Mitochondrial DNA variability and evaluation of the likely feeding grounds of the humpback whale (*Megaptera novaeangliae*) population of the Abrolhos bank, Bahia, Brazil

Running title: mtDNA diversity in Brazilian humpback whales

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Abstract

In the Southwestern Atlantic Ocean, humpback whales migrate every winter to the Abrolhos bank, on the Brazilian coast, for breeding and calving. This “stock” represents the remnants of a larger population heavily hunted during the beginning of the 20th Century. Despite its relevance to conservation efforts, the amount of genetic variation and the Antarctic feeding areas for this population are still largely unknown. In order to examine these questions, we sequenced 450 bp of the mitochondrial DNA control region from samples taken from individuals at the Abrolhos bank (n=176) and near the Antarctic Peninsula (n=77). A total of 61, 17 and 13 haplotypes were determined in Brazilian, Antarctic Area I and II populations, respectively. The proportion of shared haplotypes and the genetic distance showed a greater similarity between the two Antarctic areas than between any of these feeding areas and Brazil. These results indicate that the humpback whale populations from these portions of Antarctic Areas I and II seem to have no clear genetic differentiation and, therefore, that the boundaries between Areas I and II as currently defined by the International Whaling Commission should be considered with caution in a biological sense. We suggest that the feeding area of the Brazilian humpback whale population be located in the eastern part of Area II, near South Georgia/South Sandwich/Scotia Sea area.

Introduction

In the Southern Hemisphere humpback whales (*Megaptera novaeangliae*) usually migrate from summer feeding grounds in the Antarctic to mating and calving grounds in tropical and subtropical regions (*e.g.* Mackintosh 1945; Chittleborough 1965; Dawbin 1966). The International Whaling Commission (IWC) currently recognizes seven humpback whale breeding populations in the Southern Hemisphere (IWC 2001), with corresponding feeding grounds in high-latitude Antarctic waters (Dawbin 1966; Clapham and Mead 1999). The longitudinal boundaries of the feeding areas have led the IWC to establish political units for commercial whaling in the region (Tonnessen and Johnsen 1982), and since 1957 the limits of six feeding grounds in Antarctic waters, known as Areas I to VI, were adopted by this organization, which in the 1974/75 season included them in its "official" schedule.

Humpback whales wintering off the Brazilian coast are considered as part of the Southwestern Atlantic breeding stock "A" (e.g. see summary in IWC 2004). Previous studies (Engel 1996; Siciliano 1997; Martins et al. 2001; Freitas et al. 2004) suggesting the Abrolhos Bank (16°40´- 19°30´S and 37°25´- 39°45´W) in Brazil as the main mating and calving ground of this species in the Southwestern Atlantic Ocean were corroborated by aerial surveys between 12° 10'S and 20° 42'S (Andriolo *et al.*2006) along the coastal waters of the states of Bahia and Espírito Santo. However, the corresponding feeding ground in the Antarctic region, Areas I or II (located respectively between the meridians 120°W to 60°W and 60°W to 0°; Donovan 1991), has not yet been clearly established. Comparisons based in the photo-identification catalogs (e.g. Stevick et al. 2004) did not result in any match between Abrolhos and Antarctic Area I. On the other hand, photo-identification studies and mitochondrial DNA (mtDNA) analyses (Caballero et al. 2001; Olavarría et al. 2000) have demonstrated an evident link between the humpback populations that breed along the Pacific coasts of Colombia and Ecuador and Area I, in the western part of the Antarctic Peninsula. Studies have

been much less extensive in Antarctic Area II in the Southwestern Atlantic Ocean, another likely feeding ground of the Brazilian population. Despite this lack of information, a recent comparison with Shag Rocks, South Georgia, revealed the first photo-identification match of this population in a feeding ground (Stevick *et al.* 2005). Another recent study demonstrated that two humpback whales tagged with satellite transmitters off the Brazilian coast migrated to areas close to South Georgia and Sandwich Islands (Zerbini et al., 2006). This was recently supported with additional photographic matches from Abrolhos Bank to South Sandwich (Marcovaldi, et al., 2006)

Commercial exploitation has brought this species to the brink of extinction in many areas of the world. More than 200,000 humpback whales were hunted in the Southern Hemisphere in the past century (Clapham 2002), including several thousands in the feeding area of the stock A (Tonnessen and Johnsen 1982). Although the worldwide protection of humpback whales from hunting was established in 1966 (Rice 1978), the impact of such population reduction over the current levels of genetic variation is still unknown for the Brazilian "stock", despite the importance of genetic diversity parameters for understanding the historical demography of the species, such as its long-term effective population size (Baker and Clapham 2004). In this study we used mitochondrial DNA control-region sequencing to investigate the genetic diversity and the putative association between Brazilian and Antarctic (areas I and II) humpback whales to clarify the location of the feeding ground for the Brazilian population, and to improve current conservation policies for this species.

Materials and methods

Sampling, and mtDNA sequencing

Skin samples of humpback whales, mostly from the Abrolhos Bank, were taken periodically every week during the breeding seasons (July to November),

from 1997 through 2001 with the exception of the 2000 season, when wild animal sampling was temporarily suspended in Brazil. A few samples resulted from individuals stranded in Bahia and Espírito Santo or from other locations on the Brazilian coast (Figure 1a). Adult animals were sampled randomly among social groups. With few exceptions, it was not possible to completely avoid resampling the same animal. For each whale sampled, date, GPS coordinates, and group composition were recorded. For the sampling of free-ranging whales, a Barnett Wildcat XL crossbow was used with stainless steel biopsy darts (8mm diameter, 15mm length sampling tip). Samples were kept in 70% ethanol or DMSO, according to the protocol established by Baker et al. (1998).

Additional skin samples were obtained in the Gerlache and Bransfield Straits and in the Weddell Sea, near the Antarctic Peninsula, using similar sampling procedures as described above (Figure 1b). These samples were obtained during the expeditions by the Brazilian Antarctic Program (PROANTAR) in the austral summers of the years 1999 and 2000 (see Secchi et al. 2001).

Genomic DNA extraction was carried out following protocols modified from Baker et al. (1993a) and Palsboll et al. (1995), with tissue digestion in 1.0% SDS, 150 mM sodium chloride, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 100 μg /ml^{-1} Proteinase K at 65^0 C for a minimum of three hours, followed by phenol/chloroform extraction and ethanol precipitation. Approximately 450 nucleotides from the most variable portion of the mtDNA control region were amplified using primers Dlp-1.5 and Dlp-5 with PCR profile described in Baker et al. (1993a). Amplification conditions were as follow: approximately 100 ng of genomic DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 units of Taq DNA polymerase, 0.2 μM of each primer, and 0.2 mM dNTPs in 25 μl of reaction volume. The amplified material was purified with shrimp alkaline phosphatase and exonuclease I, and sequenced with the chain terminators method (ET terminator kit – GE Healthcare) in MegaBACE 1000 (GE Healthcare). The sequences were checked by visual inspection of the resulting chromatogram in Chromas v.2.0

(www.technelysium.com.au). The sequences have been deposited in GenBank under accession numbers AY329844-AY330096.

Statistical methods

Sequence alignment was done using ClustalX program (Thompson et al. 1994), and corrected by hand in the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit. html). Nucleotide and haplotype diversity and their standard errors, as well as Tajima's and Fu and Li's neutrality tests for each population were calculated in the DnaSP v.3.51 program (Rozas and Rozas 1999). The genetic structure was assessed using AMOVA and pairwise Φ_{st} (Excoffier et al. 1992), as implemented in the ARLEQUIN 2.0 software (Schneider et al. 2000). For this analysis, in addition to considering samples from Brazil, Antarctic area I, and Antarctic area II as three independent populations, we also studied the effect of grouping any two populations, such that all three possible pairs were tested. AMOVA was performed using Kimura's K2P distance among haplotypes (Φ_{st}) and 2000 replications. This program was also used to estimate pairwise Φ_{st} values whose significance was tested using 1000 permutations. Φ_{st} was used instead of traditional F_{st} because of the modest haplotype sharing between populations.

Results

Variability of mtDNA control region sequences

A consensus segment of about 400 bp of the mtDNA control region was assembled from 176 samples from Brazil, 46 from Antarctic Area I and 31 from Antarctic Area II. For the Brazilian sample, 57 polymorphic sites were identified defining 61 haplotypes. For the Antarctic samples, 24 and 21 segregation sites were detected defining 17 and 13 haplotypes for Areas I and II, respectively (Table 1). None of the neutrality tests performed resulted in significant deviations from neutral expectations for any of the populations studied (data not shown),

suggesting that the current diversity results mainly from the demographic history of these populations and not from adaptive evolution of the locus.

Nucleotide and haplotype diversities for each one of these areas was compared to that reported for other breeding and feeding grounds within three ocean basins (North Atlantic, North Pacific and Southern Hemisphere; Table 1). The Brazilian haplotype diversity (H=0.971) was similar to that found in the majority of the breeding grounds including the other Atlantic breeding areas (Angola, Gabon and Western South Africa), but was statistically higher than in Malaga Bay and in the Antarctic Area I sample analyzed in the present study. The very similar values for Antarctic Areas I and II (H=0.913, and H=0.912, respectively), albeit lower than the average for feeding grounds, were not statistically different from most other populations. The nucleotide diversity value found for the Brazilian population (□=0.020) was not statistically different from any other population sampled at the breeding grounds. On the other hand, the value for Antarctic Area II population (□=0.017) was statistically lower than any other Antarctic Area except Area I, whose value (□=0.018) was lower than that found in Antarctic Areas IIIE, IV and V.

Populational Comparisons

The global frequency distribution of haplotypes among the three populations was significantly different (Table 2, □□=21.934; p=0.001). There is an excess of the BR clade in Brazil, and of the AE clade in the Antarctic Area II, and a lack of the AE clade in Brazil. An adjusted pairwise comparison further reveals that while the Brazilian and Antarctic Area II populations remain statistically different (□□=17.581; p=0.001), neither differs from the Antarctic Area I population (□□=5.735; p<0.250, and □□=5.118; p<0.327, respectively).

The proportion of haplotypes that were private differed greatly among the three populations (Table 3). While in the Brazilian sample 88.5% of the observed haplotypes were restricted to that population, the proportion of exclusive

haplotypes in the Antarctic samples was only 29.4% in Area I and 15.4% in Area II. The analysis of shared haplotypes was also suggestive of a higher distinctiveness of the Brazilian population, since it revealed ten common haplotypes between Antarctic Areas I and II, but only six between Brazil and Area I, and five between Brazil and Area II, despite the much higher sample size of the Brazilian population. Four haplotypes were shared among all populations.

The AMOVA showed that when each population is considered an independent unit, 98.6% of the mtDNA variability was found within the populations (Table 4). Comparing the Brazilian population against the pooled Antarctic populations resulted in the highest among groups variation (2.2%), higher than that found for any other alternative grouping. However, the Φ_{st} was statistically significant in all comparisons (Table 4). The greater similarity between the two Antarctic grounds is also corroborated by the pairwise genetic distances, whose value between the samples for the two Antarctic areas (0.018) was, though not statistically significant, lower than that found between Brazil and any of the two Antarctic feeding Areas (0.020). This is further supported by the pairwise Φ_{st} matrix that indicates non-significant values between Antarctic Areas I and II, even though the Φ_{st} value between Brazil and Antarctic Area II was also non-significant (Table 5).

Discussion

The high mitochondrial DNA diversity (nucleotide and haplotype) observed in the Brazilian sample is in agreement with other breeding areas studied in the Southern Hemisphere and in the North Atlantic Ocean (Baker *et al.* 1993b, 1998; Rosenbaum *et al*. 1998; 2000; 2001). Despite the severe depletion of this stock due to commercial whaling (Paiva and Grangeiro 1965; 1970), its maternal genetic diversity shows no sign of a strong reduction. A likely explanation for the maintenance of such high levels of diversity in humpback whale mtDNA in general is that the major harvests were both short-lived relative to the generation time

estimated for this species (12-24 years, Roman and Palumbi 2003) and never reached too small absolute population sizes. The decay of the genetic variability in a population that was reduced in size is determined directly by interplay between the duration of the reduction in generations and the minimum absolute population size attained (Nei et al. 1975). For the Brazilian population, for example, it is estimated that the duration of the most extensive whaling period was about 46 years (Paiva and Grangeiro 1965; 1970), corresponding to only between two and four humpback whale generations. An alternative hypothesis would be that a genetic bottleneck did occurred during the harvest period, but the presently high mtDNA diversity in all Southern Hemisphere humpback populations may have been caused by recent gene flow, since a low but constant gene flow among these areas may exist (Pomilla et al. 2004). However, that estimated levels of present gene flow would be insufficient for the recovering of high diversity levels after a putative bottleneck in such a short period. Moreover, if we postulated a higher gene flow, the sharing of mtDNA haplotypes from breeding stock A with other areas would be extensive, which seems not to be the case (e.g. Pacheco de Godoy et al. 2004; Rosenbaum et al. 2004). Therefore, the most likely explanation for the high mtDNA variability presently shown in the Abrolhos Bank breeding population is that it has just maintained its historically high diversity through the whaling period. Nonetheless, gene flow has certainly played an important role in the remote past (before whaling), as it helps to explain both the long term high genetic diversity of most humpback whale populations studied in the Southern Hemisphere (Rosenbaum et al. 2004) as well as the low geographical structuring of the clades.

It is less clear why the nucleotide diversity found in both Antarctic Areas I and II were lower when compared to that found in other Antarctic areas, as the geographic range of the sampling seems to be similar to the other studies (Fig. 1B). Interestingly, one of the breeding populations sampled in the Pacific South American coast (Malaga Bay, Colombia) has both haplotype and nucleotide diversities lower than other breeding grounds (Table 1). The known migratory

connection between the Colombian population and the Antarctic Peninsula (Area I and west of Area II) (Caballero et al. 2001; Stevick et al. 2004) could explain the lower genetic diversity in these feeding grounds. However, this conjecture must be taken with caution, firstly because another Pacific South American population (Gorgona Is., Colombia) does not seem to have lower than average diversity values, and, secondly, because these estimates for the Malaga Bay population, despite lower than average, are not statistically different from those obtained for other breeding populations.

Concerning the relationship among the Brazilian breeding ground and Antarctica feeding areas, the AMOVA analyses indicate a lower differentiation between Antarctic Areas I and II when both are compared with the Brazilian population. The results of population pairwise F_{STs} also corroborate the AMOVA results, with a lower genetic difference between Antarctic Areas I and II than between Brazil and any of these feeding grounds. More significantly, the proportion of shared haplotypes between Brazil and both Antarctic Areas and between Brazil and each Antarctic Area separately (Table 2) may be considered very low when compared to the proportion between the Colombian breeding area and Antarctic Area I, whose migratory connection is well established also by photo-identification studies (Stevick et al. 2004). Of a total of 37 haplotypes found in these two areas, 17 were common to both (Caballero et al. 2001).

The Antarctic Areas I and II sampled here shown a very high similarity. Despite the lower sample sizes, both Antarctic Areas showed the highest number of shared haplotypes, while a high percentage of exclusive haplotypes (88.5%) occurred in the Brazilian population. Furthermore, in analyses such as AMOVA and the pairwise genetic distances, both Antarctic Areas showed the highest affinity, though these differences were not always statistically significant. There has been some discussion in the literature concerning the limits between feeding Areas I and II for the humpback whales. Recently, Olavarria et al. (2000) recommended changing the boundary between Antarctic areas I and II from 60ºW to at least 58ºW based on a comparison between samples from the Colombian breeding grounds

and the Antarctic Peninsula. They found that the sharing of haplotypes between the Colombian samples occurred all over the Antarctic Peninsula, independently of the longitude where the samples where taken. As in our results the populations that forages in Area I and in western Area II are genetically undistinguishable, we agree with Olavarria et al. (2000) that the 60ºW does not seem to mark any population boundary for the humpback whales. In this way, the delimitation of different feeding Areas within the Antarctic Peninsula seems not appropriate for management purposes.

In summary, all our results are very robust in pointing to the greater distinctiveness of the Brazilian population in comparison with the Antarctic Peninsula samples and indicate that Area I and the western portion of Area II, close to the Antarctic Peninsula (Figure 1), do not constitute the main feeding ground of the Brazilian humpback whales. Although the haplotypes from Colombia and the Antarctic Peninsula from Olavarria et al. (2000) and Caballero et al. (2001) are not available for direct comparison, the sampled Antarctic Peninsula area from their studies widely overlaps ours. The strong genetic and photo-identification connections between the Colombian breeding ground and the Antarctic Peninsula feeding ground for one hand and for the other the weak genetic connection and the lack of photo-identification matches (Stevick et al. 2004) between the Antarctic Peninsula and the Brazilian populations support the hypothesis that the Antarctic Peninsula is the feeding ground for the Pacific Colombian breeding population but not for the Atlantic Brazilian population. The feeding ground for the Brazilian population should therefore be located elsewhere, most likely in the South Georgia/South Sandwich/Scotia Sea area, as indicated by recent photo-identification reports in these areas (Stevick et al. 2005) and a study using radio telemetry (Zerbini et al. 2004). The use of molecular markers, including nuclear microsatellite loci,,in humpback whale samples collected around the South Georgia/South Sandwich Islands, as well as in the Scotia Sea, would be of greatest importance to access the genetic structure in these areas and to support the hypothesis that they correspond to the humpback whale Brazilian breeding

population. Of great importance would be to compare populations from all or most South Hemisphere breeding and feeding grounds using both uniparental and biparental molecular markers to get a more complete picture of the evolutionary history of these populations.

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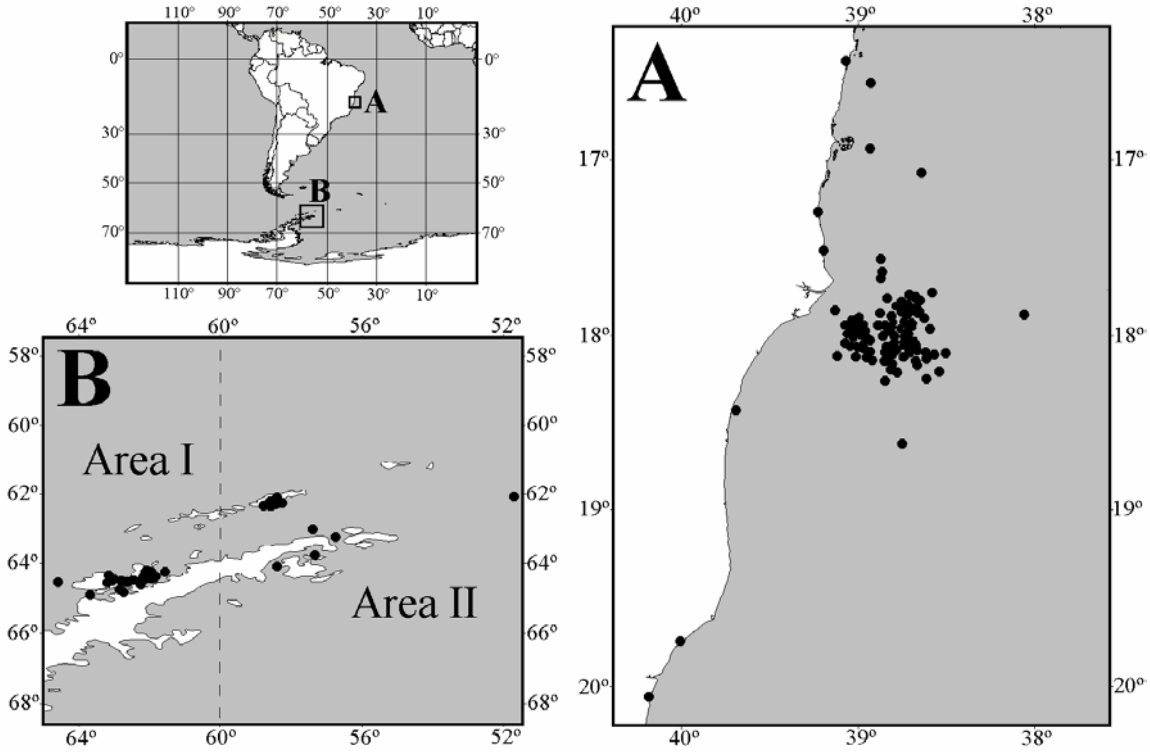

Figure 1. Map of the studied populations. A. Detail of the Abrolhos bank in the Brazilian Coast with the location of the sampled specimens. Samples resulting from strandings are represented over the coast line. B. Detail of the Gerlache and Brasfield Straits in the Antarctic Peninsula with the location of the sampled specimens. Due to the scale of the map, a sample obtained at 38.2ºW, 62.5ºS is not represented. The boundary between Areas I and II, located at the 60ºW meridian is represented as an interrupted line.

Table 1. Summary mtDNA diversity statistics from humpback whales populations samples worldwide, with emphasis on the Southern Hemisphere. n, sample size; h, number of haplotypes; L, sequence length; S, number of singletons; H(SE), haplotype diversity and standard error; and □. nucleotide diversities and standard error

Region	n	h	L	S	H (SE)	□ (SE)
Breeding (Winter) Grounds						
Gorgona Is., Colombia[a]	30	16	240	26	0.913 (0.037)	0.027 (0.015)
Malaga Bay, Colombia[a]	37	12	240	22	0.880 (0.036)	0.020 (0.011)
Abrolhos, Brazil[b]	**176**	**61**	**324**	**57**	**0.971 (0.004)**	**0.020 (0.001)**
Abrolhos, Brazil[c]	49	27	350	38	0.969 (0.010)	0.025 (0.013)
Southwest Africa[c]	23	11	350	25	0.910 (0.030)	0.023 (0.012)
Gabon[d]	70	37	340	47	0.973 (0.007)	0.027 (0.013)
Angola[d]	11	9	340	30	0.964 (0.051)	0.028 (0.016)
Mozambique[c]	8	6	350	21	0.893 (0.111)	0.021 (0.013)
Mayotte, Comoros Is.[c]	17	11	350	28	0.949 (0.033)	0.026 (0.014)
Antogil Bay, Madagascar[c]	141	51	350	50	0.976 (0.003)	0.025 (0.013)
South Madagascar[c]	35	19	350	40	0.955 (0.017)	0.027 (0.014)
Western Australia[a]	26	22	240	32	0.988 (0.014)	0.031 (0.017)
Eastern Australia[a]	15	8	240	16	0.895 (0.053)	0.022 (0.013)
New Caledonia[a]	16	12	240	23	0.967 (0.031)	0.029 (0.016)
Tonga[a]	20	14	240	25	0.932 (0.044)	0.029 (0.016)
Feeding (Summer) Grounds						
Antarctic Area I[e]	11	7	333	NA*	0.927 (NA*)	0.023 (0.004)
Antarctic Area I[b]	**46**	**17**	**324**	**24**	**0.913 (0.021)**	**0.018 (0.001)**
Antarctic Area II[b]	**31**	**13**	**324**	**21**	**0.912 (0.028)**	**0.017 (0.001)**
Antarctic Area IIIE[e]	15	14	333	NA*	0.991 (NA*)	0.024 (0.002)
Antarctic Area IV[e]	73	34	333	NA*	0.959 (NA*)	0.026 (0.001)
Antarctic Area V[e]	40	23	333	NA*	0.960 (NA*)	0.028 (0.001)
Antarctic Area VIW[e]	16	12	333	NA*	0.958 (NA*)	0.024 (0.002)
Other Oceanic Basins						
North Atlantic[f]	246	NA*	283	NA*	0.881 (0.015)	0.024 (0.001)
North Pacific[f]	109	NA*	283	NA*	0.772 (0.024)	0.046 (0.008)

*Not Available; [a] Rosenbaum et al. 1998; [b] this study; [c] Rosembaum et al. 2000; [d] Rosenbaum et al. 2001; [e] Pastene et al. 2000; [f] Baker and Medrano-Gonzales 2002.

Table 3. Private and Shared Haplotypes between Brazil (BR), Antarctic Area I (A1) and Antarctic Area II (A2) populations

Haplotypes	Number (% in parenthesis) of Haplotypes		
	Population		
	BR	A1	A2
Private	54 (88.5)	5 (29.4)	2 (15.4)
Shared with BR	-	6 (35.3)	5 (38.5)
Shared with A1	6 (9.8)	-	10 (76.9)
Shared with A2	5 (8.2)	10 (58.8)	-
Total*	61	17	13

* Four haplotypes were common to all popopulations

Table 4. AMOVA results for the pairwise comparisons between Brazil and Antarctic Areas I (AI) and Area II (AII) using mtDNA control region data

Breeding and feeding grounds	Source of Variation			Φ_{ST}*
	Among groups	Among populations within groups	Within populations	
Brazil X (AI+AII)	2.21	-0.65	98.44	0.016
AI X (Brazil +AII)	0.36	1.12	98.52	0.015
AII X (Brazil +AI)	-1.11	1.87	99.24	0.008
Brazil X AI X AII	1.35	-	98.65	0.013

*p<0.05 for all analyses

Table 5. Mean Kimura-2-Parameter genetic distances between populations and its standard error in parenthesis (below diagonal) and pairwise Φ_{ST} indices based on the same genetic distance (above diagonal)

	Antarctic Area I	Antarctic Area II	Brazil
Antarctic Area I		-0.004	0.018*
Antarctic Area II	0.018 (0.004)		0.010
Brazil	0.020 (0.004)	0.020 (0.004)	

*p<0.05 based on 2000 replications