FOR CONSIDERATION BY THE SCIENTIFIC COMMITTEE OF
THE INTERNATIONAL WHALING COMMISSION
TROMSØ, NORWAY, JUNE 2011

A minimum census of surviving maternal lineages among contemporary Antarctic blue whales: progress report

A. SREMBA[1], B. HANCOCK-HANSER[2], R. LEDUC[2], T.A. BRANCH[3], C.S. BAKER[1]

1. *Marine Mammal Institute, Oregon State University, Newport, OR*
2. *Southwest Fisheries Science Center, 3333 North Torrey Pines Court, La Jolla, CA*
3. *School of Aquatic and Fishery Sciences, Box 355020, University of Washington, Seattle WA*

ABSTRACT

Antarctic blue whales (*Balaenoptera musculus intermedia*) were intensely exploited by the commercial whaling industry between 1904 and 1972. This resulted in a demographic population 'bottleneck' that reduced the population to an estimated 360 individuals in 1972 before recovering to 2,280 (1,160-4,500) individuals in 1998. Here we report on progress with using mtDNA and microsatellite genotypes to place a lower bound on the number of maternal lineages to have survived this 'bottleneck' based on access to 218 biopsy samples collected aboard the IDCR/SOWER research cruises between 1990 and 2009 (SC/61/019). Microsatellite genotypes for up to 17 loci identified 164 individuals. Genotype matching revealed movement ranging up to 137º longitude and up to an elapsed time of 4 years. Among 164 individuals we described 51 mtDNA haplotypes, or maternal lineages. This dataset was combined with 20 previously published sequences for a total of 184 individuals and 53 haplotypes. Our minimum census of 53 haplotypes more than doubles the previous census of 26 haplotypes by LeDuc et al. (2007) and can be used to revise the estimate of surviving lineages by Branch and Jackson (2008).

INTRODUCTION

Antarctic blue whales were intensely hunted by the commercial whaling industry throughout the 20th century. Population models have estimated the population to have declined from 239,000 (202,000-311,000) to 360 (150-840; 95% Bayesian intervals) individuals in 1972, less than 1% of its original abundance estimate (Branch et al., 2004). The current population is estimated at 2,280 (1,160-4,500) individuals, increasing at 8.2% per year (Branch, 2007). An updated assessment (Branch, 2008) used methods developed by Jackson et al. (2008), to limit the minimum possible abundance in the Bayesian logistic model to 214 individuals (Branch and Jackson, 2008), which was based on the mitochondrial control region of 26 haplotypes from 47 Antarctic samples collected between 1993-2002 (LeDuc et al., 2007).

Here we update the previous number of mtDNA haplotypes of the Antarctic blue whale through analysis of an extended dataset collected on IDCR/SOWER cruises from 1990-2009 (n=218). This new estimate of contemporary diversity can be used to revise estimates of surviving mtDNA lineages using the methods of Jackson et al. (2008) and Branch and Jackson (2008). We identify replicate samples using microsatellite genotypes up to 17 loci. Through the identification of replicate individuals, we infer individual movement of Antarctic blue whales throughout the circumpolar Southern Ocean.

METHODS

Sample collection. Blue whale biopsy samples were collected during the IDCR/SOWER cruises from 1990-2009 (n=218) below the Antarctic convergence (54°-55°S) (Fig. 1). Analyses of length frequencies (Branch et al., 2007a), ovarian corpora lutea (Branch et al., 2009), and length at maturity from commercial whaling records (Branch et al., 2008) provides evidence that blue whales south of the convergence (i.e., south of 60°S) are Antarctic blue whales (*B. m. intermedia*), not pygmy blue whales (*B. m. brevicauda*). A subset of these samples (n=26) were included in the previous analysis of Antarctic blue whale mtDNA diversity (LeDuc et al., 2007) and re-analyzed in this study (i.e. mtDNA sequencing and microsatellite-genotyping were repeated). The remainder of samples included in LeDuc et al. (2007) (n=20) was collected from JARPA cruises and were not available for re-analysis in this study; however, published sequences were available in supplementary material provided in LeDuc et al (2007).

DNA extraction. IWC IDCR/SOWER biopsy samples were archived at Southwest Fisheries Science Center (SWFSC) in La Jolla, CA, where genomic DNA (gDNA) was extracted. Genomic DNA extractions were performed at SWFSC following a variety of methods, namely lithium chloride extraction (Gemmel and Akiyama, 1996), sodium chloride protein precipitation (Miller et al., 1988), silica-based filter purification (DNeasy kit, Qiagen, Valenica, CA, USA) and Xtractor gene (Corbett Robotics, San Francisco, CA, USA) or a standard phenol/chloroform extraction (Sambrook et al., 1989). Genomic DNA was transferred to the Cetacean Conservation and Genetics Laboratory (CCGL) at Hatfield Marine Science Center (HMSC) of Oregon State University (OSU) in Newport, Oregon.

mtDNA sequencing. A 560 bp region of the 5` end of the mtDNA control region was amplified and sequenced on an ABI 3730xl. Sequences were visually inspected (Phred scores >20; Morin et al., 2010) edited and aligned using Sequencher v.4.9 (Gene Codes Corporation). MtDNA haplotypes were described based on one or more substitutions within the 560 bp control region sequence and compared to a database of worldwide blue whale mtDNA control region sequences. Unique haplotypes that were not found in the worldwide database were reverse sequenced from an independent amplification for verification of all variable sites. Previously undescribed haplotypes were named according to the lab code of the first sample found to have that haplotype. Haplotypes derived from samples used previously by LeDuc et al. 2007 (n=24) were resequenced and extended to 560 bp.

Microsatellite genotyping and sex identification. To identify replicates within the dataset, samples (n=218) were genotyped for up to 17 microsatellite loci: GATA28*, GATA417*(Palsbøll et al., 1997), GT211, GT575, GT23* (Berube et al., 2000), rw4-10, rw31, rw48, rw26 (Waldick et al., 1999), Ev37*, Ev96, Ev1, Ev104, Ev14, Ev21, (Valsecchi and Amos, 1996), 464/465 (Schlötterer et al., 1991). These microsatellite markers were chosen based on LeDuc et al. (2007, indicated above by an asterisk) and a trial study of other published loci screened on 19 North Pacific blue whale samples (D. Steel, pers. comm.). All microsatellites were amplified individually (i.e., no PCR multiplexes). Microsatellite products were co-loaded for genotyping in 4 sets of up to 5 loci. Two µl of co-load in addition to size standard GS500 LIZ (Applied Biosystems) were heated to 95˚C for 5 minutes and genotyped on ABI 3730 Genetic Analyzer (Applied Biosystems) at Hatfield Marine Science Center (HMSC). Each co-load was run with negative controls to detect contamination. Genotypes were assigned after visually checking the automated calling of alleles by GENEMAPPER v.4.0 (Applied Biosystems).

Primer designed to amplify fragments of the X and Y chromosomes were used to identify sex: primers P1-5EZ and P2-3EZ on the X chromosome (Gilson et al., 1998) and primers Y53-3C and Y53-3D on the Y chromosome (Aasen and Medrano, 1990).

Replicate identification. Microsatellite genotypes were reviewed using the program CERVUS v.3 (Kalinowski et al., 2007) to identify likely replicate samples, which were also reviewed for consistency with mtDNA haplotype and genetic sex information. Replicate samples that were collected on different

days are considered to be 'recaptures'. Individual movement was inferred from the location of recaptures. Probability of identity for all replicates was calculated in CERVUS v.3.

RESULTS and DISCUSSION

mtDNA haplotype resolution at 560 bp. Of the total of 218 samples, 215 amplified for 560 bp of the mtDNA control region (98.6% success), providing high quality sequences (e.g., Phred score >40). A total of 51 haplotypes were resolved from 47 variable sites, of which 24 matched to those reported by LeDuc et al. (2007). All haplotypes found in only a single individual (singletons, n=8) were confirmed through re-sequencing from an independent PCR. The extended sequences of the mtDNA control region (560 bp) differentiated only one haplotype that would not have been found in the 414 bp fragment used by LeDuc et al. (2007).

Microsatellite genotyping and replicate identification. We attempted to amplify 17 loci and sex markers from all samples. On average, samples amplified for 13 loci out of 17 and we identified sex for 83% of all samples. Among pairwise comparisons of samples with a sufficient number of loci for confident identification (>7 matching loci), 51 replicates were identified with a probability of identity range from 4.57×10^{-25} to 2.80×10^{-7}. Given low concentrations of genomic DNA, genotypes for a small number of samples were insufficient for confident individual identification (17 samples with fewer than 7 amplified loci). For the purposes of conducting a census of mtDNA haplotypes, however, we considered these 17 samples as 'likely individuals'. Three of these 17 samples represent unique mtDNA haplotypes (i.e., singletons), which were confirmed by resquencing and reverse sequencing.

Recapture identification. Most matching genotypes represented sequencial or near sequencial samples within a single encounter (i.e., replicates). In 9 cases, however, genotype matched established 'recaptures' on different dates and in some cases, different Areas (I-VI) (Table 2). All within-year recaptures were captured and recaptured within Area III; one event occurring in 2006 and the other seven in 2007. Two individuals were recaptured between years: sample #51452, a female, was originally captured in Area V in 2002 and was re-captured in Area III in 2006; sample #62489, a male, was captured in Area III in 2006 and again in Area III in 2007. The between-year movement of two individuals can be seen in Fig. 2. Previous movements of Antarctic blue whales have been estimated from Discovery tags, and included long-range movements of up to 170° degrees of longitude around the Antarctic (Branch et al., 2007b).

Census N_{min}. After removal of replicate samples, 164 individuals remained within the dataset representing a total of 51 haplotypes. Of the 51 haplotypes, 25 had not been found previously in any of the worldwide blue whale populations. Eight of the 25 previously undescribed haplotypes were found in only one individual (singletons). Two of the previously found haplotypes were reported in the Indian Ocean and South Pacific blue whale populations (LeDuc et al., 2007) but had not previously been reported in the Southern Ocean.

Sequence information from an additional 20 samples from the Southern Ocean collected during JARPA surveys (LeDuc et al., 2007) were added to the dataset for a cumulative database of 184 individuals. Within these 20 samples, there were13 haplotypes, 11 of which were also found within the 51 haplotypes in the IDCR/SOWER dataset (n=164). Thus, in total, the combined dataset of 184 individuals represented 53 haplotypes.

Our minimum census of 53 haplotypes from 184 individuals is more than double the previous number of 26 haplotypes described from 47 samples by LeDuc et al. (2007). This number can be used to revise the estimate of surviving lineages by Branch and Jackson (2008). It should be noted that finding 53 haplotypes from just 184 individuals is a higher discovery rate than predicted by Branch and Jackson (2008), who conservatively estimated that only 51 haplotypes would be found in the entire population of 2280 individuals. On the other hand, their correction factor of 1.29 for haplotypes missed due to short mtDNA sequences is likely too high, given that only one new haplotype was discovered when extending

the mtDNA sequences from 420 to 560 base pairs. Sequencing of the whole mitogenome from a sample of individuals with identical control region haplotypes would provide an improved correction factor.

REFERENCES

Aasen, E., & Medrano, J. F. (1990). Amplification of the Zfy and Zfx genes for sex identification in humans, cattle, sheep and goats. *Nature Biotechnology, 8*(12), 1279-1281.

Berube, M., Jorgensen, H., McEwing, R., & Palsboll, P. J. (2000). Polymorphic dinucleotide microsatellite loci isolated from the humpback whale, *Megaptera movaeangliae*. *Molecular Ecology, 9*, 2181-2183.

Branch, T. A. (2007). Abundance of Antarctic blue whales south of 60°S from three complete circumpolar sets of surveys. *Journal of Cetacean Research Management, 9*(3), 87-96.

Branch, T. A. (2008). Current status of Antarctic blue whales based on Bayesian modeling. *Report SC/60/SH7 to the Scientific Committee of the International Whaling Commission*

Branch, T. A., Abubaker, E. M. N., Mkango, S., & Butterworth, S. D. (2007a). Separating southern blue whale subspecies based on length frequencies of sexually mature females. *Marine Mammal Science, 23*, 803-833.

Branch, T. A., Allison, C., Mikhalev, Y. A., Tormosov, D., & Brownell, J. R. L. (2008). Historical catch series for Antarctic and pygmy blue whales. *Report SC/60/SH9 to the Scientific Committee of the International Whaling Commision*

Branch, T. A., & Jackson, J. A. (2008). *Minimum bottleneck abundance of Antarctic blue whales based on current mtDNA diversity.* Paper presented at the Report SC/60/SH10 to the Scientific Committee of the International Whaling Commission

Branch, T. A., Matsuoka, K., & Miyashita, T. (2004). Evidence for increase in Antarctic blue whales based on Bayesian modeling. *Marine Mammal Science, 20*(4), 726-754.

Branch, T. A., Mikhalev, Y. A., & Kato, H. (2009). Separating pygmy and Antarctic blue whales using long forgotten ovarian data. . *Marine Mammal Science, 25*(4), 833-854.

Branch, T. A., Stafford, K. M., Palacios, D. M., Allison, C., Bannister, J. L., Burton, C. L. K., Cabrera, E., Carlson, C. A., Galletii-Vernazzani, B., Gill, P. C., Hucke-gaete, R., Jenner, C., Jenner, M., Matsuoka, K., Mikhalev, Y. A., Miyashita, T., Morrice, M. G., Nishiwaki, S., Surrock, V. J., Tormosov, D. D., Anderson, R. C., Baker, A. N., Best, P. B., Borsa, P., Brownell Jr., R. L., Childerhouse, S., Findlay, K. P., Gerrodette, T., Llangakoon, A. D., Joergensens, M., Kahn, B., Ljunblad, D. K., Maughan, B., McCauley, R. D., McKay, S., Norris, T. F., Group, O. W. a. D. R., Rankin, S., Samaran, F., Thiele, D., Van Waerebeek, K., & Warneke, R. M. (2007b). Past and present distribution, densities and movements of blue whales *Balaenoptera musculus* in the Southern Hemisphere and northern Indian Ocean. *Mammal Review, 37*(2), 116-175.

Gemmel, N. J., & Akiyama, S. (1996). An efficient method for the extraction of DNA from vertebrate tissues. *Trends in Genetics, 12*, 338-339.

Gilson, A., Syvanen, M., Levine, K., & Banks, J. (1998). Deer gender determination by polymerase chain reaction: validation study and application to tissues, bloodstains, and hair forensic samples from California. *Californian Fish and Game, 84*(4), 159-169.

Jackson, J. A., Patenaude, N. J., Carroll, E. L., & Baker, C. S. (2008). How few whales were there after whaling? Inference from contemporary mtDNA diversity. *Molecular Ecology, 17*(1), 236-251.

Kalinowski, S. T., Taper, M. L., & Marshall, T. C. (2007). Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology, 16*, 1099-1006.

LeDuc, R. G., Dizon, M. G., Pastene, L. A., Kato, H., Nishiwaki, S., LeDuc, C. A., & Brownell Jr., R. L. (2007). Patterns of genetic variation in Southern Hemisphere blue whales and the use of assignment test to detect mixing on the feeding grounds. *Journal of Cetacean Research Management, 9*(1), 73-80.

Miller, S. A., Dykes, D. D., & Polesky, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research, 16*(3), 1215.

Morin, P. A., Martien, K. K., Archer, F. I., Cipriano, F., Steel, D., Jackson, J., & Taylor, B. (2010). Applied Conservation Genetics and the Need for Quality Control and Reporting of Genetic Data Used in Fisheries and Wildlife Management. *Journal of Heredity, 101*(1), 1-10.

Palsbøll, P. J., Bérubé, M., Larsen, A. H., & Jørgensen, H. (1997). Primers for the amplification of tri-and tetramer microsatellite loci in baleen whales. *Molecular Ecology, 6*, 893-895.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (2nd ed.). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schlötterer, C., Amos, B., & Tautz, D. (1991). Conservation of polymorphic simple sequence loci in cetacean species. *Nature, 354*(6348), 63-65.

Valsecchi, E., & Amos, W. (1996). Microsatellite markers for the study of cetacean populations. *Molecular Ecology, 5*, 151-156.

Waldick, R. C., Brown, M. W., & White, B. N. (1999). Characterization and isolation of microsatellite loci from the endangered North Atlantic right whale. *Molecular Ecology, 8*(10), 1763.

Table 1. List of total number of IWC IDCR/SOWER Antarctic blue whale biopsy samples collected by year and Area along with the total number of samples in which mtDNA haplotype was identified, sex determined and the average number of microsatellite loci amplified for samples collected each year.

Year	Area	Samples collected	mtDNA haplotype	Sex	Microsatellite loci
1990	I	1	1	0	0
1993	III	2	2	2	9.50
1995	IV	2	2	1	7.00
1997	II	5	5	5	7.80
1998	II	6	4	3	7.67
1999	IV	10	10	8	8.90
2001	I/V/VI	22	21	15	9.32
2003	V	21	21	17	12.10
2004	V	14	14	14	11.71
2005	IV/III	5	5	2	8.80
2006	III	36	36	36	16.11
2007	III	85	85	71	15.65
2009	IV	9	9	8	16.00
TOTAL		218	215	182	13.43

Table 2. Identification of genotype recaptures of Antarctic blue whales within the 1990-2009 IWC IDCR/SOWER biopsy samples (n=218). Recaptures were identified as the same individual sampled on different days where replicates are reported where the individual was sampled twice on the same day. The p(ID) is reported for each recapture.

Event	SWFSC LAB ID	Area	Date	LAT	LONG	Matching loci	p(ID)
1) capture	51452	V	6-Jan-2002	-64.32	137.27		
recapture	62484	III	26-Jan-2006	-69.38	5.43	14	6.80E-18
2) capture	62489	III	29-Jan-2006	-67.32	12.32		
recapture	72957	III	7-Feb-2007	-69.60	5.83	14	1.31E-13
replicate	72959	III	7-Feb-2007	-69.60	5.83	16	5.15E-17
3) capture	62501	III	9-Feb-2006	-68.48	18.55		
recapture	62508	III	13-Feb-2006	-68.42	14.23	17	3.02E-23
4) capture	72903	III	7-Jan-2007	-67.58	2.75		
replicate	72904	III	7-Jan-2007	-67.58	2.75	17	6.77E-21
recapture	72908	III	8-Jan-2007	-68.70	0.45	17	6.77E-21
5) capture	72906	III	8-Jan-2007	-68.17	-0.03		
recapture	72971	III	8-Feb-2007	-69.82	4.78	17	6.51E-22
6) capture	72930	III	5-Feb-2007	-69.08	8.33		
recapture	72945	III	6-Feb-2007	-69.37	6.23	12	1.25E-13
replicate	72946	III	6-Feb-2007	-69.37	6.23	12	1.25E-13
7) capture	72935	III	5-Feb-2007	-69.08	8.33		
recapture	72941	III	6-Feb-2007	-69.32	7.22	17	2.24E-19
recapture	72955	III	7-Feb-2007	-69.60	5.83	17	2.24E-19
8) capture	72944	III	6-Feb-2007	-69.37	6.23		
recapture	72963	III	8-Feb-2007	-69.67	4.88	17	5.83E-23
recapture	72970	III	8-Feb-2007	-69.82	4.78	17	5.83E-23
9) capture	72949	III	7-Feb-2007	-69.40	5.15		
recapture	72973	III	8-Feb-2007	-69.82	4.78	17	2.49E-21

Fig. 1. The location of Antarctic blue whale IDCR/SOWER biopsy samples collected from 1990 to 2009 (map courtesy of Tomas Follet).

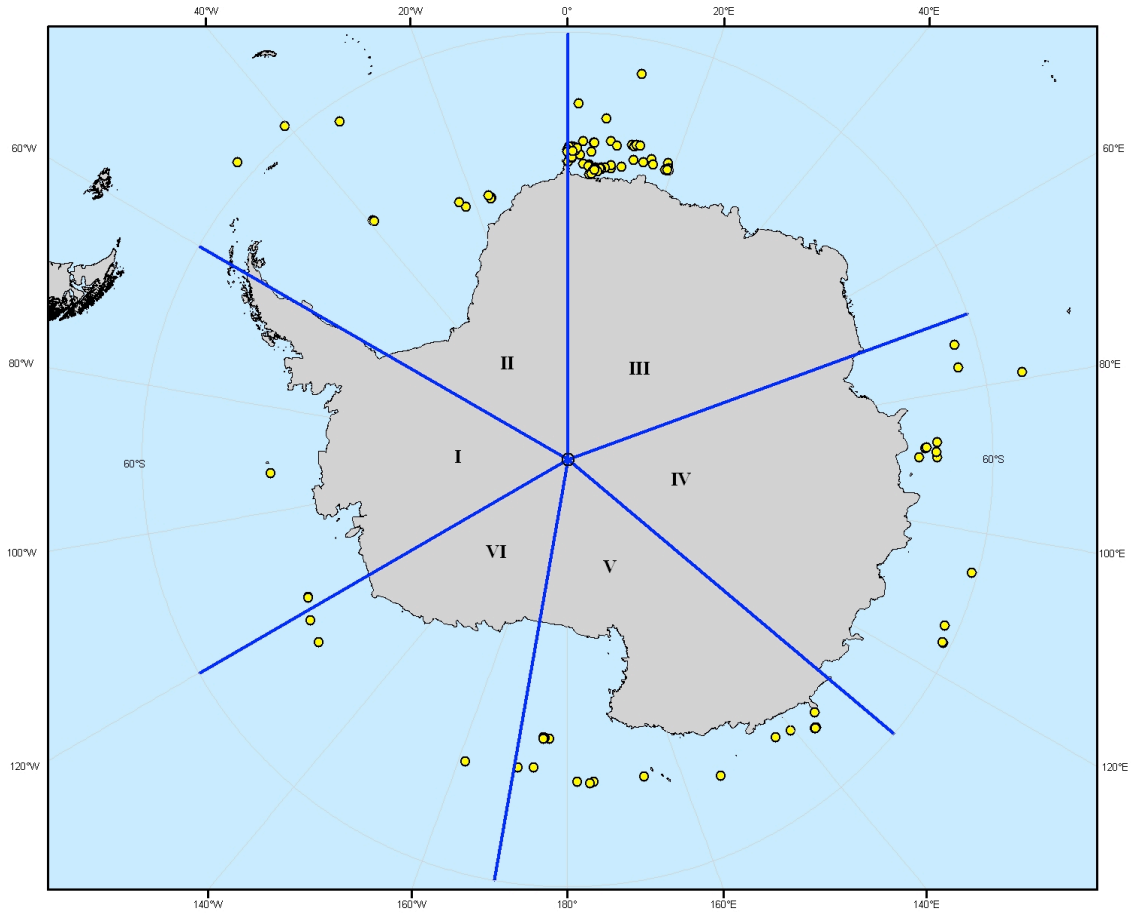

Fig. 2 The 2 between- year genotype recaptures of Antarctic blue whales collected during IDCR/SOWER cruises (see Table 2) (map courtesy of Tomas Follet).