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Mitochondrial control region haplotypes of the South American sea lion *Otaria flavescens* (Shaw, 1800)

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Abstract

The South American sea lion, *Otaria flavescens*, is widely distributed along the Pacific and Atlantic coasts of South America. However, along the Brazilian coast, there are only two nonbreeding sites for the species (Refúgio de Vida Silvestre da Ilha dos Lobos and Refúgio de Vida Silvestre do Molhe Leste da Barra do Rio Grande), both in Southern Brazil. In this region, the species is continuously under the effect of anthropic activities, mainly those related to environmental contamination with organic and inorganic chemicals and fishery interactions. This paper reports, for the first time, the genetic diversity of *O. flavescens* found along the Southern Brazilian coast. A 287-bp fragment of the mitochondrial DNA control region (D-loop) was analyzed. Seven novel haplotypes were found in 56 individuals (OFA1-OFA7), with OFA1 being the most frequent (47.54%). Nucleotide diversity was moderate (π = 0.62%) and haplotype diversity was relatively low (67%). Furthermore, the median joining network analysis indicated that Brazilian haplotypes formed a reciprocal monophyletic clade when compared to the haplotypes from the Peruvian population on the Pacific coast. These two populations do not share haplotypes and may have become isolated some time back. Further genetic studies covering the entire species distribution are necessary to better understand the biological implications of the results reported here for the management and conservation of South American sea lions.

Key words: *Otaria flavescens*; mtDNA; D-loop; Genetic variability; New haplotypes

Introduction

The South American sea lion, *Otaria flavescens* (Shaw, 1800), is widely distributed along the coasts of South America, occurring from 6°S in the Pacific Ocean to 29°S in the Atlantic Ocean. The main breeding colonies are located in Uruguay, Argentina, Falkland Islands (UK), Chile, and Peru (1). This is not a migratory species, although seasonal movements occur when adult males, after the breeding season, move to the sea searching for food (2). The South American sea lion is one of the most frequent pinniped species in Brazil, occurring mainly during winter and spring. According to Rosas et al. (3), specimens found along the Southern Brazilian coast are part of the breeding stock of Uruguayan rookeries. However, some specimens from the Argentinian stock were also recorded in Camboriú Beach (Santa Catarina, Southern Brazil) (Barreto AS, personal communication).

There are only two nonbreeding sites for the species along the Brazilian coast: Refúgio de Vida Silvestre da Ilha dos Lobos (29° 20'S, 49° 43'W) at Torres and Refúgio de Vida Silvestre do Molhe Leste da Barra do Rio Grande (32° 10'S, 52° 6'W) in the Lagoa dos Patos, both on the coast of the Rio Grande do Sul (2). Rosas et al. (2) observed that most animals present on the Rio Grande do Sul coast were males (81%), represented mainly by large numbers of immature individuals from April to June. In a previous study investigating aging by the same authors (3), skulls and teeth of *O. flavescens* found dead along the southern coast of Brazil were collected. The oldest males and females

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were 16 and 14 years old, respectively. Animals aged 3-5 years were the most frequent. Both sexes attain 95% of their maximum length by 8 years of age (3). Kinas et al. (4) suggest a strong seasonal pattern for *O. flavescens* with a maximum average number of strandings in September and a minimum in January. The estimated stranded sea lion in a typical year is 115 sea lions.

There are only few studies published about South American sea lions on the Brazilian coast. Most of them are related to seasonal movements (2), age and growth (3), patterns of occurrence (4), diet and fishery interactions (5) on the Southern Brazilian coast. However, there is no information about the genetic diversity of the species in Brazil. The only published studies on the genetics of pinnipeds from the Southern Brazilian coast are those by Ferreira et al. (6) and Oliveira et al. (7) regarding *Arctocephalus tropicalis* and *Arctocephalus australis*, respectively. Therefore, we present here, for the first time, an evaluation of the genetic diversity of *O. flavescens* found on the Southern Brazilian coast and compare the results obtained with existing data for a single population from the Southern Pacific (8).

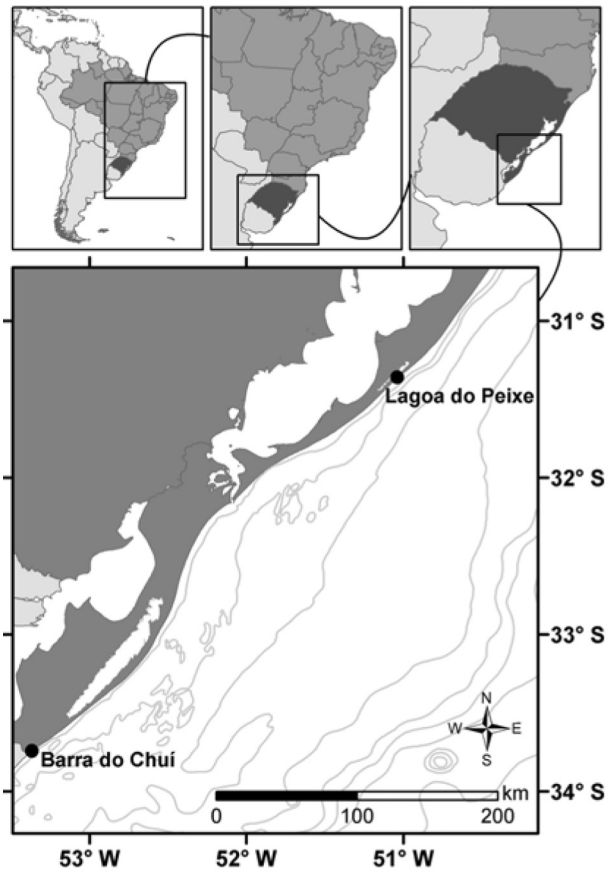

Figure 1. Sampling area of the present study. South American sea lions were collected between May 2005 and September 2006.

Material and Methods

Tissue samples (skin) were collected from 52 dead sea lions along the Rio Grande do Sul coastline between May 2005 and September 2006. Additional samples from 4 individuals were supplied by a non-governmental Brazilian organization (NEMA/Núcleo de Educação e Monitoramento Ambiental, Rio Grande, RS, Brazil). The samples were collected during beach surveys performed from June 2002 to June 2004. The area studied extends from the "Barra da Lagoa do Peixe" at Mostardas (31° 21' 31.9"S/51° 02' 19.6"W) to "Barra do Chuí" (33° 44' 37.7"S/53° 22' 10.5"W; Figure 1). For each specimen sampled, the following data were recorded: date, season, body length (from the tip of the snout to the end of the tail), geographic coordinates, sex (based on external morphology), and degree of decomposition. Tissue samples of approximately 1 cm³ were collected and preserved in 95% ethanol. Total genomic DNA was extracted using the standard phenol-chloroform and ethanol precipitation protocol by the method of Sambrook et al. (9). A 287-bp fragment from the mitochondrial DNA control region was amplified by polymerase chain reaction (PCR) using a TC-512 automatic thermal cycler (Techne, UK) with a 25-µL reaction volume: 1 µL genomic DNA (10 ng DNA template), 19.05 µL MilliQ® water, 0.75 µL (10 mM) dNTP mixture, 0.6 µL (50 mM) $MgCl_2$, 2.5 µL (10X) buffer (500 mM KCl, 200 mM Tris-HCl, pH 8.4), 0.5 µL (10 pmol/µL) of universal primers [DL-FOR (5'-TTCCCCGGTCTTGTAAACC-3') and DL-REV (5'-ATTTTCAGTGTCTTGCTTT-3')] (10) and 0.2 µL Taq DNA Polymerase (Invitrogen, Brazil; 5 U/µL). PCR conditions were a denaturation step at 94°C for 1 min, followed by 30 cycles (denaturation at 94°C for 30 s, annealing at 50°C for 1 min and extension at 72°C for 1 min) and 5 min at 72°C for final extension. Products were characterized by electrophoresis on 1% agarose gel (Invitrogen, Brazil) stained with ethidium bromide prepared in Tris-acetate EDTA. PCR products were then purified with PEG 8000 (Promega, USA) and sequencing was performed using a MegaBace 1000 automatic sequencer (GE Healthcare, Brazil).

Sequence electropherograms were checked with the Chromas software (Technelysium, Australia). The sequences obtained were aligned using Clustal X (11), manually edited with Bioedit 6.0.7 (Sequence Alignment Editor©), and corrected visually.

Genetic variability was estimated using haplotype (h) and nucleotide (π) diversities, which were calculated with the DnaSP 4.0 software (12). A median joining network method was generated to infer the relationships between the novel haplotypes from the Atlantic population and the Peruvian population on the Pacific coast (8) obtained from GenBank (accession Nos. AF384419, AF384420, AF384421, AF384422, AF384423) using Network 4.5 (13) (Figure 2).

Results

A total of 56 sequences of the mtDNA control region were obtained for analysis, which comprised 287 bp. These sequences defined seven new and different haplotypes for the species analyzed, represented by white circles in Figure 2. The most common and widespread haplotype, OFA1, was shared by 29 individuals representing 47.54% of the sample, followed by the haplotypes OFA2 (18.3%; N = 11), OFA3 (16.39%; N = 10), OFA4 and OFA5 (3.27%; N = 2), and OFA6 and OFA7 (1.63%; N = 1). These seven haplotypes (OFA1-7) were considered to be new since they are different from the previous haplotypes described for the species by Wynen et al. (8). All unique haplotypes were submitted to GenBank (accession Nos. EU044835-EU044841).

Results obtained by the median-joining network method using D-loop sequences for the haplotypes described in this study and representative haplotypes from the Pacific Ocean, represented by gray circles, are shown in Figure 2. These analyses demonstrate the reciprocal monophyly of *O. flavescens*, with a grouping of haplotypes OFA1-OFA7 being from the Atlantic Ocean population, and specimens from Peru being from the Pacific Ocean group (haplotypes OB4, OB5 and OB2) (8).

Moderate levels of genetic diversity were observed among *O. flavescens* individuals. The haplotype (h) and nucleotide (π) diversities calculated for the species were 0.67 ± 0.051 and 0.006 ± 0.00067, respectively.

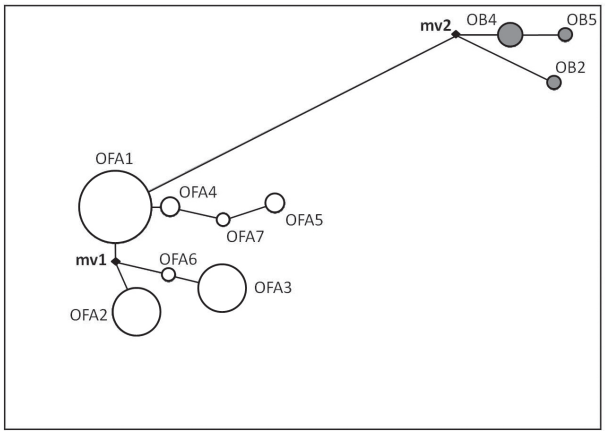

Figure 2. Median-joining network of the Atlantic (white circles) and Peruvian (gray circles) haplotypes based on polymorphic sites of the mitochondrial D-loop region. The circled areas are proportional to the frequency of the haplotypes indicated. Black diamonds symbolize median vectors (mv) produced by the network software, representing missing or not sampled haplotypes.

Discussion

The nucleotide diversity observed (0.006 ± 0.00067) is consistent with values reported by Wynen et al. (8) for the same region of mtDNA of other sea lion species and populations, such as *O. flavescens* from the Pacific coast (π = 0.008), *Eumetopias jubatus* from Alaska (π = 0.004), and *Phocarctus hookeri* from New Zealand (π = 0.004). However, other investigators found that *Zalophus californianus* from the California coast and *Callorhinus ursinus* from Alaska had significantly higher levels of nucleotide diversity (π = 0.03 and 0.027, respectively). Therefore, the result observed for *O. flavescens* in the present study is within the range of nucleotide diversity described for other sea lion species. Regarding haplotype diversity, a relatively low value was observed (h = 67%) for *O. flavescens* when compared to other pinnipeds. High levels of haplotype diversity (h = 93%) were reported for *E. jubatus* sampled in Alaska after analysis of 531 bp of the mtDNA control region (14). Also, 91% haplotype diversity was reported for *E. jubatus* individuals sampled in the North Pacific and Bering Sea, after analysis of 238 bp of the mtDNA control region (15). For *Phoca vitulina* from the North Pacific, a haplotype diversity of 97% was observed after analysis of 435 bp of the mtDNA control region (16). In fur seal samples (*Arctocephalus philippii*) from Chile, a high haplotype diversity (h = 90%) was reported after analysis of 315 bp of the mtDNA control region (17). A very similar value (h = 91.4%) was observed for the harbor porpoise, *Phocoena phocoena*, sampled in the Northwest Atlantic, when analyzing the same mtDNA region (18).

The moderate haplotype diversity observed in sea lions from Southern Brazil could be explained by the single Uruguayan origin of the individuals analyzed. This hypothesis is consistent with the reduced genetic variability of cytochrome *b* described for *O. flavescens* sea lions and *A. australis* fur seals from Uruguay and Argentina, associated with the recent colonization of these areas (19).

The molecular data reported in the present study are the first genetic information for South American sea lions from the Brazilian coast. The evidence of reciprocal monophyletic clades and the absence of shared haplotypes between Brazilian and Peruvian specimens indicated that these populations may have become isolated some time back. Median joining network analysis performed with D-loop region haplotypes (Figure 2) showed a subdivision of the *O. flavescens* population into two main clades: the Peruvian and the Brazilian groups of haplotypes. These two clades may reflect a possible differentiation between Atlantic and Pacific sea lions at some taxonomic level, as proposed by Túnez et al. (19). This separation can be characterized by the representation of the median vector (mv2), possibly an ancestral haplotype, among clades in the Pacific and Atlantic populations, which, after several changes over time, led to the Atlantic clade (Figure 2). The median vector mv1 may represent a not scored or ancestral haplotype, which gave origin to a group formed by OF6, OF3 and OF2.

Glacial episodes usually induced important evolutionary events in populations, considering that they constitute physical barriers for dispersion and genetic flow (20). Glaciations associated with geographic distances may have been the cause of a significant reduction in gene flow between Pacific and Atlantic colonies of the Southern sea lions, as suggested by the lack of shared haplotypes observed between these populations, as described by Túnez et al. (19) for the cytochrome *b* gene, and corroborated by our results for the mtDNA D-loop region.

The two populations compared in the present study are the most distant ones geographically, thus explaining the genetic difference observed between them. However, for any future comment or suggestion on the systematic, conservation and/or management plan for the studied species we strongly recommend further studies considering additional new samples to cover the entire species distribution. This should include individuals from Argentina, Falkland Islands and Chile. This action would take into account the most important breeding colonies of the species as well as the potential connection points between populations in the Southern tip of South America. In addition, we also suggest new analyses using other molecular markers such as microsatellites as well as morphological and behavioral studies to identify evolutionarily significant units of the species (5).

Finally, it is important to note that, in spite of the anthropic impacts on O. *flavescens* populations along the South American coast, this species is still considered to be of least concern in the IUCN 2008 Red List of Threatened Species. In this context, we believe that the results reported in this present study may serve as a baseline for future studies involving populations of South American sea lions and future actions for their conservation and management.

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