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Substructuring of mitochondrial, but not nuclear, markers in the "southern feeding group" of eastern North Pacific gray whales

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ABSTRACT

During fall and early winter, eastern North Pacific gray whales (*Eschrichtius robustus*) migrate south to calving grounds in the lagoons of Baja California, and from late winter to early spring they migrate north to their summer feeding grounds in the Chukchi and Beaufort Seas. Although the majority of the population makes this migration, a small subset of the population—known as the "southern feeding group"—ends their northward migration early, spending summers feeding in waters ranging from northern California to southern Alaska. Previous analyses based on photo-identification and mitochondrial DNA (mtDNA) data indicate that this seasonal substructuring results from maternally-directed site fidelity to different feeding grounds, and that this site fidelity and feeding ground preference is passed from mothers to their offspring. It is currently assumed, but not known, that the individuals of the southern feeding group mate with the rest of the population, and therefore that the eastern North Pacific gray whale represents one interbreeding population. Testing this assumption is key to making appropriate management decisions because there is an increasing interest by native groups in Washington and British Columbia to resume their traditional hunts. Such hunts could disproportionately affect whales of the southern feeding group, and therefore understanding how these whales are related to the rest of the population is necessary for properly managing such hunts. Here, we analyzed 15 nuclear microsatellite loci in 82 individuals of the southern feeding group and 51 individuals sampled in the calving lagoons—representative of the larger population—to test the hypothesis that the eastern North Pacific gray whale represents one interbreeding population. We found no indication of population substructuring based on these nuclear loci, suggesting that all sampled whales do indeed represent one interbreeding population. Combined, these data from mitochondrial and nuclear markers suggest one interbreeding population that is seasonally subdivided based on maternally-directed site fidelity to different feeding areas.

INTRODUCTION

The eastern North Pacific gray whale (*Eschrichtius robustus*) has been the focus of considerable interest and controversy over the past several years, due in large part to the proposed resumption of traditional whaling by native groups in Washington State, and potentially by others in British Columbia (B.C.). Discussion of resuming these hunts followed the removal of the population from the U.S. Endangered Species List in 1994. Given this revised status, and the treaties that are in place, the eventual resumption of at least some of these hunts seems likely. However, the history of the management of whaling is dismal (e.g. Birnie, 1981; Baker and Palumbi, 1994; Cherfas, 1996; Clapham and Ivashchenko, 2009). As a result, and to improve this record, it is important that the guidelines and management of any resumed hunts are based on appropriate scientific data.

One of the primary topics of concern is population structure: how individuals are divided in space and/or time such that some groups may be differentially affected by direct hunting or by non-intentional threats. If such structuring exists, then the different groups often require separate management/conservation consideration because the detrimental effects will not be spread evenly throughout the population, but instead will disproportionally affect the different groups (e.g. Hoelzel, 1998; Taylor, 2005; Wang, 2009). Such localized impacts on structured populations, if not considered, can nullify otherwise well-planned management/conservation initiatives. Indeed, there are several case studies where the effectiveness of conservation actions has been compromised because population structure was not taken into consideration (e.g. Daugherty *et al*., 1990; Frankham *et al*., 2002).

Previous studies have detected seasonal population substructuring in the eastern North Pacific gray whale in relation to summer feeding ground use (Frasier *et al*., 2011). As with many other baleen whales, gray whales show a seasonal migration from low-latitude calving grounds in the winter to high-latitude feeding grounds in the summer. The winter calving grounds for this population are located in the lagoons of Baja California (Swartz, 1986; Findley and Vidal, 2002; Swarts *et al*., 2006), whereas during the summer the majority of the population feeds in the Bering and Chukchi Seas (Moore and Ljungblad, 1984). However, there is a small subset of the population—estimated at roughly 200 individuals (Calambokidis *et al*., 2002)—that spend the summer in lower-latitude feeding areas ranging from northern California to southeastern Alaska (Pike, 1962; Hatler and Darling, 1974; Darling, 1984; Calambokidis *et al*., 2002; Swartz *et al*., 2006). This group is often referred to as the "southern feeding group". Data from photo-identification and mitochondrial DNA (mtDNA) show that this seasonal population substructuring results from maternally-directed site fidelity to different feeding areas, and that this differential use of feeding areas is passed from mothers to offspring (Darling, 1984; Calambokidis *et al*., 2002, 2010; Frasier *et al*., 2011). This type of seasonal substructuring is common in baleen whales (e.g. Baker *et al*., 1990; Malik *et al*., 1999).

Despite this substructuring during the summer, it has always been assumed—but not known—that individuals from all feeding areas utilize the same mating area(s), and therefore represent one interbreeding population. Unfortunately, there is a lack of clarity regarding where fertilization likely occurs, and hence where the mating grounds are. Sexual behaviour is frequently observed on the winter calving grounds (e.g. Swartz, 1986), and indeed these are often referred to as "breeding grounds" (e.g. Jones, 1990; Goerlitz *et al*., 2003; Alter *et al*., 2009). However, physiological data actually suggest that fertilization most often occurs during the southward migration, prior to arrival at the lagoons (Rice and Wolman, 1971). Thus, there is potential for differential feeding area use to also result in substructuring with respect to reproductive patterns.

There is also evidence that individuals show differential use of the calving grounds, with some females showing fidelity to particular lagoons (Jones, 1990; Goerlitz *et al*., 2003; Alter *et al*., 2009). Combined, there are enough questions regarding the timing and location of mating, as well as potential for differential habitat use of potential "breeding" grounds, to warrant skepticism of the hypothesis of

one interbreeding population. Addressing this issue is important for the development of proper management strategies for the proposed hunts, because the southern feeding group occupies the waters directly adjacent to the groups interested in resuming traditional whaling. These whales may therefore be differentially impacted by the hunts, and understanding how they are structured in relation to the larger population will be important for accounting for, and minimizing, such localized impacts.

Here, we used data from nuclear microsatellite loci to compare genetic data from individuals of the southern feeding group to samples obtained from one of the calving lagoons (Laguna San Ignacio)—used here as representatives of the larger population—to test the hypothesis that individuals of the southern feeding group interbreed with individuals from the larger population. These data, in combination with previous information regarding structuring of mitochondrial haplotypes, will identify the degree of substructuring of the southern feeding group.

METHODS

Sample Collection

Samples used for these analyses were collected over many years as part of other long-term research programs on eastern North Pacific gray whales: off Vancouver Island, Canada by Dr. Jim Darling; and off San Ignacio Bay, Mexico by Dr. Jorge Urbán-Ramirez. Specifically, 86 samples from Vancouver Island (representing the southern feeding group), and 51 samples from San Ignacio Bay (representing the larger population) were analyzed. All samples were collected using a crossbow and a modified bolt, as is common for collecting small skin samples from free-ranging large whales (Lambertsen, 1987; Palsbøll *et al*., 1991). This method of sample collection has been extensively scrutinized, has proven to be safe, and does not have any short- or long-term impacts on the whales, other than an immediate startle response (Brown *et al*., 1991; Best *et al*., 2005).

Genetic Analyses

DNA was extracted from all samples using standard phenol:chloroform procedures that are commonly used for whale skin (e.g. Wang *et al*., 2008). The quantity of DNA obtained from each sample was estimated based on spectrophotometry using a NanoDrop 2000 (Thermo Scientific Inc.) The quality of DNA obtained (i.e. the amount of DNA degradation) was assessed based on electrophoresis through 2.0% agarose gels stained with SYBR Green I (Invitrogen). Sex was determined for each sample based on PCR amplification of a region on the *X*- and *Y*- chromosomes using the primers described in Gilson *et al*., (1998).

To identify useful microsatellite markers for this study, 23 loci were screened for amplification and variability in gray whales (**Table 1**). These loci were chosen because previous experience showed that they amplify well, and are highly variable, across a wide range of whale species. Specifically, loci were initially tested based on the amplification of two gray whale samples (one from the southern feeding group and one from the larger population) using annealing temperatures of 50, 55, and 60°C. The reactions contained 10 ng of template DNA, 1X PCR Buffer (20 mM Tris pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.05 U/µl *Taq* DNA polymerase (Invitrogen), 0.2 mM each dNTP (Invitrogen), 0.3 µM each primer, and 0.1 mg/ml of bovine serum albumin (BSA) (Invitrogen). The cycling conditions were as follows: an initial denaturing step of 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, annealing temperature for 1 minute, and 72°C for 1 minute; followed by a final extension step of 60°C for 45 minutes. All PCR was conducted on Veriti® 96-well thermal cyclers (Applied Biosystems). PCR products were then size-separated and visualized via electrophoresis through 1.5% agarose gels

stained with ethidium bromide. Loci that showed a clean PCR product (without amplification of multiple regions) were selected for further development.

For primer pairs that amplified well, the forward primer was re-ordered with one of four fluorescent tags (6FAM-Blue, VIC-Green, PET-Red, or NED-Yellow). Each locus was then screened for variation by amplifying 10 samples using the optimal conditions that had previously been determined. PCR amplification was carried out using the same conditions as described for testing annealing temperatures. PCR products were de-salted via ethanol precipitation (Irwin *et al*., 2003), and size-separated and visualized on an ABI 3500xl capillary-based genetic analyzer (Applied Biosystems).

Based on these amplification data (i.e. peak height and allele ranges) multiplex reactions – where multiple loci are amplified simultaneously in the same PCR – were developed through testing the amplification of different combinations of loci. These tests resulted in combinations of loci that minimized the number of reactions that were needed to amplify the variable loci. These protocols were then used to genotype all individuals. Alleles were scored using the GeneMarker software (SoftGenetics). Each 96-well plate of samples contained 2 individuals as "standards", meaning that these same two individuals were present on all plates, to ensure consistency in genotyping across plates. All samples were scored by two individuals in a double-blind fashion to identify any potential scoring errors (e.g. Morin *et al*., 2010).

Statistical Analyses

Once genotypes for each sample had been obtained, the program CERVUS (Marshall *et al*., 1998) was used to identify any duplicate genotypes (i.e. individual that had unknowingly been sampled more than once). CERVUS was also used to obtain estimates of allele frequencies, and to estimate the frequency of null alleles for each locus. Loci were tested for deviations from linkage and Hardy-Weinberg Equilibrium (HWE) using exact tests as implemented in the program GENEPOP (Rousset, 2008). Based on these data, duplicate individuals were removed, as were any loci that showed linkage or deviated from HWE. The resulting genotypes were used for subsequent analyses of population structure.

A "classical" assessment of population differentiation was conducted using the program GENEPOP. Here, individuals were categorized as representing either the southern feeding group or the larger population, based on sampling location. Specifically, samples collected off Vancouver Island were classified as the southern feeding group, and samples collected on the calving grounds off Baja California were classified as representing the larger population. Based on this division, estimates of F_{ST} were obtained, and exact tests of population differentiation were conducted.

Population structure was also assessed without making *a priori* assumptions about the nature of population structure (e.g. how many groups there are, and which individuals represent each group). These analyses were conducted in two different ways: using the programs *structure* (Pritchard *et al*., 2000; Hubisz *et al*., 2009), and *structurama* (Huelsenbeck and Andolfatto, 2007). For the analyses in *structure*, the number of groups, and the membership of individuals within those groups, were estimated based a run length of 1,000,000 Markov Chain Monte Carlo (MCMC) steps, with 50,000 steps as the burn-in period. The program was run assuming that allele frequencies were correlated between groups, and allowing for admixture (i.e. allowing for individuals to have ancestry in more than one group). The program was run 16 times, testing for 1 – 4 populations ($K = 1 – 4$), with four iterations if each K. The average likelihood over the four iterations for each K was taken as the likelihood for that K.

The program *structurama* works in a similar manner as *structure*, but differs in how the user specifies the number of populations to be tested. With *structure*, the user must explicitly specify the number of populations considered, and then run the program independently for each hypothesized

number, and subsequently compare the probabilities associated with each. With *structurama* the number of populations considered can be a random variable within the model (Pella and Masuda, 2006), and therefore the posterior probabilities associated with a range of values for the number of putative populations can be obtained within a single run, without requiring *a priori* specification by the user (Huelsenbeck and Andolfatto, 2007). We ran *structurama* allowing the number of populations to be a random variable with a Dirichlet process prior. The alpha value was also treated as a random variable, following a gamma distribution. We first used a shape parameter of 1.0001, and a scale parameter of 0.0001, which result in a relatively flat distribution. However, to test the robustness of the subsequent posterior probabilities to our choice of shape and scale values, we also ran *structurama* with the shape and scale values both set to 1, which results in an L-shaped distribution. Each scenario was run three times, to ensure consistency between runs under the same conditions. All analyses were run for 1,000,000 MCMC steps, with 10,000 steps used as the burn-in.

RESULTS

The average yield of DNA from the extraction process was 2.80 nanograms of DNA per milligram of tissue for the samples from Vancouver Island, and 0.42 ng/mg for the samples from San Ignacio Bay. This lower yield from the San Ignacio Bay samples likely results from the fact that these samples were stored in ethanol rather than DMSO, and DNA degradation is known to occur at a higher rate in ethanol than DMSO (e.g. Michaud and Foran, 2011).

Gender could be determined for 93 of the 133 samples, with 45 males and 48 females. The DNA was too degraded from the remaining 40 samples to obtain reliable gender information.

The tests of microsatellite amplification and variability resulted in the identification of 15 loci that amplify well in gray whales, and are also variable. Multiplex reaction development resulted in all 15 loci being amplified in 5 reactions (**Table 2**), which were subsequently used for genotyping all samples.

No genotypes differed between the duplicate scoring personnel, indicating that allele calls were consistent across scorers. Analysis of the genotypes identified 29 individuals that had been sampled multiple times. All re-sampling events were from the same location (i.e. there were no cases where one individual was sampled in one location and re-sampled at the other). These duplicates were removed from the data set, resulting in genotype data for 104 individuals. No loci showed any significant signs of linkage or deviations from Hardy-Weinberg Equilibrium, and none had estimates of null allele frequencies greater than 0.05.

The "classic" tests did not show any significant signs of genetic differentiation between the genotypes of the southern feeding group and the larger population. Specifically, the F_{ST} estimate was -0.0010, with a *P*-value estimate of 0.489. The *structure* analyses also did not detect any significant genetic differentiation within the data set, with one population ($K = 1$) having the highest probability (**Table 3**). Similar results were also obtained with *structurama*, with the scenario of the data representing one single population having a higher probability than scenarios with any other putative number of populations (**Table 4**).

DISCUSSION

One of the first results that warrants discussion is the differential recovery of DNA from tissue stored in different solutions. Several studies have compared the ability of different solutions (primarily ethanol and DMSO) to preserve DNA over long periods of time (e.g. Seutin *et al*., 1991; Michaud and Foran, 2011). All such studies indicate that DMSO solutions preserve DNA at a higher quality, and

over a longer period of time, than ethanol. However, many researchers and museum staff still use ethanol for the long-term preservation of tissue. The differential yields of DNA obtained here add to the growing amount of data suggesting that DMSO is the desirable storage solution for long-term storage of tissue that may be used as a source of DNA.

The results of all analyses of population structure lead to the same conclusion: a lack of differentiation of nuclear genotypes. The "classical" tests did not detect significant differences in allele frequencies between whales of the southern feeding group and those sampled in Laguna San Ignacio, and both Bayesian approaches indicated that the probability that all samples originated from one single population was substantially higher than any other alternatives. These data suggest that the whales of the southern feeding group do indeed freely interbreed with whales that utilize other summer feeding grounds. Thus, from these data, it appears that the eastern North Pacific gray whale represents one interbreeding population.

These data based on nuclear markers add to previous photo-identification and mtDNA data to provide a more complete picture of the relationship between seasonal habitat use patterns and gene flow throughout the population. The photo-identification and mtDNA data indicate that, during the summer, whales of the southern feeding group represent a seasonal subpopulation, where this differential habitat use is driven by maternally-directed site fidelity to this feeding area that is then passed on to their offspring. However, the nuclear DNA data suggest that these whales breed with whales that show fidelity to other feeding grounds, and therefore are part of one interbreeding population. Thus, the combined picture is one of seasonal subdivision on summer feeding grounds, but with no such substructuring during the mating season, where all individuals represent one gene pool.

This result and interpretation is consistent with other data relating to known migration patterns and the timing of fertilization. Briefly, it is thought that the majority of conceptions occur at the beginning of December, during the southern migration (Rice and Wolman, 1971). Whales that utilize the northern feeding grounds migrate through the areas occupied by the southern feeding group, suggesting that whales from both known feeding grounds may migrate together the remainder of the way to the winter calving grounds (Darling 1984). The peak time of migrants passing through the southern feeding area is December (Darling 1984). Thus, the timing of fertilization coincides with when whales from different feeding grounds become intermingled during their southern migration. This pattern indicates the strong potential for interbreeding regardless of any substructuring that may exist during the summer, or on the winter calving grounds.

Despite the presence of nuclear gene flow between whales from the southern feeding group and the rest of the population, this group still represents a separate management unit that warrants separate consideration with respect to the proposed resumption of traditional whaling. The presence of long-term site fidelity to this area, that is passed on from mothers to offspring, indicates that these whales represent a seasonal subpopulation. Thus, detrimental impacts (i.e. "takes") to these whales will not have a "random" impact on the population at large, but will instead primarily impact these matrilines specifically. As a result, the resulting effect on this local subpopulation could be far greater than would be expected under the assumption of a single, unstructured population. Potential impacts could include the loss of knowledge of these feeding areas from this population, and localized extirpation. For example, if the whales that currently show this site fidelity are removed, then this information will be lost, and thus these whales will not likely be replaced by others from the larger population, resulting in localized extirpation. Indeed, the recognition of such seasonal subpopulations as separate management units is recommended, and common, for baleen whales (e.g. Dizon and Perrin, 1997).

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Table 1. Name and reference for each of the 23 microsatellite loci tested for amplification and variability in gray whales.

Locus	Reference
EV1*Pm*	Valsecchi and Amos, (1996)
EV5*Pm*	Valsecchi and Amos, (1996)
EV14*Pm*	Valsecchi and Amos, (1996)
EV37*Mn*	Valsecchi and Amos, (1996)
EV94*Mn*	Valsecchi and Amos, (1996)
EV104*Mn*	Valsecchi and Amos, (1996)
FCB1	Buchanan *et al*., (1996)
FCB4	Buchanan *et al*., (1996)
FCB5	Buchanan *et al*., (1996)
FCB14	Buchanan *et al*., (1996)
FCB17	Buchanan *et al*., (1996)
GATA028	Palsbøll *et al.*, (1997)
GATA098	Palsbøll *et al.*, (1997)
GATA417	Palsbøll *et al.*, (1997)
GT023	Bérubé *et al*., (2000)
IGF1	Barendse *et al.*, (1994)
RW31	Waldick *et al*., (1999)
RW34	Waldick *et al*., (1999)
RW48	Waldick *et al*., (1999)
SW10	Richard *et al*., (1996)
SW13	Richard *et al*., (1996)
SW19	Richard *et al*., (1996)
TexVet5	Rooney *et al*., (1999)

Table 2. Locus and multiplex reaction information. Included is the locus name, fluorescent label, reaction number, the number of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E, Nei, 1978), and the polymorphic information content (*PIC*, Botstein *et al*., 1980).

Locus	Label	Reaction	Alleles	H_O	H_E	*PIC*
EV14*Pm*	6FAM	1	10	0.804	0.846	0.823
EV37*Mn*	VIC	1	17	0.871	0.883	0.868
FCB14	VIC	1	7	0.747	0.815	0.785
GATA028	NED	1	5	0.776	0.754	0.710
FCB5	NED	1	4	0.500	0.457	0.424
GT023	VIC	2	7	0.704	0.722	0.670
FCB4	PET	2	3	0.128	0.225	0.201
EV1*Pm*	NED	2	3	0.588	0.502	0.380
TexVet5	NED	2	6	0.735	0.710	0.659
FCB17	6FAM	3	14	0.946	0.907	0.894
GATA417	PET	3	7	0.704	0.720	0.680
SW10	NED	3	8	0.765	0.791	0.755
SW13	6FAM	4	8	0.674	0.622	0.546
EV94*Mn*	6FAM	5	9	0.821	0.811	0.779
RW31	VIC	5	9	0.827	0.817	0.788
Average			**7.80**	**0.706**	**0.706**	**0.664**

Table 3. Results from *structure* analysis. Shown is the estimated natural logarithm (*ln*) of the probability of the data with the number of populations (*K*) ranging from one to four, and performing four iterations of each *K*. The bold value indicates the average *K* with the highest probability.

	K			
Iteration	1	2	3	4
1	-4219.9	-4243.6	-4273.9	-4276.9
2	-4220.9	-4243.6	-4268.5	-4272.6
3	-4220.3	-4238.9	-4266.2	-4257.1
4	-4220.6	-4228.6	-4248.7	-4263.9
Average	**-4220.4**	-4238.7	-4264.3	-4267.6

Table 4. Results from *structurama* analysis. Shown are the estimated posterior probabilities of the data representing 1 – 3 populations. The top panel shows these probabilities calculated with the alpha value for the Dirichlet process being a random variable following a gamma distribution with a shape parameter of 1.0001, and a scale parameter of 0.0001. The second panel shows these probabilities calculated when the shape and scale value parameters were both set to 1.

gamma(1.0001,0.0001)

	Number of Populations		
Iteration	1	2	3
1	0.96	0.04	0
2	0.96	0.04	0
3	0.96	0.04	0

gamma(1,1)

	Number of Populations		
Iteration	1	2	3
1	0.97	0.03	0
2	0.97	0.03	0
3	0.97	0.03	0