Genetic diversity and population structure of humpback whales (*Megaptera novaeangliae*) from Ecuador based on mitochondrial DNA analyses

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ABSTRACT

Information on the genetic characterisation of humpback whales (*Megaptera novaeangliae*) wintering off Ecuador (Breeding Stock G) is presented. Mitochondrial DNA was extracted and sequenced from 230 skin samples collected between 2002 and 2008 to establish the genetic diversity of this population. From 182 usable samples, 41 different haplotypes were found, eight of which were new and unique. Haplotype diversity *(h ± SD)* was estimated to be 0.922 ± 0.012 and the nucleotide diversity *(π ± SD)* 0.019 ± 0.009. A comparison with other areas within the Southeast Pacific (Colombia and Magellan Strait) and the Antarctic Peninsula suggested panmixia within Breeding Stock G, even though significant differentiation was found with Magellan Strait ($p < 0.0001$ in both F_{ST} and Φ_{ST}). An additional analysis with the exact test of population differentiation showed significant differences in haplotype frequencies between breeding areas in Ecuador and southern Colombia ($p < 0.01$), suggesting some level of stratification at breeding grounds as supported by photo-identification studies. The Ecuadorian dataset included haplotypes reported in all three Southern Hemisphere ocean basins indicating recent gene flow within the Southern Hemisphere. The population showed a male-biased sex ratio in adult animals of 2.16:1. Further research and a larger number of samples from breeding areas in the north (Panama and Costa Rica) are required to appropriately assess the extent of structure in this population.

KEYWORDS: HUMPBACK WHALE; GENETICS; BREEDING GROUNDS; SOUTH AMERICA; BREEDING STOCK G

INTRODUCTION

Many baleen whale populations carry out extensive migrations between summer feeding grounds in polar waters and wintering breeding grounds located in temperate and tropical waters (e.g. Mackintosh, 1942). The humpback whale (*Megaptera novaeangliae*) is one of the species in which such a migrating pattern is most evident because of their coastal distribution around continental coasts and oceanic archipelagos where they concentrate for breeding (Dawbin, 1966). For management purposes, whaling areas were traditionally divided by pragmatic boundaries based on whaling records and biological data; thus in the Southern Hemisphere baleen whale populations were assigned by the International Whaling Commission (IWC) to six management areas, I–VI (Donovan, 1991). The Eastern and Southeastern Pacific waters were included in Area I (120°W–60°W). As part of an in-depth assessment of Southern Hemisphere humpback whales, the IWC Scientific Committee has recently designated the Southeast Pacific as Breeding Stock G (IWC, 1998).

The discreteness of the Southeast Pacific humpback whale population was assumed for a long time (Kellogg, 1929; Mackintosh, 1942; Omura, 1953), despite a lack of evidence to support this. Only recently, based on photo-identification (Garrigue *et al.*, 2002; Stevick *et al.*, 2004) and genetic analyses (Caballero *et al.*, 2001; Olavarria *et al.*, 2007), this has been confirmed by comparisons with neighbouring Southern Hemisphere breeding stocks.

Within the Southeast Pacific, humpback whales are distributed during the austral winter along the Northwestern coast of South America, mainly off Colombia and Ecuador, but also further north, off Panama and Costa Rica (Acevedo-Gutiérrez and Smultea, 1995; Kellogg, 1929; Mackintosh, 1942; Townsend, 1935). Photo-identification studies have been used to investigate movements of whales among these wintering areas (e.g. Castro *et al.*, 2008; Félix *et al.*, 2009; Flórez-González *et al.*, 1998). These studies reported photo-identification matches between Ecuador and Colombia, Colombia and Panama, Ecuador and Peru, Colombia and Peru, and Ecuador and Costa Rica, indicating that exchange of individuals among these regions occur, and expanding the range of the wintering grounds of this population within the Southeast Pacific to an overall 3,000km of coastal environment (Félix *et al.*, 2009).

Breeding areas in the Southeast Pacific have been also linked to the feeding areas on the west side of the Antarctic Peninsula and the Magellan Strait in southern Chile (Acevedo *et al.*, 2007; Capella *et al.*, 2008; Castro *et al.*, 2008; Garrigue *et al.*, 2002; Rasmussen *et al.*, 2007; Stevick *et al.*, 2004; Stone *et al.*, 1990) and in a few cases to further east of the Antarctic Peninsula into the Southwestern Atlantic Ocean (Dalla Rosa *et al.*, 2008). Sightings of humpback whales almost all year round off Peru (Ramírez, 1988) and south of Ecuador suggest that not all whales from this stock complete an annual migration. Some animals may remain in between the breeding grounds or the feeding areas in the

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highly productive waters of the Humboldt Current off Peru and Chile, where there are predictable concentrations of food (Papastavrou and Van Waerebeek, 1997). This behaviour is not exclusive to this stock (e.g. Best *et al.*, 1995; Craig and Herman, 2003).

Genetic studies have been conducted in recent years in different locations in the Southeast Pacific, including breeding grounds off mainland Ecuador and the Galapagos Islands (Félix *et al.*, 2007; In press), Gorgona Island and Málaga Bay in Colombia (Caballero *et al.*, 2000; 2001; Olavarria *et al.*, 2007) and feeding areas in the Magellan Strait in southern Chile (Capella *et al.*, 2008; Olavarría *et al.*, 2006) and along the west coast of the Antarctic Peninsula (Olavarria *et al.*, 2000). Such studies based on mitochondrial DNA (mtDNA) sequence analyses have provided an overview of genetic diversity that appears to be the lowest among humpback whale stocks in the Southern Hemisphere (Olavarria *et al.*, 2007). These studies have also shown a lack of genetic differentiation between the Antarctic Peninsula feeding area and the Colombian breeding ground (Caballero *et al.*, 2001; Olavarria *et al.*, 2007; 2000) confirming the links between feeding and breeding areas as revealed previously from photo-identification data (Acevedo *et al.*, 2007; Stevick *et al.*, 2004). Interestingly, the whales inhabiting the Magellan Strait, represent a separate feeding aggregation (Acevedo *et al.*, 2007) which is genetically distinct from the Antarctic feeding area (Olavarría *et al.*, 2006). Despite this information, some knowledge gaps remain, particularly regarding population structure and migration.

This report presents new mtDNA control region analysis on the genetic diversity of humpback whales sampled off Ecuador. It expands previous analyses conducted in this region to include comparisons between neighbouring wintering areas as well as between individuals sampled in Ecuador and feeding areas in Southern Chile and the Antarctic Peninsula. Information from the other Southern Hemisphere areas has enabled a first insight regarding gene flow at a hemispheric scale in this species.

MATERIALS AND METHODS

Sampling

Humpback whale skin samples were obtained between 2002 and 2008 off Ecuador. Four samples were collected from beached animals and 225 from sloughed skin (Amos *et al.*, 1992). One sample was obtained from biopsying with a Barnett crossbow equipped with a 60cm long arrow and modified tip (Lambertson, 1987). This sample was collected in Galapagos Islands, about 1,000km off Ecuador. Sloughed skin samples were obtained during the breeding seasons 2006–2008 (July–October) from onboard whalewatching vessels departing from Salinas, Ecuador (2°10'S, 81°00'W; Fig. 1). Sampling was conducted by a research team as part of a long-term research programme (see Felix and Haase, 2005, for additional references on this study).

When sampling for sloughed skin, boat skippers were asked to approach the site where a whale entered the water after an energetic surface display. Small pieces of skin were scooped from the upper water column with a net with fine mesh (1–2mm). Pieces of skin were stored in 2mL containers

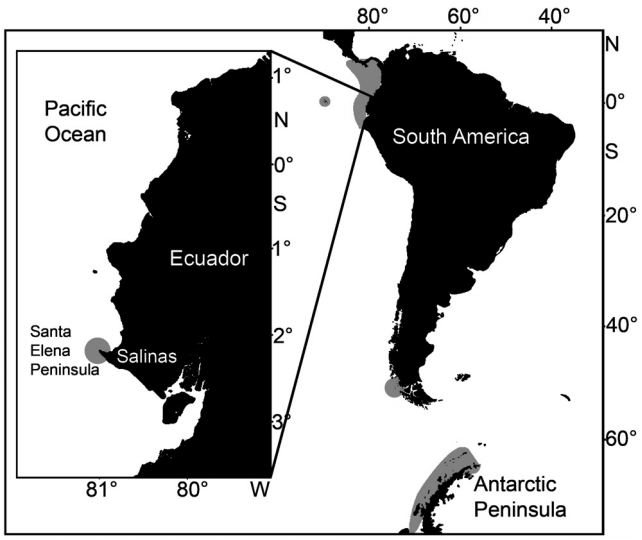

Fig. 1. The study area at Salinas off the Santa Elena Peninsula, Ecuador (left). Distribution range of the Breeding Stock G: feeding grounds at the Antarctic Peninsula and Magellan Strait and breeding grounds in the northwestern coast of South America and Central America (right).

with either a solution of DMSO saturated NaCl or 50–95% ethanol. The net was thoroughly washed with sea water until no pieces of skin were visible on its surface, and then the device was considered ready for the next sampling attempt. Once on shore, samples were stored at 4°C for up to six months prior to laboratory analysis.

Usually only one animal was sampled per group in order to minimise resampling, however, occasionally it was possible to collect two or three samples, presumably from different individuals. When more than one sample was taken from the same group, resampling was assumed if the sex and mtDNA of the samples matched and only one sample was included in the statistical analyses. This criterion was not applied when cow-calf pairs were sampled. Some 'false' duplicated samples could have been left out of the analysis when no genetic fingerprinting was undertaken.

Sampled whales were photographed for individual identification, using the pigmentation pattern on the ventral side of the flukes (Katona *et al.*, 1979). It was possible to photo-identify half of the sampled whales ($n = 83$, 47%). The bias introduced by resampling (between groups) was assumed to be comparable to the within-year resighting rate obtained by photo-identification. This rate was 3.1% in the period 2006–2008, thus we assumed a low rate of resampling. Moreover, when photo-identified individual sampled whales were compared, it was found that only one whale was sampled twice.

Molecular analyses

A fragment of approximate length 500bp of the mitochondrial DNA control region (CR) was amplified via the Polymerase Chain Reaction (PCR; Saiki *et al.*, 1988) using standard reaction conditions (Palumbi, 1996). For the PCR, we used the primer combination t-Pro-whale Dlp1.5 (5'-TCACCCAAAGCTGRARTTCTA-3') and Dlp8 (5'CCATCGWGATGTCTTATTTAAGRGGAA-3') (Baker *et al.*, 1998; Olavarria *et al.*, 2007). The PCR profile was as follows: an initial denaturation at 95°C for 2 minutes, 36

cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute and 30 seconds, and a final extension at 72°C for 5 minutes. Free nucleotides and primers were removed from the PCR products using the PCR Cleaning kit (Invitrogen). PCR products were sequenced in both directions using the standard protocols of Big Dye™ terminator sequencing chemistry on an ABI 3100 automated capillary sequencer (Perkin Elmer), using the same PCR primers.

All sequences were manually edited and aligned using Sequencher 4.1 software (Gene Codes Corporation). Sequences were trimmed to 469bp to match a consensus region analysed previously (Olavarría *et al.*, 2006; 2007). Control region sequences were aligned and compared using MacClade (Maddison and Maddison, 2000) to identify haplotypes. Ecuador haplotypes were compared with haplotypes previously identified in other five humpback whale populations in the South Pacific (Colombia-Antarctic Peninsula, New Caledonia, Tonga, Cook Is. and French Polynesia) and Western Australia (Olavarría *et al.*, 2006; 2007). A search of Genbank was made with those new haplotypes that did not match the South Pacific to define whether they were unique or reported in other populations.

Sex specific markers for gender determination followed the methodology of Gilson *et al.* (1998), which amplify a 224bp fragment of the *SRY* gene located on the Y chromosome. As internal positive control against PCR amplification failure, the homologous ZFY/ZFX region (445bp) was amplified. Thus, in the electrophoresis analysis two bands of 224 and 445bp were present in males and only one of 445bp in females.

Data analyses

Genetic diversity at haplotype (h) and nucleotide (π) levels were computed using the software Arlequin Ver 3.1 (Schneider *et al.*, 2006). Haplotype frequencies (F_{ST}) and nucleotide (Φ_{ST}) composition were compared between Ecuador and Colombia, Antarctic Peninsula and Magellan Strait (Olavarria *et al.*, 2007) using an Analysis of Molecular Variance (AMOVA) (Excoffier, 1995). A comparison based on haplotype frequencies of stratified data from 2006–2008 by sex and year, as well as between sites in the Southeast Pacific, were additionally tested with an exact test of population differentiation which test the non-random distribution of haplotypes into population samples under the hypothesis of panmixia (Raymond and Rousset, 1995). Both AMOVA and exact test were implemented using the Arlequin software.

RESULTS

Genetic diversity

From the 230 samples obtained off Ecuador, 42 were eliminated because they were considered to be duplicates or because they failed sequencing and sexing, leaving 188 samples for subsequent analyses (sequencing, sexing or both). From the successful sequenced samples (n = 182) 41 haplotypes were identified, of which eight were new and unique (GenBank accession numbers HQ241479-86) and one was recorded previously in the Magellan Strait (haplotype Mno03Ma02; C. Olavarría, unpublished data) (Table 1). The remaining 32 haplotypes were previously found either in the Southeast Pacific or in other Southern Hemisphere stocks (see below).The variable sites nucleotides included two insertion/deletions, 42 transitions and 3 transversions. Haplotype diversity ($h \pm SD$) was estimated to be 0.922 ± 0.012 and the nucleotide diversity ($\pi \pm SD$) 0.019 ± 0.009. The mean of pair-wise differences was 8.99 ± 4.16 SD.

Sex composition

The sex identification analyses revealed a significant sex bias towards males of 2.16:1 in adult animals (104 males and 48 females; $\chi^2 = 20.63$, $p < 0.01$). In the case of calves, the sex ratio was also skewed toward males (1.78:1) but the difference was not statistically significantly (16 males and 9 females; $\chi^2 = 2$, $p > 0.05$).

Population structure by sex

A comparison of haplotype composition by sex was made to examine possible variability within the population. For this purpose information from 171 individuals with known haplotype and sex (53 females and 118 males) was used. Through AMOVA tests, haplotype composition of females and males separately (two groups) was compared. Less than half of the total haplotypes in the sampled population were shared by both sexes (n = 20, 48.8%), but the two most common haplotypes (SP32 and SP90) were found in similar proportion in both sexes (Table 1). There were 15 haplotypes found only in males and six identified only in females. Still, no significant differences in haplotype frequency and nucleotide composition between sexes were found ($F_{ST} = –0.001$, $p = 0.33$ and $\Phi_{ST} = 0.0075$, $p = 0.347$).

When comparison included sex and year (six groups) significant differences in haplotype frequency and nucleotide composition were found between females in 2006 and females in 2007 ($p < 0.01$ in both cases), as well as between females in 2006 and males in the three years in haplotype frequency ($p < 0.05$ in all cases) and between females in 2006 with males in 2007 and 2008 at nucleotide composition ($p < 0.05$ in both cases) (Table 2). Similar results were obtained with the exact test of population differentiation (using 100,000 Markov chain steps); a highly significant difference in haplotype frequency between females in 2006 and females in 2007 ($p = 0.004$) and a significant difference between females in 2006 and males in all years ($p < 0.05$ in all cases) was found.

Comparisons with other areas of Breeding Stock G

Ecuadorian haplotype frequencies were compared with other locations in the Southeast Pacific including breeding (Colombia) and feeding areas (Magellan Strait and the Antarctic Peninsula), as reported by Olavarría *et al.* (2006; 2007). The frequency of the two most common haplotypes reported in Ecuador (SP90 and SP32) was similar in Colombia and the Antarctic Peninsula. The former haplotype occurred also in the Magellan Strait with much higher incidence (80.77%) but the second was absent, as were most of the haplotypes found in the Southeast Pacific and Antarctic whales. The haplotype found in Galapagos (SP61) was recorded six times off mainland Ecuador, once in Colombia and twice in the Antarctic Peninsula. Overall,

Table 1

Ecuador humpback whale haplotype diversity and frequency of mtDNA control region sequences and proportion of haplotypes by sex and year (period 2006–08, $n = 171$). Haplotype nomenclature follows Olavarría *et al.* (2006, 2007), Engel *et al*. (2008) and Rosenbaum *et al*. (2009).

Haplotype (466 pb)	Females					Males					Overall	
	2006	2007	2008	Total	%	2006	2007	2008	Total	%	*n*	%
SP1	–	1	1	2	3.8	4	1	5	10	8.5	12	7.0
SP8	–	–	–	–	–	–	–	3	3	2.5	3	1.8
SP10	–	–	–	–	–	–	1	3	4	3.4	4	2.3
SP14	–	–	1	1	1.9	1	–	–	1	0.8	2	1.2
SP16	1	–	–	1	1.9	–	–	–	–	–	1	0.6
SP19	–	–	–	–	–	–	3	–	3	2.5	3	1.8
SP25	1	–	1	2	3.8	2	2	3	7	5.9	9	5.3
SP26	1	–	2	3	5.7	–	–	–	–	–	3	1.8
SP32	–	2	3	5	9.4	3	3	4	10	8.5	15	8.8
SP33	1	–	1	2	3.8	2	–	1	3	2.5	5	2.9
SP35	–	–	–	–	–	–	–	1	1	0.8	1	0.6
SP41	–	1	–	1	1.9	–	–	–	–	–	1	0.6
SP42	1	–	–	1	1.9	1	–	–	1	0.8	2	1.2
SP43	–	–	–	–	–	–	–	1	1	0.8	1	0.6
SP50	–	1	1	2	3.8	2	–	–	2	1.7	4	2.3
SP52	–	–	–	–	–	1	–	1	2	1.7	2	1.2
SP54	–	–	1	1	1.9	–	–	1	1	0.8	2	1.2
SP60	1	–	1	2	3.8	3	1	2	6	5.1	8	4.7
SP61	1	–	2	3	5.7	1	–	1	2	1.7	5	2.9
SP62	–	–	2	2	3.8	2	1	3	6	5.1	8	4.7
SP63	–	–	1	1	1.9	–	–	2	2	1.7	3	1.8
SP68	–	2	–	2	3.8	–	1	–	1	0.8	3	1.8
SP70	–	–	–	–	–	–	–	2	2	1.7	2	1.2
SP72	–	–	–	–	–	–	1	–	1	0.8	1	0.0
SP73	1	–	–	1	1.9	–	1	2	3	2.5	4	2.3
SP90	–	4	5	9	17.0	9	8	10	27	22.9	36	21.1
SP98	–	3	1	4	7.5	1	3	2	6	5.1	10	5.8
SP100	–	–	1	1	1.9	–	1	–	1	0.8	2	1.2
SP101	–	–	–	–	–	–	1	–	1	0.8	1	0.6
Mno03Ma02	–	–	2	2	3.8	–	2	–	2	1.7	4	2.3
EC001	–	–	–	–	–	1	–	–	1	0.8	1	0.6
EC002	–	–	–	–	–	1	–	–	1	0.8	1	0.6
EC003	1	–	–	1	1.9	–	–	–	–	–	1	0.6
EC004	1	–	–	1	1.9	–	–	–	–	–	1	0.6
EC005	–	1	–	1	1.9	–	–	2	2	1.7	3	1.8
EC006	–	–	–	–	–	–	–	1	1	0.8	1	0.6
EC007	–	–	–	–	–	–	–	1	1	0.8	1	0.6
EC008	–	–	1	1	1.9	–	–	1	1	0.8	2	1.2
HBA040	–	–	1	1	1.9	–	–	–	–	–	1	0.6
HBA112/BRA15–97	–	–	–	–	–	–	–	1	1	0.8	1	0.6
HBR002/BRA03–98	–	–	–	–	–	–	–	1	1	0.8	1	0.6
Total	**10**	**15**	**28**	**53**	**100**	**34**	**30**	**54**	**118**	**100**	**171**	**100**

Ecuador humpback whales shared 21 haplotypes of 27 previously reported from Colombia (78%), 17 of 25 from Antarctic Peninsula (68%) and four from Magellan Strait (100%). There were six haplotypes shared within the breeding Stock G that have not been found in other Southern Hemisphere stocks (SP32, SP60, SP61, SP90, SP98 and SP101).

A pair-wise AMOVA between Ecuador and the other Stock G locations calculated a between variance of 5.45% and a within variance of 94.55%. The high proportion of the within variance indicates a high genetic similarity between the compared sites, as expected for a panmictic population. A significant difference was found between Ecuadorian and Magellan Strait whales in both haplotype frequency and nucleotide composition ($p < 0.0001$ in both cases) (Table 3). The exact test of population differentiation (using 30,000 Markov chainsteps) confirmed a highly significant difference between Ecuador and Magellan Strait, but also revealed a highly significant difference in haplotype frequency between the two breeding areas, Ecuador and Colombia ($p = 0.00171 \pm 0.0016$), indicating some level of structure at these breeding grounds.

Comparisons with other Southern Hemisphere stocks

When the Ecuador haplotype dataset was compared with other Southern Hemisphere humpback whale stocks, 26 haplotypes matched. There were 20 haplotypes shared with South/Southwest Pacific stocks, three with the Southeast Indian Oceanstock (SP16, SP35 and SP70) (Olavarria *et al.*, 2007) and three with Southwest Indian/Southeast Atlantic stocks (HBA040, HBA112/BRA15/97 and HBR002/BRA03-98), two of the later had been first recorded also in the Southwest Atlantic (Engel *et al.*, 2008; Rosenbaum *et al.*, 2009), indicating some level of genetic interchange across the entire Southern Hemisphere.

DISCUSSION

From our analyses of humpback whales sampled in Ecuador some interesting aspects on population structure of Breeding Stock G were revealed. In terms of genetic variability,

Table 2

Pair-wise test of differentiation for mtDNA control region sequence by sex and year based on the F_{ST} and Φ_{ST} indices (values are below and above the diagonal, respectively). F = females, M = males, period 2006–08. The significance was analysed using 5,000 non-parametric permutations of the data matrix. Significant *p*-values are highlighted in bold.

	F2006		F2007		F2008		M2006		M2007		M2008	
	Value	*p*-value	Value	*p*-value	Value	*p*-value	Value	*p*-value	Value	*p*-value	Value	*p*-value
F2006	–	–	1.622	**0.009**	0.175	0.238	0.675	0.054	0.862	**0.034**	0.603	**0.049**
F2007	0.17	**0.008**	–	–	0.22	0.157	–0.033	0.45	0.017	0.356	0.164	0.174
F2008	0.016	0.245	0.02	0.187	–	–	–0.095	0.677	–0.074	0.581	–0.16	0.953
M2006	0.076	**0.045**	–0.007	0.502	–0.010	0.662	–	–	–0.099	0.698	–0.051	0.607
M2007	0.09	**0.03**	–0.001	0.397	–0.007	0.577	–0.012	0.697	–	–	0.003	0.376
M2008	0.061	**0.043**	0.012	0.227	–0.016	0.943	0.006	0.629	–0.0001	0.392	–	–

Ecuadorian whales showed a slightly higher diversity than whales sampled in other known breeding and feeding areas in the Southeast Pacific and the Antarctic Peninsula (see Olavarría *et al.*, 2006; 2007). Although high, the diversity of this stock is one of the lowest in the Southern Hemisphere, perhaps as a result of whaling activities during the 19th and 20th centuries and/or a low gene flow with other Southern Hemisphere stocks.

The general results at regional level, as revealed by the AMOVA analysis, suggest panmixia in the Breeding Stock G. Most of the haplotypes in Ecuadorian whales were also found in other sites of the Southeast Pacific and the east of Antarctic Peninsula, the main feeding area of this stock. The proportion of the two most common shared haplotypes (SP32 and SP90) was similar between Ecuador, Colombia and the Antarctic Peninsula. However, the exact test of population differentiation revealed a significant difference in haplotype frequency between two adjacent breeding areas, Ecuador and Colombia, despite the fact that they share 78% of haplotypes. This unexpected result contradicts the F_{ST} analysis in favour of stratification at the breeding grounds. Nevertheless, our sample contains many haplotypes with low frequencies which may have reduced the degree of certainty of the exact test as it does not take into account genetic distances between haplotypes but frequencies.

A plausible explanation for the heterogeneity between adjacent breeding grounds off western South America could be related to variability in whales' migrating behaviour. When the Ecuadorian population was modelled using photo-identification data with open population models with a large sample (n = 1,511) similar inconsistencies were found, probably because sampling in the study area favoured less transient individuals (Félix *et al.*, 2011). It has been demonstrated that females tend to have a higher level of fidelity than males in both breeding and feeding grounds (Rizzo and Schulte, 2009; Rosenbaum *et al.*, 2009; Weinrich *et al.*, 2006). Therefore, if heterogeneity was introduced in our sampling process due to differences in site fidelity by sex, most probably it occurred with females, as males clearly showed absence of stratification in our dataset. In addition, differences in migratory patterns of both sexes were found in Hawaii, with males undertaking the winter migration more often than females (Craig and Herman, 2003). This may introduce another source of heterogeneity, particularly in studies with few years of data like ours. Our analysis when the dataset was broken down by sex and year, despite showing a higher level of stratification in females than in males, is not very useful at elucidating the topic because some female strata had small sample sizes and therefore results are difficult to consider as conclusive. However, genetic differentiation by sex in migrating western South Pacific whales suggest a more complex migratory pattern than previously considered in this species and highlight the necessity to conduct comparisons disaggregating data by sex (Valsecchi *et al.*, 2010).

Table 3

Pair-wise test of differentiation for mtDNA control region sequence between whales sampled in Ecuador and in other sites of distribution of the Breeding Stock G based on the F_{ST} and Φ_{ST} indices. The significance was analysed using 5,040 non-parametric permutations of the data matrix.

	Colombia (n = 148)	Magellan Strait (n = 52)	Antarctic Peninsula (n = 89)
F_{ST}	–0.0006	0.1761	0.00263
p-value	0.475	**<0.0001**	0.2221
Φ_{ST}	–0.0055	1.7400	0.0251
p-value	0.468	**<0.0001**	0.2240

Despite the significant differences at haplotype and nucleotide levels between Ecuador and Magellan Strait whales, all four haplotypes found in this small feeding area were also present in Ecuadorian samples. It is not clear whether those whales breed off Ecuador or just passed through our study area in their way to breeding areas located further north. But photo-identification studies on Magellan Strait whales showed a correspondence 10 times higher (but not significantly different) with breeding areas in Panama/Costa Rica than with Ecuador (Acevedo *et al.*, 2007), suggesting, again, some level of stratification at breeding grounds. In another study with a larger sample from Colombia, Capella *et al.* (2008) found a similar level of interchange between Magellan Strait and Colombia (0.093, n = 1,042) as the one reported by Acevedo *et al.* (2007) between Magellan Strait and Ecuador (0.09, n = 927). Even though the distinctiveness of the Magellan Strait from the Antarctic Peninsula as two different feeding areas of the Breeding Stock G had been demonstrated previously (Acevedo *et al.*, 2007; Olavarría *et al.*, 2006) regular gene flow between whales belonging to both feeding areas is expected to occur during the breeding season.

Shared haplotypes with distant populations such as the Indian and South Atlantic Oceans in the Ecuadorian sample included both sexes, demonstrating possible recent gene flow through the three southern ocean basins. While those matches could also be the result of common ancestral lineages, additional information is available on extensive

movement across humpback whale stocks in the Southern Hemisphere (Chittleborough, 1965; Pomilla and Rosenbaum, 2005; Robbins *et al.*, 2008; Rosenbaum *et al.*, 2009; SPWRC *et al.*, 2006; Steel *et al.*, 2008). Further collaboration between research groups working on this species in the Southern Hemisphere will provide a better understanding of the level of present days gene flow in this species at a hemispheric scale.

The sex bias found in this study with males outnumbering females (2.16:1) is similar to that reported in other studies carried out at breeding areas (2.4:1 in Eastern Australia, Brown *et al.*, 1995; 1.86:1 in Hawaii, Craig and Herman, 2003; 1.95:1 in the South Pacific, Olavarria *et al.*, 2007; 1.7:1 in the North Atlantic, Palsbøll *et al.*, 1997; 1.9:1 in Gabon and 2.4:1 in Madagascar, Pomilla and Rosenbaum, 2006). This difference is therefore unlikely to have been due to a variation in surfacing behaviour between the sexes. It has been postulated that the sex bias observed at breeding grounds could be related to migration behaviour (see Craig and Herman, 2003; Dawbin, 1966) given that such a difference does not occur at feeding grounds (Clapham *et al.*, 1995), neither in the unique non-migrant population of the Arabian Sea (Mikhalev, 1997). The results of our analysis by sex and haplotype composition and the absence of significant differences regarding sex proportions in calves, support the belief of differences in the migrating behaviour of adult animals in this species as a valid explanation for the skewed proportion toward males found at breeding grounds.

In summary, genetics studies confirm connections of whales belonging to the Breeding Stock G among Ecuador, Colombia, Magellan Strait and the Antarctic Peninsula, but also suggest some heterogeneity in the breeding assemblage. The current available information suggests that differences in migrating behaviour between sexes with females showing higher level of site fidelity than males would be the cause of heterogeneity in breeding individuals. If stratification at breeding grounds occurs in this population it seems to be weak, at least in the case of better sampled areas in south of Ecuador and south of Colombia (some 700km apart); still a large part of the breeding area remains under poorly surveyed. Molecular studies are required to be conducted in the northernmost part of the wintering distribution of the Breeding Stock G (Panama and Costa Rica) to appropriately assess the level of population structure.

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