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Sotalia dolphins in their potential sympatry zone: searching for hybrids in the Amazonian estuary

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The two Sotalia *species (the marine* S. guianensis *and the freshwater* S. fluviatilis*) have only recently been recognized, and both face several conservation challenges. We investigated the existence of hybridization between the two species in their possible area of sympatry in the Amazon Estuary, in northern Brazil. A fast and cheap PCR-RFLP diagnostic method using nuclear DNA was developed to discriminate between the two species, while allowing the detection of hybrids. All samples that could be identified (N = 51) were identified as* S. guianensis*, and no hybrids were detected. Our results, coupled with previous mitochondrial data, suggest that* S. fluviatilis *is not present in the Amazon delta. Thus, sympatry with* S. guianensis*, if it does occur, may be restricted to upstream areas of the Amazon River.*

Keywords: Guiana dolphin, forensics, introgression, tucuxi

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INTRODUCTION

The taxonomy of *Sotalia* dolphins remained doubtful for over a century, until morphological (Monteiro-Filho *et al.*, 2002) and genetic data (Cunha *et al.*, 2005; Caballero *et al.*, 2007) revealed two distinct species in this genus: *Sotalia guianensis* (van Bénéden, 1864), which lives in the coastal-estuarine region, and the exclusively freshwater *Sotalia fluviatilis* (Gervais, 1853). *Sotalia guianensis* is distributed from Honduras to south Brazil, whereas *S. fluviatilis* occurs in most of the Amazon Basin (da Silva & Best, 1996; da Silva *et al.*, 2010). Both species face conservation issues and *S. guianensis* has recently been listed as 'vulnerable' in Brazil (MMA, 2014). Although still considered 'data deficient', *S. fluviatilis* is also likely under threat, as are all other river dolphin species around the world (Reeves *et al.*, 2003; Secchi, 2012).

The Amazon Estuary is the only geographic area where the two *Sotalia* species may co-occur. Two factors create the scenario for a possible sympatric zone there: the fresh water from the Amazon River reaches the Atlantic Ocean, forming a wide plume a few hundred kilometres into the sea (Muller-Karger *et al.*, 1988; Goulding *et al.*, 2003). Hence, dolphins in the Amazon mouth are actually living in fresh water. At the same time, *S. guianensis* is known to enter up to hundreds of kilometres upriver (da Silva & Best, 1996). Thus, the two species may coexist in sympatry in the estuary, and this could allow hybridization between *S. guianensis* and *S. fluviatilis* (Cunha *et al.*, 2010), although there are no previous data on possible hybrids.

The existence of interspecific hybridization in cetaceans has been documented in captivity and in the wild, including intergeneric crossings (Dohl *et al.*, 1974; Nishiwaki & Tobayama, 1982; Baird *et al.*, 1998; Bérubé & Aguilar, 1998; Zornetzer & Duffield, 2003) and even between *Sotalia* and *Tursiops* (Caballero & Baker, 2010). If hybridization does occur between different species and genera, the chance that this event has occurred between the two species of *Sotalia*, which diverged only 2.3 million years ago (Cunha *et al.*, 2011), is even greater. Almost all of the samples from the Amazon Estuary analysed so far have been molecularly identified as *S. guianensis* (Cunha *et al.*, 2005, N = 21; Ruiz-Garcia *et al.*, 2013, N = 76). However, those studies relied exclusively on mitochondrial sequences, so the possibility that some samples were hybrids between the two species could not be assessed.

We investigated the occurrence of hybridization between *S. guianensis* and *S. fluviatilis* by analysing 76 specimens from the Amazon Estuary. Species identification and the test

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Fig. 1. Sampling sites in the Amazon River estuary. Samples from site 2 were of unknown origin at the time of sampling, since they were obtained from 'love charms' sold at a market in this location.

for hybrids were done using a molecular diagnostic method based on polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis using the introns of two nuclear genes.

MATERIALS AND METHODS

Samples were collected from *Sotalia* spp. dolphins that stranded in the Amazon Estuary (N = 18) and from love charms sold in Mercado Ver-o-Peso in Belém (Pará State, N = 58) (Figure 1). Previous studies using mtDNA have shown that love charms sold in the Ver-o-Peso are from *S. guianensis* (Cunha & Solé-Cava, 2007; Gravena *et al.*, 2008; Sholl *et al.*, 2008), and samples have been assigned to the Amazon Estuary population using microsatellite data (Cunha *et al.*, submitted). DNA from all samples was purified by the standard phenol-chloroform procedure (Sambrook *et al.*, 1989).

To develop PCR-RFLP diagnostic systems using nuclear DNA regions, we used sequences of the glucocerebrosidase (GBA) and the α-lactalbumin (Lac-1) genes from GenBank (EF027024-25, EF027010-11). Both genes are known to contain fixed polymorphisms between *S. guianensis* and *S. fluviatilis* (Caballero *et al.*, 2007). Sequences were aligned in the software MEGA 5.10 (Tamura *et al.*, 2011), and the program WebCutter 2.0 (http://rna.lundberg.gu.se/cutter2) was used to find restriction sites in the diagnostic region of each gene. One enzyme was selected for each gene: *Hap*II for GBA and *Tsp*RI for Lac-1. Both PCR-RFLP systems were expected to be able to diagnose the two species and to detect hybrids between them (Figure 2).

To test both systems, we used voucher DNA samples of *S. guianensis* (N = 4) and *S. fluviatilis* (N = 2), which were identified through sequencing. We also mixed the PCR products of the two species to simulate hybrid individuals. Later, we used the two systems to analyse the remaining 76 samples.

GBA and Lac-1 were PCR-amplified using primers described in Roca *et al.* (2001) and Milinkovitch *et al.* (1998), respectively. Amplifications were performed in 15 μl reactions containing 1 unit of Taq polymerase, 0.20 mM dNTPs, 2.5 mM $MgCl_2$ and 0.5 μM of each primer, and the following thermal conditions: 3 min at 95°C; 30 cycles of 1 min at 93°C, 1 min at 57°C (GBA) or 55°C (Lac-1) and 1 min at 72°C; plus 5 min of final extension at 72°C. The PCR products were visualized after electrophoresis in 1% agarose gels in TBE buffer.

Restriction analyses of the GBA and Lac-1 PCR products followed the manufacturers' procedures (*Hap*II, Amersham Biosciences and *Tsp*RI, New England BioLabs). We used 4 μl of PCR product in 20 μl final volume reactions. Digestions were performed overnight and restriction patterns were visualized after electrophoresis in 2% agarose gels in TBE buffer.

For the GBA/*Hap*II system (Figure 3A), samples of *S. guianensis* had two bands (200 and 100 bp), whereas *S. fluviatilis* samples showed only one band (300 bp), indicating that the GBA fragment remained intact. The simulated hybrids revealed three bands on the agarose gel, which were 300, 200 and 100 bp, thus presenting a third pattern.

For the Lac-1/*Tsp*RI system (Figure 3B), *S. guianensis* showed only one band (~600 bp), whereas *S. fluviatilis* produced two bands (500 and 100 bp). The mixed sample of

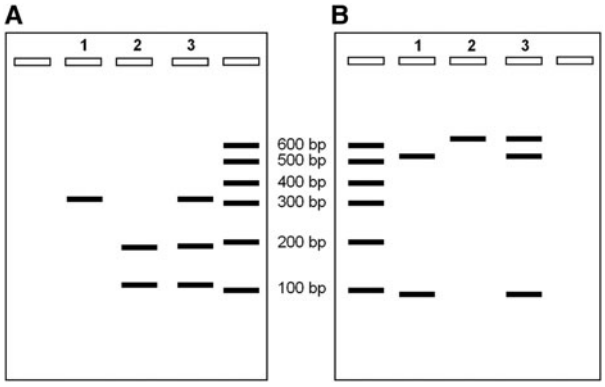

Fig. 2. Schematic diagrams with the expected results of the two diagnostic PCR-RFLP systems, (A) GBA/*HapII* and (B) Lac-1/*TspRI*, where (1) is *S. fluviatilis*, (2) is *S. guianensis* and (3) represents the pattern expected for a hybrid. The last lane of A and first lane of B represent a 100 bp DNA ladder.

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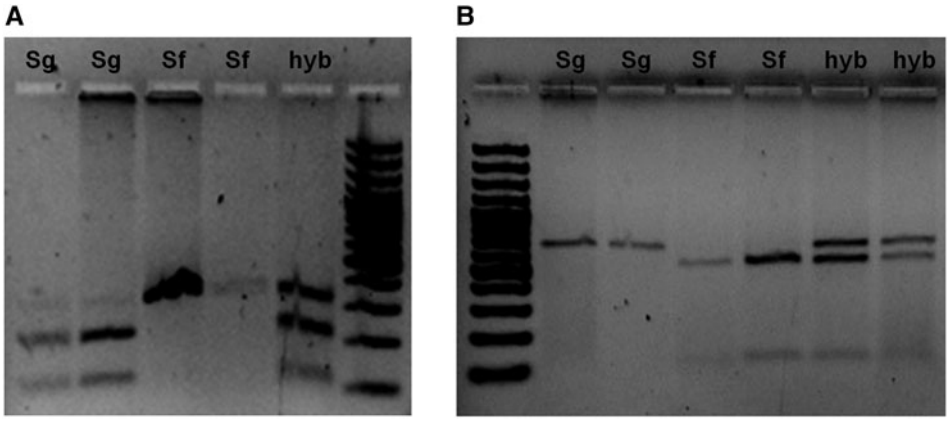

Fig. 3. PCR-RFLP diagnostic systems in 2% agarose gels. (A) GBA/*Hap*II specific patterns. Samples coded as 'Sg' are *S. guianensis*, samples 'Sf' are *S. fluviatilis* and sample 'hyb' is the simulated hybrid. (B) Lac/*Tsp*RI specific patterns. Sample codes are the same as in (A), and sample 'hyb' is a second simulated hybrid. We used a 100 bp molecular ruler.

Sotalia spp. showed the expected three bands pattern (600, 500 and 100 bp) (Figure 3B).

To confirm the patterns obtained, 15 samples (including the six individuals used as controls) had their GBA and Lac-1 introns sequenced. The PCR products were purified using magnetic beads (Agencourt AMPure XP Kit) and sequenced in both directions in an ABI 3500 platform (Applied Biosystems) using specific chemistry.

Sequences were edited using the SeqMan 7 program (DNASTAR, Inc.). Species identification was confirmed by a similarity search in GenBank (http://www.ncbi.nlm.nih.gov) using the Blastn algorithm and by alignment analysis in MEGA 5.10.

RESULTS

Using the GBA/*Hap*II we could identify 35 of the 76 samples as *S. guianensis*. One sample produced an ambiguous PCR-RFLP pattern and was sequenced for identification. The remaining 41 samples failed to amplify.

With Lac-1/*Tsp*RI, we identified 24 samples as *S. guianensis*. Eight samples generated ambiguous banding patterns and had their Lac-1 intron sequenced. The remaining 44 samples failed to amplify.

The nine samples sequenced for confirmation of the PCR-RFLP systems were identified as *S. guianensis*. Combining all identifications, we verified that of 76 samples, 51 were confirmed as *S. guianensis* (67%), whereas 25 could not be identified with the methods used in this study. No individual of *S. fluviatilis* or any hybrid between the two species was detected.

Of these 51 *S. guianensis* samples, 43 were collected in the Ver-o-Peso market and eight in the estuary. Microsatellite analysis of the Ver-o-Peso samples indicated that they belonged to different individuals (Cunha *et al.*, submitted).

DISCUSSION

All 51 samples that could be analysed using the diagnostic systems and the sequencing of GBA or Lac-1 were *S. guianensis*. This study thus confirms, through the analysis of nuclear genes, that the amulets sold in popular markets of the Amazon belong to *S. guianensis* as indicated by previous studies based on mtDNA (Cunha & Solé-Cava, 2007; Gravena *et al.*, 2008; Sholl *et al.*, 2008; Ruiz-Garcia *et al.*, 2013). Microsatellite data has further shown that the illegal trade of love charms is supplied by a population of *S. guianensis* in the Amazon Estuary (Cunha *et al.*, submitted). Therefore, all samples analysed in this study belonged to *S. guianensis* from the Amazon delta. Up to now, 150 samples from the delta have been molecularly analysed, and *S. fluviatilis* has not been detected (Cunha *et al.*, 2005; Ruiz-Garcia *et al.*, 2013; this study). Although the lack of evidence does not confirm that *S. fluviatilis* does not exist in the Amazon Estuary, it is a strong indication. It is noteworthy that other freshwater mammals, such as the Amazonian manatee *Trichechus inunguis* and the river dolphin *Inia araguaiensis*, do occur in the same area where our samples were collected (Domning, 1981; Siciliano *et al.*, 2016).

At the same time, the absence of *S. guianensis* × *S. fluviatilis* hybrids in our sample does not rule out their existence. Hybridization has been observed among other animals in the Amazon Estuary, for example, between the Antillean manatee *Trichechus manatus* and the freshwater Amazonian manatee *T. inunguis* (Vianna *et al.*, 2006). The lack of hybrids in our sample could be due to the absence or very low abundance of *S. fluviatilis* in the region, as suggested by the DNA data, or it could reflect a very infrequent event, which is usually the case.

If *S. fluviatilis* is absent from the estuary, the sympatric area for *Sotalia* dolphins may still occur upstream in the Amazon River because marine *S. guianensis* is known to enter fresh water in other areas of its distribution (da Silva & Best, 1996). How far upriver *S. guianensis* exists is presently unknown. Thus, we emphasize that studies in the Amazon delta and lower Amazon River should attempt to detect hybridization as a way to check for the existence of a sympatry zone between the two species. Another interesting avenue for research is using environmental DNA (eDNA) to try to detect the presence of *S. fluviatilis* in the Amazon estuary, and of *S. guianensis* up the Amazon river (see Foote *et al.*, 2012 for an example).

The PCR-RFLP systems developed here are recommended for this goal. They are able to successfully discriminate the two

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Sotalia species and their potential hybrids, and provide faster results than DNA sequencing at a lower cost. We estimated that the simultaneous use of the two PCR-RFLP systems is 7–25 times faster and 3.37 times cheaper than sequencing. If a single PCR-RFLP system is chosen, then the method becomes even more attractive in terms of time and cost. These diagnostic systems are ideal for fast and cheap initial screening of a large number of samples, leaving DNA sequencing for those that cannot be identified through PCR-RFLP.

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