

Insights about the genetic diversity and population structure of an offshore group of common bottlenose dolphins (*Tursiops truncatus*) in the Mid-Atlantic

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ABSTRACT. Although the genus *Tursiops* has a worldwide distribution and is globally well-studied, some dolphin populations continue to face high risks of decline. Hence, it is necessary to assess the genetic diversity and structure of this genus to properly assess its conservation status and to implement appropriate management actions. In Brazil, genetic studies on this group remain rare, particularly for

populations inhabiting offshore waters. Saint Peter and Saint Paul Archipelago (SPSPA) is a small group of islands located in the Mid-Atlantic Ridge, where recent studies of the *Tursiops truncatus* group indicate that individuals are resident throughout the year around the archipelago, exhibiting considerable site fidelity. A previous study with this group indicated that the individuals form an isolated population. To test this hypothesis, and describe the genetic diversity of SPSPA individuals, we assessed 12 microsatellite loci and a portion of the mitochondrial control region. Bayesian analysis revealed that SPSPA bottlenose dolphins form a unique population. In a phylogeographic perspective, we found that individuals from SPSPA shared mtDNA haplotypes with inshore and offshore individuals from North Atlantic, suggesting that they are not currently isolated from their conspecifics. Mirroring mtDNA findings, microsatellite analysis revealed that most of the pairs of individuals sampled seem to be unrelated (83.8%) and no indication of inbreeding, what would be expected if a small population such as SPSPA was reproductively isolated.

Key words: Microsatellite; Control region mtDNA; Offshore islands; Conservation status

INTRODUCTION

Characterizing genetic diversity and structure is fundamental for properly assessing the conservation status of populations and groups of individuals. High levels of genetic diversity may help to maintain a population's evolutionary potential (Frankham et al., 2005). This is especially true for cetacean species, as they are currently exposed to a variety of anthropogenic impacts, and are particularly vulnerable to habitat degradation and climatic changes (Simmonds and Isaac, 2007). Bottlenose dolphins are not an exception to this pattern (Currey et al., 2009; Hammond et al., 2012). The genus *Tursiops* has a worldwide distribution from temperate to tropical seas, showing great plasticity to occupy different ecosystems, in addition to being a well-studied marine mammal globally (Segura et al., 2006; Quérouil et al., 2007; Hammond et al., 2012). Yet, this genus is listed as Least Concern in the last IUCN assessment (Hammond et al., 2012), with some populations facing high risks of decline (Currey et al., 2009).

Tursiops truncatus has 2 distinct ecotypes, defined as inshore and offshore. These ecotypes have been well documented for the North Atlantic and Caribbean, where clear differences between them have been confirmed based on multiple phenotypic, genotypic, and ecological data (Segura et al., 2006; Caballero et al., 2012). Studies conducted in different regions have found that inshore populations present low levels of genetic diversity and high genetic differentiation over small geographic scales. In comparison, offshore dolphins seem to exhibit higher genetic diversity and lower potential for genetic population structure, even across considerable spatial scales (Quérouil et al., 2007; Tenzanos-Pinto et al., 2009).

Studies have extensively documented the population and social structure of bottlenose dolphins worldwide, leading to the development of management recommendations for conservation (Caballero et al., 2012). Despite this species being subject to increasing human pressure

in southern Brazil, primarily due to by-catch in gillnets (P. Fruet, unpublished data) and pelagic longline fishery (Perrin et al., 2011), genetic studies are still lacking, particularly in offshore waters.

The Saint Peter and Saint Paul Archipelago (SPSPA) is a small group of islands located on the Mid-Atlantic Ridge, straddling the northern and the southern hemispheres (0°55'N, 29°20'W). The islands are located 1010 km from the northern Brazilian coast and 1800 km from Guinea Bissau, in the African continent. The waters surrounding the islands exhibit relatively high productivity (Lessa et al., 1999); hence, the SPSPA comprise an important fishing ground for the small scale Brazilian fleet from northeastern Brazil (Vaske Jr. et al., 2005). Analyses show that on average the number of new photo-identified individuals stabilized after the fifth day of survey effort in each one of the expeditions, considering 15 days of field trips by expedition (Genoves et al., 2010), indicating that this population has a stable number of individuals. Ott et al. (2009) investigated levels of variation in dolphin individuals from the SPSPA, using sequences of the mitochondrial DNA (mtDNA) control region. The authors suggested that the dolphin group represents a single and isolated population. However, the analysis of fine-scale genetic structure, such as bi-parental markers that are able to reveal genetic admixture, is still lacking. The relationship of the SPSPA bottlenose dolphin population with populations from the North Atlantic, including both inshore and offshore ecotypes, and mid-Atlantic remains unknown.

Therefore, in this study, we used microsatellite markers and the mtDNA control region to characterize genetic variation and evaluate the population structure of SPSPA bottlenose dolphin to determine their relationship with the North Atlantic ecotypes. We also provide the first biparental microsatellite markers combined with mtDNA genetic diversity description of this offshore dolphin group, inhabiting one of the most marine isolated areas in Brazil.

MATERIAL AND METHODS

Sample collection

The study area was the Saint Peter and Saint Paul Archipelago (SPSPA), Brazil, which is located in the North Atlantic Ocean (0°56'N, 29°22'W). The archipelago lies approximately 1100 km off the coast of Rio Grande do Norte, 627 km from the archipelago of Fernando de Noronha, and 1824 km from the African coast (Campos et al., 2010) (Figure 1). The archipelago contains 6 major islands and 4 small islets predominantly basaltic rocks, with an area of approximately 17,000 m² emerged and extreme points distance is about 420 m. The islands have steep slopes, maximum elevation above sea level 18 m, and are devoid of beaches, vegetation, and freshwater.

Samples were collected using a biopsy darting system, with darts and tips specially designed for small cetaceans by F. Larsen, Ceta-Dart (ACC darts, Easton 3-71, with floats and vanes/steel sampling heads M8/25 mm). Darts were attached to a wood biopsy pole of 1.5m long (modified from Bilgmann et al., 2007) and was used by researchers to collect skin samples when the dolphins approached the boat to bowride. The individuals were photographed and identified based on their marks or scars on the dorsal fin, whenever possible. Only adult individuals were sampled; however, mothers with calves were not sampled. The tissues obtained were either preserved in 100% ethanol or dimethyl sulfoxide (DMSO), and were then stored at -20°C until use.

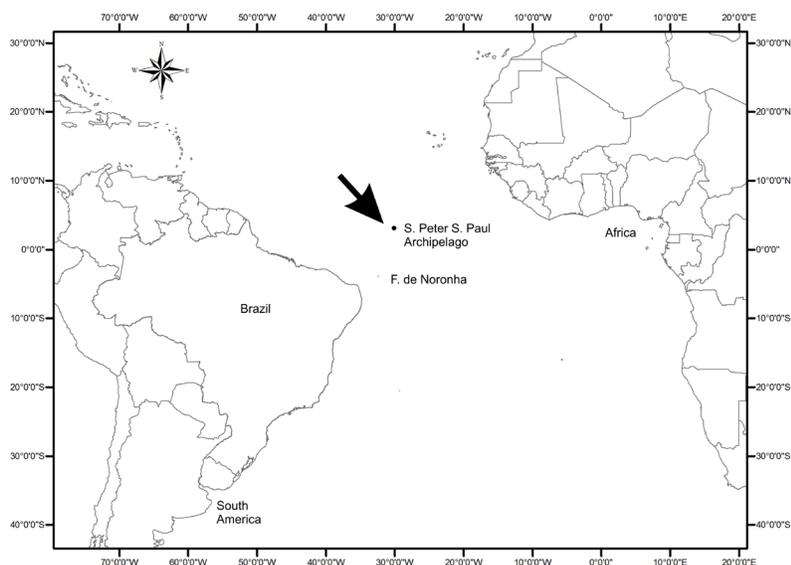


Figure 1. Study area, Saint Peter and Saint Paul Archipelago. Location indicated by an arrow.

DNA extraction, polymerase chain reaction (PCR) amplification, gender identification, and microsatellite genotyping

Total genomic DNA was extracted using the CTAB (cetyltrimethylammonium bromide) protocol (Doyle and Doyle, 1987). Individuals were sexed by the amplification of fragments from the SRY and ZFX genes via PCR (Gilson et al., 1998). All samples were PCR-amplified and genotyped for 12 cetacean microsatellite loci that have been previously described. These loci included 7 dinucleotides (EV1, Valsecchi and Amos 1996; D8, Shinohara et al., 1997; KW2, KW12, Hoelzel et al., 1998; MK6, MK8, MK9, Krützen et al., 2001) and 5 tetranucleotides (Tr4_80, Tr4_91, Tr4_105, Tr4_141, Tr4_E12, Nater et al., 2009). Amplifications were carried out according to PCR conditions reported in the literature for each locus. The genotypes were obtained by the ABI 3730 automated sequencer (Applied Biosystems Foster City, CA, USA). To define the allele sizes, we used the program PeakScanner 1.0 (<http://www.appliedbiosystems.com>).

Microsatellite analysis

The presence of null alleles, allele dropout, and scoring errors due to stutter was tested using MicroChecker 2.2.3 (Van Oosterhout et al., 2004). We used GENECAAP (Wilberg and Dreher, 2004) to search for identical genotypes, because it was not always possible to identify the individual sampled.

Genetic polymorphism was estimated as the number of alleles per locus (A), Allelic richness (A_R), observed heterozygosity (H_O), expected heterozygosity (H_E), polymorphic

information content (PIC), and allelic frequencies, using the FSTAT 2.9.3.2 (Goudet, 1995, 2001) and the ARLEQUIN 3.1 software (Excoffier et al., 2005). The ARLEQUIN 3.1 was also used to test for linkage disequilibrium (LD) and Hardy-Weinberg equilibrium (HWE). Significance levels ($\alpha = 0.05$) were corrected for multiple simultaneous comparisons with the Bonferroni's approach (Rice, 1989) to infer LD and departures from HWE. The FSTAT 2.9.3.2 was also used to calculate the inbreeding coefficient (F_{IS}).

To assess the existence of population structure, and to determine the most probable number of putative populations (K) that best explained the pattern of genetic variability, we used a Bayesian model based clustering method, as implemented in STRUCTURE 2.3.3 (Pritchard et al., 2000). We conducted 5 independent runs for each number of clusters (k) between 1 and 5, using the admixture model, no prior population information, 10^6 generations for burn-in, and 10^6 for sampling.

The multilocus genotypes of all sampled dolphins were used to estimate symmetrical pairwise genetic relatedness within the program ML-Relate, which calculates maximum likelihood estimates of relatedness and relationship (Kalinowski et al., 2006).

mtDNA control region sequencing and analysis

The mtDNA control region was amplified using the primers Dlp-1.5 and Dlp-5 (Baker et al., 1993), and following the PCR conditions described in Möller and Beheregaray (2001). The sequences were obtained in the ABI 3730 sequencer.

Additionally to the data generated in this study, control region sequences from the GenBank were also added in our analysis in order to evaluate the haplogroups of SPSPA samples. The MEGA 5.1 software (Tamura et al., 2011) was used to align the mtDNA sequences, while DNAsp 5 (Librado and Rozas, 2009) was used to perform nucleotide and haplotype diversity analysis. The Network 4.6.1.0 software (fluxus-engineering.com) was used to reconstruct relationships between haplotypes using the median joining method (Bandelt et al., 1999).

RESULTS

Samples and sex ratios

Between 2006 and 2012, we collected 19 bottlenose dolphin skin biopsy samples during some of the 14 expeditions to the SPSPA, that were carried out in a medium of 2 per year (1 to 4) in the follow years (months): 2006 (May and June), 2007 (December), 2008 (January and October), 2009 (January, May, September, and December), 2010 (March and July), 2011 (January and June), and 2012 (August).

GENECAP identified 2 re-sampling individual; thus, we excluded these samples from all analyses. From the remaining 17 samples, we identified 6 females and 11 males, with a sex ratio of 1:1.83, respectively. These 17 samples represented more than half of the individuals exhibiting site-fidelity to the SPSPA during the field trips (Hoffmann LS, personal communication).

Microsatellite genetic diversity, population structure, and parentage

The locus Tr4_141 was monomorphic for the surveyed population, presenting only

the allele 236. All of the other 11 loci were polymorphic, containing 3-7 alleles per locus, and an AR of 2.882-6.86 (mean: 4.47). Mean H_E was 0.64 (range 0.43-0.78), mean H_O was 0.59 (range: 0.47-0.70) (Table 1). None of the loci showed evidence of null alleles, nor scoring errors caused by stuttering or allele dropout. Mean PIC value was 0.56, and only 3 loci (Tr4_80 Tr4_105 Tr4_E12) had values below 0.5 (Table 1). In addition, all loci were in HWE after Bonferroni's correction (Table 1).

Table 1. Characterization of microsatellites for *Tursiops truncatus* in Saint Peter and Saint Paul Archipelago for 12 microsatellite loci.

Locus	N	Alleles	AR	PIC	H_O	H_E	P	F_{IS}
D8	17	7	6.86	0.734	0.647	0.788	0.04825	0.183
Ev1	17	5	4.754	0.525	0.588	0.622	0.62077	0.056
Kw12	17	6	5.999	0.729	0.706	0.781	0.13163	0.099
Kw2a	17	3	3	0.577	0.529	0.672	0.07698	0.217
MK6	17	6	5.871	0.695	0.529	0.759	0.01002	0.309
MK8	17	6	5.861	0.6	0.706	0.654	0.93309	-0.082
MK9	15	5	5	0.558	0.6	0.614	0.23707	0.023
Tr4_80	17	3	2.882	0.416	0.647	0.544	0.46967	-0.197
Tr4_91	17	3	3	0.554	0.529	0.643	0.68404	0.182
Tr4_105	17	3	3	0.385	0.470	0.437	1	-0.08
Tr4_E12	16	3	3	0.478	0.625	0.554	0.85576	-0.132
Mean	-	4.545	4.475	0.5683	0.598	0.642	-	0.071

Locus name, number of individuals genotyped (N), number of alleles (alleles), allelic richness (AR), polymorphic information content (PIC), observed heterozygosity (H_O), expected heterozygosity (H_E), HWE P values, and inbreeding coefficient (F_{IS}).

The Bayesian population structure analysis performed by STRUCTURE showed evidence of just 1 population based on the individuals sampled from the SPSPA ($k = 1$) (Figure 2). Kinship analysis indicated the presence of 6 pair of full-siblings (4.4%), 14 pairs of half-siblings (10.3%), and 2 pairs of parent-offspring (1.5%). However, the majority of the pairs of individuals (83.8%) were unrelated. We identified 10 related pairs of males-males (7 half-siblings, 2 full-siblings, and 1 parent-offspring), 11 related pairs of female-males (7 half-siblings, 3 full-siblings, and 1 parent-offspring), and only 1 pair of female full-siblings (Table 2).

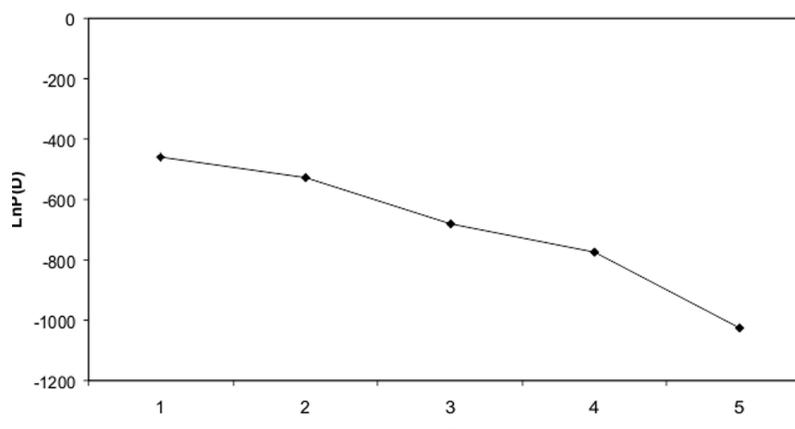


Figure 2. Bayesian clustering analyses by STRUCTURE, showing the LnP(D) values corresponding to each tested K. One is the most probable number of populations for the SPSPA population.

Table 2. Relationships between pair of individuals related to sex.

	HS	FS	PO	Total
Male-male	7	2	1	10
Female-male	7	3	1	11
Female-female	0	1	0	1
Total	14	6	2	22

HS = half-sibs, FS = full sibs and PO = parent/offspring.

mtDNA control region network and genetic diversity

In addition, we sequenced 457-bp mtDNA from the control region of the SPSPA individuals (GenBank KC896604 to KC896620). Sequencing produced 13 individuals with the H1 haplotype and 4 with the H2 haplotype (Table 3). Haplotype diversity was 0.38 (variance = 0.01, SD \pm 0.11) and nucleotide diversity was 0.0016.

We searched the GenBank database and found 264 sequences of *T. truncatus*. Thus, we built one network using all sequences (N = 264) and one network using only North Atlantic and Caribbean sequences (N = 98). However, because the sequences used in both networks were of different lengths, much information was lost. Finally, we built a haplotype network using samples just from the North Atlantic only and the SPSPA individuals (N = 57).

To reconstruct the network, we used 288 bp from a total of 57 sequences; of which, 17 sequences were from SPSPA individuals and 40 were from inshore and offshore North Atlantic individuals (Table 3). We observed that H30 from an inshore North Atlantic individual was intermediary to the H1 and H2 from SPSPA individuals. In addition, the H1 haplotype was shared by SPSPA individuals and both inshore and offshore North Atlantic individuals (Figure 3, Table 3).

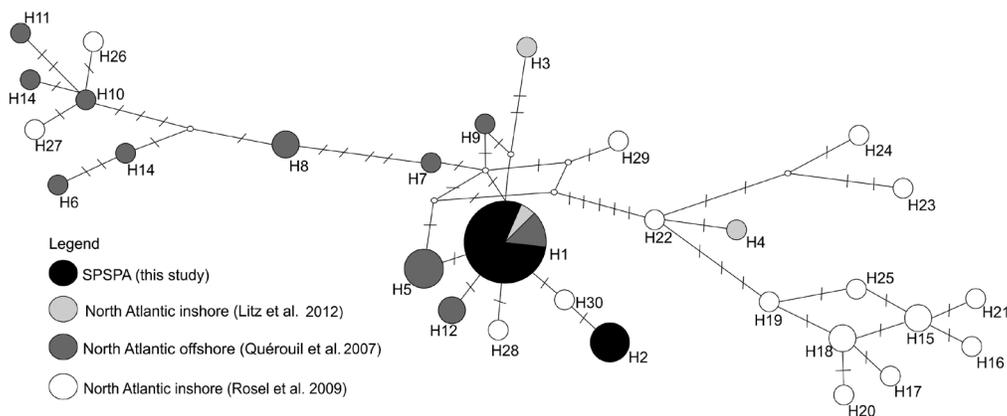


Figure 3. Minimum spanning network among haplotypes. The size of the circles is proportional to the total number of haplotypes observed. Sectors are proportional to the numbers of each haplotype observed in each population. Small white circles indicate either extinct or unsampled haplotypes.

Table 3. Sequences used in network haplotypes building showing GenBank registration.

GenBank ID	Area: ecotype	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	Reference	
ASPS001	SPSPA: offshore	X																														this study	
ASPS002	SPSPA: offshore	X																															this study
ASPS003	SPSPA: offshore	X																															this study
ASPS004	SPSPA: offshore	X																															this study
ASPS005	SPSPA: offshore		X																														this study
ASPS006	SPSPA: offshore		X																														this study
ASPS007	SPSPA: offshore	X																															this study
ASPS008	SPSPA: offshore	X																															this study
ASPS009	SPSPA: offshore	X																															this study
ASPS010	SPSPA: offshore		X																														this study
ASPS011	SPSPA: offshore	X																															this study
ASPS012	SPSPA: offshore	X																															this study
ASPS013	SPSPA: offshore	X																															this study
ASPS014	SPSPA: offshore	X																															this study
ASPS015	SPSPA: offshore	X																															this study
ASPS016	SPSPA: offshore	X																															this study
ASPS017	SPSPA: offshore		X																														this study
T101	AN: inshore			X																													this study
HQ33684.1																																	Litz et al., 2012
HQ33685.1	TT02																																Litz et al., 2012
HQ33686.1	TT03						X																										Litz et al., 2012
FJ768001.1	TT04							X																									Queroni et al., 2007
FJ768002.1	TT05								X																								Queroni et al., 2007
FJ768003.1	TT06									X																							Queroni et al., 2007
FJ768004.1	TT07										X																						Queroni et al., 2007
FJ768005.1	TT08											X																					Queroni et al., 2007
FJ768006.1	TT09												X																				Queroni et al., 2007
FJ768007.1	TT10													X																			Queroni et al., 2007
FJ768008.1	TT11														X																		Queroni et al., 2007
FJ768009.1	TT12															X																	Queroni et al., 2007
FJ768010.1	TT13																X																Queroni et al., 2007
FJ768011.1	TT14													X																			Queroni et al., 2007
FJ768012.1	TT15														X																		Queroni et al., 2007
FJ768013.1	TT16															X																	Queroni et al., 2007
FJ768014.1	TT17																X																Queroni et al., 2007
FJ768015.1	TT18																	X															Queroni et al., 2007
FJ768016.1	TT19																		X														Queroni et al., 2007
FJ768017.1	TT20																			X													Queroni et al., 2007
FJ768018.1	TT21																				X												Queroni et al., 2007
FJ768019.1	TT22																					X											Queroni et al., 2007
QS04040.1	TT23																						X										Rosel et al., 2009
QS04041.1	TT24																							X									Rosel et al., 2009
QS04042.1	TT25																								X								Rosel et al., 2009
QS04043.1	TT26																									X							Rosel et al., 2009
QS04044.1	TT27																										X						Rosel et al., 2009
QS04045.1	TT28																											X					Rosel et al., 2009
QS04046.1	TT29																												X				Rosel et al., 2009
QS04047.1	TT30																													X			Rosel et al., 2009

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DISCUSSION

This study presents the first comprehensive biparental microsatellite markers combined with mtDNA analyses of the common bottlenose dolphin offshore ecotype in the mid-Atlantic (at a Brazilian archipelago), and provides key information about the genetic diversity and population structure of this group.

A previous study using the mtDNA control region has suggested that bottlenose dolphins from SPSPA form a small and isolated population (Ott et al., 2009). However, the results obtained in the present study bring evidence that does not support this scenario. For example, the haplotype network analyses revealed that individuals from SPSPA shared haplotypes with inshore and offshore individuals from North Atlantic, indicating that this population apparently is not current isolated from their conspecifics. Supporting this finding we found no evidence of inbreeding for the population detected by the Structure analysis ($k = 1$), which would be expected if such small group of dolphins was in fact reproductively isolated. What strengthens this scenario is the fact that most of the pairs of individuals sampled seem to be unrelated (83.8%). Therefore our findings indicate that SPSPA bottlenose dolphins are part of a larger oceanic population where individual movements may provide opportunity for reproduce with individuals from other sources, preventing the formation of population structure and inbreeding. Our results support those found for offshore individuals in the Azores and Madeira Archipelago, where the authors (Qu erouil et al., 2007) observed a single oceanic population with no genetic structure with gene flow exchange. Caballero et al. (2012) compared the haplotype network of Caribbean bottlenose dolphins with the inshore and offshore individuals of the North Atlantic, and found that haplotypes were shared between the 2 ecotypes. Tezanos-Pinto et al. (2009) also observed that gene flow indicates long-distance dispersal among coastal and pelagic bottlenose dolphin populations worldwide.

Galov et al. (2011) assessed *T. truncatus* in the Croatian coast of the Adriatic Sea, using microsatellites, and suggested that the mean AR (6.835 ± 0.705) and mean H_E (0.692 ± 0.05) indicated a high level of genetic diversity. The mean H_E for SPSPA individuals (0.64258) was similar to this previous study (0.64258), although the mean AR was lower (4.475), possibly due to the small sample size.

In the SPSPA, we observed an adult female to male sex ratio of 1:1.83. In contrast, other studies obtained a sex ratio of approximately 1:1 (Hersh et al., 1990). The results obtained for the SPSPA population might be a consequence of sampling bias, as females accompanied by calves tended to avoid the boat, and were deliberately not sampled to minimize stress. This behavior is also observed in *Stenella longirostris*, where males and juveniles tended to follow the boat keeping females and calves away (Norris et al., 1985). Increasing sample size from resident SPSPA dolphins might help further elucidate these results.

We observed higher haplotype and nucleotide diversity compared to the previous study in the SPSPA, but these values were lower compared to those found for inshore Brazilian populations (Ott et al., 2009), or the Azores and Madeira (Qu erouil et al., 2007). This difference might be attributed to the sampled individuals at the SPSPA being part of a bigger population, rather than a lower genetic diversity *per se*. Hence, conducting more studies in adjacent areas might contribute toward understanding the actual diversity of bottlenose dolphins within a larger scale oceanic (meta-) population.

Our data indicate that SPSPA individuals are part of a larger oceanic population, be-

cause they did not show genetic or population structure or evidence of inbreeding; yet, photo identification (Hoffmann LS, unpublished data) indicates that the individuals from this group exhibit high site fidelity with the SPSPA surrounding waters. Dolphins inhabiting the open-ocean tend to form larger groups with less cohesive social units compared to coastal dolphins (Salinas-Zacarias, 2005). The formation of groups helps to improve hunting for prey and reduce predation risk (Gowans et al., 2008). Quérroul et al. (2007) observed resident dolphins year-round. This phenomenon might also occur at the SPSPA, helping to prevent the formation of population structure and inbreeding.

Future research in the SPSPA should be directed toward elucidating the relationships of this population with other groups/populations inhabiting other offshore islands around the SPSPA, such as Abrolhos and Atol das Rocas Archipelagos. Further studies using photo identification and parentage data should focus on clarifying the relationship between this group and the larger oceanic population to which they belong.

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