FOR CONSIDERATION BY THE SCIENTIFIC COMMITTEE OF
THE INTERNATIONAL WHALING COMMISSION
SANTIAGO, CHILE, JUNE 2008

Using Mitochondrial DNA and Mixed-Stock Analysis to estimate Migratory Allocation of Humpback Whales from Antarctic Feeding Areas to South Pacific Breeding Grounds

R. ALBERTSON-GIBB[1], C. OLAVARRÍA[2], C. GARRIGUE[3], N. HAUSER[4], M. POOLE[5], L. FLÓREZ-GONZÁLEZ[6], C. ANTOLIK[1], D. STEEL[1] and C. S. BAKER [1,7]

1 *Marine Mammal Institute and the Department of Fisheries and Wildlife, Oregon State University, 2030 SE Marine Science Dr, Newport Oregon 97365 USA*
2 *Proyecto INACH 163 Ecología de Cetáceos Antárticos. Instituto Antártico Chileno, Punta Arenas, Chile*
3 Opération Cétacés BP 12827 98802 Nouméa, New Caledonia
4 *Cook Islands Whale Research Takuvaine Valley PO Box 3069, Avarua Rarotonga, The Cook Islands*
5 *Marine Mammal Research Program, BP 698 98728 Maharepa, Moorea, French Polynesia*
6 *Fundacion Yubarta, Carrera 24F Oeste No. 3-110, Tejares de San Fernando, Cali, Colombia*
7 *School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand*

ABSTRACT

We present mixed-stock analyses of mtDNA haplotypes for allocation of humpback whales (*Megaptera novaeangliae*) from three Antarctic feeding areas to low-latitude breeding grounds. These breeding grounds include New Caledonia, Tonga, Cook Islands, French Polynesia, Colombia and Western Australia. Migratory allocation was estimated using the program Statistics Program for Analyzing Mixtures (SPAM) with an expanded dataset of mtDNA haplotypes from breeding grounds of the South Pacific and Western Australia (n = 1,072, Olavarría *et al*. (2007)) and a more limited number of samples from Antarctic feeding Areas IV, VI*, and I* (n=144). Assuming that the breeding grounds represent the 'pure stocks' and that each feeding area represent the 'mixed stocks' Area IV was allocated in nearly equal proportions to Western Australia (33.1%) and New Caledonia (31.0%), Area VI* whales were allocated primarily to Tonga (78.9%) and Area I* was allocated primarily to Colombia (78.3%).

INTRODUCTION

In the absence of direct observations of migratory pathways, molecular genetic markers have proven useful for resolving migration patterns (Bowen *et al.* 2007). Humpbacks are a highly migratory species, and describing connections between their Antarctic feeding Areas and tropical breeding grounds continues to be a challenge, especially for the allocation of historical catches and the modelling of current population dynamics (e.g., Jackson *et al.* SC/60/SH14). Here we present a summary of mtDNA haplotype frequencies from six individual breeding grounds (IWC breeding stocks D, E1, E2, E3, F and G) and three Antarctic feeding Areas (Areas IV, VI and I). This dataset revised and extends that presented previously in Olavarría *et al.* (2007), including 221 previously unanalyzed samples from Oceania and removing samples identified as replicates by genotyping (Steel *et al*. SC/60/SH13). An Analysis of Molecular Variance (AMOVA) first confirmed differentiation between breeding grounds and between the three feeding areas. We then used Mixed-Stock Analysis (MSA) to estimate the migratory allocation of each feeding Area to the six breeding grounds.

METHODS

Field collection

In addition to the samples described in Olavarría *et al.* (2007) (n=1,112), biopsy and sloughed skin tissue samples (n=221) were collected from living humpback whales on breeding grounds of the South Pacific during the austral winters of 2003 through 2005. Sampling was carried out aboard dedicated small boat surveys by members of the South Pacific Whale Research Consortium. Biopsy samples were collected using a stainless steel biopsy dart deployed from a modified veterinary capture rifle. In the feeding areas, biopsy samples (n=144) were collected from living whales during the austral summers of 1998 through 2005, as part of the International Whaling Commission's IDCR/SOWER cruise. All samples were preserved in 70% ethanol at the field site then transferred to sterile vials and stored at -80^{o}C for long-term storage.

Genetic Analysis

As described by Olavarría *et al.* (2007), an approximately 750 base-pair (bp) fragment of the 5'-end of mtDNA control region (i.e. D-loop) was amplified via the Polymerase Chain Reaction (PCR) using the primers, light strand tPro-whale Dlp1.5 and heavy strand Dlp8G (Garrigue *et al*. 2004). Amplifications were conducted in a final volume of 10ul at the following concentrations: 2.5 mM $MgCl_2$, 200 uM dNTP, 0.4 mM each primer, 0.25 U Platinum *taq* (Invitrogen), 1 x PCR reaction buffer (Invitrogen) and 1ul DNA. Unincorporated primers and nucleotides were removed from PCR products using exonuclease (Exo I) and shrimp alkaline phosphatase (SAP) and sequenced on an AB13730x1 DNA sequencer (Applied Biosystems) using the primer M13Dlp-1.5.

Data Analysis

All variable positions in sequences were aligned and edited using SEQUENCHER (version 4.1.2, Gene Codes Co.), and then checked by comparison to other chromatographs. An AMOVA was performed in the program ARLEQUIN (version 2.0, Schneider *et al.* 2000) to measure the differentiation at the haplotype (F_{ST}) level.

Mixed-Stock analyses were conducted using the Statistical Program for Analyzing Mixtures (SPAM; version 3.7b; Alaska Department of Fish and Game (2003)) with 10,000 iterations and 500 bootstrap resamples. For the future purposes of allocating historical catches to breeding stocks, we assumed that the breeding ground samples represented the 'source stocks' and the feeding area samples represented the 'mixed stocks'. In this framework, an analysis was run for each feeding area, estimating the proportional allocation to the six breeding grounds. Due to the number of zeros in any particular region's haplotype distribution (i.e., not all regions contain all haplotypes), a Bayesian method (the Pella-Masuda model) was implemented in the estimation mode. This method assumed that the absence of a haplotype from a breeding stock's baseline sample means that it is rare rather than nonexistent in the stock (Koljonen *et al.* 2005).

RESULTS AND DISCUSSION

The total sample represented 1,072 individual humpback whales from 6 breeding regions and 144 individuals from three feeding areas (Table 1, Figure 2). A total of 97 haplotypes were resolved from the 470 bp consensus region defined by Olavarría *et al*. (2007).

Population Differentiation and Diversity

Breeding Regions. Of the 97 haplotypes observed in this study, five haplotypes occurred in all six breeding grounds, five occurred in five of the breeding grounds, and 38 occurred in only one breeding ground. As reported in Olavarria *et al.* (2007), the number of haplotypes was greatest for New Caledonia (65) and the lowest for French Polynesia (22) (Table 1). The proposed subdivisions of IWC breeding stock E into E1, E2, and E3, as described previously, were supported by the AMOVA (F_{ST} = 0.0316; $p < 0.001$). Pairwise F_{ST} values (Table 2) showed a greater difference between Colombia and the Oceania breeding grounds than between Western Australia and Oceania Olavarría *et al.* (2007).

Feeding Areas. After review of sample locations, we modified the boundaries of Area VI and Area I to reflect apparent geographic groupings (Figure 1). For Area VI*, we excluded 3 samples from the eastern edge of true Area VI and included 4 samples collected in the western edge of Area I, giving an n = 28. For Area I*, we excluded one additional sample in the central region and included all samples from the Antarctic Peninsula, giving an n = 77. The two samples from Area V and the 3 samples from the western boundary of Area VI were not considered sufficient for statistical analysis. The boundaries of Area IV were unmodified, giving an n = 39.

Although we did not attempt to adjust for differences in sample size, the number of haplotypes was similar in each of the three areas, ranging from 19 in Area VI to 23 in Area IV, and lower than on the breeding grounds. The AMOVA results showed significant differences between all feeding areas (Table 2), with the greatest difference between Area I* and Area VI*.

Feeding and breeding regions
Pairwise comparisons of feeding areas to breeding grounds showed significant F_{ST} values for all but three cases: Area IV to Western Australia, Area VI* to Tonga, and Area I* to Colombia.

Migratory Allocations
Area IV. The mixed-stock analysis allocated the largest proportion of Area IV to Western Australia (33.1%) and New Caledonia (31.0%), with a substantial proportion (17.9%) allocated to an unknown (or unsampled) breeding ground (Table 3). The allocation of Area IV to Western Australia supports previous studies (Chittleborough 1965; Dawbin 1964; Jenner *et al.* 2001) using historical whaling data, *Discovery* marking and photo-identification. However, the large allocation to New Caledonia was somewhat surprising. Although Eastern Australia was not a part of this study, there has been documentation connecting Eastern Australia and Area IV (Chittleborough, 1965; Dawbin 1964), and some migratory interchange between east Australia and New Caledonia has been identified (Garrigue *et al.* 2006). Additional Area IV samples are required to confirm that New Caledonia is consistently a part of the Area IV migratory route, or if the current analysis is biased by sampling location or temporal shifts in feeding aggregations (Chittleborough, 1965).

Area VI.* The mixed stock analysis allocated the largest proportion of Area VI* to Tonga (78.9%). These results agree with recent genotype matching and limited *Discovery* marking, which have provided evidence of a connection between Tonga and Area VI (Steel *et al.* SC/60/SH13). Surprisingly, Area VI* show no allocation to French Polynesia despite the geographic proximity of these seasonal habitats.

Area I.* The mixed stock analysis allocated 78.7% of the Antarctic Area I* samples to the Colombia breeding region (Table 3). These results confirm a strong association between Colombia and the Antarctic Peninsula. Genetic markers and naturally marked individuals have confirmed that there is a connection between Colombia and Area I around the Antarctic Peninsula (Olavarría *et al.* 2007, Stevick *et al.* 2006, Caballero *et al.* 2001, Stone *et al.* 1990). Our results are in agreement with the conclusions of Olavarría *et al.* (2007) and previous investigators (Stone *et al.* 1990), who have suggested that many humpbacks from the Antarctic Peninsula migrate along the western coast of South America to Colombia.

CONCLUSIONS

The Mixed-Stock analysis showed considerable promise as a method for assisting the proportional allocation of feeding grounds to breeding stocks. The predominant allocation of Area IV to Western Australia, Area VI* to Tonga and Area I* to Colombia all agree with previous photo identification and genotype studies. The analysis also suggested some unexpected results, including the strong allocation of Area IV to New Caledonia and the absence of allocation of Area VI* to French Polynesia. Unfortunately, samples sizes for the three feeding areas were relatively small, resulting in large standard errors for some allocations. In the case of Area V, arguably the most important area for allocation of historical catches, sample size was too small for inclusion in the analysis. Sample sizes for the breeding grounds were considerably larger and more representative of the known distribution, except for the absence of samples from the eastern Australia breeding ground, assumed to be the largest in the South Pacific. However, genetic samples are available from the eastern Australia migratory corridor (Olavarría *et al.* 2006) and could be included in future analyses. A more concerted and systematic sampling of feeding areas is needed to complement the synoptic, coordinated sampling of breeding grounds in Oceania. Assignment tests using microsatellite genotypes, also promise to provide further fine-scale genetic information to better understand stock mixing on feeding grounds and the migratory routes of individual humpbacks throughout the Southern Hemisphere (e.g., Steel *et al.* SC/60/SH13).

ACKNOWLEDGEMENTS
Funding for fieldwork in Oceania was provided by the International Fund for Animal Welfare. The collection of samples around the Antarctic Peninsula was supported primarily by the Instituto Antártico Chileno (INACH). Funding for laboratory analysis was provided by the Regional Natural Heritage Program, Department of Environment and Heritage, Government of Australia, and the endowment of the Marine Mammal Institute, Oregon State University. We thank the South Pacific Whale Research Consortium for access to the data.

REFERENCES

Alaska Department of Fish and Game. 2003. SPAM Version 3.7: Statistics Program for Analyzing Mixtures. Alaska Department of Fish and Game, Commercial Fisheries Division, Gene Conservation Lab.

Bowen, B.W., Grant, W.S., Hills-Starr, Z., Shaver, D.J., Bjornadal, A., Bolten, A.B., Bass, A.L. 2007. Mixed stock analysis reveals the migrations of juvenile hawksbill turtles (*Eretmochelys imbricate*) in the Caribbean Sea. *Molecular Ecology*. 16: 49-60.

Chittleborough, R.G. 1965. Dynamics of two populations of humpback whales, *Megaptera novaeangliae* (Borowski). *Aust. J. Mar. Freshw. Res*. 16:33-128.

Dawbin, W.H. 1964. Movements of humpback whales marked in the southwest Pacific Ocean 1952 to 1962.*Norsk Hvalf. Tid*. 53:68-78.

Garrigue, C., Dodemont, R., Steel, D., Baker, C.S., 2004. Organismal and 'gametic' capture-recapture using microsatellite genotyping confirm low abundance and reproductive autonomy of humpback whales on the wintering grounds of New Caledonia. 274: 251-262.

Garrigue, C., Baker, C.S., Constantine, R., Poole, M., Hauser, N., Clapham, P., Donoghue, M., Russell, K., Paton, D., Mattila, D. 2006. Interchange of humpback whales in Oceania (South Pacific). Report SC/A06/HW55, Presented to the IWC Scientific Committee 2006. (unpublished) [Available from the office of this journal]

Jackson, J.A., Zerbini, A., Clapham, P., Constantine, R., Garrigue, C., Hauser, N., Poole, M.M., Baker, C.S. 2008. Progress on a two-stock catch allocation model for reconstructing population histories of east Australia and Oceania. SC/60/SH14. Presented to the IWC Scientific Committee.

Jenner, K.C.S., Jenne, M.N.M., and McCabe, K.A. 2001. Geographical and Temporal Movements of Humpback whales in Western Australian Waters. *APPEA Journal* 749-764.

Koljonen, M.L., Pella, J.J., Masuda, M. 2005. Classical individual assignments versus mixture modelling to estimate stock proportions in Atlantic salmon (Salmo salar) catches from DNA microsatellite data. *Can. J. of Fisheries and Aquatic Science*. 62: 2143-2158

Olavarría, C., Anderson, M., Paton, D., Burns, D., Brasseur, M., Garrigue, C., Hauser, N., Poole, M., Caballero, S., Florez-Gonzalez, L., Baker, C.S. 2006. Eastern Australia humpback whale genetic diversity and their relationship with Breeding Stocks D, E, F and G. SC/58/SH25 Presented to the IWC Scientific Committee.

Olavarría, C., Baker, C.S., Bannister, J., Brasseur, M., Caballero, S., Capella, J., Clapham, P., Dodemont, R., Donaghue, M., Jenner, C., Jenner, M.M., Moro, D.J., Florez-González, L., Hauser, N., Oremus, M., Paton, D., Poole, M., Rosenbaum, H., Russell, K. 2007. Population structure of South Pacific humpback whales and the origin of the eastern Polynesian breeding grounds. *Marine Ecology Progress Series*. 330: 257-268.

Schneider, S., Roeslli, D., Excoffier, L. 2000 Arlequin. A software for population genetics data analysis Stevick, P.T., Aguayo-Lobo, A., Allen, J.M., Chater, K., Dalla-Rosa, L., Olavarría, C., Secchi, E., 2006. Mark-recapture abundance estimates for humpback whales in the Antarctic Peninsula. SC/A06/HW54. 2006. Presented to the IWC Scientific Committee. [Available from the office of this journal].

Steel, D., Garrigue, C., Poole, M., Hauser, N., Olavarría, C., Florez-Gonzalez, L., Constantine, R., Caballero, S., Thiele, D., Paton, D., Clapham, P., Donoghue, M., and Baker, C.S. 2008. Migratory connections between humpback whales from South Pacific breeding grounds and Antarctic feeding areas demonstrated by genotype matching. SC/60/SH13. Presented to the IWC Scientific Committee. [Available from the office of this journal].

Stone, G.S., Florez-González,L and Katona, S. 1990. Whale migration record. *Nature* 346: 705.

Table 1. A summary of the number of individuals, the number of haplotypes in each region out of the total 97, and the number of unique haplotypes relative to seasonal habitats. Individual samples were collected from 1991-2005 and replicates were deleted. Area I* is solely the Antarctic Peninsula, and Area VI* includes 7 individuals from Area I/VI border (Figure 1).

Region	**Individuals**	**Haplotypes**	**Unique Haplotypes by seasonal habitat**
Breeding Regions			
Western Australia	132	30	2
New Caledonia	368	65	16
Tonga	354	53	5
Cook Islands	105	24	2
French Polynesia	113	22	2
Colombia	97	29	5
Total for breeding regions	**1072**		
Feeding Areas			
Antarctic Area I	77	20	13
Antarctic Area IV	39	23	6
Antarctic Area VI*	28	19	9
Total for feeding areas	**144**		

Table 2. Updated Fst values corrected for replicate samples. Pair-wise test of differentiation between all areas for mtDNA control region sequences. **Values in bold** represent a significant difference in the data greater than 5% of 10,000 random permutations (p> 0.05). Regions: Western Australia (WA), New Caledonia (NC), Tonga (Tg), Cook Islands (CI), French Polynesia (FP), Colombia (Col), and Antarctic Areas (I*, IV and VI*).

Region	**WA**	**NC**	**Tg**	**CI**	**FP**	**Col**	**IV**	**VI***
WA								
NC	**0.012**							
Tg	**0.013**	**0.006**						
CI	**0.020**	**0.027**	**0.014**					
FP	**0.032**	**0.038**	**0.023**	**0.014**				
Col	**0.040**	**0.051**	**0.056**	**0.074**	**0.076**			
IV	0.001	**0.006**	**0.011**	**0.035**	**0.048**	**0.058**		
VI*	**0.022**	**0.011**	0.003	**0.016**	**0.035**	**0.067**	**0.015**	
I*	**0.047**	**0.043**	**0.045**	**0.058**	**0.057**	0.001	**0.051**	**0.059**

Table 3. Results allocated by MSA from each of the breeding regions for each of the Antarctic feeding areas including (Standard Error).

Breeding ground source populations	Allocation from Area IV (Standard Error)	Allocated from Area VI (Standard Error)	Allocated from Area I (Standard Error)
Western Australia	33.1% (0.0834)	4.6% (0.0457)	0.00 (0.00)
New Caledonia	31.0% (0.1257)	12.4% (0.1112)	0.00 (0.00)
Tonga	18.0% (0.1214)	78.9% (0.1865)	0.04% (0.0005)
Cook Islands	0.00 (0.00)	4.0% (0.1342)	15.6% (0.0937)
French Polynesia	0.00 (0.00)	0.00% (0.00)	3.5% (0.0741)
Colombia	0.00 (0.00)	0.00 (0.00)	78.3% (0.0566)
Unknown	17.9%	0.1%	2.5%

Figure 1. Distribution in the Antarctic feeding grounds of samples taken during the IWC-SOWER cruise (1999, 2001, 2005). Area I = red, Area III = yellow, Area IV = blue, Area V = orange, Area VI = purple. Individuals designated by + were not used in analyses after regrouping of available samples. Circled portions designate regrouped Areas.

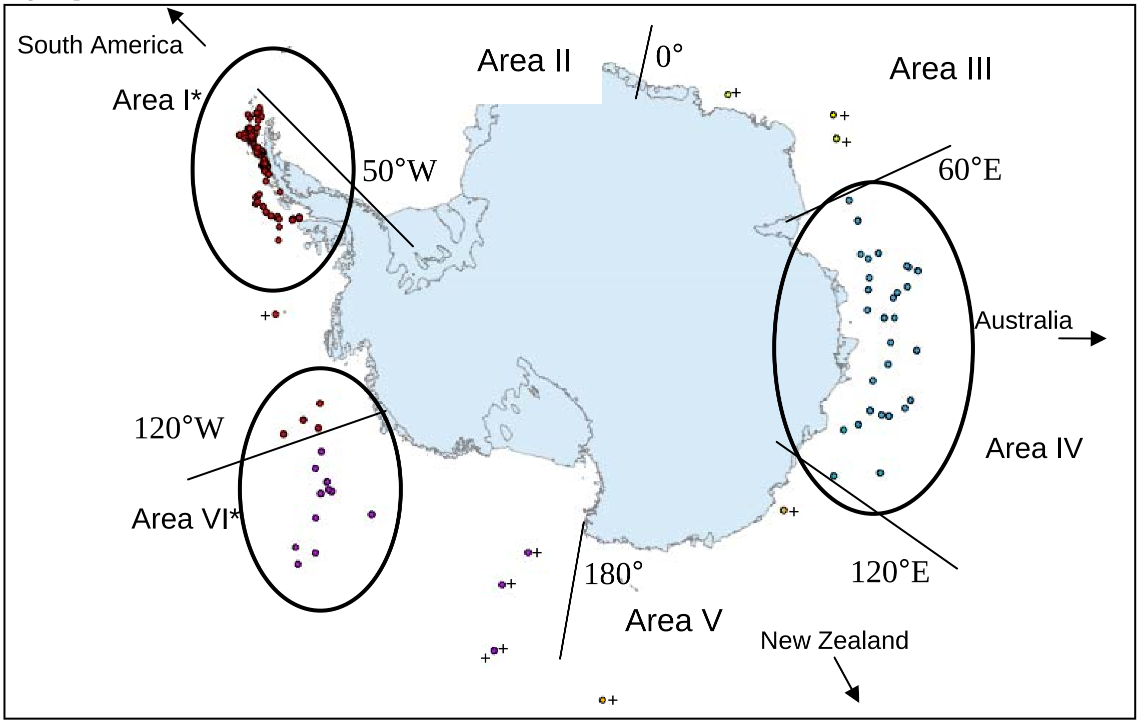

Figure 2. Geographic distribution and the number of updated mtDNA haplotypes in each breeding and feeding region. Each colour in the chart represents one haplotype and the size of each colour represents a relative number of individuals with that haplotype in that breeding or feeding region. The number represents the total number of individuals for each region used in the Mixed Stock Analysis.