STOCK IDENTITY OF ANTARCTIC PENINSULA HUMPBACK WHALES INFERRED FROM MTDNA VARIATION

C. OLAVARRÍA B.[1], C. S. BAKER[2], L. MEDRANO G.[3], A. AGUAYO L.[1], S. CABALLERO G.[4], L. FLÓREZ-GONZÁLEZ[4], J. CAPELLA A..[4], H. C. ROSENBAUM[5], C. GARRIGUE[6], J. GREAVES[6], J. L. BANNISTER[7], M. JENNER[8] & C. JENNER[8].

[1]*Proyecto INACH 163, Casilla 16521, Correo 9, Santiago, Chile. Email: colavarria@hotmail.com*
[2]*School of Biological Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand.*
[3]*Facultad de Ciencias, UNAM. A.P. 70-572. CP 04510, México D.F., México.*
[4]*Fundación Yubarta, AA 33141, Cali (Valle), Colombia*
[5]*American Museum of Natural History, Molecular Lab, New York 10024, USA*
[6]*Operation Cetaces, BP 12827, Nouméa, New Caledonia*
[7]*c/o Western Australia Museum, Francis Street, Perth, WA 6000, Australia*
[8]*Centre for Whale Research (Western Australia), P.O. Box 1622 Freemantle WA 6959, Australia*

ABSTRACT

The migratory connection for humpback whales (*Megaptera novaeangliae*) feeding near Antarctic Peninsula (AP; Bransfield and de Gerlache strait) was investigated by molecular methods using skin samples (n=64) collected in four summer seasons (1996-99) by the Instituto Antártico Chileno (INACH). A 425 bp segment of the mitochondrial DNA control region was sequenced for each individual revealing 24 unique haplotypes, defined by 41 polymorphic sites. A phylogenetic reconstruction grouped the sequences into three clades, corresponding to those previoulsy described, characteristic shared only with Colombia, the presumed Stock I breeding ground. A comparison of the AP sequences (trimmed to the *worldwide consensus* of 283 bp, Baker *et al.* 1993) with 327 individual humpback whales sampled in wintering ground of stocks I, III, IV and V, and 155 from the North Pacific and North Atlantic suggested limited connections with the other southern regions and historical interchange between oceans. An analysis of variance adapted for molecular information showed significant difference between the AP and all southern hemisphere breeding grounds, except with Colombia. These results based on a genetic approach strongly support the direct evidence of migratory connection between Antarctic Peninsula and Colombia. Further, it support the historical assumption of longitudinal division between stocks represented by both feeding and breeding grounds in the Southern Hemisphere. For Area I feeding ground modification of the eastern boundary is recommended from 60º W to at least 58º W.

KEYWORDS: Humpback whale, *Megaptera novaeangliae*, feeding ground, Antarctic Peninsula, Population structure

INTRODUCTION

For humpback whales (*Megaptera novaeangliae*, Borowski 1781) six stocks or populations have been recognized in the Southern Ocean with basis on commercial whaling data and *Discovery* marks, numbering them I to VI (Towsend 1935, Mackintosh 1942, Omura 1953, Mackintosh 1965; Figure 1). Those data let know the migratory destinations and population identities mainly for Western South Pacific humpback whales, (stock IV and stock V, Chittleborough 1965), but the migratory connections for the rest of stocks was supported with less data.

Although a marked humpback whale was never recovered in the Eastern South Pacific (stock I), it was assumed that humpback whales from the western coast of Antarctic Peninsula (AP; feeding ground) migrated to wintering grounds along Colombia and Ecuador (Omura 1953, Mackinstosh 1965). Just ten years ago this was confirmed when a humpback whale was photographed in waters adjacents to the AP in April 1986 and in Colombian waters four months later (Stone *et al.* 1990). Additional photo-identification matches have supported the migratory connection between these two regions (Capella & Flórez-González

1993, Muñoz *et al.* 1998[1]). Although samples from AP humpback whales have been used on previous works of genetic structure (Baker *et al.* 1993, Baker *et al.* 1994, Baker & Medrano *in press*, Baker *et al.* 1998, Palsbøll *et al.* 1995, Valsecchi *et al.* 1997) none of them has adressed the migratory connection issue of this feeding ground.

In this report we investigate the putative migratory connection and stock identity of AP humpback whales to Colombia and other wintering grounds by molecular methods based on mitochondrial (mt) DNA markers.

MATERIALS AND METHODS

The samples for this analysis were collected off the western coast of the AP. The study was divided in two main areas: Bransfield and de Gerlache Strait (Figure 2). The Bransfield strait is bounded by the South Shetland islands in the north and the AP in the south. The eastern north limit is set by the Clarence and Joinville islands (61ºS; 54ºW) and in the western south side is set by the Hoseason and Trinidad islands (63º40'S; 61º30'W). This strait is 240 miles long and 60 miles wide.

The De Gerlache strait includes the Palmer archipelago surrounding waters (Brabante, Anvers, Wiencke and others minors islands) and the AP. It extends 100 miles since the north mouth, limited by Trinidad and Hoseason islands, to the south opening limited by the Anvers and Wiencke islands and the AP (63º40'S; 63º30' W to 65º S; 61º30' W). This strait is bounded by the Bransfield strait in the northeast and by the Bismark strait in the southwest.

In the austral summers of 1996, 1997, 1998 and 1999 the study area was visited as part of the Scientific Antarctic Expeditions (ECA 32, 33, 34 and 35, respectively) organizated by the Instituto Antártico Chileno (INACH) developing the projects INACH 08-93 (Aguayo *et al.* 1993[2]) and INACH 163 (Aguayo *et al.* 1999[3]). The Chilean Navy ships PSG 71 *Contramaestre Micalvi* and PSG 73 *Aspirante Isaza* (45 m long, 10 m width, 15 knots max speed) were used as survey vessels.

Skin samples were obtained using a crossbow (Ranger 125 and 150 lb; Lambertsen 1987) with a biopsy dart (Medrano-González 1993[4]). The vessel and an inflatable boat (6 m long) were used for this purpose. To avoid getting samples from same individuals, biopsy collection was combined with photoidentification. Skin samples were stored in 2 mL criotube with 70% ethanol at -15º C to posterior analysis.

Extraction of genomic DNA followed Sambrook *et al.* (1989) protocol modified by Baker *et al.* (1991). A 550 pb portion of DNAmt D-loop proximal to the tPro gene was amplified by PCR (Saiki *et al.* 1988) and sequenced using the ABI 373A and ABI 377 automated sequencer. Temperature profiles for PCR reactions are described in Baker *et al* (1993), Dalebout *et al.* (1998) and Pichler *et al.* (1998).

MtDNA sequences were aligned and compared to identify the different haplotypes. To relate the AP humpback whales with Southern Ocean breeding grounds from three stocks (Colombia/stock I, Madagascar/stock III, Western Australia/stock IV, Eastern Australia, New Caledonia and Tonga/stock V) published and unpublished sequences of this wintering grounds were compared, including additionally previous sequences from AP, North Pacific and North Atlantic (Baker *et al.* 1993, Baker *et al.* 1998,

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[3] Aguayo A, D Torres, C Olavarría, R Bernal, A Larrea, MJ Perez, C Lippi, M Flores, V Vallejos & J Acevedo. 1993. Ecología de cetáceos en las aguas del Océano Austral y de sus ecosistemas asociados. Proyecto INACH 163.

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Rosenbaum *et al.* 1997, Rosenbaum *et al.* 1998[5], Caballero *et al.* 2000[6]). A phylogeny of AP humpback whales haplotypes was constructed using a distance matrix (Kimura-two-parameters, Kimura 1980) and Neighbour-Joining method (Saitou & Nei 1987), using PAUP v.4.03b (Swofford 1999).

The diversity and geographic differentiation of haplotypes was quantified using the Analysis of Molecular Variance procedure (AMOVA; Excoffier *et al.* 1992) as implemented in the computer program Arlequin ver 1.1 (Schneider *et al.* 1997). This procedure calculates standard variance components and an array of haplotypic correlations measures, referred to as Φ-statistics, for population structure. For then hierarchical analysis of six regional wintering grounds and one feeding ground representing four stocks, the statistics of interest is the Φ_{ST}, defined as the correlation of random haplotypes within a region population relative to that of the entire population; Φ_{CT} defined as the correlation of random haplotypes within a stock relative to the entire population and the Φ_{SC} defined as the correlation of random haplotypes within a stock relative to other regions of the same stock. As such, the Φ_{ST} is analogous to Wright's (1951) F_{ST} statistics and to other genotypes correlations used for study of population structure (e.g. Hudson *et al.* 1992, Weir & Cockerham 1984, Takahata & Palumbi 1985). The signification of the observed Φ_{ST} were tested using 5000 random permutations of the data matrix permutation. Following the recommendation of Hudson *et al.* (1992), we also tested regional differences in haplotypes (i.e., nucleotype, Nei 1987) frecuencies using a modified permutation procedure. These tests considers only the categorical difference (i.e., 0,1) between identified haplotypes and does not include the contribution of molecular distances.

The diversity of humpback whale mtDNA was estimed at the haplotype level (Nei 1987) using the computer program Arlequin ver 1.1 (Schneider *et al.* 1997). At the haplotype or nucleon level, diversity (*h*, Nei 1987) and its standar error were calculated without regard to the genetic distance (i.e., number of nucleotide substitutions) between two sequence of mtDNA.

RESULTS

During the four expeditions at Antarctic Peninsula 64 skin samples were collected (ECA32: n=19, ECA 33: n=27, ECA 34: n=6 and ECA 35: n=12). Thirty seven samples were collected at de Gerlache strait and 27 in Bransfield strait. Of these, 52 have been analized by Olavarría *et al.* (1998[7]), Olavarría *et al.* (1999[8]) and Olavarría (1999[9]). A common consensus of 425 bp were sequenced for the mtDNA control region in all the samples, except one. Twenty four haplotypes were defined by 41 polymorphic sites including four insertions/deletions (one bp each), one trasvertion and the rest transitions. A phylogenetic reconstruction using Neighbor-Joining grouped the AP haplotypes in three clades referred to previously as AE, CD e IJ (Figure 5; Baker *et al.* 1993, Baker *et al.* 1998). The frecuency distribution for AP haplotypes was 76,9 % (n=20) in CD clade, 19,2 % (n=5) in IJ and 3,9 % (n=1) in AE. These agreed closely with

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frecuencies previoulsy reported for the Colombian wintering grounds (Baker *et al.* 1998, Baker & Medrano *in press*).

For comparison to other published and mtDNA sequences, a consensus region of 283 bp was defined, corresponding to that described by Baker *et al.* (1993) and Baker & Medrano (*in press*). This allowed comparison with 302 sequences from south hemisphere breeding grounds (Colombia: 144, Tonga: 32, New Caledonia: 36, Eastern Australia: 14, Western Australia: 44 and Madagascar: 32; Baker *et al.* 1998, Rosembaum *et al.* 1998[5], Caballero *et al.* 2000[6]), three AP sequences analized previously (Baker *et al.* 1998) and 155 sequences from feedings and breedings grounds of North Pacific and North Atlantic. The comparison included a total of 26 AP haplotypes, because the two previous haplotypes (three samples; Baker *et al.* 1998) did not matched with the 24 new haplotypes of this work (Figure 4). Haplotype diversity of AP, $h = 0{,}9357 \pm 0{,}0168$, was somewhat higher than for the other regions previoulsy (Baker *et al.* 1998).

Of the 26 haplotypes in the AP sample, two were shared with North Pacific and one with the North Atlantic. In the Southern Ocean, 17 AP haplotypes were shared with Colombia, seven with Tonga, seven with New Caledonia, three with Eastern Australia, five with Western Australia and six with Madagascar. Some of these were shared by more than one breeding ground (Caballero *et al.* 2000[6]; Table 1). Despite the number of haplotypes shared with other wintering grounds, it is clear that the common haplotypes from AP were shared primaly with Colombia. For example the most common haplotype in both regions was the same (AP, n=13, Co, n=34). The close genetic relationship of the AP and Colombian wintering grounds was confirmed by the AMOVA. Significant differences were found between the AP feeding ground and all wintering grounds of South Hemisphere, except Colombia (Table 2). Based on the slightly negative values for the F_{ST} and Φ_{ST}, and given the reasonably large sample sizes used here, the AP and Colombia can be considered effectively panmictic. Estimated maternal gene flow to all other regions varied from 10 to 15 females per generation, based on the F_{ST}, and did not show a strong effect of distance. These estimates are consistent with those previously reported for exchange among wintering grounds.

DISCUSION

One of the most important issues in whale management is stocks identification. For southern hemisphere humpback whales six historical stocks were recognized for whaling purposes, based on mark-recapture analysis of *Discovery* marks. For stock I, photo-identification have been a useful non-lethal technique to support the suppose migratory connection between feeding ground off Antarctic Peninsula and breeding grounds off Colombia (Stone *et al.* 1990, Capella & Flórez-González 1993, Muñoz *et al.* 1998[1], Olavarría 1997[10], Aguayo *et al.* 1998[11]) and Ecuador (Garriegue *et al.* 2000[12]) in the Eastern South Pacific.

In this report we addressed from a molecular approach, the stock identity of Eastern South Pacific humpback whales using mtDNA control region sequences by direct comparison, phylogenetic reconstruction and analysis of molecular variance comparated with six southern ocean breeding grounds corresponding to four stocks. These results were consistent with the assumed migratory connection between the AP feeding grounds and the Colombian wintering grounds. Further, the combined analyses of

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[11] Aguayo A, C Olavarría, R Bernal, L Medrano, D Torres, A Larrea & CS Baker. Patrones de coloración caudal de las ballenas jorobadas *Megaptera novaeangliae* en aguas adyacentes a la Península Antártica. 8ª Reunião de Trabalho de Especialistas em Mamíferos Aquáticos da América do Sul e 2° Congresso da Sociedade Latinoamericana de Especialistas em Mamíferos Aquáticos, 25 a 29 outubro 1998, Olinda, Brasil.

[12] Garrigue, C., A. Aguayo, C.S. Baker, S. Caballero, P. Clapham, R. Constantine, J. Denkinger, M. Donoghue, L. Flórez-González, J. Greaves, N. Hauser, C. Olavarría,C. Pairoa, H. Peckham and M. Poole. 2000. Movements of humpback whales in Oceania, South Pacific. SC/52/IA6.

Area I summer grounds, presented here, and Colombian wintering grounds presented by Caballero *et al.* (2000)[6] is the most comprehensive evidence to date that genetic differentiation in both seasonal habitats for stocks in the Southern Hemisphere. This confirms an extends the previous analysis of a small sampels from Area IV and V by Pastene & Baker (1997)[13].

Interesting, it was note than eleven AP haplotypes had been observed in others south pacific breeding grounds different from Colombia. This could be the result of historical associations among Southern Ocean populations that are now isolated (Baker *et al.* 1998). Alternatively, it could suggest low levels of contemporary migratory interchange as documented by Discovery marking in Area IV and V (Chittleborough 1965). In the South Pacific, a marked whale in 1952 at Malinoa island, Tonga (21º 02' S, 175º 08' W, stock V) was captured four years later at Bellingshausen sea (68º 01' S, 95º 45' W, stock I; Dawbin 1964).

The International Whaling Commission (1998) have proposed as limits of Area I the meridians 120º and 60º W. However, Rayner (1948) considered the humpbacks surrounding South Shetland islands to be like those from the Pacific. Because samples used here were collected from whales to 58º W, we suggest the eastern limit of Area I should be moved at least 120 miles to the east, in the waters actually defined as Area II.

Obviously, it is needed to compare the genetic information with the recent data of Western South Atlantic wintering ground at Brazilian coast (Engel *et al.* 1999[14]). This could be an alternative wintering ground for humpback whales from the Antarctic Peninsula feeding grounds, although fenotipic data based on fluke patterns do not support this as a primary migratory connection (Muñoz *et al.* 1998[1]).

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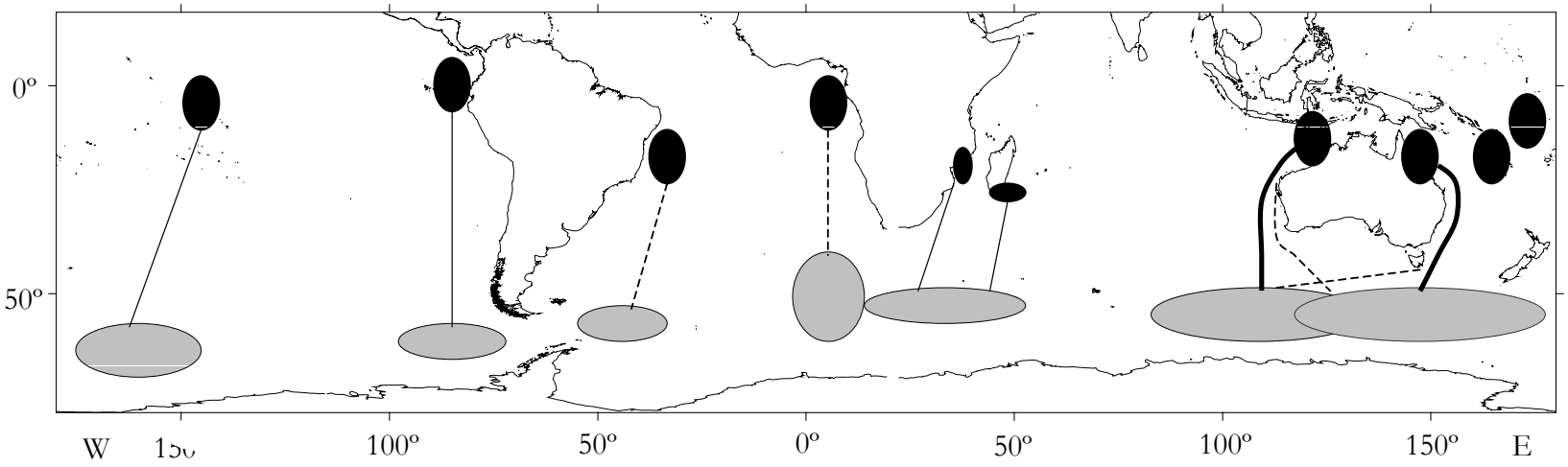

Figure 1. Historical stock divisions at Southern Ocean for humpback whales *Megaptera novaeangliae*. The symbol ⬮ show the breeding grounds and the symbol ⬭ show the feeding grounds. Lines show the putative migratory connecctions, in bold where strong data support this (stocks IV and V) in continue where some data suggest this and in dashed where there is not enough data to support (based on fig 1, Internationational Whalking Commission 1998b)

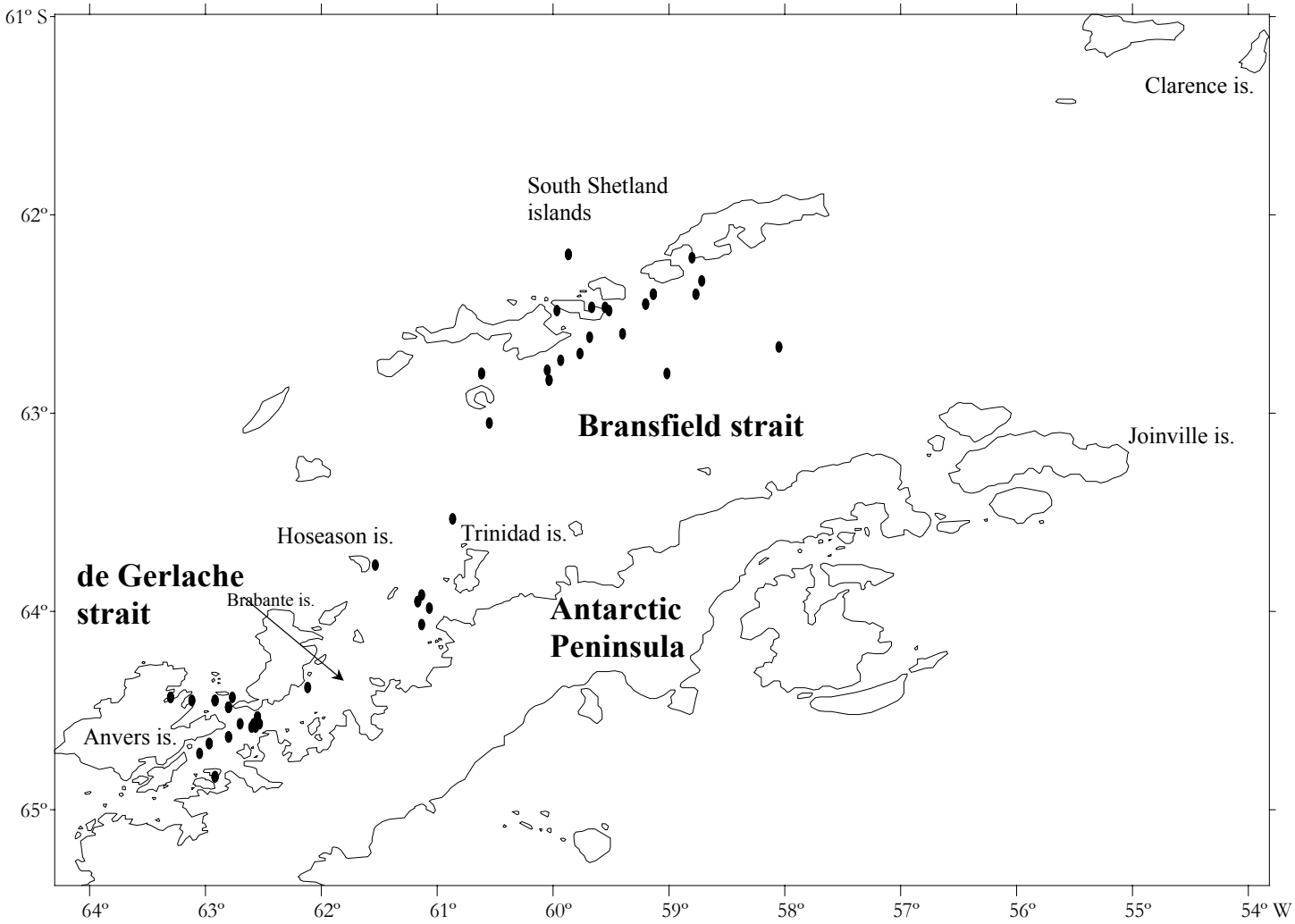

Figure 2. Study area at Antarctic Peninsula showing the location of sampled humpback whales (dots).

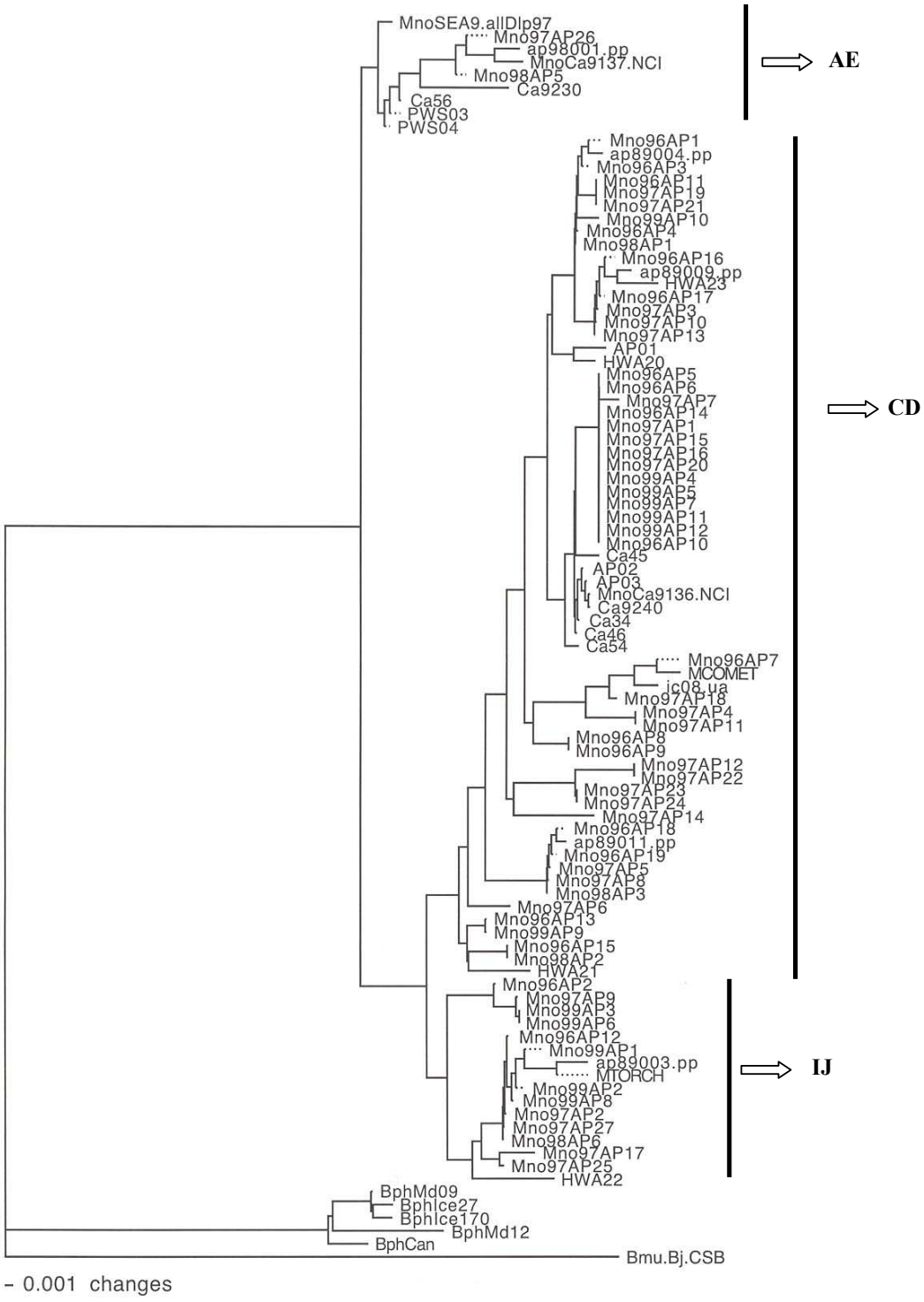

Figure 3. Neigbor-Joining phylogenetic reconstruction

Table 1. Variable sites defining haplotypes of humpback whales from the Antarctic Peninsula feeding ground and the number of individuals sharing these haplotypes in samples from other wintering grounds of the Southern Hemisphere.

[10 20 30 40 50 60 70]			Southern Hemisphere							North Pacific	North Atlantic
[. . . . . . .]			*AP*	*Col*	*Tg*	*NC*	*EA*	*WA*	*BA*		
GI9101	-CG--GCCT-C-AGCTCCACTTTTTTTAATAGTGCCCAATAGGTTCATTTGACTCTCTAGCTGCGACTCTCTC	[68]	13	34						3	
GI9105	-..--...C-.-G.T.T.....A..T.....C.G.C.A.....AC.....C...	[68]	2	3							
GI9116	-..--....-.-..T.....C...CC...C.....C...	[68]	2	2	7	1	1	1			
GI9117	-..--....-.-.A..T.....C...	[68]	4	4		2	3	3	1		
GI9112	-..--....-.-G.T.TT.....T.....T....A....C.....C...	[68]	1	3							
GI9125	-..--....-.-..T.T.....CT.....C.....C...	[68]	2	4							
GI9136	-..--....-.-....T.....A.C....C.....CT..	[68]	2	7	1						
GI9130	-..--....-.-.A..T.....T.....C...	[68]	5	12							
GI9209	-..--...C-.-....TT.....T.....T...A..C.....	[68]	7	10					1		
BM9617	-..--....-.-.A..T.....CT.....C..G.....C...	[68]	5	9		3		3	1		
BM9632	-..-T...C-.-....TT.....T....A..C.....C...	[69]	1	3	3	1		2	1		
BM9634	-..--....-.-..T.T....C.....C....C.....C...	[68]	1	2	1						
BM9614	-..--....-.-G.T.TT.....T.....T....A....C.....C.C...	[68]	3	4							
BM9619	-..--....C.-.T.T.....T.....CT.....C...	[69]	1	2							
BM9604	-..--....-.-....T.....C.....TT.....A.....	[68]	1	2							
BM9537	-..--.T..-.-....T.....T....C..A.C....C.....CT..	[68]	2	3							
BM9502	-..--....-.-..T.....T.....C...	[68]	2	2		2	4		2		
AP9607	-..--....-.-..T.....C.....T.....C....C.....C...	[68]	1								1
AP9611	-..--....-.-.A..T.....T.....C...	[68]	3		3	2					
AP9707	-..--....-.-.....T.....	[68]	1						1		
AP9717	-..-T...C-.-G...TT.....T.....T...A..C.....C...	[69]	1		1						
AP8902	-..--....-.-.....C...	[68]	2		1	2		1		14	
AP9706	-..--....C.-....T.....T.....CT.....C.....	[69]	1								
AP9909	-..--....-.C..T.T.....T.....CT.....C...	[69]	1								
AP9910	-..--....-.-.A..T.....G.....C...	[68]	1								
AP8901	-?.--....-.-.AT.....T.....C...	[68]	1								

Table 2. F_{ST} and Φ_{ST} statistics of AMOVA analysis and probability of greater value by chance (p-value) for pair-wise comparison between Antarctic Peninsula (AP) and the breeding grounds of Colombia (CO, Stock I), Tonga (Tg), New Caledonia (NC) and Eastern Australia (EA, Stock IV), Western Australia (WA, Stock V) and Madagascar (BA, Stock III). Values shown in bold are significantly greater than those founded in 5% of 5,000 random permutations of the data matrix.

AP	***CO***	***Tg***	***NC&EA***	***WA***	***BA***
F_{ST}	-0,002	**0,049**	**0,033**	**0,035**	**0,031**
p-value	0,611 ± 0,008	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,001 ± 0,0004
Nm	Inf	10	15	14	16
Φ_{ST}	-0,00447	0,04562	0,01126	0,05899	0,01605
p-value	0.777 ± 0.006	0.006 ± 0.001	0.097 ± 0.004	0.0 ± 0.0	0.085 ± 0.004
Nm	Inf	11	44	8	31
Differentiation	0,249 ± 0,029	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0	0,0 ± 0,0