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CARACTERIZAÇÃO DA VARIABILIDADE GENÉTICA E AVALIAÇÃO DAS PROVÁVEIS ÁREAS DE ALIMENTAÇÃO BASEADA NO DNA MITOCONDRIAL DA POPULAÇÃO DE BALEIAS JUBARTE, *Megaptera novaeangliae*, NO BANCO DOS ABROLHOS, BAHIA, BRASIL

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DISSERTAÇÃO DE MESTRADO

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SUMÁRIO

Para o Luca, pela paciência e companhia nesta e em outras etapas da minha vida.

2. AGRADECIMENTOS

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3. RESUMO

No Oceano Atlântico Sul Ocidental as baleias jubarte migram a cada inverno para o banco dos Abrolhos (16°40' to 19°30'S; 37°25' to 38°57'W) para acasalamento e cria de filhotes. Entretanto, esta população permanece geneticamente não caracterizada e suas áreas de alimentação são ainda desconhecidas, uma vez que não há pareamento fotográfico de indivíduos entre Abrolhos e os sítios de alimentação na Antártida. A fim de examinar estas questões, sequenciamos um segmento de 450 pares de bases do DNA mitocondrial da região controladora de baleias jubarte de Abrolhos (n=176) e das proximidades da Península Antártica (n=77). Um total de de 61, 17 e 13 haplótipos foram determinados respectivamente no Brasil e nas Áreas I e II da Antártida. A variabilidade genética da área de reprodução brasileira foi alta, similar à de outros sítios reprodutivos do Hemisfério Sul. A proporção de haplótipos compartilhados e a distância genética demonstraram uma maior semelhança entre as duas regiões antárticas que entre o Brasil e qualquer uma destas áreas. Estes resultados indicam que as populações de baleias jubarte das àreas I e II da Antártida parecem não possuir uma clara diferenciação e que os limites entre as àreas I e II correntemente definidos pela Comissão Internacional da Baleia devem ser deslocados para leste. Sugerimos que a área de alimentação da população de baleias jubarte brasileiras não estaria na Península Antártica ou próximo a ela, mas pode estar localizada na porção leste da Área II, no mar de Weddell ou próximo às ilhas Geórgias do Sul.

4. ABSTRACT

Characterization of the genetic variability and evaluation of the likely feeding grounds based on the mitochondrial DNA of the humpback whale, *Megaptera novaeangliae* population in the Abrolhos bank, Bahia, Brazil

In the Southwestern Atlantic Ocean humpback whales migrate every winter to Abrolhos bank ($16^{o}40'$ to $19^{o}30'$S; $37^{o}25'$ to $38^{o}57'$W) for mating and calving. However, this population remains genetically uncharacterized and their feeding areas unknown, as there are no photographic matches between individuals from Abrolhos and Antarctic feeding grounds. In order to examine these questions, we sequenced a 450 bp segment of the mitochondrial DNA control region from Abrolhos humpback whales (n=176) and from Antarctic Peninsula surroundings (n=77). A total of 61, 17 and 13 haplotypes were determined in the Brazilian, Antarctic Area I and II respectively. The genetic variability of the Brazilian breeding area was high, similar to that from other Southern Hemisphere breeding grounds. The phylogenetic tree using also sequences from the GenBank found a new clade (named BR) constituted by Brazilian sequences and a sequence from Eastern Australia (EA11). The proportion of sharing haplotypes and the genetic distance showed a greater similarity between the two Antarctic grounds than between Brazil and any of these areas. These results indicate that humpback whale populations from the Antarctic Area I and II seem to have no clear differentiation and that the boundaries between Areas I and II as currently defined by the International Whaling Commission may be shifted to the east. We suggest that the feeding area of the Brazilian humpback whale population is not in the Antarctic Peninsula or near it but may be located in the eastern part of Area II, in the Weddell Sea or near South Georgia Island.

5. APRESENTAÇÃO

O presente trabalho teve por objetivo estimar a variabilidade genética, a proporção sexual e determinar o sítio de alimentação do " stock" (subpopulação) de baleias jubarte, *Megaptera novaeangliae*, que utiliza o banco dos Abrolhos para reprodução e cria de filhotes. O conhecimento das áreas de alimentação e reprodução e da rota migratória das jubartes entre estas áreas agregam além disso subsídios para a defesa de proposta brasileira que será reapresentada em 2003 à Comissão Internacional da Baleia (IWC), órgão máximo que regulamenta a caça e conservação de grandes cetáceos no mundo, de criação do "Santuário do Atlântico Sul", local onde as baleias estarão protegidas da caça comercial em uma das fases mais vulneráveis de sua vida.

As ações de pesquisa e conservação destes animais na região de Abrolhos são desenvolvidas desde 1988 pelo Projeto Baleia Jubarte, com o apoio do Parque Nacional Marinho dos Abrolhos/IBAMA, mais antiga unidade de conservação federal do país. Estudos de fotoidentificação, estimativa populacional, observações de comportamento, monitoramento do turismo e outros têm sido desenvolvidas e os estudos de genética iniciados em 1997 vêm agregar informações importantes para a ecologia e manejo da espécie.

As jubartes de ambos os hemisférios formam sazonalmente diferentes concentrações reprodutivas em latitudes tropicais. Cada subpopulação, apesar da ausência de barreiras geográficas evidentes, possui grande fidelidade a seu local de acasalamento, realizando migrações verticais para uma também específica área de alimentação em águas árticas ou antárticas. No hemisfério sul, a espécie possui seis sítios reprodutivos distintos, entre os quais o banco dos Abrolhos, no sul da Bahia, considerado a principal área de reprodução da espécie em todo o Oceano Atlântico Sul Ocidental. A Comissão Internacional da Baleia divide as áreas de alimentação das baleias na Antártida em também seis regiões principais, numeradas de I a VI. Considerando-se o padrão migratório vertical da espécie, as áreas definidas como I e II, situadas respectivamente entre os meridianos 60ºW e 120ºW e 0ºW e 60ºW constituiriam o provável local de alimentação das jubartes brasileiras.

As análises da região controladora do DNA mitocondrial constituem uma ferramenta importante para o manejo e conservação das espécies. O DNA mitocondrial constitui uma das mais estudadas regiões do genoma de mamíferos para a construção das relações filogenéticas e análise dos padrões de distribuição e variabilidade genética de populações,

subpopulações ou espécies. Devido a suas elevadas taxas de mutação, transmissão somente pelas fêmeas e ausência de recombinação, o DNA mitocondrial oferece vantagens sobre outras regiões do genoma como a nuclear nos estudos de variabilidade genética, padrões de distribuição e fluxo gênico entre populações.

A determinação do sexo dos indivíduos e da proporção sexual em populações de cetáceos também proporcionam parâmetros essenciais para seu manejo, fornecendo informações acerca de seu comportamento e estrutura social. A determinação do sexo em cetáceos na natureza, entretanto, costuma ser complicada, já que em muitas espécies o dimorfismo sexual está limitado ao tamanho e peso do corpo e localização das regiões genital e anal. Nas jubartes as fêmeas também exibem outra característica sexual secundária, que consiste na presença de um lobo hemisférico na porção posterior da região genital, mas que só pode ser observada em animais encalhados, através de imagens submarinas ou quando o animal expõe a nadadeira caudal acima da superfície da água. A análise de regiões sexo-específicas no DNA constitui então uma alternativa eficiente para a obtenção destas informações.

A dissertação, escrita em formato de artigo científico, está sendo submetida à "Conservation Genetics" e pretende trazer contribuição importante para o conhecimento e conservação de uma espécie presente em todas as listas oficiais de fauna brasileira ameaçada de extinção, protegida da caça e captura através de moratória internacional e que constitui um dos maiores ícones da conservação mundial.

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

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Running title: mtDNA diversity of the humpback whales from Brazil

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Key words: humpback whale, mitochondrial DNA, Abrolhos bank, genetic variability, migration

Abstract

In the Southwestern Atlantic Ocean humpback whales migrate every winter to Abrolhos bank (16°40' to 19°30'S; 37°25' to 38°57'W) for breeding. However, this population remains genetically uncharacterized and their feeding areas unknown. In order to examine these questions, we sequenced a 450 bp segment of the mitochondrial DNA control region from Abrolhos humpback whales (n=176) and from Antarctic Peninsula surroundings (n=77). A total of 61, 17 and 13 haplotypes were determined in the Brazilian, Antarctic Area I and II respectively. The genetic variability of the Brazilian breeding area was high, similar to that from other Southern Hemisphere breeding grounds. The phylogenetic tree using also sequences from the GenBank found a new clade (named BR) constituted by Brazilian sequences and a sequence from Eastern Australia (EA11). The proportion of sharing haplotypes and the genetic distance showed a greater similarity between the Antarctic grounds than between Brazil and any of these areas. These results indicate that humpback whale populations from the Antarctic Area I and II seem to have no clear differentiation and that the boundaries between Areas I and II as currently defined by the International Whaling Commission may be shifted to the east. We suggest that the feeding area of the Brazilian humpback whale population is not in the Antarctic Peninsula or near it but may be located in the eastern part of Area II, in the Weddell Sea or near South Georgia Island. However, further analyses are needed and will be conducted before specific recommendations for boundaries are made.

Introduction

In the Southern Hemisphere, it is generally accepted that humpback whales (*Megaptera novaeangliae*) have seven distinct breeding areas, distributed along continents or islands or in shallow banks in tropical latitudes.

At the beginning of the austral summer, each of these 'stocks' migrate to specific feeding grounds in high-latitude Antarctic waters (Dawbin 1966; Whitehead & Moore, 1982; Baker *et al.* 1995; Clapham & Mead 1999). The longitudinal boundaries of such areas – as well as the knowledge about the distribution of blue whales (*Balaenoptera musculus*) - have led the International Whaling Commission to establish political units for commercial whaling in the region (Tonnessen & Johnsen 1982). Since 1957 the limits of six feeding grounds in Antarctic waters, known as Areas I to VI, were accepted by the International Whaling Comission and in 1974/75 season they were included in the "official" schedule of the Commission. The defined Antarctic Areas I and II were located respectively between the 120° to 60° W and 60° to 0° W (Donovan 1991).

In the Southwestern Atlantic Ocean, humpback whales are found in the Abrolhos Bank (16º40´- 19º30´S and 37º25´- 39º45´W) their main mating and calving grounds (IBAMA/FUNATURA 1991; Engel 1996; Martins et al. 2001). Their corresponding feeding ground in the Antarctic region, however, remains unknown, since comparisons based in the photo-ID catalogs from the College of the Atlantic Program and Projeto Baleia Jubarte/Humpback Whale Project – Brazil, did not result in any match between Abrolhos and Antarctic Area I (Muñoz, pers. comm.; Projeto Baleia Jubarte, unpubl. data). The bad conditions for navigation in the Weddell Sea have not allowed researchers to develop comparisons with photoidentified individuals from Antarctic Area II, their most likely feeding ground, as it is generally located due south below the Southwestern Atlantic Ocean. At the same time, photoidentification studies (Stone et al. 1990; Capella & Flórez-González 1993; Muñoz et al. 1998) and mtDNA analyses (Caballero et al 2000; Olavarría et al, 2000) demonstrated an evident link between the subpopulation of humpbacks that breed along the Colombian coast and Area I in the Western part of the Antarctic Peninsula.

Commercial hunting has brought this species to the border of the extinction: the major harvests were relatively recent and short-lived, between 1920 and 1986, a period in which, according to Allen (1980) and Evans (1987), more than a million humpbacks, blues, fins

and sei whales were killed. The worldwide protection of humpback whales from hunting was established in 1966 (Rice 1978) so the species experienced around 46 years of intensive killing. As example of what happened in many of these concentration areas, is actually known that the humpback whale "stock" of the Brazilian coast was extensively hunted until the international moratorium in 1966, despite the existence of many gaps in these records (Paiva & Grangeiro 1965; 1970).

Mitochondrial DNA has become one of the most studied regions of the mammalian genome for the reconstruction of phylogenetic relationships and analyses of distribution patterns within populations or species genetic variation. The advantages of the mtDNA against other portions of the genome are due its high substitution rate, maternal inheritance and absence of recombination (Wilson *et al.* 1985; Avise 1986; Lyrholm *et al.* 1996). The effective population size of mtDNA genomes is one fourth that of autosomal nuclear genes, allowing a higher rate of regional variability through random drift. (Wilson *et al.* 1985; Avise 1986; Thomas *et al.* 1996). The analyses of the mtDNA control region provide a high resolution view of intraspecific genetic structure in a variety of taxa, including whales (Baker *et al.* 1993a), despite the hypothesis that the mtDNA rate of evolution may be lower for some cetaceans (Baker *et al.*, 1993b). Transition between these 2 paragraphs needed

The visual determination of gender in wild cetaceans is usually difficult, because in most species sexual dimorphism is limited almost exclusively to body weight and lengths, and to the location of their genital and anal regions. However, sex determination of individuals in a population of cetaceans and the resulting knowledge of the sexual rate constitute essential parameters to their management (Clapham 1995), as they provide important information regarding their behavior and social structure. Glockner (1983) described that humpback whale females exhibit a secondary sexual characteristic consisting in a hemispheric lobe in the posterior portion of their genital region. This lobe is absent in males. Consequently, the determination of gender of the individual humpbacks can be done through the observation of their genital region and recorded through submarine photography or when such animals expose this part of the body out of the water. As an alternative to this methodology, which is very difficult to apply to animals in the wild, it was found that the gender of cetaceans can be determined by the analysis of tissue samples, through its karyotyping (Winn *et al.* 1973; Pallsboll *et al.* 1992) or through the cell culture

of such biopsies (Lambertsen *et al.* 1987; Pallsboll *et al.* 1992). In recent years, new techniques based in sex-specific DNA sequences have been used to determine gender in cetaceans (Baker *et al.* 1991; Pallsboll *et al.* 1992).

In this report we investigated the genetic diversity and putative migratory connection of the Brazilian humpback whales, through molecular methods based on mitochondrial DNA. It was also determined the sex-biased rate of the Brazilian stock and the gender of each individual as a complement of the Projeto Baleia Jubarte/Humpback Whale Project – Brazil catalogue, which includes 183 whales photoidentified and sexed by molecular techniques until 2001.

Materials and methods

Sampling and mtDNA sequencing

201 skin samples of humpback whales from different social groups were collected during the breeding seasons of 1997 through to 2001, most of it from the Abrolhos Bank and a few as a result of strandings in Bahia and Espírito Santo States, or other locations in the Brazilian coast (Figure 1). For each whale sampled, date, location (by Global Positioning System), group composition, number of animals, and presence of calf were also recorded. For sampling of live animals a Barnett Wildcat XL crossbow was used with stainless steel biopsy darts with a 8mm diameter and a 15 mm length sampling tip. Samples were kept in 70% ethanol or DMSO, according to the protocol established by Baker *et al.* (1998).

Additional 79 skin samples were obtained in the Gerlache and Bransfield Straits and in the Weddell Sea near the Antarctic Peninsula using the same methods described above. These samples were obtained during the expedition organized by the Antarctic Brazilian Program (PROANTAR) in the austral summers of the years 1999 to 2000. Following a recent proposal of Caballero *et al.* (2000) of changing the limits between Antarctic areas I and II from 60°W to 58°W, in this study were considered as belonging to Antarctic area II a total of 25 samples collected very near the 58°W boundary.

The DNA extraction of the tissue samples followed protocols modified from Baker *et al.* (1993a) and Palsboll *et al.* (1995), with lisys of cells in 1.0 % SDS, 0.15 M of sodium chloride, 10 mM Tris-HCl (pH 8.0), 1.0 mM de EDTA and digested with proteinase

K (100 μg /ml^{-1}) at 65^{0} C for a minimum of three hours, followed by the extraction with phenol/chloroform and precipitation with ethanol.

A region of approximately 450 nucleotides from the most variable portion of the control region in mtDNA was amplified, using primers Dlp-1.5 and Dlp-5 (as described in Baker et al., 1993). Approximately 100 ng of total DNA were submitted to 35 cycles/25 μl of reaction volume with 0.5 units of the Taq DNA polymerase enzyme, 0.2 μM of each primer, 1.5 mM of magnesium chloride, 0.2 mM of DNTPs and 1X buffer (20 mM Tris-HCl ph 8.4; 50 mM KCl). The amplified material was purified with shrimp alkaline phosphatase and exonuclease I (Amersham Biociences) and sequenced with the chain terminators method (Amershan Biosciences ET terminator kit) in a thermocycler, purified afterwards (precipitation with ethanol) and taken to the automatic sequencer MegaBACE 1000.

Molecular determination of sex

The molecular determination of sex of 183 individuals from Abrolhos Bank and the Brazilian coast was obtained following the protocol of Pallsboll et al. (1992) modified by Berube and Palsboll (1996). The oligonucleotide primers ZFY0097 and ZFY1204 were used to amplify by PCR an 1100 base pair sex-specific homologue region of the X and Y chromosomes. One μl of total cellular DNA was amplified in a 30 μl reaction volume by 40 cycles of standard PCR. Sixteen μl of the amplified DNA was digested with TAQ I restriction enzyme in a total solution of 20 μl. The restricted DNA was separated by agarose gel (2%) eletrophoresis and observed by UV light after exposure to ethidium bromide (0,5 μg/ml). The total and the social groups sexual rates were estimated and compared with that obtained in other breeding and calving areas, and the Pearson chi-square test with Yate´s correction was used to calculate the statistical significance of these rates. The calves were not biopsied in this study.

Statistical methods

Each sequence was manually checked and validated using the software Chromas (available at http://www.technelysium.com.au/index.html) and the automatic alignment was performed using program Clustal (Thompson *et al.*, 1994) with manual adjustments with GENEDOC (Nicholas e Nicholas, 1997) program.

Some analyses were done using different dataset. In order to classify the new haplotypes according to the three previously described clades, referred to as "AE", "CD" and "IJ" (Baker *et al.* 1990), one set of phylogenies were undertaken using the sequences from Brazil and the Antarctic described here and 48 other humpback whale sequences obtained from GenBank (Baker *et al.* 1993; 1998). To confirm the position of the root in the haplotype tree of *M. novaeangliae,* published sequences of *Balaenoptera edeni, B. musculus, B. physalus, B. acutorostrata, Balaena mysticetus, Eubalaena* and *Caperea marginata* from Genbank were used as outgroups. In this case only approximately 250 positions could be used. To maximize the information, in all other phylogenies estimated and analyses performed, only the 253 sequences described here that include the full 431 nucleotides alignment were used (324 sites were present in all sequences).

Haplotype and nucleotide diversity were calculated using the MEGA software version 2.1 (Kumar *et al.* 2001) with the standard error estimated by 500 boostrap replicates. The phylogenies were estimated using the neighbour–joining method with Kimura-2 parameter (K2p) distance (Saitou e Nei 1987) also using MEGA. Other distances were also used but as all gave essentially the same results, only the K2p results were presented. The 'bootstrap' method (Felsenstein 1985) was used to estimate the statistical validity of the clades. The TCS 1.13 software (Clement *et al.* 2001) was used to produce a haplotype network.

The structure of the genetic diversity in these areas was studied using the AMOVA approach (Excoffier et al. 1992) as implemented in ARLEQUIN 2.0 software (Schneider et al. 2000). For this hierarchical method the Abrolhos bank and Brazilian coast samples, Antarctica area I and Antarctica area II were considered as three populations. To study the relationships among the breeding area and the two feeding grounds, three grouping of populations were considered. The AMOVA was performed using the k2p distance among the haplotypes (Φst) and also not using the distance among haplotypes (Fst) and the significance of the differences was tested using 1000 no-parametric permutations.

The DnaSP 3.51 program (Rozas & Rozas 1999) was used to estimate other statistics, such as the neutrality tests.

Results

Variability of mtDNA control region sequences

A consensus segment of 431 bp of the mtDNA control region was assembled from 176 sequences from Brazil, 46 from Antarctic Area I and 31 from Antarctic Area II. A total of 59 polymorphic sites were identified defining 62 haplotypes in the Brazilian sample. For the Antarctic samples, 33 and 27 segregation sites were detected defining 20 haplotypes for Area I and 14 for Area II, respectively. The table 1 presents these data and the nucleotide and haplotype diversities of each one of these three sampled areas, in comparison with that reported for other breeding and feeding grounds within the three ocean basins (North Atlantic, North Pacific and Southern Hemisphere). The Brazilian haplotype diversity (h=0.971) was high and similar to that found in the majority of the breeding grounds such as the African Gabon and Antogil Bay, Madagascar (Rosenbaum et al., in prep). The nucleotide diversity (in Brazil was low, only comparable to Colombia and the overall North Pacific sample. The Antarctic Areas I and II presented haplotype diversity values (h=0.913 and 0.912, respectively) lower than all other feeding grounds studied, but higher than North Atlantic and North Pacific and some Southern Ocean breeding areas. Regarding the nucleotide diversities, we found in both Antarctic Areas a value (= 0.017) lower then all other area studied.

Considering together all sequences from the three populations described here it were identified 74 different haplotypes (figure 2), the three areas sharing four of them. The number of sharing haplotypes and of individuals sharing haplotypes between the areas (table 2), clearly indicate that Antarctic Areas I and II have a closer connection that any one of them has with the Brazilian area.

mtDNA Phylogeny and Clade distribution

Firstly, we constructed a phylogenetic tree (data not shown) of all our humpback whale data from Brazil and Antarctic together with the other haplotypes available in GenBank (Baker *et al.*1993; Baker *et al.* 1998) and with the outgroups. Although the number of common sites reduced to only about 250 in this analysis, this phylogeny helped to place our data in the context of the previously described clades. In this global tree, the monophyletic *M. novaeangliae* haplotypes clustered in a few different clades (although with low bootstrap confidence) that could be associated to the previously described AE, IJ

and CD clades (Baker *et al.* 1990; 1993; 1998). We found a new clade, holding a few Brazilian sequences and EA11 (a sequence from Eastern Australia not included in any of the other three clades in the previous studies). This clade was found in a basal position near the outgroups and the AE clade. We named this new clade as "BR.

The phylogenetic tree built using only the Brazilian and Antarctic haplotypes described here and one representative of each set of identical sequences presented a very similar result (Figure 2). The same four clades were found but the bootstrap values for the AE and BR clades were relatively high.

Similarly to what was found in other studies (what other studies), the haplotype clades were dispersed among the populations. However, the frequency distribution for the clades among the three populations (table 3) was significantly different (X^2=21.934; p=0.001). There is a predominance of the CD clade in all three areas while the IJ clade presents an intermediate frequency. The AE clade had a much higher frequency in Antarctic II in contrast to its lower occurrence in Antarctic I and in Brazil, while the BR clade contained DNA sequences that were only found in Brazil.

Within-Population Variation

An initial AMOVA analysis without structuring the areas in groups resulted that 98% to 99% of the mtDNA variability was found within the populations (table 4). Considering the three different grouping of these populations, the higher among groups variation, 2.2%, was obtained when we compare Brazil against the two Antarctic feeding areas. However, in all the simulations, the FST fixation indices were statistically significant (table 4). Corroborating these results, the analysis of the Kimura 2-parameter genetic distance between the two Antarctic areas (0.018) was lower than between Brazil and any one of the two Antarctic feeding Areas (0.02) (table 5).

Molecular sexing

A total of 183 individuals were sexed from Abrolhos and Brazilian coast and classified according their social group (Table 5). The observed overall proportion of 55.2% males and 44.8% females did not differ significantly (p>0.05 for chi-square distribution) from the 1:1 sex ratio generally accepted for humpback whales (Chittleborough 1965; Clapham & Mayo 1987; Medrano et al. 1994).

Discussion

The high mitochondrial DNA diversity (nucleotide and haplotype) observed in the Brazilian sample in this study is in agreement with that described for other areas studied in the Southern Hemisphere and North Atlantic Ocean (Baker et al. 1993b; 1998; Rosenbaum et al. 1998, 2000, 2001) (Table 1). Therefore, despite the severe effects of commercial hunting in this stock (Paiva & Grangeiro 1965;1970), its maternal genetic diversity was not probably strongly reduced. A possible explanation for this maintenance of diversity in humpback whales in the Brazilian area and in general is that the major harvests was relatively recent and short-lived in relation to the other whales.

Furthermore, differently from other mysticeti species, humpback whales have a minimum age at sexual maturity of 4 to 6 years (Klinowska, 1991) and a long generation time between 5 to 10 years (Baker *et al.* 1993b), what may have allowed reduced populations of humpbacks to overlap during and consequently pass through a bottleneck without much mtDNA variability loss. According to Nei *et al.* (1975), the decay of mtDNA variability is slowed down if the period of time of the population bottleneck is short and the species has a long generation time. This seems to be the case with most of the humpback populations, including the Brazilian one, which recovered to an estimated number around 2500 individuals in the present (Freitas *et al.* 2001; Andriolo *et al.* 2001).

Another explanation for the high diversity maintained in most breeding sites is that a low but consistent gene flow among these areas exists. It is not clear why the nucleotide diversity we found in Antarctic Areas I and II were so low when compared with other estimates (Table 1) but it may be related to the very small region where samples were taken.

The frequency of CD clade haplotypes in the Brazilian area was high (61.4%), similar to that found in Eastern Australia and Tonga, and Western Australia region (Baker *et al.* 1993a). The clade IJ in the Southern Hemisphere was most frequently observed in Western Australia but was found in 32.4% of the Brazilian samples. Colombia and the Antarctic Peninsula were the only regions of the Southern Hemisphere where the clade AE has been found so far (Caballero *et al.* 2000). Our results corroborate its occurrence in the Antarctic and include also the Brazilian breeding region in its distribution, although in a very low frequency (0.05%). We suggest the existence of a new mitochondrial clade

labeled BR, comprising five haplotypes from 10 individuals found in the Brazilian coast plus the Eastern Australia haplotype EA11. This suggests that this clade may occur in other southern hemisphere populations, but this conclusion should wait until more sequences from this region render publicly available for comparisons.

The AMOVA analyses indicate a lower differentiation between Antarctic Areas I and II when both compared with the Brazilian population. The results of population pairwise FSTs also corroborate the AMOVA results, obtaining a lower genetic distance between Antarctic Areas I and II than between Brazil and any of these feeding grounds. Moreover, the proportion of shared haplotypes between Brazil and both the Antarctic areas (n=4) and between Brazil and each Antarctic areas separately (table 2) may be considered very low if we take the example of the Colombia breeding area and Antarctic Area I (17 over 37), whose migratory connection is well know. Such results imply that Antarctic Areas I and Area II as sampled here, near the Antarctic Peninsula (Figure 1) do not constitute the feeding ground of the Brazilian humpback whales.

However, the cause of the greater similarity found between the Antarctic populations and its dissimilarity with the Brazilian animals may be related to the collecting places and the limits between Area I and II. In the present study and all other we know about, the sampling of biopsies were in the west of the Antarctic Peninsula (Area I) and in Area II, almost always very near the peninsula (in most of cases between the 60ºW and 56ºW), as the bad conditions of navigation in most of that region difficult the development of research cruises to collect biopsies and photoidentification data. It is possible that the Brazilian population of humpback whales feed somewhere in the middle of the Weddell Sea or near to South Georgia Island, in the east part of the Antarctic Area II, far from the place where the majority of the collects of samples were done. Therefore, our data suggests that, at least for the humpback whale, the 60ºW or even the 58ºW limit (as suggested by Olavarria et al. 2000) between Areas I and II stocks may be inappropriate and that this limit should be pushed somewhere farther to the east(too early to state this based on the analysis and level of sampling). The tagging of whales with satellite transmissors during the breeding season close to Abrolhos bank constitute another way to help to test this hypothesis.

Although the observed proportion of males and females in the Brazilian sample did not differ significantly, an overall higher number of males was found (table 5), as usually observed in humpback whale breeding areas due to the different pattern of migration in males versus females (Palumbi & Baker 1994; Craig & Herman 1997). According to the first authors, females apparently visit the winter grounds less frequently than do males, resulting in a surplus of males. This strategy aim to increase the female's probability of reproductive success by maximizing the time spent on the feeding grounds, as the energy costs of migration and lactation for long periods, plus the absence of food sources, is formidable.

The sex composition of the various social groups studied here were in agreement with the expected values. For example, the high predominance of males in surface-active groups and trios corroborates the observations described in Clapham (1996) such as that mature females usually are distributed separately each other aiming to increase the possibility of interaction with many males and that the males are usually forming short-period groups where they are engaged in the competition to copulate with the female. The four single females registered probably were immature or already pregnant animals; otherwise escorts or calves likely would accompany them. An almost equal proportion of male and females composed the dyads, which were often observed in mating and courtship behaviors. Of the nine stranded animals, one was rescued alive but the other eight were dead whales and many were in an advanced state of decomposition, preventing visual sex identification.

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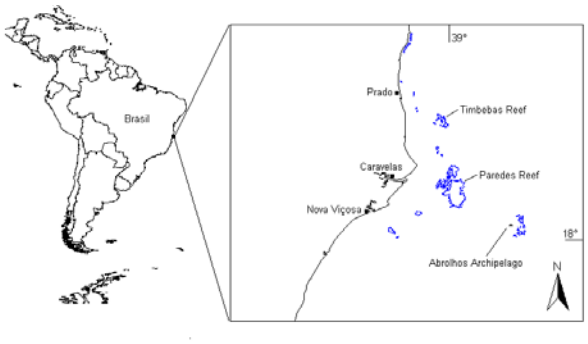
a



b

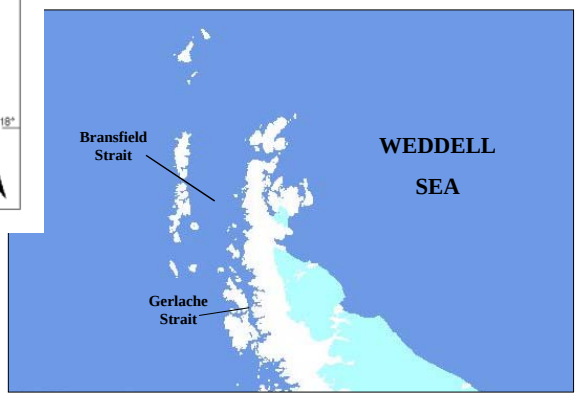


Figure 1. a) Abrolhos bank, the Brazilian breeding ground and, b) the likely feeding areas I and II in the surroundings of the Antarctic Peninsula.

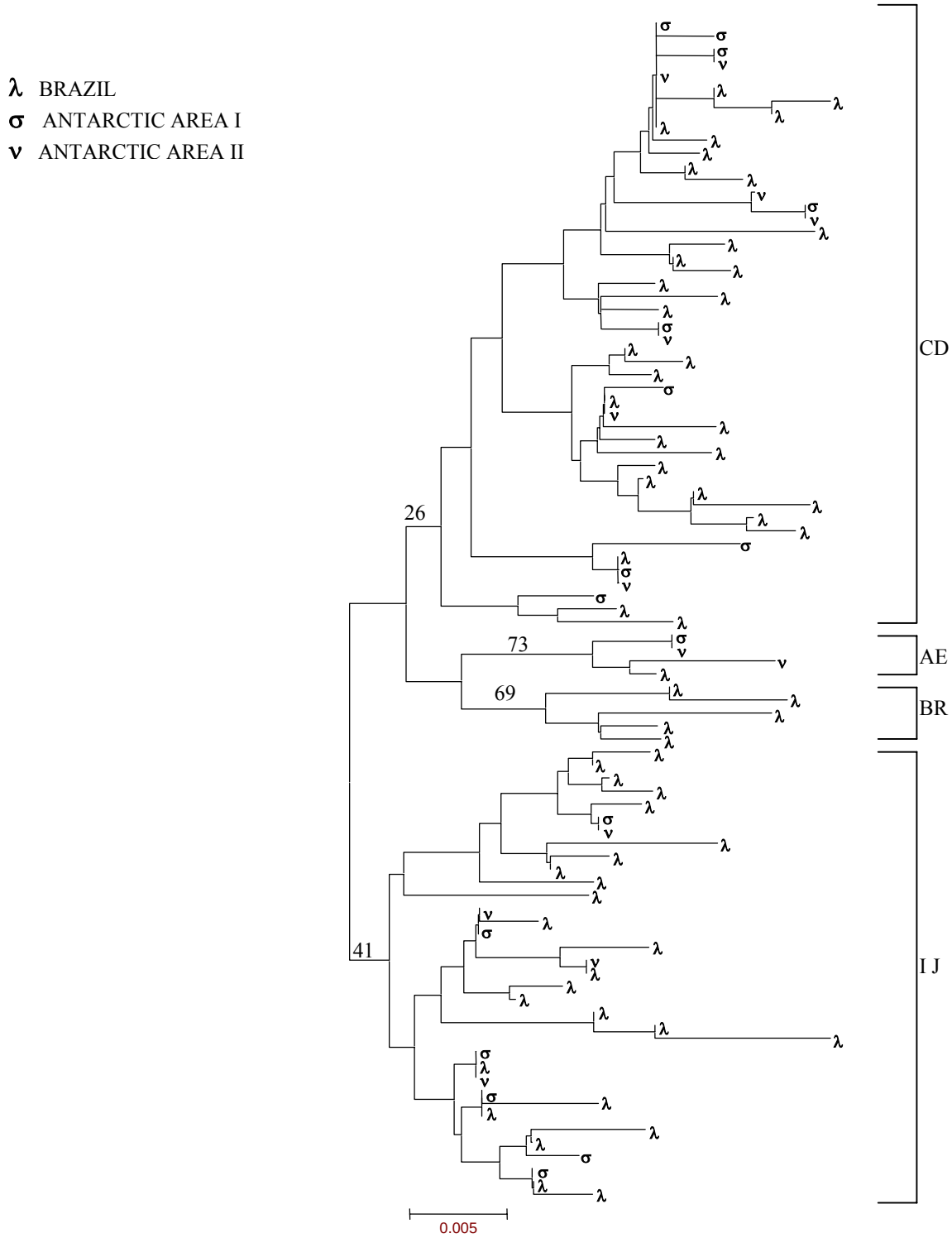


*Figure 2.*Unrooted phylogenetic tree of shared haplotypes between the three areas and clades division

Table 1. Sample by region and information of variability mtDNA control region of humpback whales from Brazil and Antarctic Areas I and II and its comparisons with other breeding and feeding grounds. Haplotype (h) and nucleotype (¶) diversities, as well as their standard deviations are reported.

REGION	N	HP	ND	P	h+/-SD	π+/-SD	REFERENCE[a]
BRAZIL	49	27	350	38	0.969+/-0.010	0.025+/-0.013	1
BRAZIL	176	61	324	57	0.971+/-0.004	0.02040+/-0.001	this study
ISLA GORGONA, COLOMBIA	30	16	240	26	0.913+/-0.037	0.027+/-0.015	2
MALAGA BAY, COLOMBIA	37	12	240	22	0.880+/-0.036	0.020+/-0.011	2
ANTOGIL BAY, MADAGASCAR	141	51	350	50	0.976+/-0.003	0.025+/-0.013	1
SOUTH MADAGASCAR	35	19	350	40	0.955+/-0.017	0.027+/-0.014	1
MAYOTTE, COMOROS	17	11	350	28	0.949+/-0.033	0.026+/-0.014	1
MOZAMBIQUE/SE AFRICA	8	6	350	21	0.893+/-0.111	0.021+/-0.013	1
WESTERN AUSTRALIA	26	22	240	32	0.988+/-0.014	0.031+/-0.017	2
EASTERN AUSTRALIA	15	8	240	16	0.895+/-0.053	0.022+/-0.013	2
TONGA	20	14	240	25	0.932+/-0.044	0.029+/-0.016	2
NEW CALEDONIA	16	12	240	23	0.967+/-0.031	0.029+/-0.016	2
GABON	70	37	340	47	0.973+/-0.007	0.027+/-0.013	3
ANGOLA	11	9	340	30	0.964+/-0.051	0.028+/-0.016	3
WEST SOUTH AFRICA	23	11	350	25	0.910+/-0.03	0.023+/-0.012	1
ANTARCTIC AREA I	11	7	333	***	0.9273	0.0230+/-0.0039	4
ANTARCTIC AREA I	46	17	324	24	0.913+/-0.021	0.01779+/-0.001	this study
ANTARCTIC AREA II	31	13	324	21	0.912+/-0.028	0.01740+/-0.001	this study
ANTARCTIC AREA IIIE	15	14	333	***	0.9905	0.0244+/-0.0018	4
ANTARCTIC AREA IV	73	34	333	***	0.9593	0.0256+/-0.0008	4
ANTARCTIC AREA V	40	23	333	***	0.9603	0.0281+/-0.0014	4
ANTARCTIC AREA VIW	16	12	333	***	0.9583	0.0243+/-0.0020	4
NORTH ATLANTIC	246	***	283	***	0.881+/-0.015	0.0236+/-0.00015	5
NORTH PACIFIC	109	***	283	***	0.772+/-0.024	0.046+/-0.0081	5

N = Sample size, HP = Number of haplotypes, ND = Number of nucleotides, P = Polymorphic sites
*** data not available
[a] 1=Rosenbaum et al. 2000, 2=Rosenbaum et al. 1998, 3=Rosenbaum et al. 2001, 4=Pastene et al. 2000, 5=Baker & Medrano-González 2002.

Table 2. Shared Haplotypes between Brazil (BR), Antarctic Area I (A1) and Antarctic Area II (A2)

	Number of Haplotypes			Number (Frequency %) of Individuals		
	Populations			Populations		
Haplotypes	BR	A1	A2	BR	A1	A2
Restricted to 1 pop	54	5	2	136 (77.27)	10 (21.74)	4 (12.90)
Shared only with BR	-	2	1	-	2 (4.35)	1 (3.23)
Shared only with A1	2	-	6	9 (5.11)	-	16 (51.61)
Shared only with A2	1	6	-	1 (0.57)	22 (47.82)	-
Common to all pops	4	4	4	30 (17.05)	12 (26.09)	10 (32.26)
Total	61	17	13	176 (100)	46 (100)	31 (100)

Table 3. Frequency (%) of occurrence of each clade in the three areas analyzed.

Groups/Clades	CD	IJ	AE	BR
Brazil	61.4	32.4	0.6	5.7
Antarctic I	67.4	30.4	2.2	0
Antarctic II	58.1	25.8	16.1	0

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			Fixation index F_{ST}*
	Among groups	Among populations within groups	Within populations	
BRAZIL X (AI+AII)	2.21	-0.65	98.44	0.01556
AI X (BRAZIL+AII)	0.36	1.12	98.52	0.01480
AII X (BRAZIL+AI)	-1.11	1.87	99.24	0.00755
BRAZIL X AI X AII	1.35	-	98.65	0.01349

*For all values p<0,05

Table 5. Mean genetic distances[a] between populations (below diagonal) and pairwise F_{ST} indices based genetic distance between haplotypes[a] (above diagonal)

	ANT 1	ANT 2	BRAZIL
ANT 1		-0.00434	0.01801*
ANT 2	0.01792		0.01061
	(0.00407)		
BRAZIL	0.01995	0.01961	
	(0.00416)	(0.00414)	

[a] Kimura 2-parameter distance, standard errors in parenthesis; * p<0.05, based on 20000 replicates.

Table 6. Molecular genetic identification of sex in different social groups of the Brazilian humpback whale population.

SOCIAL GROUP	MALES	FEMALES
SINGLE	9	4
MOTHER-CALF PAIR*	0	17
DYAD	22	19
TRIO	13	3
MOTHER-CALF PAIR & ESCORT*	22	24
4 OR MORE ADULTS	18	5
3 OR MORE ADULTS & CALF*	12	5
STRANDING	5	4
NON-IDENTIFIED	0	1
TOTAL	101	82

* Calves were not sampled.