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[1]JOHN W. BICKHAM, [2]VLADIMIR A. BRYKOV, [3]J. ANDREW DEWOODY, AND [4]CÉLINE A. J. GODARD-CODDING

[1]*Batelle Memorial Institute, Houston, TX, USA*
[2]*A.V. Zhirmunsky Institute of Marine Biology, Russian Academy of Sciences, Vladivostok, Russia*
[3]*Department of Forestry and Natural Resources, Purdue University, West Lafayette, IN, USA*
[4]*The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX, USA*

Contact email: bickhamj@battelle.org

ABSTRACT:

The western gray whale subpopulation is listed as critically endangered, as designated by the IUCN. The IWC is currently conducting a Rangewide Review of the Population Structure and Status of North Pacific Gray Whales (IWC, 2015) in which multiple stock structure hypotheses are being considered within the framework of a population dynamics model. Genetics analyses play an important role in the development and evaluation of plausible stock structure hypotheses. Here, we report on the molecular sexing and mtDNA control region (CR), cytochrome b (cyt b), and COI haplotype sequences, of 32 gray whales biopsied off Sakhalin Island in 2011, 2012, and 2013. As with previous studies of the genetics of this population, appreciable genetic variation was observed for mtDNA. Two control region haplotypes predominate in frequency, A and B, with many more haplotypes observed at very low frequencies. Many of the low frequency haplotypes are males. Haplotype networks confirm that the whales summering near Sakhalin Island do not comprise a specific maternal lineage. Statistically significant differences were observed in haplotype frequencies for the years 2012 and 2013 that might reflect groups of related individual of unknown provenance.

INTRODUCTION:

Two North Pacific populations of gray whales (*Eschrichtius robustus*) are currently recognized; the large eastern gray whale population (ca. 19,000, Laake et al., 2009) and the small western gray whale population (ca. 179, Cooke et al., 2014; IUCN, 2014). Western gray whales inhabit summer feeding grounds in the Sea of Okhotsk, off the northeast coast of Sakhalin Island. Their wintering grounds are poorly known but include the South China Sea (Weller and Brownell, 2012). Recent photographic and genetic matches along with satellite tagging reports have also indicated the presence of western gray whales in North America (Lang et al., 2011; Weller et al., 2011; Urban et al., 2012; Mate et al., 2015). The western gray whale is listed as critically endangered, as designated by the IUCN; the most recent estimate of its population size is N = 179 for the age 1+ population (Cooke et al., 2014; IUCN 2014). The IWC is currently conducting a *Rangewide Review of the Population Structure and Status of North Pacific Gray*

Whales (IWC, 2015) in which multiple stock structure hypotheses are being considered within the framework of a population dynamics model. Genetics analyses play an important role in the development and evaluation of plausible stock structure hypotheses. Here, we report on the molecular sexing and mtDNA control region (CR), cytochrome b (cyt b), and COI haplotype sequences of gray whales biopsied off Sakhalin Island in 2011 (N = 6 biopsies; 6 whales), 2012 (N = 20 biopsies, 17 whales), and 2013(N = 9; 9 whales) for a total of 35 biopsy samples which sampled 32 whales. Samples from 2012 and 2013 were analyzed separately at Purdue University and at the Institute of Marine Biology.

METHODS:

Genetic analyses

Somewhat different methods and slightly different numbers of individuals were used at Purdue University and the Institute of Marine Biology. Differences in methods used between the labs are explained below.

DNA extractions.—DNA was extracted from tissue samples using standard potassium acetate or phenol-chloroform extraction methods (Sambrook and Russell, 2001).

Control region.— Primers for the gray whale control region amplification were 5'-TACCAAATGTATGAAACCTCAG-3' and 5'- CCTCCCTAAGACTCAAGGAAG-3' (Alter and Palumbi 2009). Amplification conditions were as follows: denaturation for 2.5 minutes at 90°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 48°C for 1 minute and a 1.5 minute extension at 72°C.

Cytochrome b.—At Purdue University, primers for gray whale cytochrome b were 5'-CCTCATGATGAAACTTCGGTTCCC-3' and 5'- AAGAGGAAGTAGAGGATGGATGCG-3' (Alter and Palumbi 2009). This amplified an 852 bp segment of the cytochrome b gene. Amplification conditions were as follows: denaturation for 2 minutes at 94°C, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds and a 1.5 minute extension at 72°C. Low-sodium clean-up was conducted on all PCR products. Sequencing reactions were performed with the primers given above. Additionally, two internal primers were used in cytochrome b sequencing reactions: 5'- ATATCATTCTGAGGCGCAACCGTCA-3' and 5'-CCCAGATTCATTCGACTAGGGTAG-3'. Amplified products were cleaned using Sephedex and sequenced in both directions on an automated sequencer. Sequence data were edited and aligned in Sequencher 5.0. NCBI BLAST was used to align sequences to previously described haplotypes from Alter and Palumbi (2009).

At the Institute of Marine Biology, the full gene sequence from cytochrome b (Cytb) and tRNA region 1,445 base pairs long was obtained with the following overlapping pairs of primers 3'developed in the genetic laboratory at the Institute of Marine Biology: GW-CYTB F 5'-TACCATTAACCCCAGAAACGAACCAC-3' and GW-CYTB R 5'-GAGTCTTAGGGAGGTGTGGTTTGTCT-3' and GW-CYTB F2 5'-ATGGGTCTGAGGCGGTTTTTCTGTAG-3' and GW-CYTB R2 5'-GAAGTGGAAGGCAAAGAAGCGTGTTA-3'. The amplification conditions were as follows:

denaturation at 94°C for 2 min, following 34 cycles at 94°C for 30 sec, primer annealing at 54° for 30 sec, and chain extension at 72°C for 90 sec.

COI.—The cytochrome C oxidase subunit I (COI) gene segment of 650 base pairs was amplified using the following primers developed in the genetic laboratory at the Institute of Marine Biology: GW-COI F 5'- ACCTACTCGGCCATCTTACCTA -3' and GW-COI R 5'- AAGCCTAAGAACCCGATGGATA -3'.

Molecular sex determination.—Several primer sets were used to molecularly sex the whales. The following primers from Fain and LeMay (1995) were used: 5'- ATAATCACATGGAGAGCCACAAGCT-3' and 5'-GCACTTCTTTGGTATCTGAGAAAGT-3' (Zfy/x gene) and 5'- CCCATGAACGCATTCATTGTGTGG-3' and 5'- ATTTTAGCCTTCCGACGAGGTCGATA-3' (SRY gene). Two products (bands) are amplified in males and one product (band) is amplified in females. Methods used previously for sexing gray whales were generously provided by Dr. Aimee Lang (NOAA). Amplification conditions were as follows: denaturation for 3 minutes at 94°C, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and a 1 minute extension at 72°C. The primers 5'-TTCAGCTTGCWGCTAGRTTCCTCT-3' and 5'- AWGRTGAAGRACAAGGCCCATCT-3' were developed by Dr. John Patton (Purdue University) for use in bowhead whales. Two products (bands) are amplified in males, and one product (band) is amplified in females. Primers 5'- GCATTGTGCATTCTACTCCGTCAC-3' and 5'- MACTTCCCTTCTSAGGAGATTTARYACTG-3' which amplify SRY were also developed by Dr. John Patton for use in bowhead whales. A single product is amplified only in males. Amplification conditions were as follows: denaturation for 2 minutes at 94°C, followed by 35 cycles of denaturation at 96°C for 30 seconds, annealing at 57°C for 30 seconds and a 2 minute extension at 72°C.

DNA sequencing and alignment.—Amplified products were cleaned using Sephedex and sequenced in both directions on an automated sequencer. The sequencing reaction was carried out using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's recommendations. Capillary electrophoresis was carried out in a 50-cm capillary unit of automatic genetic analyzer ABI Prism GA3130 in POP-7 polymer. Sequence data were edited and aligned in Sequencher 5.0, Geneious 8.0.4 (Biomatters Ltd., www.geneious.com) and Unipro UGENE:1.12 (Okonechnikov et al., 2012). The species of obtained sequences was compared to the NCBI Genbank using the algorithm BLASTn (NCBI GenBank).

Statistical analysis.—The programs ARLEQUIN 3.5 (Excoffier and Lischer, 2010) and DNAsp5 (Rozas, 2009) were used for statistical processing of genetic data and calculation of mtDNA haplotype networks, haplotype diversity, and statistical significance of differences in haplotype frequencies between pairs of samples.

RESULTS:

Genetic analyses

Table 1 lists the results of our sequence analyses of 3 mtDNA genes and molecular sexing of 32 western gray whales. We analyzed a total of 35 biopsies, many of which shared identical sex and mtDNA haplotypes. To determine how many represented potential duplicates we scored two microsatellite loci (EV-37 and EV-94) for 28 individuals, and compared the photographs taken of the biopsied whales to the catalog. The combination of microsatellite genotype and photo ID revealed 4 and 5 ostensible duplicate pairs, respectively. These could be resolved to 3 duplicate pairs of samples by combining the photo IDs with microsatellite genotypes. There were 2 pairs of biopsies with different genotypes that were photographic matches (KOGW050 and KOGW167; see Table 1). This reduced the number of whales sampled to N = 32. The sex ratio of 31 gray whale samples was determined to be 14 males:17 females. The sex of whale KOGW221 sampled in 2013 was not determined because the results differed between labs.

Upon sequencing the mitochondrial control region, 10 distinct haplotypes were observed among the 32 whales including 9 of the 36 haplotypes defined by LeDuc, et al. (2002) and 1 of the 5 haplotypes described by Lang, et al. (2011, 2014). For the cytochrome b region, the 4 of the 5 previously defined haplotypes were identified (Alter and Palumbi 2009) in 34 of the samples. A single individual was found to have a new haplotype that differs at a single newly variable site from previously described haplotype 6. A summary of the haplotype counts for all 32 whales is below (Table 2).

Attached as a supplementary file is a spreadsheet listing the results from all 35 gray whales by ID number and year, including sex, amplified sequences for the mitochondrial control region and cytochrome b region, the BLASTn link to the 100% match from each amplified sequence, and haplotype names, and the alleles present at two microsatellite loci (EV-37 and EV-94) is attached as a supplementary file. EV-14 alleles were not able to be reliably produced and therefore removed from analysis. A summary of the results is as follows:

Table 1.—Results of sequence analysis of three mtDNA genes and molecular sexing of 35 Sakhalin Island western gray whale biopsy samples.

Biopsy No.	Catalog No.	Control Region Letter[a]	Control Region No.[b]	Cytochrome b[c]	Cytochrome b[d]	COI	Sex	Comments
2011								
Z112743		E	5		1 or 5		M	
Z112744		AI	35		1 or 5		F	
Z112745		A	1		1 or 5		F	
Z112746		B	2		2		F	
Z112747		B	2		2		F	
Z112748		B	2		2		F	
2012								
1	KOGW015	W’	38		4		F	
2	KOGW019	B	2		2		F	
3	KOGW179	B	2		2		F	
4	KOGW059	D	4		6		M	
5	KOGW021	B	2		2		F	
6	KOGW135	W’	38		4		F	
9	KOGW005	B	2		2		F	
10	KOGW133	B	2		2		M	
11	KOGW213	B	2		2		M	calf
12	KOGW182	B	2		2		M	
13	KOGW050?	A	1		1 or 5		F	Not duplicate of 2014 (8)
14	KOGW216	AC	29		1 or 5		M	calf
15	KOGW028	H	8		1 or 5		M	
16	KOGW189	A	1		1 or 5		F	
17	KOGW209	A	1		1 or 5		F	calf
19	KOGW168	B	2		2		M	
20	KOGW167?	B	2		2		M	Not duplicate of 2014 (9)
2013								
1	KOGW094	A	1		1 or 5		M	
2	KOGW055	A	1		1 or 5		M	

3	KOGW223	B	2		2		M	calf
4	KOGW172	C	3		New Haplotype		M	
5	KOGW174	AB	28		1 or 5		F	
6	KOGW221	W’	38		4		M/F	calf with mother KOGW135
7	KOGW173	A	1		1 or 5		F	calf with mother KOGW050 (9)
8	KOGW050?	A	1		1 or 5		F	Not duplicate of 2013 (13)
9	KOGW167?	B	2		2		M	Not duplicate of 2013 (20)

Control region sequences.— Upon sequencing the mitochondrial control region, 10 haplotypes were observed among the 32 whales including 9 of the 36 haplotypes defined by LeDuc et al. (2002) and 1 of the 5 haplotypes described by Lang, et al. (2011, 2014). Haplotype W' is a recently observed haplotype for the western gray whale. It was first described from one eastern and 3 western gray whales by Lang et al. (2011, 2014) who called it haplotype 38. We refer to it as W' because it is mutational event distant from haplotype W of LeDuc et al. (2002). Control region haplotype summaries by collection year are given in Table 2. The haplotype network for control region sequences is shown in Figure 1. As in previous studies, the observed haplotypes from western gray whales are found throughout the tree and do not represent a group of animals related through maternal lineage.

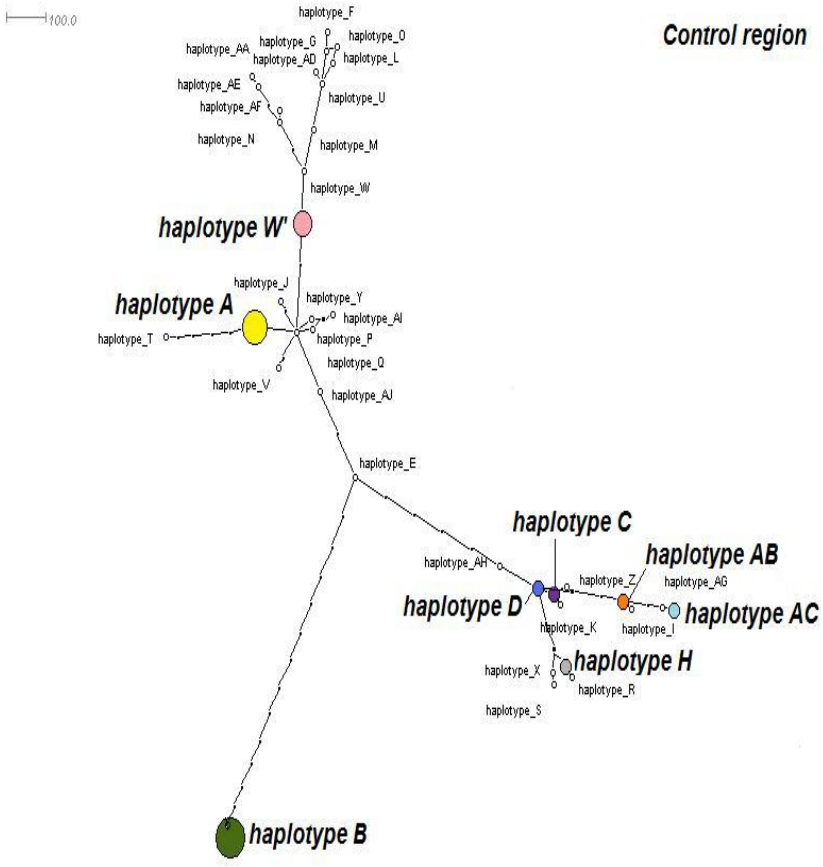

Figure 1.—Minimum spanning network showing the relationships among gray whale control region haplotypes. Haplotypes with colored circles are those reported in this study from western gray whales. The sizes of the colored circles are proportional to the number of observed haplotypes. Haplotype W' is the same as haplotype 38 of Lang et al. (2011, 2014).

Table 2.—Control region haplotypes from 32 western gray whales observed in this study.

Haplotype	2011	2012	2013	Total
A	1	3	4	8
B	3	9	2	14
C	0	0	1	1
D	0	1	0	1
E	1	0	0	1
H	0	1	0	1
AB	0	0	1	1
AC	0	1	0	1
AI	1	0	0	1
W’	0	2	1	3
Total	6	16	9	32

Cytochrome b and COI sequences.—Table 3 gives the combined protein coding gene haplotypes. This includes the full cytochrome b sequence and partial COI sequence done at the Institute of Marine Biology. We observed a total of 7 haplotypes among 23 whales (COI sequences are not available for the whales collected in 2011). Haplotype frequencies were statistically significant between whales biopsied in 2013 and 2014 (χ^2=13.91, p=0.012). Figure 2 shows the haplotype network for the protein coding gene haplotypes. In this figure a deep split between haplotype D and the remaining haplotypes is well illustrated. This same differentiation has previously been observed in studies of gray whale mtDNA and it has been noted that both parts of the tree are typically present within any gray whale population. The implications of this deep sequence divergence have not been fully explored in terms of the historical demography of the species.

Table 3.—Haplotypes observed in the combined cyt b and COI sequences for 2012 and 2013 samples only.

Haplotype	2012	2013
A	0	4
B	0	1
C	2	0
D	7	1
E	1	0
F	1	0
G	4	1
Total	16	7

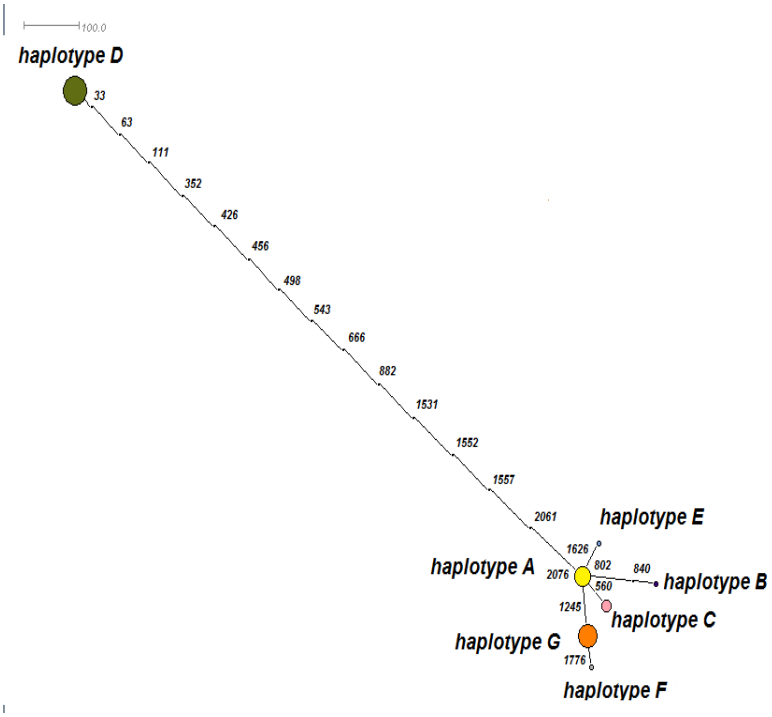

Figure 2.—Minimum spanning network of haplotypes from the combined cytochrome b and COI gene sequences. Sizes of the circles are proportional to their frequencies. Numbers identify nucleotide positions with base substitutions.

Combined 3-gene sequences.—Nine haplotypes were observed among 23 whales when all three mtDNA genes (COI+Cytb+CR) were combined (Table 4). Again, statistically significant differences (χ^2 =15.26, p=0.027) were observed for haplotypes between samples collected in 2012 and 2013. Analysis of genetic separation of temporal samples, if considered as distinct populations, show significant population subdivision (F_{st}=0.036, $p< 0.05$).

Table 4.—Haplotypes of combined 3-gene sequences (COI+Cytb+CR) of the mtDNA for 23 whales collected 2012 and 2013.

Haplotype	2012	2013
DB	8	1
AA	0	3
ED	1	0
CW	2	0
GAC	1	0
FH	1	0
BC	0	1
AAB	0	1
GA	3	1
Total	16	7

DISCUSSION:

Sequences for three mitochondrial genes from 35 biopsies representing 32 gray whales taken near Sakhalin Island on their summer feeding grounds show appreciable genetic variation. The data reported here are consistent with previous studies of mtDNA from this population. The two most frequently observed control region haplotypes are A and B (Table 2). Only one other haplotype, W' with three individuals, was observed more than once. Of the 7 singleton haplotypes, 5 were from males.

The observation of statistically significant differences among samples collected in 2013 and 2014 is unexpected. Although this might be due to sampling error and small sample sizes, there may be another explanation. It is possible that groups of related whales travel together to the feeding area of Northern Sakhalin, which leads to formation of temporal heterogeneity that we detected. If it is not due to sampling error, it is yet unknown whether or not such groups of whales belong to different stocks (eastern and western gray whales), different feeding groups of eastern gray whales, or just related individuals within a group.

Observation of larger samples over several seasons and analysis of nuclear loci are required for understanding the composition of the Sakhalin Island population of gray whales and its migration near North Sakhalin. Currently, we are analyzing biopsy samples (N = 30 whales) taken in 2014 and a similar number is planned to be collected in the field season of 2015.

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