Mitochondrial and Nuclear Genetic Variation across Calving Lagoons in Eastern North Pacific Gray Whales

(Schrichtius robustus)

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Abstract

Accurate knowledge of population structure in cetaceans is critical for preserving and managing breeding habitat, particularly when habitat is not uniformly protected. Most eastern gray whales return to their major breeding range each winter along the Pacific coast of Baja California, Mexico, concentrating in 3 major calving lagoons, but it is unknown whether genetic differences exist between lagoons. Previous photo-identification studies and genetic studies suggest that gray whales may return to their natal lagoons to breed, potentially resulting in the buildup of genetic differences. However, an earlier genetic study used only one genetic marker and did not include samples from Bahia Magdalena, a major calving lagoon not currently designated as a wildlife refuge. To expand on this previous study, we collected genetic data from the mitochondrial control region (442 bp) and 9 microsatellite markers from 112 individuals across all 3 major calving lagoons. Our data suggest that migration rates between calving lagoons are high but that a small but significant departure from panmixia exists between Bahia Magdalena and Laguna San Ignacio (Fisher's Exact test, $P < 0.0001$; $F_{ST} = 0.006$, $P = 0.025$). Coalescent simulations show that the lack of extensive population structure may result from the disruption of structure due to whaling. Another possibility is that rates of migration have always been high (>10% per generation). In addition, microsatellite data showed evidence of a severe population bottleneck. Eastern gray whales are still recovering from the impacts of whaling on their breeding grounds, and these populations should be protected and monitored for future genetic changes.

Key words: cetacean, microsatellite, population structure
display long-term fidelity to particular lagoons, based on photo-identification data (e.g., Jones 1990). In addition, a previous genetic study found small but significant differences between gray whales using lagoons and whales outside of the lagoons (Goerlitz et al. 2003), suggesting the possibility of fidelity to natal lagoons across generations.

Although interyear fidelity to particular lagoons has been documented in gray whales based on photo-identification data, the proportion of animals that is resighted is relatively low, and even among those resighted, the interval between return trips varies among individuals (Swartz and Jones 1983; Jones 1990; Urbán and Gómez-Gallardo 2000a, 2000b). A 6-year survey of gray whales in Laguna San Ignacio during the years 1977–1982 found that 179 out of 562 whales identified (or 32%) were resighted in other years, and out of 55 mature females identified, 10 were sighted in all 6 years (Jones 1990). Similar resighting analyses are currently unavailable for the other lagoons (though photo-identification data are in the process of being analyzed). Although females are generally thought to return to the same lagoon to calve over the course of their lifetimes, it is not known if individuals generally return to their own birthplaces to calve. Unfortunately, using photo identification to document fidelity to particular lagoons across generations is not possible because of the large size of the population, long generation time, and the fact that newborn calves do not yet carry identifying scars and marks. In addition, the sex of single whales (e.g., those without calves) cannot generally be determined in the field.

However, genetic data are well suited to detecting intergenerational philopatry in gray whales. Natal philopatry, defined as returning to the birthplace to reproduce (Pearce 2007), has been detected using genetic data for a number of cetacean species, including humpback whales (Clapham and Mayo 1987), right whales (Schaeff et al. 1993), and beluga whales (Brown Gladden et al. 1997), as well as for other marine animals such as green turtles (Bowen et al. 1992). Though such fidelity is relatively common at large geographic scales, the degree to which cetaceans may faithfully return to breeding grounds at smaller geographic scales (e.g., specific inlets, bays, or bathymetric features such as seamounts) is less well known. For example, southern right whales may return to the broader South African coastline to breed but may not necessarily come back to specific geographic features such as particular bays (Best 2000). To date, one genetic study has examined genetic variation in

Figure 1. The 3 major breeding lagoons of eastern North Pacific gray whales and sampling locations: Laguna Ojo de Liebre (OL), Laguna San Ignacio (SI), and the Bahia Magdalena complex (BM).
eastern North Pacific gray whales on their breeding grounds using a single genetic marker (the mitochondrial control region) and samples from 2 breeding lagoons (San Ignacio and Ojo de Liebre) (Goerlitz et al. 2003). This study found significant genetic differences between cows and single females from both of these lagoons compared with a random sample of nonlagoon females, but no significant difference was detected between the 2 lagoons in either cows or single females. The third major breeding lagoon, Bahia Magdalena, is the largest geographically and, unlike the first 2 lagoons, is not currently designated as a wildlife refuge (Urba ´n et al. 2003), but until now no population genetic study of this lagoon has ever been conducted.

Together, photo-identification and genetic studies raise the possibility that populations among breeding grounds in Mexico may be genetically differentiated. A better understanding of gray whale population substructure and long-term habitat use is critical for assessing threats to particular lagoons. Whereas both Lagunas San Ignacio and Ojo de Liebre are designated as whale refuges under Mexican law, anthropogenic threats to gray whales remain in areas adjacent to the protected lagoons, along their migration routes, and in the last unprotected breeding lagoon, Bahia Magdalena. In particular, genetically differentiated subpopulations would indicate that movement among breeding lagoons was low over multiple generations.

Even if gray whales display strong fidelity to natal areas like many other baleen whales, observable genetic structure among different lagoons today may also reflect the ecological and genetic consequences of whaling. The lagoons of Baja California were the first areas where whalers targeted gray whales, using skiffs launched from large sailing ships (Henderson 1984). Intense whaling in the lagoons occurred between 1855 and 1874 and then subsequently from 1913 to 1929, a period in which at least 3500 gray whales are known to have been taken from the lagoons alone. Based on historical records, the largest number of whales were killed in Bahia Magdalena, which was whaled more heavily and for a longer period of time than the other 2 lagoons (Urba ´n et al. 2003). Although Bahia Magdalena appears to have been the most important historical whaling ground, today more whales use Laguna Ojo de Liebre than the other 2 lagoons. The importance of this lagoon appears to have grown as the population has increased: during the 1980 breeding season, Laguna Ojo de Liebre contained 58% of cow–calf pairs and 44% of single whales across all lagoons, whereas during the 1997 breeding season, it represented 72% of cow–calf pairs and 50% of single whales (Urba ´n et al. 2001). Since whaling stopped, gray whales have rebounded from as few as 1500 individuals at the end of the 19th century (Butterworth et al. 2002) to about 22 000 individuals today (Rugh et al. 2005), though the original population was probably far larger (Alter et al. 2007).

Many demographic scenarios are possible with regard to how population structure may have changed over time during recovery from whaling. The replenishment of breeding lagoons in Baja California might have been the result of regrowth of the original 3 lagoon populations, each of which suffered a whaling bottleneck (Figure 2a). In this case, each lagoon appears to have grown to a nearly constant size (Figure 2b).
case, any original population structure across lagoons might have persisted. Alternatively, after whaling stopped, lagoons might have been recolonized by random representatives from previous populations (Figure 2b). If so, then any population structure observed today would have regenerated from that point. If whaling completely disrupted any population structure that existed between lagoons in the past, therefore, we might expect relatively equal levels of genetic diversity across lagoons today or slightly higher levels in Laguna Ojo de Liebre. If, on the other hand, philopatry to particular lagoons persisted such that the prewhaling signature might still be evident, we might expect the greatest genetic diversity in Bahia Magdalena (assuming that the larger whaling effort in that lagoon reflected a larger population of whales). Of course, many alternative or intermediate scenarios are possible, for example, fidelity may have varied between lagoons such that a smaller component of the population always used Laguna San Ignacio, whereas a larger component used multiple lagoons. In the following analyses, we primarily consider the first 2 simpler demographic scenarios.

In order to assess population structure in gray whales in the context of habitat protection and whaling impacts, we collected and analyzed DNA samples from all 3 lagoons and analyzed them at 10 genetic markers (9 microsatellite markers and the mitochondrial control region). Whereas microsatellite markers are primarily useful for longer term signals on evolutionary timescales, microsatellite data are often utilized to assess demographic processes on shorter timescales (see, e.g., Goossens et al. 2006). The ability to detect demographic signals over shorter time frames is particularly important in the case of populations that have undergone recent changes due to anthropogenic impacts such as whaling or hunting. The specific goals of this research were as follows: 1) determine whether gray whales in the 3 major breeding lagoons in Baja California exhibit genetic differentiation (population substructure), 2) assess whether these populations show evidence of population bottleneck or other genetic consequences of whaling on each lagoon, and 3) use coalescent modeling to determine the level of genetic differentiation expected under different historical scenarios and to analyze whether the sample size used in this study can accurately measure these levels.

### Materials and Methods

#### Sample Collection, DNA Extraction, and Sex Determination

Skin biopsies were collected from breeding females, single adult females, and males in each of 3 major breeding lagoons in Baja California (Bahia Magdalena, Ojo de Liebre, and San Ignacio) during the winter breeding seasons (February to March) of 2001, 2002, 2005 (Ojo de Liebre and San Ignacio), and 2006 (Bahia Magdalena). All samples from Bahia Magdalena were collected from both the Santo Domingo channel and the Bahia Magdalena proper. Biopsies were obtained using small stainless steel darts deployed from a crossbow (e.g., Lambertsen 1987). All biopsied individuals were photographed on their right side to ensure that the same individual was not biopsied twice and to facilitate integration of genetic information with photo-identification programs (photo-identification data are currently being compared with the larger interyear database of gray whale photos at the Universidad Autonoma Baja California Sur). We extracted genomic DNA from 66 individuals using a QIAamp kit (QIAGEN, Valencia, CA). Previously extracted DNAs from an additional 49 individuals were supplied by Southwest Fisheries Science Center (La Jolla, CA). We initially collected genetic data from these 115 individuals (SI = 57, OL = 24, and BM = 34) at the mitochondrial control region and 9 microsatellite loci (described in Table 1); the number used for data analysis was reduced to 112 individuals (SI = 56, OL = 24, and BM = 32), after the removal of 3 individuals sampled twice (see Results). In addition, because determining the sex of gray whales in the field is difficult without direct sighting of genitalia or proximity to a calf (in which case the adult individual is assumed to be the mother), we determined the sex of unknown individuals using a modified version of the method of Fain and LeMay (1995), which amplifies both ZFX/Y and SRY protein encoding genes.

#### Mitochondrial Control Region Sequencing and Analysis

We amplified and sequenced a 442-bp fragment of the mitochondrial control region following Rosel et al. (1995), using the primers 5'-TACCAAATGTATGAAACCTCAG-3' and 5'-CTCACAGAGGGACCCGTG-3' (described in Table 1). We then determined whether these sequences fell into one of 7 geographic clusters or were unique to a single individual.

### Table 1. Microsatellite markers used in the analysis

<table>
<thead>
<tr>
<th>Locus</th>
<th>Ta</th>
<th>Repeat size</th>
<th>N</th>
<th>Ho</th>
<th>He</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV94</td>
<td>60</td>
<td>2</td>
<td>10</td>
<td>0.72973</td>
<td>0.78934</td>
<td>Valsecchi and Amos (1996)</td>
</tr>
<tr>
<td>Gata417</td>
<td>61</td>
<td>4</td>
<td>7</td>
<td>0.64865</td>
<td>0.66901</td>
<td>Palsboll et al. (1997)</td>
</tr>
<tr>
<td>Gt023</td>
<td>61</td>
<td>2</td>
<td>9</td>
<td>0.86842</td>
<td>0.7593</td>
<td>Berube et al. (2000)</td>
</tr>
<tr>
<td>D17</td>
<td>54</td>
<td>2</td>
<td>14</td>
<td>0.97368</td>
<td>0.88561</td>
<td>Buchanan et al. (1996)</td>
</tr>
<tr>
<td>RW31</td>
<td>54</td>
<td>2</td>
<td>9</td>
<td>0.79412</td>
<td>0.83363</td>
<td>Waldick et al. (1999)</td>
</tr>
<tr>
<td>AC137</td>
<td>61</td>
<td>2</td>
<td>5</td>
<td>0.60526</td>
<td>0.66281</td>
<td>Berube et al. (2005)</td>
</tr>
<tr>
<td>Gata028</td>
<td>54</td>
<td>4</td>
<td>6</td>
<td>0.89744</td>
<td>0.77189</td>
<td>Palsboll et al. (1997)</td>
</tr>
<tr>
<td>TR3G1</td>
<td>68/55</td>
<td>4</td>
<td>11</td>
<td>0.83211</td>
<td>0.8331</td>
<td>Frasier et al. (2006)</td>
</tr>
<tr>
<td>TR3G2</td>
<td>50</td>
<td>4</td>
<td>8</td>
<td>0.78788</td>
<td>0.74592</td>
<td>Frasier et al. (2006)</td>
</tr>
</tbody>
</table>

Ta, annealing temperature; N, number of alleles; Ho, observed heterozygosity; He, expected heterozygosity.
(Rosal et al. 1995) and 5’-CCTCCCTAAGACTCAAGGAGAAG-3’ (LeDuc et al. 2002). Amplification conditions and cycling were as follows: denaturation for 2 min at 94°C, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and a 1.5-min extension at 72°C. Reactions were performed in a total volume of 10 μl, with 1.5 mM MgCl₂, 200 μM each deoxynucleoside triphosphate (dNTP), 2 pmol each primer, 0.5 unit of Econotaq DNA polymerase (Lucigen, Middleton, WI), and approximately 50–100 ng of template DNA. Amplified products were sequenced on an automated sequencer (ABI 3100, Applied Biosystems, Foster City, CA). Sequence data were edited and aligned in SEQUENCHER 4.5 (Gene Codes Corporation, Ann Arbor, MI).

Genetic Diversity

We used the genetic data analysis program ARLEQUIN (Scheider et al. 2000) to obtain diversity indices [Ω(S), π, and haplotype diversity (HD)] for mitochondrial data. The program DNASP v. 4.10 (Rozas et al. 2003) was used to estimate several estimates of departure from neutrality, including Tajima’s D (Tajima 1989), F and D of Fu and Li (Fu and Li 1993), Fu’s Fs (Fu 1997), and the R2 statistic (Ramos-Onsins and Rozas 2002). In the case of neutral loci such as the mitochondrial control region, these summary statistics are used to test for demographic expansion or contraction. In particular, Fu’s Fs statistic measures the probability of having a number of haplotypes greater or equal to the observed number in the sample given no demographic growth or decline. DNASP was also used to measure Hudson’s nearest neighbor distance (Snn) (Hudson 2000). Finally, we used DNASP to measure the R2 statistic of Ramos-Onsins and Rozas (2002), which is a more powerful test for detecting population growth when sample size is small.

Population Structure

ARLEQUIN (Scheider et al. 2005) was used to obtain pairwise F statistics and to perform exact tests of population differentiation for mitochondrial data between lagoons. In addition, in order to compare our data with the earlier analysis of Goerlitz et al. (2003), we compared control region haplotypes from the lagoons with a set of previously collected haplotypes from individuals sampled outside of the lagoons (“nonlagoon” samples), intended to represent a random selection of haplotypes in the population. As noted above, this previous study found significant differences between lagoon and nonlagoon whales but not between the 2 primary lagoons sampled. Nonlagoon sequences as first reported in Steeves et al. (2001) were obtained from GenBank (accession numbers AF369762–AF369785) and were identical to those used in Goerlitz et al. (2003). We used ARLEQUIN (Scheider et al. 2005) to measure F statistics for comparisons between the data set of Steeves et al. (2001) and our sequences. Because information on reproductive status (cow vs. single female) was not available for SI or OL, we compared all females from each lagoon with nonlagoon females (n = 25). We also compared BM cows (n = 16) with nonlagoon females (n = 25) and all males from each lagoon with nonlagoon males (n = 29).

Microsatellite Genotyping and Analysis

We amplified 112 individuals at 9 previously described microsatellite loci (Table 1), using forward primers labeled with fluorescein dye 6-FAM, NED, or HEX (ABI). Polymerase chain reaction amplifications were performed on thermal cyclers in 10-μl reactions under the following conditions: 5 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 55–65°C (annealing temperatures specified in Table 1), 45 s at 72°C, and a final step of 10 min at 72°C. Reactions were performed in a total volume of 10 μl, with 1.5 mM MgCl₂, 200 μM each dNTP, 2 pmol each primer, 0.5 unit of Econotaq DNA polymerase (Lucigen), and approximately 50–100 ng of template DNA. Successful amplifications were genotyped on a 3730 Genetic Analyzer (ABI) and scored using GENEMAPPER v. 3.7 (ABI). Approximately 10% of samples were reamplified and scored to verify results.

Genetic Diversity

We used ARLEQUIN v. 3.01 (Excoffier et al. 1992) to calculate expected and observed heterozygosity for each locus and to determine allele frequencies in each lagoon. Departure from Hardy–Weinberg equilibrium was tested using exact tests (using hypotheses of either heterozygote excess or deficiency) for each locus in GENEPOP v. 3.3 (Raymond and Rousset 1995a,b). GENEPOP was also used to test for linkage disequilibrium across loci using a Markov chain method. The program ML-RELATE (Kalinowski et al. 2006) was used to assess the probability of any individuals in the sample being closely related (parent–offspring or sibling pairs).

Population Structure

We used ARLEQUIN to calculate the fixation index FST (Weir and Cockerham 1984), with permutation tests of 1000 randomized runs. Because assignment tests and contingency tests often have higher power to detect departure from panmictia than F statistics (Waples and Gaggiotti 2006), we utilized an assignment test approach. In order to determine probabilities for assigning each individual back to the lagoon in which it was sampled, we used a Bayesian approach (Rannala and Mountain 1997) as implemented in GENECCLASS (Cornuet et al. 1999; Piry et al. 2004). In this method, each individual is assigned to the population in which the likelihood of its genotype is the highest. The Bayesian method has been shown to outperform structure in cases in which migration is high or sample size or numbers of loci are low (Waples and Gaggiotti 2006). Assignment probabilities were calculated using a resampling algorithm with 10 000 simulated multilocus genotypes and a threshold of P < 0.025 for excluding populations. To further visualize the likelihoods for each individual genotype being drawn from each population, we plotted the pairwise log likelihoods of the genotype of each individual in every population. For each individual genotype, the likelihood of that genotype being drawn from a particular population sample given its
estimated allele frequencies was calculated using ARLEQUIN v. 3.0.1 (Petkau et al. 1995, 1997a; Waser and Strobeck 1998), and likelihood values for individuals from each pair of populations were plotted against each other.

Finally, we used GENEPOP to test for allelic and genotypic differentiation between lagoons. These analyses estimate the unbiased P values of a log-likelihood (G)–based Fisher’s Exact test (Goudet 1995); for P values across loci, the quantity \(-2\sum \ln P_i\) was assumed to be distributed as \(\chi^2\) with 2i degrees of freedom (Ryman and Jorde 2001), where i is the number of single-locus contingency tests. Multiple comparisons were corrected for with a sequential Bonferroni correction. Fisher’s Exact test has been shown to provide high resolving power for multiallelic loci such as microsatellites (Ryman et al. 2006).

### Bottleneck Analyses

Populations that have undergone significant demographic change (bottleneck or expansion) may display characteristic signature of allele size class distribution and frequency. In particular, severe demographic bottlenecks should erode allelic diversity more rapidly than heterozygosity. In order to test for a signature of population bottleneck or expansion, we used the method of Cornuet and Luikart (1997), which compares allelic diversity to heterozygosity under mutation models of infinite alleles model sites (IAM), stepwise mutation model (SMM), or a mixed two-phased model (TPM). Though microsatellites are thought to evolve under the SMM, most loci show at least slight departures from the SMM toward the IAM, and an empirical test of this method using 8 microsatellite loci showed that the IAM gave a better fit to the data than the SMM (Spencer et al. 2000), indicating a mixed model is preferable. We used the program BOTTLENECK (Piry et al. 1999), which calculates the standardized difference for each locus \([\text{H-hw} - H_{exp}/SD = H_{IS}]\), where H-hw is the heterozygosity under Hardy–Weinberg expectations, Hexp is the expected heterozygosity given number of alleles per locus, and SD is the standard deviation of Hexp. The distribution of expected heterozygosity was calculated under the TPM model with a variance of 30% and the proportion of SMM set to 70% (default values), using a coalescent process. BOTTLENECK implements a Wilcoxon signed-rank test used to evaluate the hypothesis that the average standardized difference is not significantly different from zero.

Garza and Williamson (2001) showed that the mean ratio of the number of alleles to the range of allele size (M) is sensitive to population bottlenecks, dropping in size when a population is reduced. The statistic M therefore measures the proportion of unoccupied allelic states that should be occupied given the range of allele sizes. We measured M using ARLEQUIN and compared it with the M values for reduced and stable populations reported by Garza and Williamson (2001).

### Population Simulations

The power to detect population structure can be heavily influenced by sample sizes per population and by the choice of markers (Ryman et al. 2006). Thus, it is important to assess the degree to which our sample sizes and number of markers may have impacted our ability to detect population structure below certain levels. In addition, it is also possible that population structure across lagoons has been impacted by whaling history. To determine the level of population structure we might expect to be able to detect given our sample sizes and different historical scenarios, we used a coalescent simulation approach using SIMCOAL (Excoffier et al. 2000).

First, we determined whether our data set would capture different levels of population structure given the sample sizes and markers used in our study by simulating a scenario in which whaling from 1850 to 1930 removed 90% of the population but did not disrupt philopatry to natal lagoons (Figure 2a). In other words, in this scenario, whales that survived whaling continued to return to their natal lagoons to breed. We modeled 3 populations (each of Ne = 10 000) that diverged 1000 generations ago, with subsequent migration rates of m = 0.001, 0.01, and 0.1 between them, where m represents the proportion of migrants per subpopulation per generation, using SIMCOAL (Excoffier et al. 2000) to simulate data sets. We chose 1000 generations ago for a divergence time as this time frame roughly corresponds to the beginning of the Holocene epoch, when important geographic features relating to gray whale habitat and ecology were established (e.g., the flooding of the Bering Strait). Each population is reduced to Ne = 1000 individuals approximately 10 generations ago to represent reduction due to whaling, but migration matrices remain unchanged. We then measured the power (Pg) of our data set (in other words, the power of a simulated data set identical to ours in terms of sample sizes and markers) to detect these migration rates, defined as the proportion of significant overall \(F_{ST}\) values across 1000 simulation runs \((P < 0.05)\). Although it would perhaps be more ideal to assess simulated data sets using assignment methods rather than \(F\) statistics (because they would potentially detect differences across shorter time frames), these methods are computationally intensive, which limits the number of simulations possible to assess. \(F\) statistics, however, are conservative and easily calculated over thousands of simulations.

Next, we modeled a scenario in which 3 populations of size Ne = 11 000 each are collapsed by whaling into a single population of size Ne = 3300. After this collapse, the populations split into 3 again with no subsequent migration between them (Figure 2b). Again, we measured Pg.

### Results

#### Genetic Diversity, Hardy-Weinberg Equilibrium, and Linkage Disequilibrium

We obtained 442 bp of sequence data for the control region of 112 whales, in addition to microsatellite data from 9 markers. High levels of genetic variability were observed in all 3 lagoons for both mitochondrial and microsatellite data. Gene diversity across all 10 loci differed slightly between lagoons, with average haplotype diversity highest in BM and
lowest in OL (Tables 2 and 3). For the mitochondrial data, 26 haplotypes were defined by 30 segregating sites (Table 4) and 1 indel. Two private haplotypes (found in 2 individuals each) from the 26 occurred in BM. Tests for deviation from equilibrium (Tajima’s D, F and D of Fu and Li, Fu’s Fs, R2 test) were nonsignificant for all lagoons. Hudson’s nearest neighbor test was also nonsignificant (Snn = 0.33).

No deviation from Hardy-Weinberg Equilibrium was detected in any of the loci in SI or BM using Fisher’s Exact test, but 2 loci (D17 and Gt023) showed heterozygote deficiency in OL. One pair of loci was found to be in significant linkage disequilibrium across all 3 populations (D17 and TR3G1), so analyses were repeated with one of the markers (TR3G1) removed. Three pairs of samples were found to have identical alleles at all microsatellite markers (2 in BM and 1 in SI), so the duplicates were removed from all data sets. No 2 individuals were found to have a more statistically significant chance of being close relatives (parent–offspring or sibling) than of being unrelated.

Sex Determination

Using a modified version of the method of Fain and LeMay (1995), we determined sex for 105 individuals sampled from the 3 lagoons. We were unable to amplify 7 samples using ZFX/Y primers, so these individuals were left out of sex-specific analyses. Samples in each lagoon were primarily composed of adult females (SI = 35, OL = 14, and BM = 28), with males composing a much smaller fraction (SI = 18, OL = 6, and BM = 4). Based on field data available from BM (individuals observed with calves), we determined that 16 of the 28 BM females were cows and the remainder (N = 12) were single females. Because of the number of males sampled was too small to draw statistically significant conclusions, we did not perform male-only analyses.

Population Structure

All tests for population structure (F statistics, assignment tests, and exact tests of allelic and genotypic differentiation) indicated high levels of migration (i.e., low differentiation) between all 3 lagoons for both mitochondrial and microsatellite data, but significant differentiation was detected in one case using microsatellite data (Table 5). Significant differences in allele and genotype frequencies were observed between SI and BM: exact tests of allelic and genotypic differentiation rejected the null hypothesis that genotypes from SI and BM are drawn from the same distribution but did not reject the null hypothesis for SI–OL or OL–BM genotypes (though the significance value for the genotypic differentiation test for OL–BM was borderline at P = 0.041). These results did not differ substantially when the TR3G1 marker was removed from the analysis.

These analyses were repeated using a female-only data set, but there were no large differences between these and the overall results. Of the F statistics results for microsatellite data, only the comparison between BM and SI neared significance with an FST value of 0.006 (P = 0.025). The global FST value among the 3 lagoons was not significant (FST = −0.006, P = 0.99). Because we tested multiple hypotheses about population structure (e.g., pairwise tests between lagoons), we adjusted the a priori significance value (α) for Type I error using a Bonferroni correction in interpreting these results. However, because Bonferroni corrections are highly conservative and diminish power, we also used a modified false discovery rate (FDR) correction (Benjamini and Yekutieli 2001), which provides increased power (Narum 2006). We found that the level of differentiation between BM and SI was significant at the adjusted significance level using the modified FDR method (P = 0.027).

Mitochondrial data showed no significant differentiation between any of the lagoons, either in F statistics (Table 6) or in exact tests (OL–SI: P = 0.588, OL–BM: P = 0.205, SI–BM: P = 0.952). The global FST value among lagoons was also not significant (FST = −0.016, P = 0.954). In addition, no significant differentiation was observed in comparisons between BM or SI and sequences from Steeves et al. (2001) representing nonlagoon individuals. However, one comparison (between OL females, n = 14, and nonlagoon females, n = 25) resulted in a relatively large FST value (FST = 0.11, α = 0.016).

### Table 3. Genetic diversity values for microsatellite markers

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Average #alleles</th>
<th>Hobs</th>
<th>Hexp</th>
<th>Range</th>
<th>GW</th>
</tr>
</thead>
<tbody>
<tr>
<td>OL</td>
<td>24</td>
<td>8</td>
<td>0.77361</td>
<td>0.79566</td>
<td>22.444</td>
<td>0.37882</td>
</tr>
<tr>
<td>SI</td>
<td>56</td>
<td>8.333</td>
<td>0.81549</td>
<td>0.78096</td>
<td>24.222</td>
<td>0.36303</td>
</tr>
<tr>
<td>BM</td>
<td>32</td>
<td>8.333</td>
<td>0.80474</td>
<td>0.79043</td>
<td>22.889</td>
<td>0.37949</td>
</tr>
</tbody>
</table>

N, number of samples; Hobs, observed heterozygosity; Hexp, expected heterozygosity; range, mean allelic range across markers; GW, mean Garza–Williamson statistic (Garza and Williamson 2001).
Although this result was not significant once corrected for multiple comparisons using the Bonferroni correction ($P = 0.0125$), the level of genetic differentiation was significant when the modified FDR method is used ($P = 0.027$).

Very few individuals were assigned back to the lagoon in which they were sampled using GENECLASS with a rejection threshold of 0.025. Out of 112 individuals sampled, 4 were assigned to only one lagoon, and 3 of the 4 were correct assignments (1 correct assignment per lagoon). An additional 9 individuals were assigned with equal probability to 2 lagoons, and in each of these cases, one of the assigned locations was correct. Despite the low number of individuals assigned to particular populations, the likelihood of being drawn from the correct lagoon was found to be higher than the likelihood of being drawn from the other 2 lagoons for the majority of individuals (77% in SI, 56% in OL, and 93% in BM) (Figure 3).

Testing for a Population Bottleneck

Across the 3 populations, overall heterozygosity excess given the number of alleles per locus across the 9 microsatellite loci was found to be significantly greater than zero based on a one-tailed Wilcoxon signed-rank test ($P = 0.0098$). None of the populations showed a significant Tajima’s $D$ value using control region data, though the overall value across all samples was large and positive ($D = 0.782$), consistent with a population bottleneck. The Garza–Williamson index across all 9 loci was 0.374, indicative of a large proportion of missing alleles. Garza and Williamson (2001) show that, given the

### Table 4. Unique mitochondrial control region haplotypes and sampling frequencies in the 3 lagoons

<table>
<thead>
<tr>
<th>Hap</th>
<th>Sequence</th>
<th>Sampling location</th>
</tr>
</thead>
</table>

Numbers above correspond to polymorphic nucleotide positions. All haplotype sequences were submitted to GenBank (accession numbers EU807842–EU807866).

### Table 5. Genetic comparisons between populations including allelic and genotypic differentiation and $F$ statistics

<table>
<thead>
<tr>
<th>POP1</th>
<th>POP2</th>
<th>Allelic $\chi^2$</th>
<th>$P$ value</th>
<th>Genotypic $\chi^2$</th>
<th>$P$ value</th>
<th>Pairwise $F$ statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>OL</td>
<td>SI</td>
<td>12.749</td>
<td>0.806</td>
<td>13.289</td>
<td>0.7741</td>
<td>$-0.0168$ ($0.99)$</td>
</tr>
<tr>
<td>OL</td>
<td>BM</td>
<td>23.751</td>
<td>0.163</td>
<td>29.647</td>
<td>0.041</td>
<td>$-0.0217$ ($0.99)$</td>
</tr>
<tr>
<td>SI</td>
<td>BM</td>
<td>&gt;197</td>
<td>&lt;0.0001</td>
<td>&gt;197</td>
<td>&lt;0.0001</td>
<td>0.0057 ($0.025$)</td>
</tr>
</tbody>
</table>

$P$ values for $F$ statistics (based on 10 000 permutations) are given in parentheses. Msat, microsatellite; CR, mtDNA control region.
appropriate mutational model, data sets with 7 or more loci that show an $\Delta F < 0.68$ can be assumed to have experienced a reduction in population size.

Coalescent Simulations

The results of the coalescent simulations indicated that, given a low to moderate amount of migration between populations ($m < 0.1$) and no disruption of migration due to whaling, our data set should have adequate power to detect any population structure that persisted from prewhaling days ($P_g > 0.75$) (Table 6). However, under a scenario in which the populations have been reshuffled by whaling (e.g., local lagoon differences have been rebuilding only for the past 6–10 generations), we expected much lower genetic structure on the order of $F_{ST} = 0.003–0.005$. In this case, our data set has much lower power to detect significant genetic differentiation. The observed value of $F_{ST}$ between SI and MB was similar to predictions ($F_{ST} = 0.006$), though of borderline significance. Values of $F_{ST}$ for comparisons with OL were negative and/or close to zero.

Discussion

Natal philopatry or maternally directed fidelity has been well documented in several cetacean species, but whether such philopatry exists over multiple generations in gray whales has been controversial. Though photo-identification studies have shown that many females do return to the same lagoons in subsequent years, the large size of the eastern North Pacific gray whale population (which has grown at a rate of roughly 1.9% per year between 1967 and 2002 [Rugh et al. 2005]) and long generation time of these animals (approximately 15.5–22.28 years [Alter et al. 2007]) have made direct observation of philopatry difficult. If philopatry occurs over long timescales and migration (or infidelity to the natal lagoon) is rare, we expect that genetic differences will evolve between lagoons such that whales in each lagoon will be more closely related to one another than expected by chance, and these differences will be detectable from even a relatively small sample of the populations. However, if a moderate proportion of the population breeds in lagoons other than the natal lagoon, these genetic differences become undetectable. Likewise, if philopatry has been occurring for only a short period of time (e.g., if lagoons were abandoned after whaling and recolonized after the cessation of hunting), measurable genetic differences may not yet have accrued.

Data from 9 microsatellite loci and 1 mitochondrial marker indicate that gray whales on their breeding grounds in Baja California are characterized by very high genetic diversity and high rates of migration between lagoons. We detected low but significant genotypic differentiation between 2 of the lagoons (Bahia Magdalena and San Ignacio) using exact tests, but no strongly significant differences in haplotype frequencies ($F_{ST}$) were observed, and assignment tests were unable to assign most individuals to the lagoon in which they were collected with high confidence. However, log-likelihood plots confirm the difference in frequencies between SI and BM and show a greater amount of overlap between SI and OL. Coalescent simulations suggest that in the absence of disruption from whaling (or another cause), our sample size and marker set would be expected to detect differences in haplotype frequencies with reasonable power even when migration is high ($m > 10\%$ per generation, roughly corresponding to $F_{ST} = 0.002$) but that the power to detect differences given total disruption from whaling is fairly low, even when subsequent migration is zero (e.g., all individuals display total fidelity to natal lagoons) because such a small number of generations have elapsed. It is important to note that the coalescent simulations we performed explored only several scenarios out of a large number of possible intermediate or alternative scenarios (e.g., differential migration to each lagoon), and additional genetic data and simulations would be valuable for further exploration. Nevertheless, taken together, our results suggest that 1) there is slight but detectable population differentiation between Bahia Magdalena and San Ignacio and 2) this structure may have persisted through whaling or could have accrued after the cessation of whaling given absolute fidelity to lagoons. Based on photo-identification evidence indicating that many individual female gray whales return to the same lagoon in multiple years to calve, we would expect to detect much higher levels of genetic differentiation if migration patterns had been consistent for many generations (with the caveat that philopatry across generations has never been directly observed). Given the current understanding of gray whale ecology, therefore, our observation of slight genetic differentiation between lagoons is perhaps most consistent with a scenario in which differences that have begun to accrue again after the cessation of whaling. However,

Table 6. $P_g$ (the proportion of significant $F_{ST}$ values across 1000 simulated data sets) for different historical scenarios ($A = \text{no disruption of migration due to whaling}; B = \text{total disruption of migration due to whaling}$) and different amounts of migration between lagoons

<table>
<thead>
<tr>
<th>Scenario</th>
<th>$F_{ST}$ significant</th>
<th>Mean $F_{ST}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Scenario A</td>
<td>Scenario B</td>
</tr>
<tr>
<td>$m = 0$</td>
<td>1</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>0.02603</td>
<td>0.00568</td>
</tr>
<tr>
<td>$m = 0.001$</td>
<td>1</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>0.01808</td>
<td>0.00524</td>
</tr>
<tr>
<td>$m = 0.01$</td>
<td>0.94</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>0.01327</td>
<td>0.00518</td>
</tr>
<tr>
<td>$m = 0.1$</td>
<td>0.75</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>0.00861</td>
<td>0.00382</td>
</tr>
</tbody>
</table>
we cannot rule out the possibility that migration between lagoons has always been high.

The issue of statistical power in conservation genetics is an important one that has been addressed in depth by previous studies (see, e.g., Taylor and Gerrodette 1993; Taylor and Dizon 1996; Ryman et al. 2006), but despite this attention, most studies continue to lack any quantitative assessment of power. We have addressed the issue of power through simulations in order to assess the ability of our data set to capture the expected level of differentiation given various levels of migration and historical scenarios, with a focus on faster evolving microsatellite markers. However, it is important to emphasize that the nonsignificant $F_{ST}$ values observed between Laguna Ojo de Liebre and the other 2 lagoons, particularly at the slower evolving mitochondrial control region, should not be interpreted as evidence for a lack of genetic differentiation in OL animals. As noted above, a broader range of simulation scenarios and a greater number of samples will be required to more fully test additional hypotheses of genetic differentiation.

Despite known instances of female fidelity to calving lagoons, little is known about fidelity to breeding grounds in male whales or about assortative mating. Mating is thought to take place during the southbound migration. If this is the case, in the absence of assortative mating, paternal genotypes would be random with respect to the natal lagoon, which would slow the accrual of genetic differences between lagoons. If no assortative mating takes place, we would expect that stronger structure would be observed in maternally inherited markers such as mtDNA as compared with nuclear markers. Such a pattern has been observed previously in matrilineal or matrifocal cetaceans (e.g., sperm whales, Lyrholm et al. 1999) and has been interpreted as a signal of greater female than male philopatry. In contrast, we have observed slight structure in nuclear markers but no observable structure in mtDNA, and results were similar for females and males (though the low sample size of males in our study precluded statistically rigorous conclusions). This pattern most likely results from the fact that multilocus, multiallelic data generally have much greater power to detect low levels of population structure (Ryman et al. 2006); however, these results are also consistent with the hypothesis of philopatry in both males and females and possibly assortative mating along the migration route. Additional data from sex-linked markers would be useful for distinguishing between these 2 possibilities.

Though our primary goal in this study was to test for differences between lagoons, we also compared lagoon samples with a random sample of nonlagoon individuals in order to compare our results with those of a previous genetic study (Goerlitz et al. 2003). The disparities between our results and those of Goerlitz et al. (2003) may result from differences in both sampling and analytical methods. This earlier study compared samples from SI and OL collected in 1996–1997 with a set of random nonlagoon representatives from the eastern North Pacific population (25 females and 28 males), using 306 bp of the mitochondrial control region. It is possible that a substantial fraction of the eastern gray whale population does not use lagoons for calving (see, e.g., Shelden et al. 2004), and a direct comparison between these animals and those sampled in lagoons would be valuable. However, Goerlitz et al. (2003) used samples collected from the North Pacific with unknown lagoon affiliation, reducing the usefulness of the comparison for testing genetic differentiation between lagoon and nonlagoon animals. Although we were unable to directly compare SI and OL cows with unknown lagoon females as in Goerlitz et al. (2003), the authors found significant haplotype frequency differences between both SI
cows and single females versus unknown lagoon females, suggesting that our comparison between all SI females and unknown lagoon females might also have detected a difference. That we do not observe any differentiation may result from the fact that our SI samples were collected over a greater number of years and thus may have a better chance of capturing interannual variability but also could potentially be due to our smaller sample size of SI females (35 vs. 53). Another possibility is that genetic differences between SI cows and SI single females (as observed by Goerlitz et al. 2003) may have masked differences between SI and nonlagoon females in our analysis. In the one case in which we were able to directly compare cows to nonlagoon females (BM vs. unknown lagoon), we saw no differentiation, although the power to detect differentiation would be small given the relatively small sample size of BM cows (N = 16). Additional multiyear sampling, as well as comparisons with samples from true nonlagoon individuals, will be needed to resolve these issues in the future.

Evidence for a population bottleneck was apparent in the microsatellite data set using 2 methods, which rely on different features of the data. Both the method of Cornuet and Luikart (1996), which tests for excess heterozygosity, and the method of Garza and Williamson (2001), which tests for missing allelic states, indicate a recent reduction in population size. This pattern is evident in spite of the rapid expansion of the eastern North Pacific gray whale population over the last several decades. However, this signature of a severe bottleneck is concordant both with historical data documenting intensive whaling that resulted in “commercial extinction” (Henderson 1984) and a previous genetic study (Alter et al. 2007), which showed that the prewhaling abundance of Pacific gray whales was approximately 3-5 times larger than today’s population in the centuries before whaling. Estimates of the minimum population size at the height of whaling based on historical and census data and population modeling vary from 1500 to 1900 individuals (Butterworth et al. 2002) to 12 000 to 15 000 individuals (Reilly 1992). The data presented here indicate that although the whaling bottleneck at the end of the 19th century was extensive enough to leave a genetic signature, it was not so prolonged or severe as to strip a great deal of genetic variation from the population.

In all, these data confirm that gray whales today have maintained high genetic diversity across all 3 breeding lagoons, implying that migration either has been very high in the past (given no disruption by whaling) or if it was lower in the past has been severely disrupted by whaling. We find some evidence for philopatry to natal lagoons by gray whales based on differences in allelic and genotypic frequencies between lagoons. Given the recent impacts of whaling in these areas, it seems unlikely that population structure in gray whales is at equilibrium. Rather, if the majority of gray whales return to their natal lagoons to breed, we expect that genetic differentiation between lagoons will continue to increase in the future. The data presented here will be valuable as a baseline for comparison with genetic samples from future generations, which should provide a clearer understanding of whether the patterns we have observed represent long-term migration patterns or whether they are reflective of populations still recovering from whaling.

Finally, this study is the first to examine genetic variation in individuals from Bahia Magdalena, a large complex that represents the southernmost major breeding ground of gray whales and the only major lagoon that is not currently protected as a wildlife reserve. In addition to significant genetic differences between Bahia Magdalena and San Ignacio, we found that HD in BM slightly exceeds that of the other lagoons. Two of the 26 mitochondrial haplotypes were private to BM, though because HD is so high relative to the number of samples collected, we cannot be sure that this difference is not simply a reflection of undersampling in other locations. Fewer studies have focused on whales in BM as compared with the other 2 lagoons, perhaps in part because individual whales tend to be more spread out across space and thus harder to sample, but these results suggest that this lagoon harbors high and possibly distinct genetic diversity in spite of intense whaling in this location. The importance of Bahia Magdalena as a unique ecosystem and as one of the most important wetland areas of Baja California Sur for turtles, birds, and marine mammals (Lluch-Belda 2000; Morgan et al. 2005; Koch et al. 2006) suggests that conservation attention to this area is warranted. The data presented here provide additional reasons to ensure that this area continues to be protected from development, additional boat traffic, and other anthropogenic threats.

Funding
American Cetacean Society; Dr Earl H. and Ethel M. Myers Oceanographic and Marine Biology Trust.

Acknowledgments
We are grateful to Francisco Ollervides and the staff and students at the School for Field Studies (San Carlos, Mexico), as well as the staff of the Vizcaino Biosphere Reserve (CONANP) and the Compania Exportadora del Sal S. A. for logistical aid. We also wish to thank Kelly Robertson and Carrie LeDuc at the Southwest Fisheries Science Center, National Oceanic and Atmospheric Administration, La Jolla, for assistance in exporting and processing samples. The manuscript was improved by comments from C. Scott Baker and 3 anonymous reviewers. Samples were collected under research permit No. INE0669/07 (Secretaria del Medio Ambiente, Recursos Naturales y Pesca).

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Received January 28, 2008; Revised September 15, 2008; Accepted September 18, 2008

Corresponding Editor: C. Scott Baker