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Microsatellite Genetic Characterization of the Humpback Whale (*Megaptera novaeangliae*) Breeding Ground off Brazil (Breeding Stock A)

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Abstract

The Southwestern Atlantic Ocean humpback whales wintering ground (breeding stock A) are distributed along the Brazilian coast (5–23°S), and their main mating and calving ground is in the Abrolhos Bank. We investigated genetic diversity, population structure, and relatedness of individuals sampled from the entire Southwest Atlantic humpback whale population. A total of 275 individuals sampled from 2 subregions (Abrolhos Bank, $n = 229$ and Praia do Forte, $n = 46$) were screened for 9 microsatellite loci. This population showed a high level of allelic diversity ($A = 12.1$) and a high mean observed heterozygosity ($H_O = 0.733$). No signal of significant genetic bottleneck was detected in accordance with the mitochondrial DNA data. We find no evidence of temporal (between years) genetic structure as well as no genetic differentiation between whales from the 2 subregions of the Brazilian breeding ground. We observed that the proportion of males and females in this population was approximately 1:1, which differs from the male-biased sex ratio observed in other breeding grounds. The data obtained through this study provided no evidence of kinship associations within social groups. Finally, a female sampled off South Georgia Islands showed a putative parent–offspring relationship with a female off Abrolhos Bank, supporting the migratory link between these 2 areas.

Key words: *bottleneck, conservation, demography, genetic diversity, migration, relatedness*

Humpback whales (*Megaptera novaeangliae* Borowski, 1781) are found throughout the world's ocean basins undertaking annual migrations between the high-latitude waters, where they feed during the summer, and the low-latitude waters, where they breed and calve during the winter months (Dawbin 1966). Currently, the International Whaling Commission (IWC) divided the Southern Hemisphere humpback whale stocks into 8 breeding grounds (termed A-G plus X in the Arabian Sea) based on low-latitude distributions (IWC 2005). The Antarctic waters were also divided into 6 feeding grounds, known as Management Areas I–VI, which have served as political units for commercial whaling in the region (Donovan 1991).

In the Southwestern Atlantic Ocean, the humpback whale breeding stock "A" (BSA) is presently distributed along the coast of the Brazil from approximately 5–23°S (Andriolo A, personal communication), with additional sightings to the north and east of 5°S, near the Archipelago of Fernando de Noronha (3°51′S) (Lodi 1994). The main mating and calving ground of this population is in the Abrolhos Bank (16°40′ to 19°30′S and 37°25′ to 39°45′W) in the southern Bahia and northern Espírito Santo States (Martins et al. 2001; Freitas et al. 2004; Andriolo et al. 2006). In recent years, sightings of females with calves have also been reported along the Brazilian coast north of the Abrolhos Bank (Martins et al. 2001; Zerbini et al. 2004;

Andriolo et al. 2006; Rossi-Santos et al. 2008). Although Freitas et al. (2004) reported 3 photographic matches between humpback whales of the Abrolhos Bank and Praia do Forte, the relationship of these 2 subregions is not clear and could represent some genetic structure within the BSA, similar to that found among subregions within some South Pacific breeding grounds (Olavarría et al. 2007).

Previous studies of the population structure of humpback whales have suggested that temporal patterns in genetic differences occur in some stocks (Rosenbaum et al. 2002; Stevick et al. 2003). In the North Atlantic Ocean, Stevick et al. (2003) described a temporal difference in occupancy pattern of the West Indies breeding area between individuals arriving from different feeding areas. Furthermore, some individuals that shared the same feeding ground may change wintering destinations from season to season (Darling and Cerchio 1993; Garrigue et al. 2002). Darling and Souza-Lima (2005) observed song similarity between Gabon (breeding stock B) and Brazil, which suggests that these stocks may share a common feeding ground, allowing possible interchange of individuals and/or song between these breeding grounds (Clark and Clapham 2004). Another possible behavioral pattern that may lead to temporal genetic structure is if some individuals do not migrate to winter grounds each year, remaining on the feeding ground (Brown et al. 1995). If temporal genetic structure occurs in the humpback whale population wintering off Brazil, we should expect to find structured annual migrations.

A mitochondrial DNA (mtDNA) study showed a significant differentiation between the humpback whale population that breeds along the Brazilian coast and the populations that feed in the western and eastern part of the Antarctic Peninsula (Antarctica Area I and II, respectively), suggesting that the latter does not constitute the main feeding ground of the Brazilian humpback whales (Engel et al. 2008). Furthermore, 2 whales sampled near South Georgia Islands matched with haplotypes from the Brazilian breeding ground (Engel et al. 2008), supporting the migratory link between these 2 areas, as indicated previously by photo identification (Stevick et al. 2006) and satellite telemetry data (Zerbini, Andriolo, et al. 2006).

The commercial whaling during the 20th century reduced the humpback whale population of the world to less than 10% of the original size, before worldwide protection in 1966 (Tønnessen and Johnsen 1982). The species is listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora, it is considered "least concern" by the International Union for the Conservation of Nature and Natural Resources (IUCN 2008 Assessment), and it is in the Official List of Threatened to Extinction Species of the Brazilian Fauna (IBAMA 2001).

mtDNA variability of the Brazilian humpback whales showed high level of haplotypic and nucleotidic diversity (Engel et al. 2008), in agreement with other breeding areas studied in the Southern Hemisphere (Baker et al. 1993, 1998; Olavarría et al. 2007). This result supports the prediction that for most stocks of whales, the population size was not sufficiently reduced by commercial whaling or this exploitation did not last for enough generations to significantly reduce their genetic diversity (Amos 1996). In the case of the Brazilian humpback whale population, the most intense exploitation seems to have occurred during only 3–6 generations (Engel et al. 2008). Another hypothesis is that gene flow between breeding grounds after the whaling period could have contributed to the current high genetic variability in these populations, but this has not been tested so far.

The genetic structure of humpback whale populations is complex reflecting a seasonal pattern of long-distance migration between summer feeding and winter breeding grounds and complex social organization (Baker et al. 1994). Although group formation has been described for this species both in feeding and breeding grounds, its social structure is still controversial (Pomilla and Rosenbaum 2006). Whereas on the summer grounds, humpback whales form cooperative feeding associations, typically small fission–fusion groups (Clapham 1993; Weinrich et al. 2006), on the breeding grounds males form groups to compete for access to females (Clapham et al. 1992). These competitive groups (CGs) consist of 3 or more individuals, including multiple males and a single female (Clapham et al. 1992).

Genetic studies have produced significant information about sexual proportions (Brown et al. 1995; Clapham et al. 1995) and kinship between individuals within groups in humpback whale populations (Clapham and Palsbøll 1997; Valsecchi et al. 2002; Cerchio et al. 2005; Pomilla and Rosenbaum 2006). Studies of relatedness have showed no association based in kinship within social groups, except pairs of mothers and calves (Valsecchi et al. 2002; Pomilla and Rosenbaum 2006). Whereas the sex ratio observed on the feeding grounds is approximately 1:1 (Clapham et al. 1995), most studies on the breeding grounds observed a sex ratio highly skewed toward males (Baker et al. 1994, 1998; Brown et al. 1995; Olavarría et al. 2007). The social structure and sex ratio of the Brazilian humpback whale population are still unknown.

The present study aims to investigate the genetic diversity of the Brazilian humpback whale population based on the analysis of genotypes constructed from 9 microsatellite loci for 275 individual whales sampled at 2 subregions (Abrolhos Bank, $n = 229$; Praia do forte, $n = 46$) off the Brazilian coast. We also investigated the existence of temporal (annual) structure, whether genetic differentiation exists between these 2 subregions, and if humpback whale associations are based on kinship. Molecular sexing was performed to verify sexual proportion in the population. Finally, we also investigated the relatedness between 2 humpback whales sampled off South Georgia Islands and the Brazilian whales. Bahia and Espírito Santo States, where most of the sampling was done, represent approximately 93% of the BSA population, according to the results of the aerial surveys that were performed by Andriolo and colleagues in 2005 (Andriolo A, personal communication). Thus, the present study covers

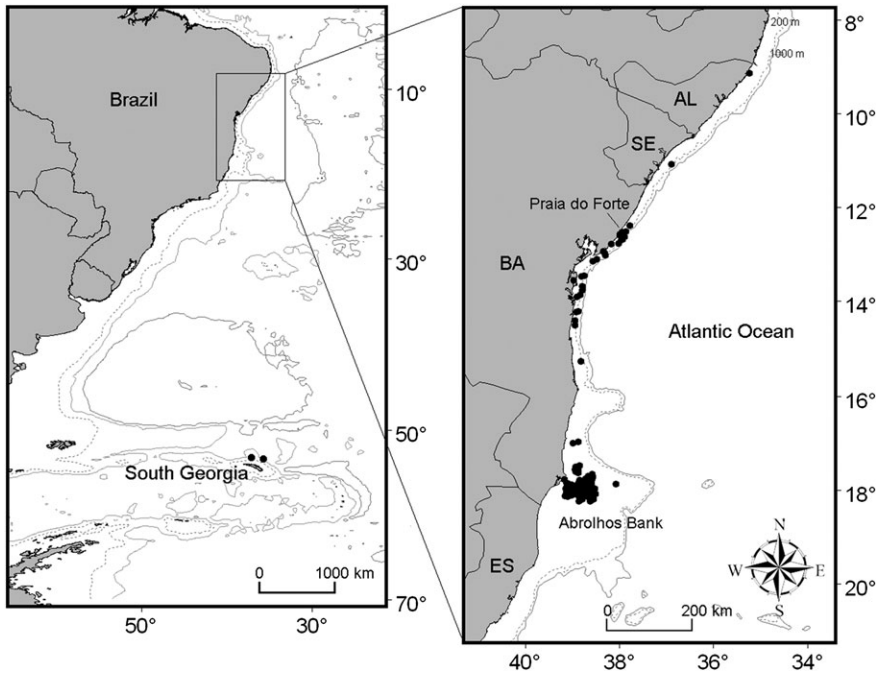

Figure 1. Map of the surveyed areas. Sampling sites comprise the breeding ground off the Brazilian coast (Abrolhos Bank and Praia do Forte) (inset) and the Antarctic feeding area off South Georgia, in the Scotia Sea. Black dots are geographic location of specimens sampled.

a significant portion of the Southwest Atlantic wintering ground.

Materials and Methods

Sample Collection, DNA Extraction, and Sex Determination

During 7 breeding seasons (July to November), from 1999 through 2005, a total of 291 tissue samples of humpback whales (1999, $n = 91$; 2000, $n = 7$; 2001, $n = 41$; 2002, $n = 23$; 2003, $n = 58$; 2004, $n = 32$; 2005, $n = 39$) were obtained along the coast of the Brazil (1999–2001 samples were reported previously in Engel et al. 2008). Most skin samples ($n = 268$) were collected by biopsy dart procedure (Lambertsen 1987) mainly at 2 geographic locations off the Brazilian coast, the Abrolhos Bank ($n = 229$), in the southern Bahia and northern Espírito Santo States, and Praia do Forte ($n = 37$), northern Bahia, with additional samples collected in the coast of Sergipe ($n = 1$) and Alagoas ($n = 1$) States (northeastern coast of the Brazil) (Figure 1a). The remaining samples ($n = 23$) resulted from individuals stranded along the southern Bahia and northern Espírito Santo coast (Abrolhos Bank, $n = 16$), along the northern Bahia coast (Praia do Forte, $n = 6$) and in the Pernambuco coast (northeastern coast of the Brazil, $n = 1$). Additionally, during the feeding summer season in 2006, 2 skin biopsies were obtained 4 mi off South Georgia Islands (Figure 1b). Samples were kept in 70% ethanol or dimethyl sulfoxide, according to the protocol established by Amos and Hoezel (1990) and later stored at −20 °C until processed.

Only adult animals were sampled within the social groups. It was not always possible to biopsy all the individuals in a group due to the behavior of the animals, size of the group, or weather conditions. It was also not possible to completely avoid resampling the same animal. For each whale sampled, date, global positioning system coordinates, and group composition were recorded. The social groups were classified in 8 classes: singletons (S), mother–calf pairs (MC), mother–calf–escort (MCE), adult pairs (AP), groups of 3 or more individuals engaged (CG) or not (non-CG [NCG]) in competitive behaviors, and MC with more than 1 escort (CG with mother and calf [CGMC]) engaged in competitive behaviors, based on the behaviors described for this species (Clapham et al. 1992).

Genomic DNA of the additional 154 samples available for this study was extracted following Engel et al. (2008). Molecular sex determination was carried out by polymerase chain reaction (PCR) amplification followed by *Taq*I digestion of the ZFX/ZFY region of the sex chromosomes following the protocol of Palsbøll et al. (1992) modified by Bérubé and Palsbøll (1996). The Pearson chi-square test with Yate's correction was used to test the statistical significance of the sex ratio against the 1:1 expected ratio.

Microsatellite Genotyping

Initially, all samples were genotyped for 10 microsatellite loci: 7 dinucleotides (EV1Pm, EV37Mm, EV94Mm, and EV96Mm, Valsecchi and Amos 1996; 199/200, 417/418,

and 464/465, Schlötterer et al. 1991) and 3 tetranucleotides (GATA028, GATA053, and GATA417, Palsbøll et al. 1997). Forward primers were 5′-tailed with the M13 sequence (5′-CACGACGTTGTAAAACGAC-3′) that is used in combination with an M13 primer marked with fluorescence (FAM, HEX, and NED) (Boutin-Ganache et al. 2001). PCRs were carried out in 20 or 10 μl with the following conditions: 2.5–3.25 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 0.125 μM of reverse primer, 0.1 μM of M13 fluorescent primer, 0.0083 μM of the M13-tailed forward primer, 0.05 U of *Taq* DNA polymerase, 1× PCR buffer (Invitrogen), and 1 μl of DNA (~50 ng). All loci were amplified in separate reactions optimized from published profiles. The PCR products were pooled in 2 sets and genotyped on a MegaBACE 1000 automated sequencer (Amersham Biosciences), and the allele size in base pairs was identified with the software GENETIC PROFILER v. 2.2 (Amersham Biosciences).

To minimize genotyping errors, specific guidelines were followed during laboratory and scoring procedures. First, negative controls were run at the PCR step to control for contamination. Second, scoring was automated in GENETIC PROFILER, and allele sizing was subsequently checked by hand. Finally, the program MICRO-CHECKER v. 2.2.3 (Van Oosterhout et al. 2004) was used to identify possible nonamplified alleles (null alleles), large allele dropout, and scoring errors due to stutter peaks. The analysis indicated that null alleles may be present at locus 417/418 as also was suggested by the general excess of homozygotes for most allele size classes and by the significant ($P < 0.01$) combined probability of observed homozygote class frequencies using the binominal test. Thus, this locus was discarded from all analyses. No evidence of null alleles was seen at other loci. Further tests for errors in the data showed no evidence for stuttering or large allele dropout.

Genetic Variation

Searches for genotype identity were performed using the program GENECAP (Wilberg and Dreher 2004) that compares each multilocus genotype with all other genotypes within the data set to identify matching samples. Pairs of matching were further compared for sex and with photographic matches. The probability of identity statistic $P_{(ID)}$, the probability that 2 individuals within the population shared the same multilocus genotype by chance, was estimated using 2 different formulations (the Hardy–Weinberg [HW] $P_{(ID)}$ and a more conservative measure, the Sib $P_{(ID)}$) through the GENECAP program. Putative duplicated samples were excluded for the estimation of statistics of genetic variation and allele frequencies.

Genetic diversity was measured as the number of alleles per locus (K), the mean number of alleles per locus (allelic diversity, A), observed heterozygosity (H_O), and expected heterozygosity (H_E) under Hardy–Weinberg assumptions (Nei 1978) using FSTAT v. 2.9.3 (Goudet 2002) and GENEPOP v. 3.4 (Raymond and Rousset 1995). The program FSTAT was used to calculate the measure of F_{IS} of Weir and Cockerham (1984). The loci were tested for deviations from Hardy–Weinberg equilibrium (HWE) (Guo and Thompson 1992) and linkage disequilibrium using the program ARLEQUIN 3.11 (Excoffier et al. 2005), corrected for simultaneous comparisons with the sequential Bonferroni test (Rice 1989).

To evaluate whether the BSA population experienced historical demographic reduction, we used 2 methods implemented in BOTTLENECK v. 1.2.02 (Piry et al. 1999): 1) the test for a deficit of rare alleles in a sample of loci (graphic method) (Luikart et al. 1998) and 2) the test for excess of observed heterozygosity based on the 2-phase mutation (TPM) model (Di Rienzo et al. 1994) estimated based on 50 000 replications and tested using a one-tailed Wilcoxon signed-rank test. A third method is based on the M value (calculated using the program AGARst, Harley 2001), where bottlenecked populations have values of $M < 0.68$ (Garza and Williamson 2001).

Genetic Structure

To evaluate the existence of temporal genetic structure in BSA caused by any structured annual migrations, we tested 3 alternative schemes for the analyses of molecular variance (AMOVA) using ARLEQUIN: 1) 1 year interval (1999–2001–2003 and 2000–2002–2004), 2) 2 years interval (1999–2002, 2000–2003, and 2001–2004), and 3) 3 years interval (1999–2003, 2000–2004, and 2001–2005). These schemes correspond to the hypotheses that some humpback whales may return to the breeding ground with 1, 2, or 3 years intervals, respectively. For this analysis, we used only individuals sampled in the Abrolhos Bank.

Geographic structure between the 2 subregions off the Brazilian coast (Abrolhos Bank and Praia do Forte) was investigated by pairwise F_{ST} (Weir and Cockerham 1984) in ARLEQUIN. The 3 samples obtained in the northeastern coast of Brazil (northern from Praia do Forte) were regarded as belonging to the Praia do Forte subregion.

We evaluated potential population subdivision in our samples using a Bayesian model–based clustering method implemented in STRUCTURE 2.1 (Pritchard et al. 2000). We conducted 4 independent runs for each K (number of cluster) between 1 and 6 using no prior information, the admixture model, and the correlated allele frequencies model. Burn-in length and length of simulation were set at 500 000 and 1 000 000 steps, respectively. Additionally, we used the program STRUCTURAMA (Huelsenbeck and Andolfatto 2007) that infers population genetic structure from genetic data by allowing the number of populations to be a random variable with a Dirichlet process prior (Pella and Masuda 2006). We ran 1 000 000 cycles and we let α (the prior mean of the number of populations) be a random variable. The first 100 000 cycles were discarded as burn-in.

Relatedness Analysis

The associations (affiliation between 2 individuals) for each social group containing a minimum of 2 sampled individuals

without known calves (i.e., MCE, AP, CG, NCG, and CGMC) were classified into 3 types according to the sex of the animals: female–female (F-F), male–female (M-F), and male–male (M-M). The within-population and within-group coefficient of relatedness r_{LR} (Lynch and Ritland 1999) were calculated for each association using the program IDENTIX v. 1.1 (Belkhir et al. 2002), which computes relatedness between any 2 individuals by comparing the alleles shared by these individuals with the allele frequency in the population. The significance of the difference between the average genetic relatedness within groups and within the population as a whole was evaluated using the 2-sample randomization test with 10 000 randomizations in the program RT v. 2.1 (Manly 1997). A similar procedure was applied to test if same sex individuals (M-M and F-F associations) are more related than individuals from different sex (M-F associations) within the groups.

Parent–offspring or full-sibling relationships within the BSA plus 2 whales sampled off South Georgia Islands (only on individuals genotyped for all loci) were searched using the program AGARst (Harley 2001). The matches yielding a high relatedness coefficient ($r > 0.4$) for r_{LR} and r_{QG} (Queller and Goodnight 1989) implemented in IDENTIX and KINSHIP 1.3.1 (Goodnight and Queller 1999), respectively, were considered likely parent–offspring or full siblings. These highly related pairs were tested using a maximum-likelihood approach implemented in KINSHIP. Three hypotheses were tested: (I) parent–offspring relationship was the primary hypothesis against the null hypothesis of unrelated individuals, (II) full-sibling relationship against the null hypothesis of unrelated individuals, and (III) parent–offspring relationship against the null hypothesis of full-sibling relationship. The significance level was calculated by simulating 10 000 pairs of individuals using the primary hypothesis settings and the observed allele frequencies and determining the ratio needed to reject the null hypothesis with $P = 0.05$, 0.01, and 0.001.

We also compared the mtDNA haplotypes available in the previous analysis of the 139 samples (Engel et al. 2008) for individuals from putative female–female and male–female matches. Shared haplotypes add weight to the parent–offspring or full-sibling association, whereas different haplotypes either reject the relationship (e.g., 2 females) or suggest another relationship (e.g., a male and female with different haplotypes could be a father with his daughter but not a mother and her son).

Results

Genetic Variability

Two hundred and ninety three samples had data in 6 or more loci. Individual multilocus genotypes were on average 97.9% complete. The power of these loci to discriminate between individuals was very high; HW $P_{(ID)}$ was 2.32×10^{-12}, and the most conservative measure, Sib $P_{(ID)}$, was 8.98×10^{-5}. Thus, different samples, which produced duplicated multilocus genotypes, can be assumed with high confidence to represent the same individual and were excluded from further analyses. Based on genotype identity and accessory information such as photographic and gender matches, the genotypes were assigned to 277 different individuals (Abrolhos Bank, $n = 229$; Praia do Forte, $n = 43$; northeastern coast of Brazil, $n = 3$; and South Georgia, $n = 2$). Given the fact that the 2 individuals sampled off South Geogia were used only in the relatedness final analysis, the remaining individuals ($n = 275$) were used in all analyses. Most ($n = 13$) of the 16 replicate samples were biopsied twice in the same group (resampling of same animal) due to the error sampling. Three replicate samples were resightings in the Abrolhos Bank, 1 female resampled in the same year (after 12 days), and the others were 2 resightings of another female with 2-year intervals. The 3 samplings of the latter female were in 2000, in a group of 4 adults engaged in competitive behavior (CG); 2002, in an MCE group; and 2004, in a group of 3 adults engaged in competitive behavior (CG).

Table 1. Summary statistics for 9 microsatellite loci genotyped for humpback whale population off Brazil

Locus	Rep	Allele range	K	H_O	H_E	F_{IS}
GATA 28 ($n = 273$)	4	143–203	15	0.637	0.624	−0.021*
GATA 53 ($n = 273$)	4	231–287	12	0.777	0.820	0.053*
GATA 417 ($n = 264$)	4	186–280	18	0.905	0.906	0.001*
199/200 ($n = 273$)	2	102–118	8	0.590	0.574	−0.028*
464/465 ($n = 267$)	2	130–152	9	0.573	0.606	0.055*
EV1Pm ($n = 273$)	2	123–129	4	0.527	0.519	−0.017*
EV37Mn ($n = 270$)	2	190–224	18	0.904	0.922	0.020*
EV94Mn ($n = 268$)	2	201–221	11	0.813	0.815	0.002*
EV96Mn ($n = 272$)	2	185–215	15	0.871	0.871	−0.001*
Total	—	—	110	6.597	6.657	0.064
Average	—	—	12.22	0.733	0.739	0.007

n, Number of individuals for each locus; Rep, repeat motif length in base pairs; F_{IS}, inbreeding coefficient (*$P > 0.005$ based on 180 randomizations).

All 9 microsatellite loci were highly polymorphic and showed genetic variability in the Brazilian humpback whales (Table 1), with the number of alleles per locus ranging from 4 (EV1) to 18 (GATA417 and EV37) with a mean of 12.1. The observed (H_O) and expected (H_E) heterozygosity ranged from 0.527 (EV1) to 0.904 (EV37) with a mean of 0.733 and from 0.519 (EV1) to 0.922 (EV37) with a mean of 0.739, respectively (Table 1). Population-wide F_{IS} values were low for the most of loci (below 0.05), except for the locus GATA053 ($F_{IS} = 0.053$) and for the locus 464/465 ($F_{IS} = 0.055$), but these values were not significant (Table 1). No significant deviation of HWE expectations was seen at the 9 loci. Pairwise comparisons of allele frequencies revealed no significant linkage disequilibrium after Bonferroni correction.

BOTTLENECK analysis did not provide evidence for a recent population decline. The test for excess heterozygosity based on the TPM model was not significant ($P = 0.455$), and the distribution of allele frequencies was clearly L shaped (Figure 2). Furthermore, the M index for the 9

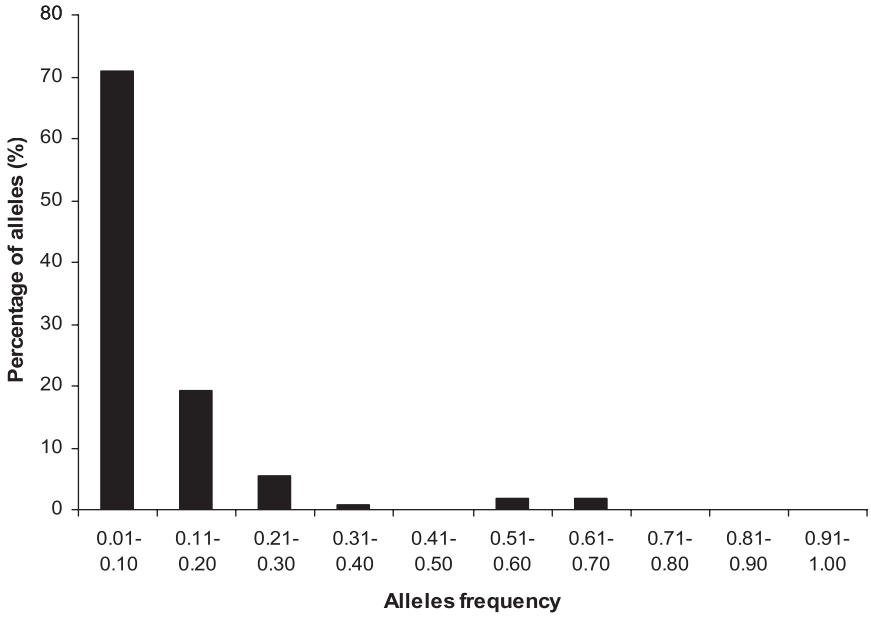

Figure 2. Allele frequency distribution in the humpback whale Brazilian population. The bars represent the percentage of all alleles detected in each allele frequency class.

polymorphic loci ($M = 0.85 \pm 0.20$) was higher than the critical value ($M = 0.68$) indicated by Garza and Williamson (2001).

Population Genetic Structure

The AMOVA results for the 3 alternative schemes of structured annual migrations devised to test the existence of temporal genetic structure in the BSA did not find evidence for differentiation between the years in this breeding ground, as almost all genetic variation was apportioned within the populations (Table 2). Likewise, the pairwise F_{ST} between the 2 subregions (Abrolhos Bank and Praia do Forte) within BSA was very small ($F_{ST} = -0.00241$) and nonsignificant ($P = 0.918$).

Table 2. AMOVA results for 3 alternative schemes of structured annual migrations in the Abrolhos Bank subregion

	Source of variation		
	Among intervals	Within intervals	F_{ST} (P)
1 year intervals			
1999, 2001, and 2003 2000, 2002, and 2004	0.08	99.92	0.00076 (0.30)
2 years intervals			
1999 and 2002 2000 and 2003 2001 and 2004	−0.00	100.00	−0.00003 (0.49)
3 years intervals			
1999 and 2003 2000 and 2004 2001 and 2005	−0.12	100.12	−0.00122 (0.83)

For the STRUCTURE clustering analyses, the highest posterior probability of the genetic data, consistent between the 4 replicate runs, was obtained when the individuals were grouped in a single population ($K = 1$, ln P [X/K] = −9791.4; Supplementary Figure S1). A similar result was obtained with STRUCTURAMA ($K = 1$, P [K/X] = 0.9940), where all the pairs of individuals shared high posterior probabilities (>0.9) of being grouped in the same population and their Bayes factors were >10 (167.976), supporting the hypothesis that all individuals belong to the same population.

Molecular Sex Determination

Sex was determined for 253 individuals resulting in 140 males (55.3%) and 113 females (44.7%). The observed overall proportion of 1.2:1 males to females did not differ significantly ($\chi^2 = 1.12$, $\alpha = 0.05$, degrees of freedom = 1) from a 1:1 sex ratio.

Relatedness within Social Groups

A total of 37 social groups were surveyed, of which 22 (59.4%) had all group members sampled (2 or 3 individuals). The groups contained 30 females and 46 males, totaling 76 whales. Table 3 shows the number of individuals (n), groups (N), and associations (A) analyzed by group class and sex combination. Among the sampled pairs of adults, more than half were M-F associations (70.0%) followed by M-M (27.5%), and F-F (2.5%) associations. The only instance of F-F association was found in an MCE group (all other escorts were males).

The mean within-group pairwise coefficient of relatedness ($r = -0.0588 \pm 0.1000$) was lower than the mean

Table 3. Number of individuals (*n*), groups (*N*, minimum of 2 individuals), and associations (*A*, affiliation between 2 individuals within group) included in the genetic relatedness analysis for all group classes and sex combinations

Association type	*n* (%)	*N* (%)	*A* (%)
F-F			
MCE	2 (2.6)	1 (2.7)	1 (2.5)
F-M			
MCE	20 (26.4)	10 (27.0)	10 (25.0)
CG	10 (13.2)	5 (13.5)	5 (12.5)
CGMC	8 (10.5)	4 (10.8)	4 (10.0)
AP	18 (23.7)	9 (24.4)	9 (22.5)
M-M			
CG	13 (17.1)	6 (16.2)	7 (17.5)
NCG	3 (3.9)	1 (2.7)	3 (7.5)
CGMC	2 (2.6)	1 (2.7)	1 (2.5)
Total	76 (100.0)	37 (100.0)	40 (100.0)

F, female; M, male.

within-population pairwise relatedness ($r = -0.0038 \pm 0.1058$), but this difference was not significant ($P = 0.091$). The mean relatedness for F-M and M-M classes of sex comparison (F-F comparison was excluded because it presents a single association) were −0.0721 (±0.1310) and −0.0682 (±0.1030), respectively. The difference between the mean pairwise relatedness of the different sex classes within the groups was not significant ($P = 0.923$). AGARst identified 76 matches (at least 1 allele shared at each locus), of which 17 yielded a high relatedness coefficient ($r > 0.4$) in the analysis of the 2 programs. Most of these 17 highly related pairs were found in the Abrolhos Bank, 4 pairs were sampled in the same years (1999, 2 pairs; 2001, 1 pair; and 2003, 1 pair), but the individuals belong to distinct social groups. Three of the 17 pairs have been formed by individuals found in different subregions (2 pairs sampled in 2003 in the Abrolhos Bank and Praia do Forte and 1 pair formed by 1 individual sampled off Abrolhos Bank in 2001 and other sampled off South Georgia Islands in 2006). All the 17 pairs presented high and significant levels ($P < 0.01$ or $P < 0.001$) of relatedness compatible with parent–offspring or full-sibling relationships (hypothesis I and II, respectively; Table 4). Fourteen pairs present significant parent–offspring relationship when full sibling was the null hypothesis (hypothesis III; Table 4), as expected because full sibling was very unlikely considering that each female invariably gives birth to a single calf and the absence of any lasting bounds between mating individuals between breeding seasons (Clapham and Palsbøll 1997).

We have mtDNA sequence for 5 of the related pairs and between them 4 female–female pairs had identical haplotypes, adding weight to a parent–offspring association, whereas the other female–female pair had different haplotypes, refuting a parent–offspring relationship (Table 4). Significantly, one of the related pairs included a female sampled off Abrolhos Bank in 2001 (ID = 177) and a female sampled near South Georgia Islands in 2006 (ID = GS02), with identical mtDNA haplotypes.

Discussion

Genetic Diversity and Demography

Our data reveal high nuclear diversity in the humpback whale population that overwinter off the Brazilian coast

Table 4. Putative parent–offspring or full-sibling relationships and their respective relatedness coefficients r_{QG} and r_{LR} calculated in KINSHIP 1.3.1 and IDENTIX, respectively

ID	Sex	r_{QG}	r_{LR}	Shared mtDNA haplotype	Hypothesis I	Hypothesis II	Hypothesis III
58/300	M-M	0.43	0.45	N/C	***	**	**
64/69	F-F	0.55	0.53	Yes	***	***	*
64/188	F-M	0.44	0.44	N/C	***	***	**
99/128	F-F	0.53	0.48	Yes	***	***	*
100/153	F-F	0.55	0.55	Yes	***	***	**
119/327	M-M	0.86	0.66	N/C	***	***	NS
127/310	F-F	0.40	0.41	N/C	***	**	***
153/366	F-M	0.48	0.42	N/C	***	***	**
177/GS02	F-F	0.40	0.64	Yes	***	***	**
195/197	F-F	0.42	0.53	No	***	***	**
205-02/363	M-F	0.42	0.50	N/C	***	***	*
282/335	F-M	0.40	0.40	N/C	***	**	**
282/346	F-M	0.42	0.41	N/C	***	**	**
284/21	?-F	0.58	0.64	N/C	***	***	*
289/361	?-M	0.58	0.48	N/C	***	***	NS
302/327	F-M	0.48	0.48	N/C	***	**	*
321/10	F-M	0.46	0.42	N/C	**	**	NS

NS, not significant; ?, sex not determined.

Hypothesis I tested parent–offspring relationship against unrelated individuals, hypothesis II tested full-sibling relationship against unrelated individuals, and hypothesis III tested parent–offspring against full-sibling relationship. Yes, identical mtDNA haplotype; No, different mtDNA haplotype; N/C, at least 1 mtDNA haplotype absent.

*$P < 0.05$, **$P < 0.01$, and *** $P < 0.001$ based on 10 000 simulated pairs of individuals.

(BSA), in agreement with its mtDNA high variability (Engel et al. 2008) and similar to that of other large baleen whales, such as fin whales (Bérubé et al. 1998), sei whales (Kanda et al. 2006), Bryde's whales (Kanda et al. 2007), and right whales (Waldick et al. 2002). This high diversity is in agreement with the other humpback whale breeding areas studied. For the same loci analyzed in this study, Pomilla and Rosenbaum (2006) found in the breeding areas of regions B and C a high mean observed heterozygosity for both Gabon (H_O = 0.745) and Northeastern Madagascar (H_O = 0.751). A similar high H_O was found in the 2 breeding areas in region E, Eastern Australia (H_O = 0.726; Valsecchi et al. 2002), and New Caledonia (H_O = 0.768; Garrigue et al. 2004). Cerchio et al. (2005) reported H_O of 0.710 in the Mexican Pacific breeding area based on 13 loci.

Before the exploitation by modern whaling, the BSA estimated population size was nearly 24 500 (95% confidence interval [CI] = 22 804–31 220) individuals (Zerbini, Ward, et al. forthcoming). This stock reached its lowest numbers in the late 1950s when there were less than 500 individuals (95% CI = 152–3 687), nearly 2% (95% CI = 0.7–11.9%) of its historical size (Zerbini, Ward, et al. forthcoming). Despite this severe depletion, no significant signal of genetic bottleneck was detected in this population with the different procedures used here. This result is similar to other studies that tested for genetic bottlenecks in large baleen whale species that had been the target of commercial whaling worldwide (e.g., sei whale, Kanda et al. 2006; bowhead whale, Rooney et al. 1999; and right whale, Waldick et al. 2002), corroborating the prediction that commercial whaling did not last long enough to significantly reduce the nuclear genetic diversity for most of these species (Amos 1996; Engel et al. 2008).

Population Genetic Structure

Notwithstanding that temporal differences in occupancy of wintering areas may exist in some populations (Stevick et al. 2003) or that humpback whales may occasionally change destinations between different wintering seasons after migration to shared feeding grounds (Darling and Cerchio 1993; Garrigue et al. 2002), our results do not present evidence for any temporal genetic structure in BSA considering the years of sampling, with up to 3 years of interval.

The geographic structure analyses (pairwise F_{ST}) showed nonsignificant differentiation between samples collected at the 2 subregions with sufficient sample size. Moreover, both Bayesian clustering analyses yielded the highest posterior probability of the data when all individuals were grouped into a single population. The nonsignificant differentiation between the Abrolhos Bank and Praia do Forte off the Brazilian coast agreed with matched photographic data between the 2 locations (Freitas et al. 2004), which together with the increasing sightings further north on the Abrolhos Bank, indicating the likely return to areas occupied by the species before the depletion of this stock (Martins et al. 2001; Zerbini et al. 2004; Andriolo et al. 2006).

Sex Ratio and Relatedness of the Groups

The proportion of males and females reported in tropical catches during commercial whaling (Mackintosh 1942; Chittleborough 1965) and in other breeding grounds has shown a high bias toward males (Baker et al. 1994, 1998; Brown et al. 1995; Olavarría et al. 2007). By contrast, the sex ratio observed on the feeding grounds in a contemporary study is approximately 1:1 (Clapham et al. 1995) or biased toward females during commercial whaling (Chittleborough 1965). Interestingly, the sex ratio in the Brazilian breeding ground did not differ significantly from 1:1, although an overall higher number of males was found (55.3% males). We do not know why the BSA is in disagreement with other breeding ground studies because their convincing explanations included patterns of migration that should be common to all winter breeding grounds (Brown et al. 1995; Craig and Herman 1997). Brown et al. (1995) suggest that not all females migrate to the winter grounds each year due to the energetic costs of reproduction and migration. In addition, immature females or sexually mature, but physically immature, may remain on the feeding grounds all year to maximize growth (Brown et al. 1995). Furthermore, female residency on the breeding ground is shorter than that of males and is temporally alternated among females, resulting in an excess of males in this region (Craig and Herman 1997). This difference is due to the short female estrous relative to residency time and is likely to be broadly asynchronous among females (Cerchio et al. 2005). Another explanation is that this is a strategy aimed at increasing the female's probability of reproductive success by maximizing the time spent on the feeding grounds and so accumulating energy that will be spent in migration and lactation for long periods without food sources (Craig and Herman 1997).

Most of the association types were of male–female pairs, in agreement with observations in other breeding grounds, where most of the groups found are M-F pairs in dyads or in large CGs (Pomilla and Rosenbaum 2006). All dyads were formed by unrelated males and females, generally associated with courtship and mating behavior. As male–female dyads also occur on the feeding grounds, Clapham (1993) suggested that bonds of males with females before the breeding season might increase a male's reproductive success, although this hypothesis has not been tested yet. With a single exception, all escorts were males, suggesting that escorting behavior is related to mating opportunities, that is, males in temporary groups competing for female access, indicating that males secure bonds with females near estrus in the migration to wintering areas or they guard the mate in the migration to feeding areas (Valsecchi et al. 2002).

In our study, a single social group had more than 1 female, found in an MCE group of unrelated individuals. Associations of females are known on the feeding grounds (Clapham 1993; Weinrich et al. 2006), but they are rarely observed during the migrations and on the breeding grounds (Valsecchi et al. 2002; Pomilla and Rosenbaum

2006). On feeding grounds, this behavior seems to indicate cooperation during feeding (Clapham 1993). Although a study on feeding grounds in the southern Gulf of Maine indicated that females with the same mtDNA haplotype are more often associated than predicted by chance, these specimens were not tested for relatedness. These results could be explained by spatial and temporal effects due to the influence of maternal experience on feeding styles and prey preferences (Weinrich et al. 2006). Our results corroborate the low frequency of female associations on breeding grounds as observed elsewhere. For example, none of the 380 groups sampled on the Hawaii breeding ground included more than 1 adult female (Craig et al. 2002), and only 2 and 10 of the 270 groups total analyzed in the Northeastern Madagascar and Gabon breeding grounds, respectively, presented associations between females (Pomilla and Rosenbaum 2006). None of these associations showed strong evidence for relatedness between females, with a single exception, where 2 females shared the same mtDNA haplotype and were related at the level of half siblings in Madagascar. Pomilla and Rosenbaum (2006) suggested that females could travel together from the same feeding area to the breeding grounds or they could associate on arrival, diluting their chance of harassment by males.

Despite the interactions among males within CGs being typically agonistic, with more than 2 males competing for a single female, pairs of cooperating males have been observed within these groups (Clapham et al. 1992). Such observations suggest a social bond based on kin, where related males cooperating enhance the reproductive success of relatives. However, our results provided no evidence for this, as pairs of males within CGs were not more related on average than random individuals in the population. This is in agreement with the associations among males in the Gabon breeding ground, where the males showed low mean relatedness (Pomilla and Rosenbaum 2006). Alternatively, a reduced mean relatedness within these groups could be explained by kin avoidance to minimize competition among relatives (Pomilla and Rosenbaum 2006).

Finally, the mean relatedness within social groups was not significantly different than the mean relatedness within the whole population, indicating no support for the hypothesis that social groups are formed by related individuals (other than mother–calf), corroborating other studies (e.g., Clapham and Palsbøll 1997; Valsecchi et al. 2002; Cerchio et al. 2005; Pomilla and Rosenbaum 2006). All related pairs were composed of individuals sampled in different groups, indicating that social groups are not formed by related individuals.

The putative parent–offspring or full-sibling relationships found in this study provide the first information on relatedness of Brazilian humpback whales. Most of these pairs were found in Abrolhos Bank, which might be expected due to its greatest sample size. The 2 pairs of highly related individuals sampled in Abrolhos Bank and off Praia do Forte subregions in the same year (2003) are concordant with the lack of genetic structure between these 2 subregions.

Finally, an interesting finding was a putative parent–offspring relationship between 1 female sampled off Abrolhos Bank in 2001 and 1 female sampled near South Georgia Islands in 2006, both with the same mtDNA haplotype. This result is compatible with the migratory link between BSA and South Georgia Islands feeding ground as indicated by satellite telemetry (Zerbini, Andriolo, et al. 2006) and mtDNA (Engel et al. 2008). This is further corroborated by recent photographic matches between whales from Abrolhos Bank and Shag Rocks off South Georgia Islands (Stevick et al. 2006) and between the Abrolhos Bank and the Sandwich Islands (Engel and Martin forthcoming). However, a larger sampling effort off South Georgia/South Sandwich Islands is necessary to better test genetically this hypothesis.

Conservation Implications

Our analyses showed high genetic variability and no evidence of significant genetic bottleneck for the humpback whales that breed off the Brazilian coast. These findings indicate that this population has a good chance of long-term viability, something that is indicated by signs of population recovery in this breeding stock (Engel MH, unpublished data). Conservation efforts must therefore focus on the maintenance of this genetic diversity through demographic stability and habitat protection. Furthermore, it is very important to monitor not only the level of genetic diversity but also changes of life-history traits.

A management concern is the risk of depleting genetically distinct populations when exploiting the stock under the assumption of a single population. Exploitation of migratory whales is mainly conducted on feeding grounds where genetically distinct populations might co-occur (Hoelzel 1998). The identification of management units needs to account for temporal and spatial factors. Moreover, the issue of potential structure also has important implications for understanding the biology and demography of this species. A better understanding of the pattern and rates of gene flow, as well as an accurate identification of population admixture, are important for conservation and management of humpback whale populations. Our results support the hypothesis that the Southwestern Atlantic humpback whale is a single population with no evidence of spatial or temporal differentiation.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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