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Microsatellite data reveal fine genetic structure in male Guiana dolphins (*Sotalia guianesis)* in two geographically close embayments at south-eastern coast of Brazil

Claudia Hollatz · Leonardo Flach · C. Scott Baker · Fabrício R. Santos

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Abstract A large macrogeographic differentiation has been observed among *Sotalia guianensis* populations along the South American coast. However, no genetic structure has been detected so far in closely distributed populations of this species, even though it has been observed in other cetaceans. Here, we examined the fine scale population structure for the largest populations of *S. guianensis* inhabiting Sepetiba and Paraty embayments at the south-eastern coast of Rio de Janeiro, Brazil. Analysis of mitochondrial DNA (mtDNA) control region sequences failed to detect variability among sequences. Conversely, evidence of significant male population structure was found on the basis of ten nuclear microsatellite loci. Surprisingly, the microsatellite markers were able to distinguish between individuals from the two embayments located 60 km apart. The results suggest that differences in habitat type and behavioral specializations are likely to explain the patterns of genetic structure. These findings should provide baselines for the management of communities exposed to increasing human-driven habitat loss.

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C. Hollatz · F. R. Santos (✉)
Laboratório de Biodiversidade e Evolução Molecular, Departamento de Biologia Geral, ICB, Universidade Federal de Minas Gerais, Caixa Postal 486, Belo Horizonte, MG 31270-010, Brazil
e-mail: fsantos@icb.ufmg.br

L. Flach
Projeto Boto-cinza, St Terezinha 531, Vila Muriqui, Mangaratiba, Rio de Janeiro 23860-000, Brazil

C. Scott Baker
Department of Fisheries and Wildlife, Marine Mammal Institute, Oregon State University, Newport, OR, USA

Introduction

Sotalia guianensis, also called Guiana dolphin, is widely distributed in the Atlantic coast from Central America to south of Brazil (Borobia et al. 1991; Carr and Bonde 2000). This species are mostly confined to estuarine environments with high site fidelity reported for different populations (Flores and Bazzalo 2004; Azevedo et al. 2004; Rossi-Santos et al. 2007; Flach et al. 2008; Nery et al. 2008a; Dias et al. 2009). Along the Brazilian coast, Sepetiba Bay encloses the largest population of *S. guianensis* so far documented, which is estimated between 739 and 2,196 individuals (Flach et al. 2008). Similar population size has also been reported in the neighboring Paraty embayment (Lodi 2003), making these areas an important site of occupation for this species. Moreover, both bays are of great environmental interest, comprising one of the Brazilian systems with greatest primary productivity (Nogara 2000).

Human-driven habitat loss has been observed in the Sepetiba Bay. This region is highly degraded by chemical contamination, pesticides, and mercury due to its proximity to a metropolitan region with increased industrial development (Seixas et al. 2009). Potential threats to the dolphins in the Sepetiba Bay are arising from intense fishing activities and the construction of a large harbor system. Indeed, injuries and fatalities such as entanglement of these animals in fishing gears and collisions with boats have been reported in Sepetiba Bay (Nery et al. 2008a). In addition, overfishing has been considered as the main threat to the dolphins in the Paraty Bay (Lodi 2003), which is also a popular tourism destination in Brazil with an intense recreational boating traffic that is currently focus of major concern as it may influence changes in dolphin behavioral and group stability.

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The species is listed in the Appendix I of the Convention in International Trade of Endangered Species (CITES) and as Data Deficient (2008) by the World Conservation Union (IUCN). Although some information regarding behavioral ecology and abundance has been reported (Flach et al. 2008; Nery et al. 2008b, Dias et al. 2009), little is known about the genetic status of *S. guianensis* in the Brazilian coast (Cunha et al. 2005, Caballero et al. 2007).

Understanding population structure is crucial to inform management actions (Avise et al. 1995; Frankham et al. 2010) toward the maintenance or recovery of the genetic diversity of a given species within its historical range area. For the marine environment, this is particularly challenging, as low intra-specific genetic variation between closely distributed populations is expected where the lack of geographic barriers may enhance the interchange of individuals. However, some studies have shown that marine populations of cetaceans are genetically more structured than might be expected even over small geographic scales (Hoelzel 1998). Some characteristics of coastal areas (estuaries and embayments) such as habitat type, local fidelity, and behavioral specializations may drive genetic divergence among populations (Möller et al. 2007). Furthermore, oceanographic features such as surface salinity, temperature, and productivity may also affect genetic differentiation (Bilgmann et al. 2007). From long-term observational studies, it appears that Sepetiba Bay harbors discrete socially structured groups of *S guianensis* along two different environments: the entrance of the bay, influenced by the open waters resulting in sandy and gravel substrates, higher salinity and water transparency and the interior part, characterized by mud and silt substrates, lower salinity and water transparency due to contact with river drainage systems (Flach et al. 2008, Dias et al. 2009). On the other hand, Nery et al. (2008b) stated that Paraty and Sepetiba Bay are environmentally similar and predicted that both embayments are used by individuals recorded in Sepetiba Bay. Since social structure is a potential force shaping intraspecific genetic differentiation in discrete populations of cetaceans, we have analyzed mitochondrial DNA control region sequences and microsatellite markers in order to assess the spatial population structure of *S. guianensis* in Sepetiba and Paraty Bays in the coast of the Rio de Janeiro state of Brazil, both located approximately 60 km apart.

Materials and methods

A total of 99 samples were obtained from animals found dead, and biopsy darting collected along 2006 and 2009 in Sepetiba (526 km^2) and Paraty (243 km^2) Bays. During 2006, only samples obtained from animals found dead were available and a subset of fifty individuals from both bays was successfully sequenced at mtDNA control region (CR), including males and females. For microsatellites, only male samples obtained from biopsy ($n = 58$) were successfully genotyped for each marker due to DNA availability and quality. Furthermore, sex ratio was biased toward male dolphins during biopsy hits (L. Flach, unpublished). Samples from Sepetiba were later divided into samples coming from the entrance ($n = 20$) and interior ($n = 23$) of the bay; a total of 15 samples were obtained from Paraty Bay (Fig. 1). All samples were preserved in 95% ethanol and stored at −20°C. DNA was extracted following phenol–chloroform extraction protocol, as described in Sambrook and Russell (2001).

A 480-base pair (bp) fragment of mtDNA control region (CR) was amplified via the polymerase chain reaction (PCR) using primers CR-4 (Southern et al. 1988) and CR-5 (Southern et al. 1988; Palumbi et al. 1991). Each PCR mix contained 20 ng of genomic DNA, 1× *Taq* reaction buffer 1B (Phoneutria®–1.5 mM $MgCl_2$, 10 mM Tris–HCl pH 8.4, 50 mM KCl, 0.1% Triton X-100), 200 µM dNTPs, 0.5 µM of each primer, and 1 unit of *Taq* DNA polymerase (Phoneutria®). The PCR cycling for CR was performed in

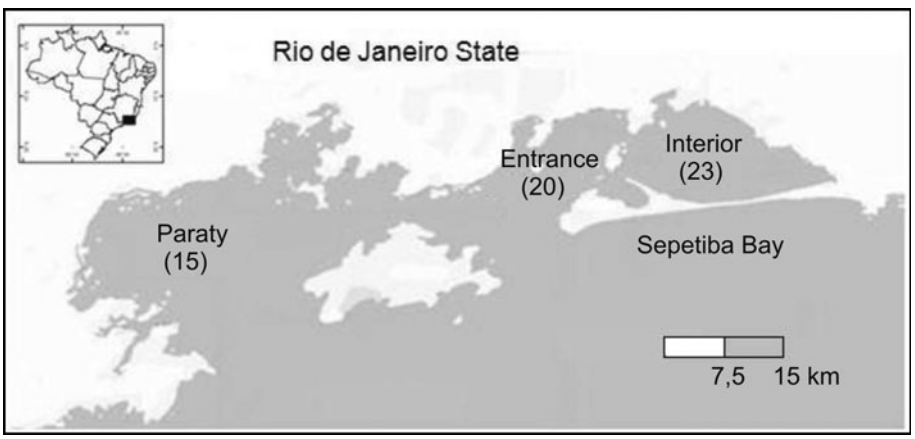

Fig. 1 Map of three sampling sites of estuarine dolphins in Sepetiba and Paraty Bay, southeastern coast of Brazil. Numbers between parentheses indicate number of sampled individuals

an Eppendorff Gradient thermocycler following the conditions described by Vianna et al. (2006). PCR products were cleaned using 20% PEG (Polyethyleneglycol) + 2.5% NaCl, and both strands were sequenced using an ET® dye terminator kit (GE Healthcare®) on a MegaBACE automated capillary sequencer following the temperature cycling profile: 95°C for 1 min, 35 cycles at 95°C for 25 s, annealing at 50°C for 15 s, and extension at 60°C for 3 min.

Samples were genotyped at ten microsatellite loci either developed directly for *Sotalia guianensis* (Cunha and Watts 2007) or using heterologous primers isolated from the other cetacean species (Table 1) and standardized to amplify *S guianensis* samples. Amplification reactions contained 20–40 ng DNA, 20 mM Tris–HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 μM of each primer, 200 μM dNTPs, and 0.25 units of Platinum-*Taq* (Invitrogen). Each forward primer had an M13 sequence tail added to its 5′ end to allow for fluorescent labeling (HEX, NED, FAM) following Schuelke (2000). The thermal cycle profile for microsatellites isolated from another species were carried out following standard protocols, in 10-μl volumes, with 2.5 mM $MgCl_2$ and annealing temperature varying by locus (50°C for MK6, MK9, EV94, and GATA98; 55°C for PPHO142 and AAT44). Microsatellite loci isolated from *Sotalia* were amplified following the procedure described by Cunha and Watts (2007). PCR products were measured using an Applied Biosystems 3730 automated sequencer. Allelic sizes were scored against the size standard GS500 LIZ (Applied Biosystems) and the peaks were analyzed using *GeneMapper* v4.0 (Applied Biosystem).

Individuals whose gender was previously unknown were submitted to sex identification by PCR amplification of a fragment of the *SRY* gene, multiplexed with fragments of the *ZFY/ZFX* genes as positive control, as described by Gilson et al. (1998).

Mitochondrial DNA Sequences were "base called" with the software *Phred* 0.020425.c (Ewing et al. 1998). High-quality consensus sequences (480 bp for CR) for each individual were produced from alignments of forward and reverse strand sequences with the software *Phrap* 0.990319 (Green 1994) and visualized/edited in *Consed* 15.0 (Gordon et al. 1998). The sequences were aligned using *Clustal-X* v1.83 (Thompson et al. 1997) to allow the identification of nucleotide variation and different haplotypes. Microsatellite genotypes were screened for likely scoring errors, allele dropout, and presence of null alleles using the program *Micro-Checker* (Van Oosterhout et al. 2004). The matching genotypes (to identify replicate individuals) and the number of private alleles for each sampling site were estimated as implemented in the program *GenAlEx* v6.3 (Peakall and Smouse 2006). Departures from Hardy–Weinberg equilibrium, tests of linkage equilibrium for each locus, genetic differentiation among populations (using F_{ST} and R_{ST}) and inbreeding coefficients (F_{IS}) were carried out using *Arlequin* v3.1 software (Excoffier et al. 2005). The

Table 1 Microsatellite *loci* used for *Sotalia guianensis* in this study

Locus	Sequence	Repeat	Range	Species	Author
GATA98	F-TGTACCCTGGATGGATAGATT R-TCACCTTATTTTGTCTGTCTG	(GATA)n	92–134	*Megaptera novaeangliae*	Palsbøll et al. (1997)
EV94Mn	F-ATCGTATTGGTCCTTTTCTGC R-AATAGATAGTGATGATGATTCACAC	(TC)n and (AC)n	198–261	*Megaptera novaeangliae*	Valsecchi and Amos (1996)
PPHO142	F-GAAGGCTCAGGGTATTG R-CAGTTACTTTCCTCGGG	(CA)n	127–161	*Phocoena phocoena*	Rosel et al. (1999)
MK6	F-GTCCTCTTTCCAGGTGTAGCC R-GCCCACTAAGTATGTTGCAGC	(GT)n	145–189	*Tursiops aduncus*	Krützen et al. (2001)
MK9	F-CATAACAAAGTGGGATGACTCC R-TTATCCTGTTGGCTGCAGTG	(CA)n	168–180	*Tursiops aduncus*	Krützen et al. (2001)
Ttru AAT44	F-CCTGCTCTTCATCCCTCACTAA R-CGAAGCACCAAACAAGTCATAGA	(AAT)n	92 product size	*Tursiops truncatus*	Caldwell et al. (2002)
SGUI03	F-TCCAATCTCCAACCAAATCCC R- GTCGCTAAGTTCATCATCTGC	(GT)28	148–162	*Sotalia guianensis*	Cunha and Watts (2007)
SGUI11	F-ACAGAGAAGCAAGTGGGAAACC R-TTCCCCGCCACTAAGATTCC	(GT)26	398–446	*Sotalia guianensis*	Cunha and Watts (2007)
SGUI17	F- GTGGTGGAGTAGAGGATAGG R-ACATTGGGCTTCAACGCACG	(CA)22	150–166	*Sotalia guianensis*	Cunha and Watts (2007)
SGUI18	F-CTGGAAAAAGAGTAGTTGGC R- GTGCAAGACCTCAAAATCC	(GT)29	234–252	*Sotalia guianensis*	Cunha and Watts (2007)

Table 2 Summary of genetic variation based on microsatellite data

Locus	SBE				SBI				Paraty			
	n	n_A	H_O	H_E	n	n_A	H_O	H_E	n	n_A	H_O	H_E
EV94	20	2	0.500	0.507	19	2	0.522	0.487	15	2	0.400	0.404
GATA98	20	5	0.650	0.605	19	5	0.783	0.625	15	5	0.600	0.542
PPHO142	19	2	0.474	0.462	17	2	0.571	0.483	14	3	0.500	0.632
MK6	20	2	0.250	0.296	19	4	**0.500**	**0.601**	15	2	**0.200**	**0.508**
MK9	20	2	**0.850**	**0.501**	19	2	**0.957**	**0.510**	13	2	**0.846**	**0.508**
AAT44	20	4	0.550	0.619	19	3	0.652	0.587	14	4	0.714	0.672
SGUI03	20	2	0.050	0.050	19	2	0.043	0.043	11	1	ND	ND
SGUI11	17	6	**0.353**	**0.531**	17	5	0.409	0.453	13	5	0.615	0.514
SGUI17	20	5	0.800	0.599	18	4	0.696	0.564	13	4	**0.308**	**0.748**
SGUI18	20	5	0.500	0.517	19	7	0.609	0.712	15	7	0.800	0.694
Average gene diversity	0.449 ± 0.251				0.490 ± 0.270				0.512 ± 0.287			
F_{IS} (P value)	−0.093 (0.915)				−0.159 (0.987)				−0.014 (0.606)			

n sample size for each region, n_A number of alleles at each locus, H_O and H_E observed and expected heterozygosity, *SBE* Sepetiba Bay Entrance, *SBI* Sepetiba Bay Interior

Significant deviations from Hardy–Weinberg equilibrium are shown in bold

Bayesian clustering program *Structure* v.2.3 (Pritchard et al. 2000) was also used to identify structured populations among the three sampling sites. We assumed the admixture model and performed the analysis considering correlated allele frequency models. Burn-in length and length of simulation were set at 10^5 iterations and 10^6 Markov Chain Monte Carlo (MCMC). Six independent runs were performed for each value of K (for 1–3), as suggested by Pritchard et al. (2000). The program Bottleneck v1.2 (Piry et al. 1999) was used to evaluate deviations of mutation-drift equilibrium in populations from the three different sites. Three different mutation models of microsatellite evolution were employed: (1) Infinite Allele model–IAM; (2) Stepwise Mutation Model–SMM, and (3) Two-Phased model–TPM, an intermediate to the IAM and SMM which better fits to the most of microsatellite datasets. The significance was assessed with the Wilcoxon sign-rank test, a more powerful and robust test to use with few polymorphic loci (<20).

Results

A fragment of 480 bp of the mtDNA CR was sequenced in 50 individuals and no variability was found across all samples. For microsatellites, all ten loci amplified successfully in 58 individuals with no matching genotypes identified. All loci were polymorphic, except one: Sgui03, which was monomorphic only in the Paraty Bay. The number of alleles per locus ranged from 2 to 9. Heterozygosities were similar for all groups with low to medium level of allelic diversity at each locus (Table 2). Sepetiba Bay displayed five private alleles (2 in the entrance and 3 in the interior) compared to the six found in Paraty. Four loci exhibited significant deviations of Hardy–Weinberg equilibrium possibly due to null alleles (Table 2). However, we did not find significant differences in the F statistics results when the four loci were excluded from the analyses. Also, no evidence of linkage disequilibrium was found for any loci pair. The analyses of population subdivision using F_{ST} and R_{ST} showed significant and marked genetic structure between Sepetiba and Paraty embayments, although no significant structure was detected within Sepetiba Bay (see Table 3). The pattern of genetic structure was confirmed when pooling samples from Sepetiba embayment ($n = 43$) against Paraty ($n = 15$). F_{ST} and R_{ST} values were 0.040 and 0.076, respectively, between two localities ($P < 0.01$). Bayesian clustering analysis using *Structure* showed no strong pattern of population differentiation (data not shown), and only one population was identified. However, this result probably relates to the performance of *Structure* being highly dependent on the number of available *loci* (Evanno et al. 2005). In addition, negative and non-significant values of

Table 3 Pairwise F_{ST} (matrix below) and R_{ST} (above matrix) values among three sampling locations based on eleven microsatellite loci

Pairwise F_{ST}, R_{ST}	SBE	SBI	Paraty
SBE	–	0.008	0.081**
SBI	0.009	–	0.076*
Paraty	0.056**	0.032*	–

SBE Sepetiba Bay Entrance, *SBI* Sepetiba Bay Interior

* P value < 0.05, ** P value < 0.001

F_{IS} were obtained for the three sampling sites. The program Bottleneck did not indicate any sign of recent population decline in either Sepetiba or Paraty Bay. One-tailed Wilcoxon sign-rank tests for heterozygote excess indicated that each group was in mutation-drift equilibrium for all mutation models: IAM, TPM, and SMM ($P > 0.05$).

Discussion

The present study failed to find any variability in the control region over 50 individuals sampled. This result is consistent with the previous studies reported by Cunha et al. (2005) and Caballero et al. (2007) for the south-eastern part of Rio de Janeiro. However, the number of samples previously analyzed by these authors was small, both analyzed seven samples each ($n = 14$), considering the large population of dolphins in those areas. For marine mammals, mitochondrial DNA is often expected to reflect population structure more rapidly than nuclear *loci*, due to its relatively low effective population size and high substitution rate (Hoelzel et al. 2002). Altogether, microsatellite *loci* analyses revealed moderate levels of genetic diversity and low allelic diversity across the embayments, reinforcing the role of founder effects in the colonization of the area suggested by Cunha et al. (2005). The same pattern was also showed by Möller et al. (2007) for bottlenose dolphin communities living in close proximity in south-eastern part of Australia. Our analysis based on microsatellite data found no evidence of a population bottleneck in Sepetiba or Paraty Bay. Indeed, geological data also indicates that the formation of Sepetiba embayment would have occurred during the Holocene postglacial period around 6000 years ago (Pereira et al. 2009). The partial closure of the bay leading to the formation of a highly productive estuarine ecosystem would have provided a suitable habitat for the establishment of *S. guianensis*. Given the likely recent colonization of the Sepetiba Bay, we hypothesized that not enough time has elapsed to allow mtDNA control region sequences to capture signals of population structure. Consider a likely recent colonization in the area and our microsatellite results that indicate a marked genetic structure between two localities about 60 km apart, we suggest that dolphins from both bays might not be currently interbreeding or if so, very rarely. For bottlenose dolphins, several studies have shown genetically differentiated dolphin populations inhabiting different habitats over large and small distances (Krützen et al. 2004; Natoli et al. 2005; Sellas et al. 2005; Möller et al. 2007; Bilgmann et al. 2007; Wiszniewsky et al. 2009). These studies strongly suggest that genetic distinctiveness is predominantly governed by habitat type and resource specializations. Influence of different oceanographic features (e.g. water salinity, and temperature) on the genetic structure has also been reported for bottlenose dolphins (*Tursiops truncatus*) (Natoli et al. 2005; Bilgmann et al. 2007). These parameters have a direct influence on the distribution of preys and as a consequence, on the spatial distribution of the dolphins. However, in Sepetiba, a preliminary study indicated that any of these variables affected the activity patterns of the dolphins in either the entrance or the interior of the bay (Flach et al. 2008). On the other hand, for *S. guianensis* in Paraty, high fluctuations in sea surface temperature and food resources influenced seasonal distribution of the dolphins within the embayment (Lodi 2003). Although more information is required regarding the habitat features and feeding ecology of dolphins from both Sepetiba and Paraty, it is possible that behavioral specializations are acting in population differentiation across these areas. Mating and breeding strategies are another possible explanation for the differential male genetic structure observed in both embayments. Male bottlenose population structure was also observed in Shark Bay, Australia (Krützen et al. 2004). Connor et al. (1992) suggested that alliance formation among males would facilitate the access to females more successfully. Thus, males may gain inclusive fitness benefits cooperating in this manner. Furthermore, long-range dispersal of males to other areas would minimize the chance of allying with a related partner (Connor et al. 1992). Most samples, acquired in social/sexual activities during this study, were from males, indicating that large groups of males around females might be commonly observed for *S. guianensis* in Sepetiba Bay. However, a more detailed ecological study is being conducted to better investigate this hypothesis (L. Flach, personal communication).

Our results corroborate previous observations that dolphins living in protected environments with strong habitat boundaries are genetically more structured than open water populations (Möller et al. 2007). To date, localized genetic studies were mostly reported for bottlenose dolphins (*T. truncatus*). Here, we documented the first localized genetic study for *S. guianensis* adding evidence that this pattern of genetic differentiation is not restricted to a particular species (e.g. *Tursiops* sp.) or to a particular estuarine environment.

Further studies taking into account more individuals and including females should provide more accurate inferences regarding Guiana dolphin population structure. Our results showing a significant population differentiation across the embayments, in addition to the increasing human-related habitat degradation, especially in the Sepetiba Bay, due to the construction of two large harbors, should help to inform specific conservation plans for each stock of Guiana dolphin in order to minimize anthropogenic disturbances in the south-eastern coast of Rio de Janeiro. This study has

important implications for understanding evolutionary processes leading to genetic structuring in delphinids and for the management of Guiana dolphin populations in Brazil and worldwide.

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