ± 0.250; *P* = 0.010, t-test). Only 40% of western males had $Q \geq 0.50$ for the cluster representing the western population, in contrast to 75% of western females.

DISCUSSION

Genetic variability

Populations reduced to small sizes can suffer from a loss of genetic diversity, which in turn may compromise their ability to respond to changing environmental conditions (Willi *et al.* 2006) and negatively influence long-term viability (Spielman *et al.* 2004, Frankham 2005). Although little is known

about the level of genetic diversity maintained in the western gray whale population prior to its depletion by commercial whaling, comparison of the levels of diversity found in this small population with those maintained in the much larger population in the eastern Pacific can provide some insight into whether reduced genetic diversity may influence its recovery. Previous studies utilizing mtDNA indicated that while the western gray whale population had retained a relatively high number of mtDNA haplotypes and levels of nucleotide diversity which were concordant with those found in the eastern population, the population had reduced haplotype diversity when compared to its eastern counterpart (LeDuc *et al.* 2002). Our results, using an extended sample set that included ~83% of photographically identified western gray whales, support these earlier findings. As previously noted, the reduced haplotype diversity found in the western population was not a reflection of the number of haplotypes present but rather of the skewed distribution of those haplotypes (LeDuc *et al.* 2002). This skew was even more marked with the added samples. While the frequencies of the two most common haplotypes changed little, new low frequency haplotypes were added, with 14 of the 22 western gray whale haplotypes being found in only one or two animals.

While approximately half (49%) of the mtDNA haplotypes identified in the eastern population were shared with animals sampled in the western North Pacific, a much larger proportion (77%) of the mtDNA haplotypes found in the western population were also identified in eastern animals. Given the relatively thorough sampling of animals on the western feeding ground, it is likely that most if not all haplotypes present in that area have been identified, indicating that the mtDNA haplotypes found only in the eastern Pacific are likely to be unique to that population. In contrast, the low proportion of animals sampled in the eastern population suggests that those haplotypes currently identified only among western animals (n=5) might also be discovered in the eastern population with additional sampling.

Although the relationship between population size and mtDNA diversity is not straightforward (Bazin *et al.* 2006; Nabholz *et al*. 2008), the number of haplotypes (n=22) found in the western gray whale population is surprising given its small size and history of exploitation. In a similar study of endangered North Atlantic right whales (*Eubalaena glacialis*), which are thought to number approximately 400 individuals, only five haplotypes have been documented (n = 180 samples, Malik *et al.* 2000). While sampling in other populations has been less comprehensive, similar patterns have been found in other small mysticete populations, including the Okhotsk Sea bowhead whale population (*Balaena mysticetus*), in which only four different haplotypes were found (n = 25 samples, LeDuc *et al.* 2005), as well as the Sea of Cortez fin whales (*Balaenoptera physalus*), in which three haplotypes have been identified (n = 56 samples, Berube *et al.* 2002). The number of haplotypes found in the western gray whale population is more consistent with numbers found in larger populations, such as the stock of right whales (*Eubalaena australis*) breeding off South Africa, which contains 21 haplotypes (n = 41 samples, Patenaude *et al.* 2007) and has an estimated abundance of 3400 animals (Best *et al.* 2005).

Although the number of haplotypes currently found in the western population is higher than might be expected, this pattern may not persist into the future. Eleven of the 14 haplotypes found in low frequencies have been identified only in a single male. Although little specific information is available on gray whale longevity, they are generally thought to live for approximately 40 to 60 years. It is possible that some of these "rare haplotype" males could be animals that escaped being killed by whalers which hunted gray whales until at least 1966. Given the maternal inheritance pattern of mtDNA, and assuming that these males are indeed the only animals in the population with these haplotypes, the eventual loss of these individuals has the potential to substantially decrease levels of mtDNA diversity in the future.

The level of nuclear genetic diversity found in the western population was slightly lower than, but very similar to, that found in the much larger eastern population. The number of microsatellite alleles found exclusively in the eastern population, however, was markedly higher than the number found in the western population. Given that the western population has been relatively thoroughly sampled, these results suggest that the western population's depletion and continued small size may have resulted in the loss of rare alleles from the population. No genetic signature of a bottleneck was detected in the western population using the microsatellite data. However, simulations have shown that detection of bottlenecks using genetic methods is dependent on a wide range of conditions, including duration of the bottleneck, mutation rate, pre-bottleneck size, and post-bottleneck recovery (Williamson-Natesan 2005), and many studies have failed to

detect the genetic signature of a bottleneck even when demographic data indicate population size collapse (*e.g.*, Queney *et al.* 2000, Spong and Hellborg 2002).

Overall, the western population appears to have retained relatively high genetic diversity despite its history of exploitation and continued small population size. In other populations, the maintenance of genetic diversity in the face of population decline has been attributed to long generation times (Dinerstein and McCracken 1990, Hailer *et al.* 2006, Lippe *et al*. 2006), which are characteristic of baleen whales and may have buffered the population against the rapid loss of variation. However, the relatively high level of genetic diversity that appears to have been maintained in the western population could also be the result of dispersal of eastern animals onto the western feeding ground. Even at low levels, dispersal has been shown to obscure bottleneck signatures (*e.g.,* Kellar *et al.* 2001, Busch *et al.* 2007) and genetically "rescue" populations from the loss of genetic diversity (Vila *et al.* 2003). Further exploration of this possibility is detailed below.

Population structure

The inclusion of additional samples to analyses employing mtDNA supported the previous conclusion that the two populations are genetically distinct (LeDuc *et al.* 2002). Nuclear differentiation estimates further confirm differences between the two populations and indicate that genetic separation between populations is not derived solely from female philopatry. These measures of differentiation remained significant after known first degree relatives (*i.e*., the calf from sampled mother-calf pairs) were removed from the dataset, suggesting that such differences are not solely an artifact of the inclusion of highly related individuals in the analysis.

Although highly significant, the degree of nuclear differentiation, as measured by F_{ST} values, between the two populations is relatively small. This pattern of differentiation is similar to that found in North Pacific bowhead whale populations, which also demonstrate a significant but small degree of differentiation between a smaller western population inhabiting the Okhotsk Sea and a much larger eastern population in the Bering-Chukchi-Beaufort Seas (LeDuc *et al.* 2005). The relatively small but highly significant genetic differences observed in gray whales, particularly when combined with the similar pattern observed in North Pacific bowhead populations, suggests that past Arctic environmental changes may have played a role in influencing patterns of historic mixing and separation of eastern and western animals. Both stranding records and radio-carbon dating of remains have indicated that changes in sea ice distribution may have mediated bowhead whale distribution in the Canadian Arctic (Dyke *et al.* 1996, SaVelle *et al.* 2000). Within the North Pacific, Arctic-wide cooling and glaciation brought on by the "Little Ice Age" (~400-750 years ago) may have resulted in a southern shift in sea ice distribution and reduced sea level (Overpeck *et al.* 1997), potentially facilitating mixing between eastern and western whales. Sea ice expansion during the Neoglacial (~4700 to 2500 years ago) may also have limited access to parts of the Bering Sea and has been hypothesized to have altered the distribution of North Pacific pinnipeds and cetaceans (Crockford and Frederick 2007).

A second explanation for the low level of differentiation is that some limited gene flow could be occurring between the two populations. Given the small size of the western population, it seems likely that even minimal gene flow from the eastern to the western population would quickly homogenize allele frequencies. However, genetic drift also acts more strongly on small populations, allowing differences between populations to develop more rapidly. As such, genetic drift could be acting to counterbalance some restricted degree of genetic interchange between populations.

A third scenario which might also explain our results involves dispersal of whales between feeding areas without genetic exchange. Since breeding in gray whales is thought to primarily occur along migratory corridors (Rice and Wolman 1971), movement between feeding regions does not necessarily imply gene flow between the populations. Given that all of the western gray whale samples were obtained on the feeding ground, low differentiation levels could potentially be generated by a small number of eastern gray whales traveling to the western gray whale feeding ground during summer months and consequently being sampled while mixed with members of the western population. If these eastern dispersers visit the western

feeding ground but return to the eastern Pacific to breed, such extralimital movements would act to reduce measured levels of genetic differentiation between populations in the absence of significant gene flow.

Some support for a limited degree of dispersal and/or gene flow between populations can be derived from the results of the sex-specific comparisons. If the observed low level of differentiation were due to recent divergence, similar patterns of differences should be observed for males and females. Contrary to this expectation, all measures of differentiation were at least twice as high for female-only versus male-only comparisons. In addition, although comparisons between males remained significant for mtDNA, the F_{st}-based comparison of microsatellite allele frequencies did not identify significant differences among males, suggesting that some degree of male-biased dispersal may be occurring between populations. Such a pattern could also provide an explanation for the large proportion of mtDNA haplotypes (11 of 22) in the western population which are represented only by a single male. Given the higher diversity and number of mtDNA haplotypes found in the eastern population, any dispersers from the east would have a relatively high probability of carrying haplotypes considered "rare' in the west (LeDuc *et al.* 2002). Eight of the eleven haplotypes carried by only a single male in the west were also found in the east; given the low proportion of sampled animals in the east, it is plausible that the other three haplotypes would also be identified among eastern animals with additional sampling.

Although the analyses summarized here are not able to discriminate between gene flow and feeding ground dispersal, a combination of genetic assignment tests and parentage analysis in the future may be useful to distinguish between these two possibilities. In addition, simulation modeling could be utilized in the future to determine the degree of gene flow or feeding-ground dispersal which could occur while still allowing the two populations to maintain genetic distinctiveness.

Conclusions and conservation implications

The results presented here support past work indicating that eastern and western populations are genetically distinct, further highlighting the need for continued conservation and expanded protection of the critically endangered western gray whale population. Although highly statistically significant, the level of differentiation between the two populations is relatively low, which may reflect recent divergence of the two populations, perhaps mitigated by past environmental changes, but could also suggest that some limited degree of dispersal and/or gene flow may occur between the two populations. Discrimination between these proposed explanations is important, given that each scenario could have different effects on the recovery of the critically endangered western population. If a restricted amount of gene flow is taking place, that interchange could be important in providing "genetic rescue" for the western population, helping to maintain relatively high levels of genetic diversity in a small population which would otherwise likely suffer from inbreeding and a subsequent loss of fitness. However, if dispersal between feeding grounds without any gene flow is occurring, then any eastern dispersers are not contributing to the gene pool but could be artificially inflating our estimates of both genetic diversity and population size, which would suggest that the western population is even more vulnerable than currently thought. Given the wide range of threats, including entrapment in fishing nets as well as expanding oil and gas development, which challenge the recovery of the western gray whale population, further exploration of possible mechanisms of intermixing is needed to better understand the dynamics of this critically endangered population.

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

Locus	Source Species	Repeat Size (bp)	T_a (°C)	Size Range (bp)	Reference
DlrFCB17t*	*Delphinaptera leuca*	2	54	183-213	Buchanan *et al.* 1996
EV14t*	*Megaptera novaeangliae*	2	55	138-156	Valsecchi and Amos 1996
EV37	*Megaptera novaeangliae*	2	55	183-231	Valsecchi and Amos 1996
EV94t*	*Megaptera novaeangliae*	2	52	209-237	Valsecchi and Amos 1996
Gata028	*Megaptera novaeangliae*	4	54	159-187	Palsboll *et al.* 1997
Gata098	*Megaptera novaeangliae*	4	54	67-103	Palsboll *et al.* 1997
Gata417	*Megaptera novaeangliae*	4	54	198-222	Palsboll *et al.* 1997
Gt023	*Megaptera novaeangliae*	2	54	94-116	Palsboll *et al.* 1997
RW31	*Eubalaena glacialis*	2	54	114-136	Waldick *et al.* 1999
RW48	*Eubalaena glacialis*	2	55	112-124	Waldick *et al.* 1999
SW10t*	*Physeter macrocephalus*	2	55	119-151	Richard *et al.* 1996
SW13t*	*Physeter macrocephalus*	2	55	168-196	Richard *et al.* 1996
SW19t*	*Physeter macrocephalus*	2	55	122-142	Richard *et al.* 1996

* The sequence for the reverse primer has been modified from the original design by the addition of a tail (Brownstein *et al.* 1996)

Table 2. Frequency of mtDNA haplotypes in each population.

	# of Individuals	
Haplotype	**East**	**West**
A	15	51
B	10	44
C	13	9
D	7	5
E	4	3
F		1
G	9	2
H	1	2
I		1
J		1
K	5	
L	6	1
M	6	2
N	5	1
O	1	
P	2	
Q	1	1
R	7	
S	1	
T	7	1
U	3	
V	3	1
W	1	
X	6	
Y	3	1
Z	2	1
27	2	
28	2	3
29	2	
30	3	
31	1	
32	1	
33	1	1
34	1	
35		7
36	2	
37	1	
38		3
41	1	
42	1	
Total	136	142

Table 3. Genetic diversity estimates based on mtDNA control region sequences. Includes number of individuals (n), number of haplotypes (k), haplotype diversity (h) and percent nucleotide diversity (π). For haplotype diversity and nucleotide diversity, standard deviations are included in parentheses.

Population		n	k	h	π (%)
East	All	136	35	0.95 (±0.006)	1.57(±0.810)
	Females	49	23	0.95 (±0.014)	1.41 (±0.744)
	Males	87	30	0.96 (±0.008)	1.66 (±0.856)
West	All	142	22	0.77 (±0.025)	1.82 (±0.932)
	No known relatives	84	22	0.82 (±0.030)	1.83(±0.937)
	Females*	36	10	0.77 (±0.050)	1.89 (±0.984)
	Males*	42	15	0.83 (±0.041)	1.82 (±0.944)
Both		278	40	0.89 (±0.012)	1.81 (±0.922)

* Excludes animals first identified as calves

Table 4. Microsatellite data for gray whales. Includes number of alleles per loci (k), expected heterozygosities (H_e), observed heterozygosities (H_o), and number of private alleles (K_p). The overall results include averaged values over all loci for k, H_e, and H_o, and the sum of all private alleles for K_p.

	East				West			
Locus	**K**	**H_e**	**H_o**	**K_p**	**K**	**H_e**	**H_o**	**K_p**
D17t	15	0.89	0.90	1	15	0.88	0.85	1
EV14t	9	0.81	0.78	1	9	0.76	0.74	1
EV37	17	0.88	0.89	1	17	0.85	0.91	1
EV94t	11	0.79	0.74	2	9	0.75	0.75	0
Gata028	8	0.78	0.82	3	5	0.75	0.78	0
Gata098	10	0.65	0.65	3	7	0.63	0.61	0
Gata417	7	0.71	0.71	0	7	0.63	0.65	0
Gt023	9	0.72	0.76	1	8	0.68	0.68	0
RW31	10	0.82	0.83	1	9	0.82	0.85	0
RW48	5	0.40	0.42	0	5	0.36	0.34	0
SW10t	9	0.77	0.76	1	9	0.75	0.77	1
SW13t	8	0.63	0.67	1	8	0.67	0.68	1
SW19t	10	0.71	0.67	3	7	0.64	0.67	0
Overall	9.8	0.74	0.74	18†	8.8	0.70	0.71	5†

Table 5. Analysis of molecular variance (AMOVA) and pairwise comparisons among gray whale populations from microsatellites and mtDNA control region sequences. Significant P values (<0.05) are shown in bold. Comparisons using only females and only males did not include known relatives.

Comparison	**Microsatellites**			**mtDNA**	
	Genotype frequency			**Haplotype frequency**	
	F_{ST}	F_{ST} probability	Exact test probability	F_{ST}	F_{ST} probability
All individuals	0.009	**≤ 0.001**	**≤ 0.001**	0.068	**≤ 0.001**
No known relatives	0.005	**≤ 0.001**	**≤ 0.001**	0.045	**≤ 0.001**
Females	0.013	**≤ 0.001**	**≤ 0.001**	0.078	**≤ 0.001**
Males	0.002	0.117	**0.039**	0.033	**≤ 0.001**

Table 6. Results of STRUCTURE analyis using a model incorporating admixture with correlated allele frequencies. Includes the inferred number of genetic clusters (K), the estimated log likelihood value (after averaging across runs) for the data given K (Ln P(X|K)), and the posterior probability of K (Pr (K|X)). The value of K with the highest posterior probability is shown in bold. Details about the parameters incorporated in each model are described in the text.

K	**Ln P (X|K)**	**Pr (K|X)**
1	-11612.18	~0
2	**-11469.22**	**~1**
3	-11682.32	~0
4	-11808.74	~0
5	-12214.70	~0

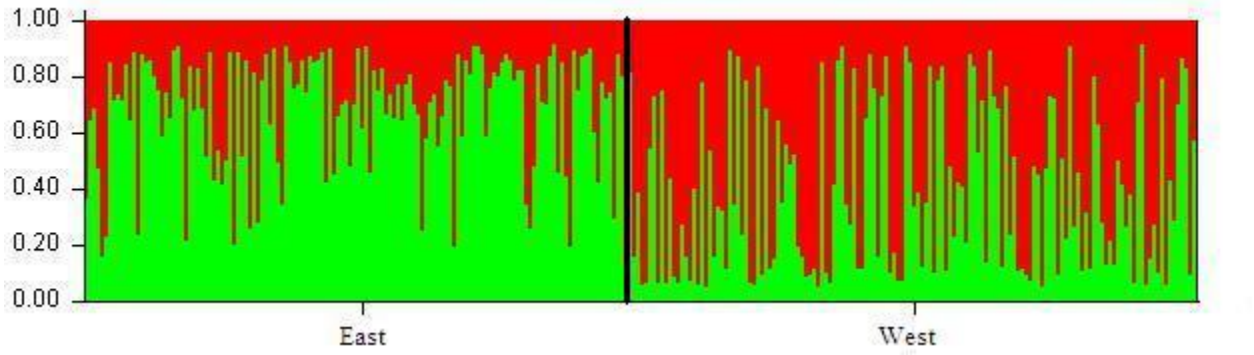

Figure 1. STRUCTURE barplot for K=2 using a model based on admixture with correlated allele frequencies. Individuals are represented by vertical bars, and the different colors of the bars represent the proportion of admixture (*Q*), or ancestry, from a each inferred genetic cluster. Individuals are grouped according to the population in which they were sampled, and the black line denotes the boundary between animals sampled in the eastern and western Pacific.