NOTE

Genetic analysis of right whales in the eastern North Pacific confirms severe extirpation risk

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ABSTRACT: Genetic analysis of 49 biopsy samples from North Pacific right whales *Eubalaena japonica* in the eastern (48) and western (1) North Pacific revealed 24 individual whales with 7 mitochondrial haplotypes. Three pairs of large and small individuals were identified in the field; genotype analysis indicated that 2 of these could represent mother–offspring pairs; for the third small individual, no sampled female genetically qualified as a potential mother. In aggregate, the population appears to have lost some genetic diversity, though not to the degree of North Atlantic right whales *E. glacialis*, and males outnumber females 2:1. A comparison of the eastern Pacific samples to a single Russian sample suggested that the 2 populations are isolated to some degree. The effective population size for the eastern North Pacific was calculated to be 11.6 (95% CI: 2.9–75.0), based on the estimated linkage disequilibrium. These results further indicate that this population is at immediate risk of extirpation.

KEY WORDS: Critically Endangered species · Mammal · IUCN Red List category · *Eubalaena japonica* · Genetics · Right whales

INTRODUCTION

The eastern North Pacific right whale *Eubalaena japonica* is perhaps the rarest large whale in the world (Wade et al. 2011a), and is designated on the IUCN Red List as 'Critically Endangered.' A recent population estimate, based on photo-ID and genetic mark-recapture, was 31 individuals (Wade et al. 2011a), and while this estimate may reflect a sub-population using the Bering Sea, the paucity of sightings elsewhere suggests that the overall population is unlikely to be much larger. This situation is the result of intensive historical (19th century) whaling, exacer-bated by illegal catches by the USSR in the 1960s; these latter catches are now known to be larger than previously thought, and likely removed the bulk of the remaining population (Ivashchenko & Clapham 2012).

In addition to the low numbers of right whales, potential loss of genetic diversity and the paucity of observed females with calves have been causes for concern regarding the ability of this population to recover (LeDuc et al. 2001). Another factor, largely unexamined to date, has been the relationship of the eastern Pacific right whales to those in the western Pacific. Although no reliable population estimates are

available for the western population, it is surmised that it is greater than the population in the eastern Pacific. According to Brownell et al. (2001, p. 283):

> In the western North Pacific, Soviet catches nullified any increases that occurred during the 20th century. Although quantitative analysis is impossible, recent sightings suggest that this population may number at least in the low hundreds and may therefore be large enough to survive.

The greater abundance of the western population of right whales is in contrast to the pattern shown by other species of whales in the North Pacific, such as gray whales *Eschrichtius robustus* and bowhead whales *Balaena mysticetus*, whose eastern populations are the ones that survive in greater numbers. Nevertheless, gene flow from the larger western population of North Pacific right whales could potentially mitigate some of the problems faced by those in the east. Using 4 samples collected in 2008 and 2009 plus samples analyzed by Wade et al. (2011a), which included 4 samples identified in the field as calves or juveniles, we conducted a genetic analysis to examine levels of diversity and possible parent–offspring relationships. In addition, a single sample from the western Pacific allowed us to make a preliminary examination of the relationship of that animal to those sampled in the eastern Pacific.

MATERIALS AND METHODS

We obtained a total of 49 biopsy samples, including 47 from the southeastern Bering Sea (43 of which were also used by Wade et al. 2011a), and single samples from the Gulf of Alaska and from Russian waters (off eastern Kamchatka). For each sample, an approximately 400 base pair region of the mitochondrial control region was amplified from extracted genomic DNA (Qiagen DNeasy #69506) using primers H16498 (5′-CCT GAA GTA AGA ACC AGA TG-3′; Rosel et al. 1994) and L15812 (5′-CCT CCC TAA GAC TCA AGG AAG-3′; developed at the Southwest Fisheries Science Center, La Jolla, CA). Using the same primers, both strands of the amplified DNA product were sequenced independently using standard protocols on the Applied Biosystems (ABI) model 3100 sequencer. Each sample was sexed according to the methods of Fain & LeMay (1995). Data from 8 microsatellite loci were generated for each sample. Markers Rw48, Rw410, Rw 45, Rw 31, and Sam25 were from Waldick et al. (1999), EV37 was from Valsecchi & Amos (1996), GATA028 was from Palsbøll et al. (1997), and DlrFCB5 was from Buchanan et al.

(1996). Minor modifications were made to the published microsatellite protocols; these can be obtained from K. M. Robertson. These genetic data were used in a previous publication (Wade et al. 2011a) to determine the number of individuals sampled, but were not subjected to their own genetic analyses.

The microsatellite data allowed us to look at possible parent–offspring relationships. We used a simple comparison of genotypes for exclusion of potential parents, where at least 1 shared allele at each locus would be required for a pair of individuals to be considered possible parent and offspring. A mother–offspring relationship would also require a shared mitochondrial haplotype. This criterion of course does not confirm the relationship; other relatives (or even non-relatives) may also have that pattern of shared alleles and haplotypes. In the field, a number of whales were sometimes sampled during the same sighting, and notes were made about larger individuals accompanying smaller ones. This allowed us to evaluate those observations.

Allele counts and heterozygosity levels were calculated and compared to published data from other right whale species. The inclusion of the samples from outside the Bering Sea, particularly the sample from Russian waters, allowed us to consider whether all of the sampled whales are members of the same breeding population. We did not have nearly enough whales to test for differentiation between eastern and western Pacific right whales directly. As a preliminary examination, therefore, we conducted a genetic exclusion test (Cornuet et al. 1999). In this analysis, by sampling with replacement from the observed allele frequencies, 10 000 replicate genotypes are produced, and the likelihood of assignment to the population is calculated for each. The assignment likelihoods of the observed genotypes are then compared to the distribution of likelihoods from the replicates, and are scored on a percentile basis. While not definitive, this test can provide a probabilistic basis for decisions as to whether a given sample is a member of the same population as the rest of the sample set. Genetic exclusion tests were conducted using the program DOH (www2.biology.ualberta.ca/jbrzusto/Doh.php).

The effective population size N_e for the eastern populations was calculated using the program LDNe (Waples 2006, Waples & Do 2010). This program uses estimates of linkage disequilibrium (LD) to infer the N_e. For this calculation, the lowest allele frequency of 0.02 was used and random mating was assumed. Ninety-five percent confidence intervals were calculated using the jackknife procedure proposed by Waples (2006).

RESULTS AND DISCUSSION

The genotypes indicated that the 49 biopsy samples represented 24 individual whales. None of the microsatellite markers was statistically out of Hardy-Weinberg equilibrium. Four of the whales sampled were recorded in the field notes as possibly being calves or juveniles, based on their smaller size or association with a larger whale. Three of these individuals were seen in association with a larger whale, and in each case the larger individual was also sampled. In all, 7 mitochondrial haplotypes were recorded (GenBank accession nos.: JX441356–JX441362). The most frequent haplotype occurred in 7 individuals, while 1 haplotype occurred in 5, another in 4, 2 in 3 each, and another 2 were seen only in single individuals. One of the singletons was represented by the Russian sample, while the Gulf of Alaska sample had 1 of the haplotypes with a frequency of 3. The ratio of males to females in the entire sample set was 2:1. This includes the Russian sample, which was a female. This ratio is more encouraging than the greater bias reported by LeDuc et al. (2001), although the paucity of females is still a cause for concern. Interestingly, a male bias was also observed in right whales killed illegally in the Gulf of Alaska by the USSR in 1963, with 82 (73.2%) males in a catch of 112 whales (Ivashchenko & Clapham 2012). Of the 4 smaller individuals sampled, only one was female.

Potential parentage

For one of the smaller whales (Individual 13), no potential mother could be detected in the dataset, and all but 2 of the males could be eliminated as potential fathers. The whale identified in the field as accompanying Individual 13 was a female, but had a different mitochondrial haplotype and was therefore excluded as a possible mother. The individual identified in the field as accompanying another smaller whale (Individual 23) did qualify as a potential mother, the only female in the dataset that did. For Individual 23, there were 4 potential fathers among all the males in the dataset. For the remaining 2 smaller whales (Individuals 16 and 17), the genetic data eliminated all but one of the females as a potential mother — the same mother (Individual 18) for both. This whale was indicated in the field notes as accompanying Individual 16. Field notes identified Individual 17 as larger than 16, with no apparent adult accompanying it. It is possible that 17 is a pre-

vious offspring of 18, independent enough to be away from its mother, who now has a new calf (16). On the paternal side, no potential fathers could be found for Individual 16, and 2 of the males sampled qualified for Individual 17.

Genetic diversity

In aggregate, the right whale genetic dataset had an average heterozygosity of 0.68 and an average number of alleles per locus of 4.6. In comparison, Waldick et al. (2002) gave average heterozygosity and allele counts of 0.31 and 3.2 for North Atlantic right whales *Eubalaena glacialis* and 0.72 and 6.9 for southern right whales *E. australis*. Given that the array of markers was not the same between the present study and Waldick et al. (2002), we then compared heterozygosity values across the 6 loci in common (Table 1). Qualitatively, the comparisons are the same as above, with the North Pacific right whales being intermediate in both number of alleles and average heterozygosity. The results are similar for mitochondrial diversity, in that the level in *E. japonica* is intermediate between that of *E. glacialis* and of *E. australis*. Although the number of samples and the population size of North Atlantic right whales are an order of magnitude greater than those of the eastern North Pacific right whales, the present 6 recorded mitochondrial haplotypes (the seventh haplotype was only seen in the Russian sample) are greater than the 5 seen in *E. glacialis* (Malik et al. 2000). For *E. australis*, Patenaude et al. (2007) found 37 distinct haplotypes. These results suggest that the North Pacific right whales have lost some genetic variability, but not to as low a level as seen in the right whales in the North Atlantic. However, it is noteworthy that of the 5 haplotypes in *E. glacialis*,

Table 1. *Eubalaena australis*, *E. glacialis*, and *E. japonica*. Measures of microsatellite diversity across the 3 species of right whale for the 6 loci common to Waldick et al. (2002) and the present study. *Ea*: *Eubalaena australis*; *Eg*: *E. glacialis*; *Ej*: *E. japonica*

Locus	No. of alleles			Heterozygosity		
	Ea	*Eg*	*Ej*	*Ea*	*Eg*	*Ej*
Ev37	11	3	5	0.90	0.52	0.70
Rw48	7	5	4	0.86	0.42	0.75
Rw410	5	2	5	0.71	0.31	0.63
Sam25	8	2	5	0.89	0.38	0.63
Rw45	5	2	3	0.87	0.10	0.58
Rw31	7	5	4	0.89	0.48	0.58
Average	7.2	3.2	4.3	0.85	0.37	0.65

one has not been recorded in females (Malik et al. 2000), whereas 2 of the 6 haplotypes of the eastern population of *E. japonica* were only recorded in males. The potential for further loss of haplotypic diversity is very real. Waldick et al. (2002) suggested that low variability may be characteristic of *E. glacialis* and not completely attributable to population decline. However, if low population levels have led to a loss of diversity in *E. glacialis*, the extent of the loss may be a function in part of the longer duration of their population bottleneck in comparison to *E. japonica*. The greatest decline in the numbers of *E. glacialis* took place prior to 1750 (Reeves et al. 2007), and the population has remained at low numbers since then, whereas the exploitation of *E. japonica* has been more recent, occurring mainly in the mid-19th century and then again in the 1960s by illegal pelagic Soviet whaling (Brownell et al. 2001, Ivashchenko & Clapham 2012). Although *E. japonica* has retained more genetic diversity than *E. glacialis*, the very low population estimate for the eastern population reported by Wade et al. (2011a) indicates that further loss of diversity is a very real concern, especially in light of the fact that some of the rare haplotypes could soon disappear from the population.

Exclusion test

In the exclusion test, 2 of the individuals had p-values (representing their percentile rank among replicate genotype assignment probabilities) below 0.05. One of these was the Russian sample (Individual 19), with a p value of 0.011. The other (Individual 12) had an even lower p-value (2.0×10^{-4}); this individual was a male sampled once in the Bering Sea in 2004. While the Russian sample had a haplotype that was unique within the sample set, Individual 12 had a haplotype that was shared with 2 other samples, including one of the calves, and also qualified as a potential father for one of the other calves. The low p-values for both of these samples reflect their possession of several relatively rare alleles for these microsatellite markers. Individual 12 had a unique allele at 2 different markers, as did Individual 19 (for different markers). No other samples possessed unique alleles at any marker. The results of the exclusion test are inevitably inconclusive, but combined with the unique haplotype shown in this individual, suggest that the Russian sample (Individual 19) is a member of a different population. The low p-value of Individual 12, for which the prior assumption is that it is a member of the same population as the

other Bering Sea samples, may simply represent a false negative. Having a single sample falling below the 0.05 threshold is not unexpected in a dataset of >20 samples. The low p-value of Individual 12 may also seem inconsistent with its status as a potential father to one of the smaller whales, but this reflects the differences in the 2 analyses. The exclusion test is a statistical test, incorporating the relative frequencies of all the alleles in the genotype. When overall diversity is low, rare or unique alleles are few in number, so their presence in an individual can have a large influence on the resulting p-value. In the paternity test conducted here, on the other hand, all that is required is a shared allele at each locus, and a single unique allele at a locus will be irrelevant if the other allele at that locus is a shared common one. Another caveat to the exclusion test is that the unique alleles shown in Individual 19, if rare in the western population as well, could result in this individual also having a low percentile score in its own population; the conclusion that it is unlikely to be a member of the eastern population cannot be compared to any probability of membership to a different population. The results of the exclusion test could also change considerably with the addition of more loci, the collection of new samples and a recalculation of allele frequencies, as well as any findings of segregation on the feeding grounds, or any other such structure within the eastern population.

The abundance estimate of Wade et al. (2011a) was for the eastern Bering Sea Shelf and eastern Aleutian Islands. There have been few sightings of right whales elsewhere in the eastern North Pacific, so the total eastern population may not be much larger, although little or no survey effort has been directed at the Gulf of Alaska, where right whale catches were distributed in offshore waters during both the 19th century and the 1960s (Townsend 1935, Ivashchenko & Clapham 2012). Wade et al. (2011b) reported on several recent sightings from near Kodiak Island in the Gulf of Alaska. Two whales were photo-identified but did not match to any photos from the Bering Sea. One of these whales was biopsied, and that sample was included in the present study. The genetic test did not exclude that whale from the Bering Sea population, suggesting that the small numbers of whales found in the Gulf of Alaska may be a part of the same population found in the Bering Sea. The fact that all the sightings and samples used here were obtained during the summer feeding season highlights a major gap in our knowledge of Pacific right whales, namely their movements and location during other times of the year (but see Kennedy et al. 2011).

Effective population size

The calculated Ne, based on estimated LD, was 11.6 (95% CI: 2.9–75.0). This is alarmingly low. Although there are some potential biases in this estimate due to violations of assumptions such as a closed population and non-overlapping generations (Waples 2006), the degree of bias is not likely to be large, and the estimated Ne is consistent with the biased sex ratio and the estimated total population size of 28 to 31 based on mark-recapture (Wade et al. 2011a). This low Ne value, being consistent with the total population size also indicates that gene flow from the western population is likely to be low, and that the eastern population is a closed one. If the eastern population were interbreeding with the much larger western population, the calculated Ne would have been higher. This result, combined with that of the exclusion test, suggests some separation from the western population.

Taken together and in light of the low population estimate given by Wade et al. (2011a), the present findings of an apparent loss of genetic diversity, low frequencies of females and calves, extremely low effective population size, and possible isolation from conspecifics in the western Pacific indicate that right whales in the eastern North Pacific are in severe danger of immediate extirpation from the eastern North Pacific. These new data provide strong support for the IUCN Red List designation as Critically Endangered.

LITERATURE CITED

Brownell RL Jr, Clapham PJ, Miyashita T, Kasuya T (2001) Conservation status of North Pacific right whales. J Cetacean Res Manag 2(Spec Issue):269–286

Buchanan FC, Friesen MK, Littlejohn RP, Clayton JW (1996) Microsatellites from the beluga whale *Delphinapterus leucas*. Mol Ecol 5:571–575

Cornuet JM, Piry S, Luikart G, Estoup A, Solignac M (1999) New methods employing multilocus genotypes to select or exclude populations as origins of individuals. Genetics 153:1989–2000

Fain SR, LeMay JP (1995) Gender identification of humans and mammalian wildlife species from PCR amplified sex-linked genes. Proc Am Acad Forensic Sci 1:34

Ivashchenko YV, Clapham PJ (2012) Soviet catches of right whales *Eubalaena japonica and* bowhead whales *Balaena mysticetus* in the North Pacific Ocean and Okhotsk Sea. Endang Species Res (in press)

Kennedy AS, Salden DR, Clapham PJ (2011) First high- to low-latitude match of an eastern North Pacific right whale (*Eubalaena japonica*). Mar Mamm Sci doi:10.1111/j.1748-7692.2011.00539.x

LeDuc RG, Perryman WL, Gilpatrick JW Jr, Hyde J, Stinchcomb C, Carretta JV, Brownell RL Jr (2001) A note on recent surveys for right whales in the southeastern Bering Sea. J Cetacean Res Manag (Spec Issue 2):287–289

Malik S, Brown MW, Kraus SD, White BN (2000) Analysis of mitochondrial DNA diversity within and between North and South Atlantic right whales. Mar Mamm Sci 16: 545–558

Palsbøll PJ, Bérubé M, Larsen AH, Jørgensen H (1997) Primers for the amplification of tri- and tetramer microsatellite loci in baleen whales. Mol Ecol 6:893–895

Patenaude NJ, Portway VA, Schaeff CM, Bannister JL and others (2007) Mitochondrial DNA diversity and population structure among southern right whales (*Eubalaena australis*). J Hered 98:147–157

Reeves RR, Smith TD, Josephson EA (2007) Near-annihilation of a species: right whaling in the North Atlantic. In: Kraus SD, Rolland RM (eds) The urban whale: North Atlantic right whales at the crossroads. Harvard University Press, Cambridge, MA, p 39–74

Rosel PE, Dizon AE, Heyning JE (1994) Genetic analysis of sympatric morphotypes of common dolphins (genus *Delphinus*). Mar Biol 119:159–167

Townsend CH (1935) The distribution of certain whales as shown by logbook records of American whaleships. Zoologica 19:1–50

Valsecchi E, Amos W (1996) Microsatellite markers for the study of cetacean populations. Mol Ecol 5:151–156

Wade PR, Kennedy A, LeDuc R, Barlow J and others (2011a) The world's smallest whale population? Biol Lett 7:83–85

Wade PR, De Robertis A, Hough KR, Booth R and others (2011b) Rare detections of North Pacific right whales in the Gulf of Alaska, with observations of their potential prey. Endang Species Res 13:99–109

Waldick RC, Brown MW, White BN (1999) Characterisation and isolation of microsatellite loci from the endangered North Atlantic right whale. Mol Ecol 8:1763–1765

Waldick RC, Kraus S, Brown M, White BN (2002) Evaluating the effects of historic bottleneck events: an assessment of microsatellite variability in the endangered, North Atlantic right whale. Mol Ecol 11:2241–2249

Waples RS (2006) A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. Conserv Genet 7:167–184

Waples RS, Do C (2010) LDNE: a program for estimating effective population size from data on linkage disequilibrium. Evol Appl 3:244–262