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RESEARCH ARTICLE

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Riverine and marine ecotypes of *Sotalia* dolphins are different species

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Abstract The current taxonomic status of *Sotalia* species is uncertain. The genus once comprised five species, but in the twentieth century they were grouped into two (riverine *Sotalia fluviatilis* and marine *Sotalia guianensis*) that later were further lumped into a single species (*S. fluviatilis*), with marine and riverine ecotypes. This uncertainty hampers the assessment of potential impacts on populations and the design of effective conservation measures. We used mitochondrial DNA control region and cytochrome *b* sequence data to investigate the specific status of *S. fluviatilis* ecotypes and their population structure along the Brazilian coast. Nested-clade (NCA), phylogenetic analyses and analysis of molecular variance of control region sequences showed that marine and riverine ecotypes form very divergent monophyletic groups (2.5% sequence divergence; 75% of total molecular variance found between them), which have been evolving independently since an old allopatric fragmentation event. This result is also corroborated by cytochrome *b* sequence data, for which marine and riverine specimens are fixed for haplotypes that differ by 28 (out of 1,140) nucleotides. According to various species definition methods, we conclude that marine and riverine *Sotalia* are different species. Based on priority criteria, we recommend the revalidation of *Sotalia guianensis* (Van Bénéden 1864) for the marine animals, while riverine dolphins should retain the species name *Sotalia fluviatilis* (Gervais 1853), thus becoming the first exclusively riverine delphinid. The populations of *S. guianensis* show a strong subdivision ($\Phi_{ST} = 0.628$) along the Brazilian coast, with at least three evolutionarily significant units: north, northeastern and south/southeastern.

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Introduction

The taxonomic status of the tucuxi dolphins (genus *Sotalia*) has been a matter of controversy for more than a century. Up to five species and two subspecies were described for South America during the second half of the nineteenth century. However, because of inconsistencies in their diagnoses and later extension of known ranges, all riverine animals were subsequently regarded as one species (*Sotalia fluviatilis*) and the two marine species were grouped under *Sotalia guianensis* (True 1889; Cabrera 1961; Carvalho 1963). Later, it was suggested that even the distinction between those two species was too subtle, and that the genus *Sotalia* should be considered as monotypic (Mitchell 1975; Leatherwood and Reeves 1983). Additional evidence for lumping the marine and riverine forms came from a morphometrics

study, which concluded that differences between them were due to size variation only, and that they should be considered the same species without subspecific differentiation (Borobia 1989). Since then, most authors have accepted the binomial *Sotalia fluviatilis*, considering the marine and riverine populations as ecotypes (Borobia et al. 1991; Jefferson et al. 1993; da Silva and Best 1996; Flores 2002). Riverine *Sotalia* occur in the Amazon River drainage as far inland as Ecuador, Colombia and Peru, while marine *Sotalia* inhabit coastal waters of the West Atlantic, from Santa Catarina in southern Brazil to Honduras (Borobia et al. 1991; da Silva and Best 1996; Flores 2002). Recently, Monteiro-Filho et al. (2002) suggested that marine and riverine forms should be treated as separate species, based on a geometric morphometrics study.

Molecular data have proved to be useful sources of information on species boundaries (Knowlton 2000; Avise 2004). Recently, numerous molecular studies have provided additional or novel evidence for the recognition of new cetacean taxa (Rosel et al. 1994; Wang et al. 1999; Dalebout et al. 2002; Wada et al. 2003). There is no published genetic information to date on the specific status of the two ecotypes of *Sotalia*.

Although information on tucuxis is still too scarce to evaluate their conservation status (Reeves et al. 2003), threats to the persistence of both ecotypes have been identified (Siciliano 1994; da Silva and Best 1996; IBAMA 1997, 2001; Lailson-Brito et al. 2002), and it is important to gather ecological and biological data to evaluate possible impacts on their populations. To achieve this, and to design effective management policies, it is imperative that evolutionary significant units be distinguished (Dizon et al. 1992, 1997; O'Brien 1994; Avise 1997; Crandall et al. 2000). Thus, two issues are in great need of investigation: the specific status of *Sotalia* ecotypes, which was assigned as a first priority in a recently held workshop on cetacean systematics (Reeves et al. 2004), and the delimitation of marine *Sotalia* populations in Brazil, which was recommended in the last Action Plan for Cetaceans (Reeves et al. 2003).

The aim of this study was to investigate the evolutionary patterns that shaped genetic variation in marine and riverine populations of *Sotalia* in Brazil. Mitochondrial DNA control region and cytochrome *b* sequences were analysed to evaluate genetic differentiation between the two ecotypes and among marine populations. The results show a clear distinction between marine and riverine animals, as well as a strong population division among coastal samples.

Materials and methods

We analysed 56 samples of skin, muscle and liver of *S. fluviatilis* collected from stranded carcasses, by-caught specimens, biopsy darting and a capture/release programme, plus one skin sample of *Steno bredanensis* (G. Cuvier in Lesson 1828) for use as an out-group. Samples were stored in a 20% DMSO saturated NaCl solution (Amos and Hoelzel 1991), in ethanol or frozen at −196 or −20°C. Riverine samples were collected in the Amazonas State. Marine samples were collected from sites at the Brazilian States of Pará (north), Rio Grande do Norte (northeast), Rio de Janeiro and São Paulo (southeast), Paraná and Santa Catarina (south) (Fig. 1).

Total genomic DNA of all samples was extracted by maceration and incubation at 65°C for 3 h in a lysis buffer containing 1% SDS; 0.15 M NaCl; 1 mM EDTA; 0.1 mg/ml proteinase K; in 10 mM Tris–HCl (pH 8.0) (Palsbøll et al. 1992) followed by the standard phenol–chloroform procedure (Sambrook et al. 1989).

A fragment of 550 bp of the mitochondrial DNA, including the 5′ end of the control region plus part of \the proline tRNA, was PCR-amplified using primers H00034—5′TACCAAATGTATGAAACCTCAG3′ (Rosel et al. 1994) and light-strand Dlp 1.5—5′TCACC CAAAGCTGAARTTCTA3′ (modified from Pichler et al. 1998). Amplifications were carried out in 40 µl reactions, containing 1 U of Taq polymerase (Amersham Pharmacia); 0.20 mM dNTPs; 1.5 mM $MgCl_2$ and 0.5 µM of each primer. PCR amplifications were per-

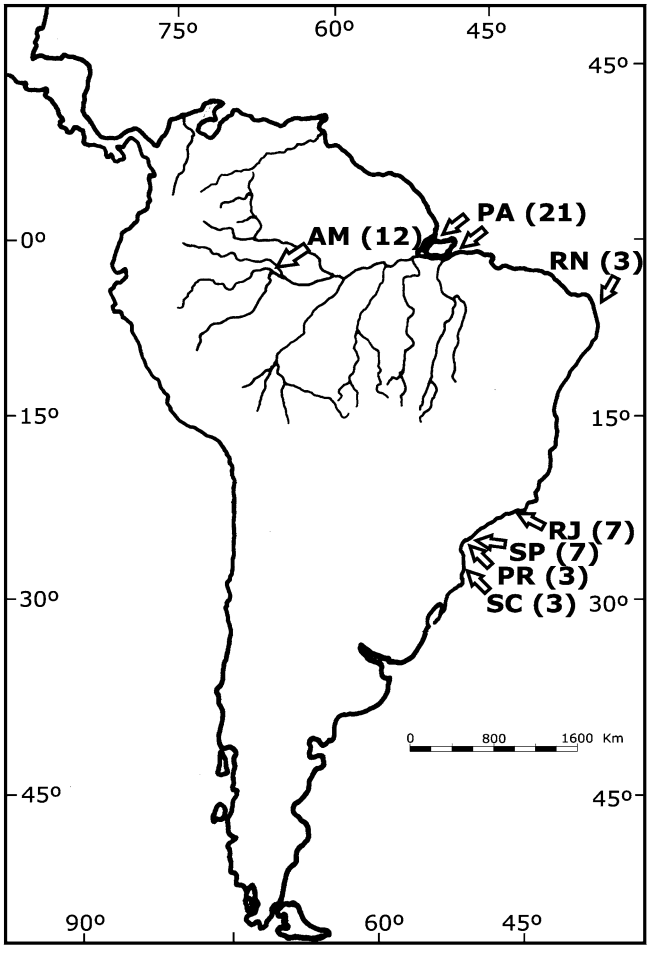

Fig. 1 *Sotalia* spp. Sampling locations and sample sizes. *AM* Amazon, riverine samples, *PA* Pará, *RN* Rio Grande do Norte, *RJ* Rio de Janeiro, *SP* São Paulo, *PR* Paraná, *SC* Santa Catarina

formed in an automated thermocycler (Hybaid) as follows: 3 min at 94°C; 38 cycles of 1 min. at 92°C, 1 min at 48°C and 1 min at 72°C; plus 5 min of final extension at 72°C.

The entire cytochrome *b* gene (1,140 bp) of 20 individuals (eight riverine and 12 marine) was amplified using primers L14724—5′TGACTTGAARAACCAY CGTTG3′ (Palumbi et al. 1991) and an unnamed primer designed by Le Duc et al. (1999)—5′CCTTTTTTGGT TTACAAGAC3′. Cytochrome *b* amplifications were conducted under the same conditions adopted for the control region. Three primers were used for sequencing: the unnamed forward external primer, and two internal: L15129—5′TAACAGTCATAGCYACTGCATT3′ (Le Duc et al. 1999) and H15149—5′CAGAATGATATTT GTCCTCA3′ (Kocher et al. 1989).

PCR products were purified and both strands were sequenced in automated sequencers (ABI 377 and ABI 3100). The different haplotypes obtained were deposited in GenBank under numbers AY842455–AY842471 (control region) and DQ086827–DQ086828 (cytochrome *b*).

Sequences were edited using DNAStar and aligned in ClustalW v.1.82. Diversity indices (haplotype and nucleotide diversities, Nei 1987) were calculated with DnaSP v. 4.0 (Rozas et al. 2003). MEGA v. 2.1 (Kumar et al. 2001) was used to calculate *p*-distances and to construct a neighbour-joining (NJ) gene tree, which was tested with 1,000 bootstraps. Additionally, maximum-likelihood (ML) and parsimony (P) trees were built using PAUP 4 (Swofford 2002). For ML analysis, we used the HKY + G + I evolution model, as chosen by Modeltest v. 3.5 (Posada and Crandall 1998). ML and P phylogenies were tested with 500 and 1,000 replicates, respectively.

A haplotype network was generated by TCS (Clement et al. 2000) according to the 95% parsimony method of Templeton et al. (1992). Clades were then sequentially nested from haplotypes (0-step) to the highest level, each separated by one substitution step (Templeton 1998, 2001). Two observed ambiguities were resolved following the rules of Crandall and Templeton (1993). The geographic association of nested clades was statistically tested with GeoDis v.2.0 (Posada et al. 2000) with 1,000 replications. A pairwise geographic distance matrix between locality centres was used because *Sotalia* dolphins are either coastal or riverine, and thus mostly distributed along a one-dimensional environment. NCA results were interpreted using the most recent inference key available (Templeton 2004).

The geographic subdivision of genetic variation was also investigated through an AMOVA (Excoffier et al. 1992), performed with Arlequin v. 2000 (Schneider et al. 2000). This software computes the *F*-statistics analogues, Φ-statistics, which incorporate information on molecular distances to partition the information of molecular variance into different hierarchical levels. One thousand permutations were run to test the significance of variance differences among hierarchical levels and genetic partitioning hypotheses.

Results

Sequence variation

The 56 *Sotalia* control region sequences were aligned and 484 bp were compared, revealing 22 polymorphic sites, which defined 16 haplotypes (Fig. 2). The most common haplotype was shared by all 18 individuals from south/southeastern Brazil (SC, PR, SP, RJ). All mutations were transitions (73% C/T). Seven fixed differences were found between coastal and riverine animals.

Overall average pairwise sequence divergence (*p*-distance) was 0.012, with 0.005 among marine and 0.004 among riverine animals. Average divergence between marine and riverine samples was 0.025 ± 0.006, and 0.063 ± 0.010 between all *Sotalia* sequences and the outgroup (*Steno bredanensis*).

Haplotype and nucleotide diversities for the entire data set were $Hd = 0.863 \pm 0.033$ and $\pi = 0.0120$. Marine samples showed haplotype ($Hd = 0.792 \pm 0.049$) and nucleotide ($\pi = 0.0052$) diversities similar to freshwater samples ($Hd = 0.788 \pm 0.090$, $\pi = 0.0043$).

Cytochrome *b* sequences displayed only two haplotypes: one was exclusive of all marine samples and the other of the riverine animals. Those haplotypes diverged by 28 substitutions, three of which were non-synonymous. Divergence (*p*-distance) between marine and freshwater forms was 0.025 ± 0.005. Due to the existence

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    [        111122 2222223333 33]
    [    7788347822 4456675577 99]
    [    2607084669 4603451434 02]
#PA27   CCCACTATCA AACTCTTTTT CG
#PA14   .......... ......C... ..
#PA29   ..T....... ......C... ..
#PA13   .......... ...C..C... ..
#PA11   .......... ...C..C..C T.
#PA08   .......... ...C.....C T.
#PA25   .......... ......C..C T.
#PA21   .......C.. ....T.CC.. T.
#PA24   .......C.. ..T.T.C... T.
#S/SE01 .......... ....T.CC.. T.
#RN04   .......... ....T.CC.. ..
#AM75   TT.GTCG..G GG...CC... TA
#AM802  TT.GTCG..G GG....C... TA
#AM113  TT..TCG..G GG...CC... TA
#AM805  TT..TCG.TG GG......C. TA
#AM109  TT..T.G..G GG...CC... T.
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Fig. 2 *Sotalia* spp. Polymorphic sites among *Sotalia* control region haplotype sequences. The first two letters of haplotype codes refer to locations (abbreviations as in legend of Fig. 1, *S*/*SE* south/southeastern—includes samples from *RJ*, *SP*, *PR* and *SC*)

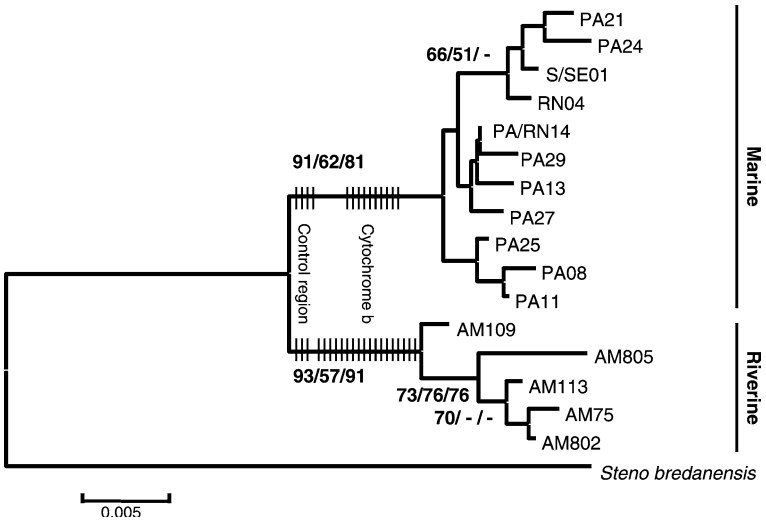

Fig. 3 *Sotalia* spp. Neighbour-joining phylogenetic tree (*p*-distance) between the observed haplotypes of the mitochondrial control region. Maximum likelihood and parsimony analyses recovered similar topologies. Bootstrap values (*NJ*/*ML*/*P*) higher than 50% are shown. Putative synapomorphies of control region and cytochrome *b* haplotypes of the marine and riverine species are indicated by *vertical bars*

of only two haplotypes, other analyses were not conducted for the cytochrome *b* sequences.

Phylogenetic analyses

All phylogenetic methods (NJ, ML and P) depicted two highly supported main groupings, comprising marine and riverine groups (Fig. 3). Shallower relationships were not well resolved.

Control region haplotype network and NCA

The marine and freshwater control region haplotypes formed different clusters, separated by eight mutational steps (Fig. 4). A single additional mutation between them would result in splitting the network, since it would violate the 95% threshold of connection. The final network contained four nested levels. Four clades exhibited significant association between haplotypes and their geographical distribution, thus rejecting the null hypothesis of random distribution of haplotypes across sampled localities. These were clades 1-2 ($P < 0.004$), 2-2

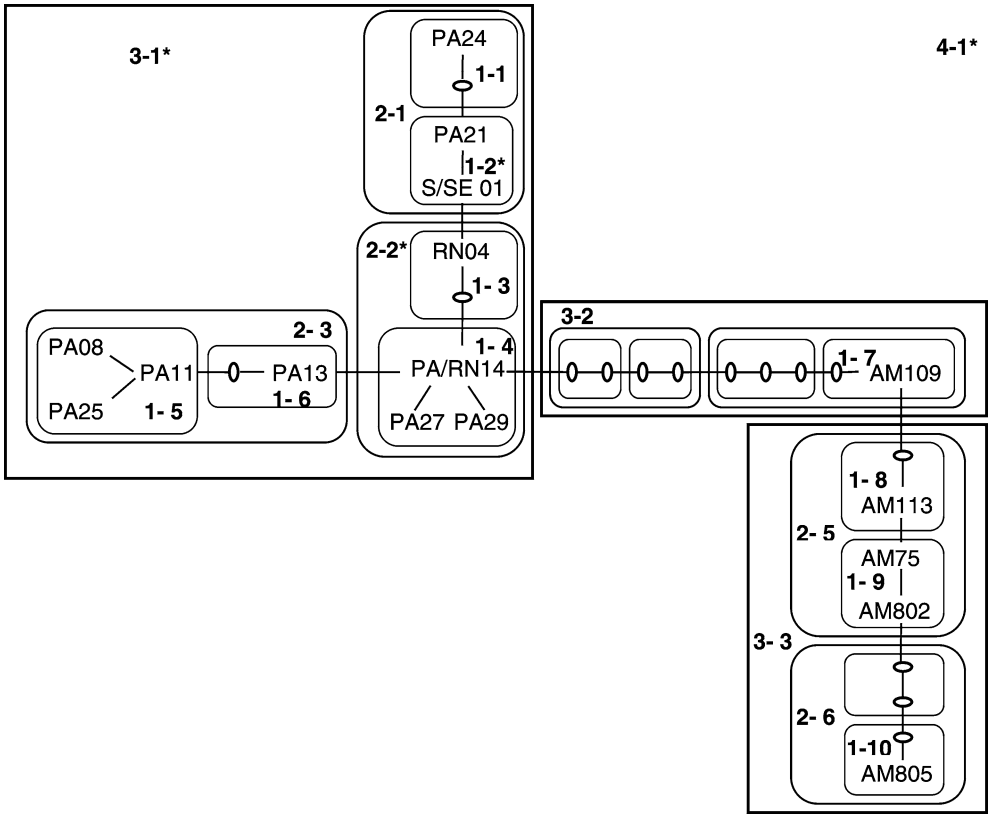

Fig. 4 *Sotalia* spp. Control region haplotype network with the nested clade design. Significant ($P < 0.05$) clades are marked with an *asterisk*. *Ovals* represent missing intermediates. Location codes are as in the legend of Fig. 2. Nesting level is denoted as 1-*x* for first level, 2-*x* for second level and so on, where *x* identifies each clade

($P < 0.038$), 3-1 ($P < 10^{-4}$) and 4-1 ($P < 10^{-4}$). Figure 5 summarizes these results and the consequent interpretations of the NCA.

Analysis of molecular variance

AMOVA analysis of control region sequences showed that 75% $(\Phi_{CT} = 0.750,\ P \ll 10^{-5})$ of the molecular variance was found between marine and riverine individuals. It also indicated that a significant percentage of variation was found among populations/within groups $(14.14\%,\ \Phi_{SC} = 0.435,\ P \ll 10^{-5})$. Thus, we performed a second analysis, stratifying our samples into four groups (one fluvial and three marine: south/southeastern, northeastern and northern). This division accounted for almost 83% $(P \ll 10^{-5})$ of total genetic variation. When only marine samples were analysed, the differences among localities along the coast explained 63% $(P \ll 10^{-5})$ of the molecular variation. For the cytochrome *b* data, we found that 100% of the variation $(\Phi_{CT} = 1,\ P \ll 10^{-5})$ is attributable to differences between the marine and riverine groups.

Discussion

All analytical approaches used support the same conclusion: the riverine and the marine populations of *Sotalia* are deeply divergent. This result, along with distinct ecological and geographical distributions and the morphometric differentiation observed between them (Monteiro-Filho et al. 2002), clearly shows that the marine and riverine forms of *Sotalia* belong to different species.

NCA is a powerful tool that quantitatively and qualitatively investigates population structure and recent evolutionary history, including speciation (Templeton 1998, 2001; Clement et al. 2000; Posada and Crandall 2001; Sites and Marshall 2003). The NCA of *Sotalia* samples indicates a relatively old allopatric fragmentation event, which separated marine and riverine populations. Fragmentation events are evidence of speciation, especially if they: (a) are in higher level (older) clades; (b) reflect the separation of two clusters by several mutational steps and (c) coincide with independent evidence from other type of data (Templeton 2001). The fragmentation observed between the two *Sotalia* ecotypes meets all the three conditions.

Although *F*-statistics, AMOVA and phylogenetic methods may fail to detect differentiation between recently separated taxa (Templeton 1998; Clement et al. 2000; Posada and Crandall 2001), they all corroborated the speciation evidenced by NCA. The NJ, ML and P trees resulted in two reciprocally monophyletic clades with high bootstrap support. AMOVA and *F*-statistics also indicated that marine and riverine animals were highly differentiated $(\Phi_{CT} = 0.75;\ P \ll 10^{-5})$.

The delimitation of species using the NCA has been regarded as a robust method. Besides statistically testing a series of null hypotheses, it also detects inadequacies of sampling design that might prevent unambiguous biological interpretation of data. Additionally, it can be used even without a priori evidence of speciation, and has the power to detect species limits that are not clear (Templeton 2001; Sites and Marshall 2003). In the case of *Sotalia*, there is previous evidence and limits are sharp. Moreover, three other criteria for the recognition of taxa as distinct species are met by our results (morphological and molecular population aggregation analysis, cladistic haplotype aggregation and Templeton's test of cohesion—Sites and Marshall 2003).

The lumping of *Sotalia* species made by former authors was due to the diagnoses of those species being incomplete, owing to the small number of specimens available at the time of their description (True 1889). In the 1960s, although one author recognized three species (Hershkowitz 1966), only two species—*S. fluviatilis* and

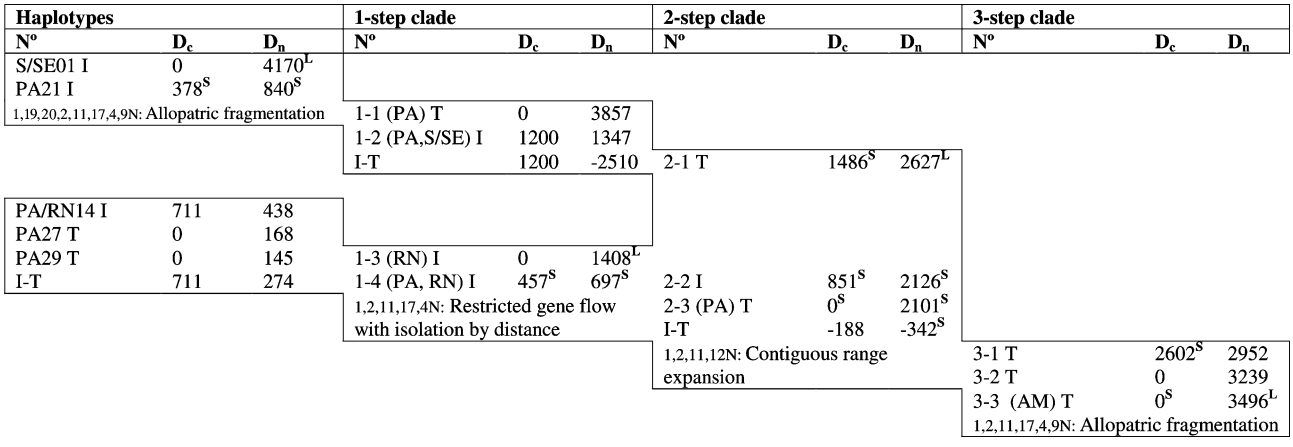

Fig. 5 *Sotalia* spp. Results of the NCA. The clade and nested clade distances are given after the haplotype or clade number. In clades containing tip and interior nested clades, the average difference between interior vs tip clades is also given. Clades with distance values that were significantly larger (*L*) or smaller (*S*) than zero ($P < 0.05$) were interpreted with the inference key (Templeton 2004). Numbers in the *bottom* of significant clades indicate the chain of inference that led to the biological interpretation

S. guianensis—were considered valid by most authors (Cabrera 1961; Rice 1998). Later, a morphometrics study found significant differences between skulls of marine and freshwater *Sotalia*, but since most of the differences were related to size rather than shape, it was decided that *S. fluviatilis* and *S. guianensis* should not be treated as distinct species or subspecies (Borobia 1989). One of the reasons that led Borobia (1989) to reach that conservative conclusion was that she did not analyse skulls from the Amazon delta, and consequently the possibility of a transitional zone between the two forms could not be excluded. The grouping of the two ecotypes under *S. fluviatilis* suggested by Borobia (1989) was accepted and used by most authors until now (Borobia et al. 1991; Jefferson et al. 1993; da Silva and Best 1996; Rice 1998; Flores 2002). The matter of skull distinctiveness was recently readdressed with geometric morphometrics, which showed that the two putative ecotypes could, indeed, be separated by shape (Monteiro-Filho et al. 2002). That study also did not include skulls from the Amazon estuary.

The present study is the first to include samples from the Amazon delta in analysis of differentiation between the two ecotypes. Our results revealed that dolphins from Pará, at the mouth of the Amazon River, are genetically much closer to dolphins from Santa Catarina (4,700 km South, along the coast) than to the geographically closer (2,000 km) riverine dolphins.

Recently, a workshop on cetacean taxonomy defined guidelines for species and subspecies definitions, and recognized that there is a "traditional tendency to err in the direction of avoiding designating too many taxa rather than making sure that all potentially recognized taxa have been designated" (Reeves et al. 2004). According to the workshop's guidelines, an argument for species status should be accepted if there are at least two independent primary lines of evidence for its existence (Reeves et al. 2004). Marine and riverine species of *Sotalia* can be separated not only on the basis of two primary types of evidence (morphology and genetics), but also of a secondary one (i.e., distribution).

Since there is molecular and morphological evidence now on the specific status of both ecotypes, and following the guidelines developed by the workshop on cetacean systematics and other standards, we conclude that the two forms should be treated as different species. Based on priority criteria, we recommend that *Sotalia guianensis* (van Bénéden 1864) be revalidated for the marine ecotype, while the riverine form should hold the binomial *Sotalia fluviatilis* (Gervais 1853—for a discussion on authorship, see van Bree 1974).

To date, there are only four species of cetaceans known to live exclusively in freshwater, all of them belonging to the Super family Platanistidea (*Platanista gangetica*, *Platanista minor*, *Lipotes vexillifer*, *Inia geoffrensis*–this latter probably (Banguera-Hinestroza et al. 2002) includes a fifth species, *Inia boliviensis*). At least four other species may also be found in rivers, but there is no agreement about the degree of differentiation between their marine and riverine populations. Among those species, three are delphinids (*Sousa chinensis*, *Sousa teuszii* and *Orcaella brevirostris*) and the other is a phocoenid (*Neophocaena phocaenoides*). Therefore, *Sotalia fluviatilis* is the first non-platanistoid dolphin to live exclusively in freshwater, and it should be included in the riverine dolphins category for conservation purposes.

Timing of speciation

The evolutionary rates of the control region of delphinids (Hoelzel et al. 1991) and mysticetes (Baker et al. 1993) have been estimated at between 0.5 and 1%/My. A much faster rate, i.e., 6.3–7%/My, has been given by Harlin et al. (2003) for the delphinid *Lagenorhynchus obscurus*. However, this value may have been inflated (Hayano et al. 2004), so we will not use it here, for dating *Sotalia* divergence. The cytochrome *b* is believed to evolve at 0.5%/My (Irwin et al. 1991).

The divergence between *S. fluviatilis* and *S. guianensis* is 2.5%, for both markers. Hence, the speciation event that separated both lineages probably happened between 5 and 2.5 My bp, during the Pliocene. At that time, the Amazon River was already flowing along its present course, with its outlet to the Atlantic (since 8 My bp, Hoorn et al. 1995; Lundberg et al. 1998). For the last 4 My, several sea level oscillations occurred, as a consequence of glacial and interglacial periods. During the periods of sea level rise, river discharge was prevented, and freshwater inflow into the Amazon basin increased, causing the inundation of the Amazon crater (Lundberg et al. 1998). The highest marine transgression happened around 2.5 My bp (Klammer 1984). It is possible that *Sotalia* colonized the Amazon basin during one of these transgression/inundation events. Regardless of the putative timings of speciation, it seems likely that dolphins that colonized the Amazon river system had an Atlantic origin, because the alternative explanation (entrance from the Caribbean via present day Maracaibo Lake and Paleo-Orinoco system) would involve a much older divergence (>10 My).

Sequence variation

Sequence divergence between *S. fluviatilis* and *S. guianensis* (0.025) was within the range reported for comparisons between congeneric delphinid species (control region: 0.011–0.044, Rosel et al. 1994; Cipriano 1997; Wang et al. 1999; cytochrome *b*: 0.008–0.045, Rosel et al. 1994; Le Duc et al. 1999; Hare et al. 2002). Control region haplotype and nucleotide diversities were also similar to those found for other delphinids (Rosel et al. 1994; Pichler and Baker 2000; Parsons et al. 2002; Harlin et al. 2003; Natoli et al. 2004).

Conversely, the lack of diversity (Hd and $\pi = 0$) among control region sequences from south/southeast-

ern Brazil, which encompasses 900 km of coast, was only observed previously in two other cetacean populations: the 134 individuals of the North Island population of *Cephalorhynchus hectori*, and samples from the 567 extant individuals from *Phocoena sinus* (Rosel and Rojas-Bracho 1999; Pichler and Baker 2000). While the lack of diversity in *C. hectori* was credited to recent population depletion (Pichler and Baker 2000), a historical low population size has been demonstrated for *P. sinus*, and its complete homogeneity was attributed to a founder effect in its origin (Taylor and Rojas-Bracho 1999). Current effective population sizes of *Sotalia* along the south/southeastern coast of Brazil are unlikely to be small, but those populations may be the result of colonization by a small number of individuals, as a consequence of a recent range expansion as suggested by the NCA.

Population structure of *S. guianensis*

The NCA suggests that a contiguous range expansion, involving all marine haplotypes, took place (clade 3-1 on Figs. 4 and 5) before isolation by distance and restricted gene flow separated some northern and northeastern lineages (clade 2-2). The most recent event detected was an allopatric fragmentation isolating two northern haplotypes from the south/southeastern one (clade 1-2).

Thus, after the separation of riverine and marine species, *S. guianensis* may have expanded along the Brazilian coast to the South, where biogeographic barriers probably limited further expansion. Later, gene flow along the coast would have diminished between north and northeastern haplotypes.

Joining NCA and AMOVA results, it becomes clear that there is subdivision among *S. guianensis* populations. The most plausible hypothesis ($\Phi_{CT} = 0.628$, $P < 10^{-5}$) is that there are at least three populations of *S. guianensis* in Brazil: northern, northeastern and south/southeastern. Notwithstanding the fact that mitochondrial molecular markers only contain information on female lineages, their use in population delimitation has been considered biologically sound (Dizon et al. 1997). The reasoning is that females are the demographically important components, and that managing stocks in terms of their female groups should present no ambiguity. Thus, the evidence of female philopatry is sufficient to establish management units, regardless of the possibility of male mediated gene flow (Dizon et al. 1997). However, caution is required before the south/southeastern populations are pooled together for management purposes. As discussed above, the existence of a single haplotype along this region may result from recent past evolutionary events rather than current gene flow, and hence populations may actually be adapted to different selective regimes. For instance, recent studies have found differences in age and growth parameters between *Sotalia* from southern and southeastern Brazil, but it is still not clear if those are due to methodological/sampling differences (Rosas et al. 2003; Santos et al. 2003; Di Beneditto and Ramos 2004). Other aspects, such as parasite or contaminant load, may provide data on population limits within south/southeastern Brazil.

Conservation aspects

The genetic analyses of *S. fluviatilis* and *S. guianensis* have profound consequences for their conservation. First, they show that the freshwater lineage that lives in the Amazon has been isolated from the marine species for over 2 My, having an evolutionary history intimately associated to that of the river. Second, they indicate that, along the Brazilian coast, there are three evolutionary significant units, which should be managed separately. The north and northeast Brazil populations are genetically variable, but the south/southeastern population is genetically very homogeneous. This indicates that there has been a recent range expansion of *S. guianensis* towards the south, perhaps linked to the warming up of the Western Atlantic during the Holocene.

Another interesting finding is the presence of the marine *S. guianensis* at the mouth of the Amazon River (samples from Pará). The freshwater load of the Amazon reaches hundreds of kilometres into the sea (Muller-Karger et al. 1988), so the animals sampled in Para were possibly living much of their lives in fresh water. It would be interesting to analyse samples from intermediate locations along the Amazon River, to detect how far upriver *S. guianensis* occurs, and verify if there is sympatry in any region with *S. fluviatilis*. The potential of *S. guianensis* to live in freshwater also begs the analysis of populations attributed to *S. fluviatilis* in other large rivers, like the Orinoco, to investigate if *S. fluviatilis* is endemic of the Amazon.

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