TAXONOMIC STATUS OF RIVERINE AND COASTAL DOLPHINS *SOTALIA* SPP.

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Abstract - Dolphins of the genus *Sotalia* spp. are found along the Caribbean and Atlantic Coast of Central and South America and in the Amazon River and most of its tributaries. Although five species were described in the late 1800's, during the last decades only one species was recognized (*Sotalia fluviatilis*) with two ecotypes or subspecies, the coastal subspecies (*Sotalia fluviatilis guianensis*) and the riverine subspecies (*Sotalia fluviatilis fluviatilis*). Morphometric analyses suggested recognition of each subspecies as separate species, *Sotalia guianensis* and *Sotalia fluviatilis*, which was recently confirmed by mitochondrial DNA sequence analyses. Here we review the history of the classification of this genus, present genetic evidence from nuclear introns sequences (Lactalbumin, Actin and Glucocerebrosidase) (n = 28) and sequences from three mitochondrial genes (Control Region (n = 132), Cytochrome *b* (n= 107) and NADH dehidrogenase subunit 2 (n=51)) from samples collected throughout the South American distribution range of *Sotalia*, and review additional evidence for this taxonomical revision from previously published and unpublished morphological and ecological studies supporting the elevation of each subspecies to the species level under the Genealogical/Lineage Concordance Species Concept (GCC). Based on priority criteria, the authors recommend that freshwater animals should retain the binomial *Sotalia fluviatilis*, while *Sotalia guianensis* should be revalidated for the coastal dolphins. It is noteworthy that *S. guianensis* could be found in the Amazon River, yet it is not known how far upriver it may occur, nor if they can be found in sympatry with *S. fluviatilis*.

Resumo - Golfinhos do gênero *Sotalia* spp. ocorrem nas costas caribenha e atlântica da América Central e do Sul e no Rio Amazonas e em vários de seus afluentes. Apesar de cinco espécies terem sido descritas no final do século XIX, durante as últimas décadas apenas uma espécie era reconhecida (*Sotalia fluviatilis*), com dois ecótipos ou subespécies, uma costeira (*Sotalia fluviatilis guianensis*) e a outra fluvial (*Sotalia fluviatilis fluviatilis*). Análises morfométricas sugeriram que as duas subespécies constituem espécies diferentes, *Sotalia guianensis* e *Sotalia fluviatilis*, o que foi recentemente confirmado por análise de seqüências mitocondriais. Neste trabalho nós revisamos a história de classificação do gênero, apresentamos dados de seqüências de introns nucleares (Lactalbumina, Actina e Glucocerebrosidase, n = 28) e seqüências de três genes mitocondriais (região controle, n = 132; citocromo *b*, n = 107; e subunidade 2 da NADH desidrogenase, n = 51) de amostras coletadas ao longo de toda a distribuição de *Sotalia* na América do Sul, e revisamos evidências morfológicas e ecológicas publicadas e não publicadas que sustentam a elevação de cada subespécie ao nível de espécie pelo Conceito de Espécie por Concordância Genealógica (GCC). Com base nos critérios de prioridade, os autores recomendam que os animais fluviais retenham o binômio *Sotalia fluviatilis*, enquanto que *Sotalia guianensis* deve ser revalidado para os marinhos. É importante ressaltar que *S. guianensis* pode ser encontrado no Rio Amazonas, mas ainda não se sabe o quanto a montante ela ocorre, nem se existe simpatria com *S. fluviatilis*.
Keywords: *Sotalia fluviatilis*, *Sotalia guianensis*, molecular taxonomy, mitochondrial DNA, nuclear DNA.

Introduction

The South American dolphins *Sotalia* spp. are endemic to the Caribbean and Atlantic Coasts of South America, ranging from Nicaragua to Southern Brazil (Borobia *et al.*, 1991, Carr and Bonde, 2000), and the Amazon River and most of its tributaries (Borobia *et al.*, 1991, da Silva and Best, 1996). The taxonomy of this genus has been controversial. In the late 1800's up to five species were described, three from riverine specimens and two from coastal specimens (Rice, 1998). The first species was described by Gervais (1853) as *Delphinus fluviatilis*, from a specimen collected in the Peruvian Amazon, close to Pebas (van Bree, 1974, Robineau, 1990). Gray placed this species in the genus *Sotalia* in 1866 (Robineau, 1990). Two additional riverine species, *Delphinus pallidus* described by Gervais in 1853 (Hershkovitz, 1966, Robineau, 1990) and *Steno tucuxi,* described by Gray in 1856 (Hershkovitz, 1966, da Silva and Best, 1994), were classified in the genus *Sotalia*, but they were later considered

synonyms of *Sotalia fluviatilis* (da Silva and Best, 1994). P. J. Van Bénéden initially described one coastal species, *Delphinus guianensis* (Van Bénéden, 1864) based on three specimens collected from the mouth of the Marowijna River in the border between Surinam and French Guiana (Williams, 1928, Hershkovitz, 1962, Husson, 1978). This species was also re-classified by Gray as a member of the genus *Sotalia* sp. in 1866 (Hershkovitz, 1966, da Silva and Best, 1994). E. Van Bénéden described a second coastal species in 1875, designated as *Sotalia brasiliensis,* but it is now considered a synonym of *Sotalia guianensis* (da Silva and Best, 1994). Recent reviews have resulted in a series of revisions (Cabrera, 1961), first reducing the number of species to two, one riverine, *Sotalia fluviatilis*, and one coastal, *Sotalia guianensis*, and later to one species, *Sotalia fluviatilis*, with two ecotypes or subspecies, *Sotalia fluviatilis fluviatilis* (riverine subspecies) and *Sotalia fluviatilis guianensis* (coastal subspecies) (Borobia *et al.*, 1991, da Silva and Best, 1994, Rice, 1998). Tridimensional morphometric analysis of skull shape showed significant differences between riverine and coastal specimens suggesting the designation of each subspecies as separate species again (Monteiro-Filho *et al*., 2002). Analysis of two mitochondrial genes demonstrated that those two species were indeed genetically different, the freshwater and marine specimens forming monophyletic groups supported by 14 and 21 synapomorphic characters, respectively (Cunha *et al*., 2005).

Here we present genetic evidence further supporting the recognition of each *Sotalia* subspecies as full species, under the GCC Genealogical/Lineage Concordance Species Concept (GCC) (Avise and Ball, 1990, Avise and Wollenberg, 1997, Avise, 2000). We include analysis of DNA sequences from 10 independent nuclear introns, three of which have fixed site differences between coastal and riverine *Sotalia*, and three gene fragments from the mitochondrial genome. In support of this proposal, we also review published and unpublished morphometric data (Borobia, 1989, Monteiro-Filho *et al.*, 2002).

Materials and Methods

Sample collection and DNA extraction

A total of 132 samples of skin, liver, muscle, bone or teeth were obtained from coastal and riverine *Sotalia* in 22 locations, grouped into 9 geographic regions throughout its range (Figure 1 and Table 1). Tissue samples were obtained from dead stranded animals or animals captured in fishing nets or from captive and free raging animals (six samples from the Colombian Caribbean). Bones and teeth were obtained from skeletal remains found in the field (n=10) or from museum specimens (n=3). Two DNA samples were obtained from the DNA and Tissue Archive at the Southwest Fisheries Science Center (NOAA, La Jolla, California). DNA extraction from tissue samples followed the protocol of Sambrook *et al*. (1989), modified for small samples by Baker *et al*. (1994). DNA was extracted from bones following a silica-guanidinium thiocyanate based protocol described by Pichler *et al.* (2001).

PCR amplification and sequencing

Three mitochondrial genetic markers were amplified and sequenced from all available samples; a 550 or 627 base pair (bp) portion of the mitochondrial DNA control region (CR), the complete cytochrome *b* (Cyt-*b,* 1,140 bp) or a 425 bp fragment of it, and a 1,044 bp fragment of NADH dehydrogenase subunit 2 (ND2). Primer combinations are summarized in Table 2. Based on initial screening of ten samples of *Sotalia* spp., seven introns (including four Y chromosome introns, Table 2) showed no variation. The other three were the first exon and first partial intron of the α-Lactalbumin gene (Lac-1), the first Actin intron (Act-1) and the Glucocerebrosidase intron (GBA), all of which showed fixed nucleotide differences between coastal and riverine *Sotalia* and were sequenced for the remaining 18 samples. A total of 28 *Sotalia* samples with high DNA quality (21 coastal and 7 riverine) from 7 geographic locations (Table 1) were amplified following previously published protocols (Table 2). DNA extracted from bones, teeth or degraded skin proved unsuitable for amplification of nuclear genes. However most of these were suitable for amplification of mitochondrial genes. To protect against contamination, samples were run in at least two separate PCR reactions, including extraction blanks. PCR products were directly sequenced in both

directions using the standard protocols of Big Dye™ terminator sequencing chemistry on an ABI 3100 or MegaBACE (Amersham Biosciences) automated capillary sequencer.

Data analyses

Sequences were edited manually and aligned using DNAStar and ClustalW v1.82 or Sequencher 4.1 software (Genes Code Corporation). For the combined mitochondrial dataset (1,052 bp), as well as for the combined nuclear dataset (4,312 bp), haplotypes (in the case of mtDNA) or genotypes (in the case of nuclear DNA (nuDNA)) were defined using MacClade (Maddison and Maddison, 2000). A polymorphic site was indicated by a secondary peak with a height ≥ 30% of the height of the primary peak as well as by the presence of a secondary peak at the same position in both the forward and reverse sequence. We calculated the number of variable and fixed site differences for all genes, as well as the proportion of synonymous and non-synonymous substitutions, in the case of the two protein coding genes studied (Cyt-*b* and ND2) using the program MEGA2 (Kumar *et al*., 2001). The model of substitution for the two combined datasets was tested in Modeltest v3.06 (Posada and Crandall, 1998) and the settings for this model were used in the Neighbour-Joining phylogenetic reconstruction with bootstrap re-sampling performed in PAUP version 4.0b1 (Swofford, 2002). Additionally, Maximum Parsimony and Maximum Likelihood phylogenetic reconstructions were also performed, but these results are not included in this document. *Steno bredanensis* and *Sousa chinensis* were used as outgroups for these analyses. Further details and information about additional phylogenetic analysis (Nested Clade Analysis) can be obtained from the authors or consulted in Cunha *et al*. (2005).

Results

Mitochondrial DNA

A minimum of 1,052 bp of the mitochondrial genome (CR and Cyt-*b*) was amplified and sequenced from 107 *Sotalia* representing coastal and riverine subspecies. An additional 1,044 bp of the mitochondrial protein-coding gene ND2 were amplified and sequenced for 51 samples. An additional 25 samples of poorer quality were sequenced for only a short fragment of the mtDNA CR (350-400bp). 38 different mitochondrial combined haplotypes (CR+Cyt-*b)* were identified within the 107 samples (Table 3), 36 of which were distinguished by substitutions in the CR, and two that were distinguished by additional variable sites in the Cyt-*b* gene. A total of 46 variable sites were found along the 1,052 bp of the combined mtDNA dataset (Table 3). Nine of the CR variable sites represented fixed differences between coastal and riverine *Sotalia*. For Cyt-*b*, twelve variable sites were found, five of which were fixed site differences between the two species. Eight additional CR haplotypes were determined in the shorter fragment amplified from bone samples. These were excluded from the present analysis, but further information is available from the authors. For the ND2 gene, 36 variable sites were found, 20 of which were fixed site differences between the two species. Four of these corresponded to non-synonymous substitutions (Figure 2).

Nuclear DNA

A 591 bp fragment of Lac-1, 949 bp of Act-1, and 307 bp of GBA were successfully amplified and sequenced from a total of 28 *Sotalia* samples. One fixed site difference was found between coastal and riverine *Sotalia* in each of these three introns, for a total of three in 1,847 bp. The presence of the three fixed sites was confirmed in a larger sample of coastal and riverine *Sotalia,* after the initial screening. All were transitions (A→ G or G→ A) (Figure 2). No heterozygotes were found in the screening, except for one individual from the Colombian Caribbean (at position 32 in the first Actin intron). One fixed site difference in the Y chromosome intron UBE1Y7 was found to be diagnostic for *Sotalia* spp. when compared to *Sousa chinensis* and *Steno bredanensis*. Three additional fixed site differences

in the Y chromosome intron DBY8 and in the autosomal introns GBA and CAT were shared between *Sotalia* spp. and *Sousa chinensis* relative to *Steno bredanensis* (Figure 2).

Phylogenetic analyses
For the two mitochondrial genes, Modeltest indicated the best-fit model of substitution to be the HKY+I+G. The Partition of Homogeneity Test found no conflict in phylogenies (p = 0.97) for the individual and combined mitochondrial dataset. Phylogenetic reconstructions by Maximum Parsimony, Maximum Likelihood and Neighbour-Joining showed clear reciprocal monophyly for individual and combined genes between haplotypes of the two *Sotalia* species (data for the Neighbour-Joining reconstruction based on the combined mitochondrial dataset shown only, Figure 2). For the three nuclear loci, the best-fit model of nucleotide substitution was HKY. A Partitioning of Homogeneity Test showed no conflicting phylogenies (p = 0.96) for the combined nuDNA dataset or the total combined dataset (mtDNA + nuDNA, p = 0.99). The Parsimony and Neighbour-Joining phylogenies showed clear reciprocal monophyly for individual and combined gene fragments (including the combined mtDNA + nuDNA) between haplotypes and genotypes of the two species (data not shown) with high bootstrap support. The small number of fixed site differences found between both species (synapomorphies) at the nuclear level were mapped as cladistic characters onto the phylogenetic reconstruction for the combined mitochondrial haplotypes (Figure 2).

Discussion
Genetic evidence presented confirms the recognition of the two *Sotalia* species based on the four criteria of the Genealogical/Lineage Concordance Concept (GCC) (Avise and Ball, 1990, Avise and Wollenberg, 1997, Avise, 2000):

i) Concordance across sequence characters within genetic locus leading to conclusive exclusion.
In the combined mitochondrial dataset (38 CR+Cyt-*b* haplotypes), 14 fixed sites differences were found between coastal and riverine *Sotalia*. An additional 20 were found in the mitochondrial gene ND2, four of which corresponded to non-synonymous substitutions. Three fixed non-synonymous substitutions have also been observed in the complete Cyt-*b* (Cunha *et al.*, 2005). The presence of non-synonymous substitutions in these two mitochondrial protein-coding genes would suggest some functional as well as neutral divergence. Reciprocal monophyly of coastal and riverine *Sotalia* mitochondrial haplotypes, as well as a large number of mitochondrial fixed differences between them, have previously been reported by Cunha *et al*. (2005). Three additional fixed differences were found among the combined nuclear gene fragments. This low number of fixed site differences at the nuclear level was expected, due to the slower rates of evolution of the nuclear genome (Hare *et al.*, 2002).

ii) Concordance in genealogical patterns across multiple loci, both mitochondrial and nuclear.
MtDNA lineages (i.e haplotypes) and nuclear introns showed a pattern of reciprocal monophyly and fixed characters satisfying the Exclusivity Criterion and Cladistic Haplotype Aggregation method of species delimitation for the GCC (Sites and Marshall, 2003). For this method, a population is considered a species if the haplotypes of all its members are joined in a contiguous section of an unrooted parsimony cladogram, forming monophyletic groups, which are separated and distinct from other such clades by a single branch, along with character-state changes leading to fixed character differences (Sites and Marshall, 2003).

iii) Concordance with biogeographical patterns

The distribution of *Sotalia* spp. showed complete concordance with the phylogenetic patterns observed in our analysis. Coastal and riverine populations occur in physical isolation (allopatry) with little overlap, possibly only at the mouth of the Amazon River and the Amazonian Estuary (Borobia *et al.*, 1991, da Silva and Best, 1996). We cannot exclude the possibility of some hybridization in this area of overlap but there appears to be little or no gene flow or introgression across this boundary (Caballero *et al.*, 2003, Cunha *et al.*, 2005)

iv) Concordance with morphological characters

Coastal and riverine *Sotalia* differ in various morphological characteristics, including slight differences in body coloration, dimensions of the orbital region and number of teeth. Borobia (1989) recorded and compared a total of 37 cranial characters measurements from 58 *Sotalia* skulls, including 21 riverine and 38 coastal specimens, using paired t-tests. Out of these 37 cranial characters, 29 were significantly different with a p value < 0.001. These characters included the length of the rostrum, the number of teeth in the upper maxilla, the internal length of the braincase and the length of the left tympanic cavity. Characters such as the preorbital width, supra and postorbital widths, as well as the condylobasal length provided best discrimination between riverine and coastal specimens (Borobia, 1989). Overall, coastal specimens tended to have larger skulls than riverine ones as well as larger body sizes. Monteiro-Filho and collaborators (2002) applied a geometric method of tridimensional morphometric analysis of 22 landmarks from 104 specimens, including 92 coastal and 13 riverine of unknown age. They found significant shape differences between coastal and riverine *Sotalia*. In riverine specimens, the rostrum and the occipital condyle pointed downwards relative to the anteroposterior axis of the skull. In coastal specimens the rostrum and the condyle were aligned along this axis.

Conclusions

Genetic, morphological and biogeographical evidence suggest species level ranking recognition for each of the two *Sotalia* subspecies (coastal and riverine). Based on priority criteria, the authors recommend that freshwater animals should retain the binomial *Sotalia fluviatilis*, while *Sotalia guianensis* should be revalidated for the coastal dolphins.

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Table 1. Sampling locations and tissue type and number of mitochondrial and nuclear sequences obtained for coastal and riverine *Sotalia*. Numbers in parenthesis before each sampling location correspond to the number of this sampling location in Figure 1.

Geographic region	*Sampling location*	*Subspecies*	*Sample size and type*	*mtDNA*	*nuDNA*
Colombian Caribbean	(1) Morrosquillo Gulf (Córdoba province)	Coastal	4 skin 1 tooth	5	4
	(2) Santa Marta (Magdalena province)	Coastal	3 skin	3	1
	(3) La Guajira province	Coastal	4 skin	4	3
Maracaibo Lake	(4) Zapara Island	Coastal	12 skin 2 bone	14	1
Trinidad and Tobago	Unknown	Coastal	1 tooth*	1	-
French Guiana	(5) Cayenne	Coastal	6 skin	6	4
Amazonian Estuary	(6) Pará state	Coastal	23 skin	23	-
Brazilian Coast	(7) Ceará state	Coastal	1 liver	1	-
	(8) Rio Grande do Norte state	Coastal	2 skin	3	-
	(9) Bahía state	Coastal	2 skin	2	1
	(10) Espírito Santo state	Coastal	3 skin	2	1
	(11) Rio de Janeiro state	Coastal	9 skin	9	-
	(12) Cananéia estuary (São Paulo state)	Coastal	19 skin	19	6
	(13) Paraná state	Coastal	3 skin	3	-
	(14) Santa Catarina state	Coastal	3 skin	3	-
	Unknown	Coastal	1 DNA**	1	-
Peruvian Amazon	(15) Curaray River	Riverine	1 skin	1	1
Colombian Amazon	(16) Caquetá River	Riverine	2 bone	2	-
	(17) Puerto Nariño (Amazonas province)	Riverine	2 skin 4 tooth	6	-
	(18) Leticia (Amazonas province)	Riverine	1 skin 1 tooth	2	1
Brazilian Amazon	unknown	Riverine	1 DNA**	1	1
	(19) Mamirauá Preserve, Japurá River (Amazonas state)	Riverine	12 skin	12	-
	(20) Tefé (Amazonas state)	Riverine	7 skin	7	4
	(21) Santarém (Pará state)	Riverine	1 bone	1	-
	(22) Araguaia River	Riverine	1 bone	1	-

* Sample donated by the USNM: United States National Museum Smithsonian Institution, (Washington D.C, U.S.A)

**Sample donated by the SWFSC: Southwest Fisheries Science Center (La Jolla, CA, U.S.A)

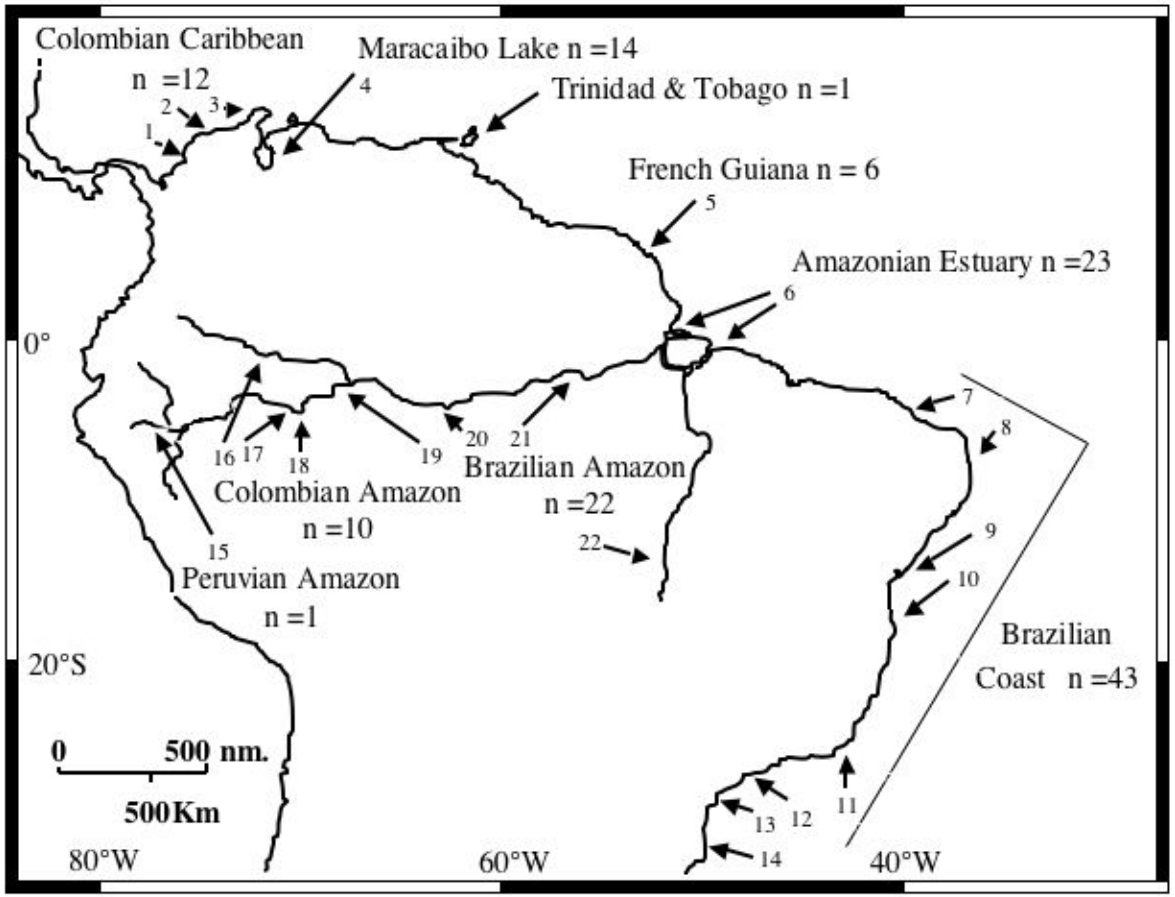

Figure 1. Distribution of coastal and riverine *Sotalia* showing geographic regions, sampling location (numbers refer to Table 1) and sample sizes included in this study.

Table 2. Summary of loci and amplification conditions used in study of *Sotalia* spp. and outgroups.

Locus	*Primer name*	*Annealing temperature*	*Observed product size*	*Reference*
				MTDNA
CR	TRO D	52°C	531 bp	R. LeDuc (SWFSC)
CR	t-Pro-whale M13Dlp1.5 Dlp8	55°C	800 bp	DNA surveillance (www.dna-surveillance.ac.nz)
CR	t-Pro-whale M13Dlp1.5 Dlp4	55°C	400 bp	Baker *et al*. (1998)
CR	Modified Dlp1.5 H00034	48°C	550 bp	Cunha *et al*. (2005) Rosel *et al*. (1994)
Cyt- *b*	Tglu CB2	55°C	464 bp	Palumbi (1996)
Cyt-*b*	L14724 Unnamed	48°C	1140 bp	Palumbi *et al.* (1991) LeDuc *et al.* (1999)
Cyt-*b* (sequencing)	L1529 H15149	48°C	———	LeDuc *et al.* (1999) Kocher *et al.* (1989)
ND2	ILP5100R BatH4823tRNA-metF	55°C	1050 bp	T. Mclenachan (Alan Wilson Centre, Massey University)
ND2 (sequencing)	BatL4235 BatH4461	55°C	———	T. Mclenachan (Alan Wilson Centre, Massey University)
nuDNA (autosomal introns)				
Act-1	Act-3 Act-1385	55°C	980 bp	Palumbi and Baker (1994) Conway (2005)
Lac-1	LacIR LacIIF	54°C	600 bp	Milinkovitch *et al.* (1998)
GBA	GBA-F **GBA-R**	55°C*	310 bp	Roca *et al.* (2001)
CHRNA1	CHRNA1-F CHRNA1-R	55°C*	360 bp	Roca *et al*. (2001)
CAT	CAT-F CAT-R	55°C*	520 bp	Lyons *et al*. (1997)
IFN	IFN-F IFN-R	55°C*	340 bp	Lyons *et al.* (1997)
nuDNA (Y chromosome introns)				
DBY7	DBY7-F DBY7-R	55°C*	400 bp	Hellborg and Ellegren (2003)
DBY8	DBY8-F DBY8-R	55°C*	200 bp	Hellborg and Ellegren (2003)
SMCY7	SMCY7-F SMCY7-R	55°C*	500 bp	Hellborg and Ellegren (2003)
UBE1Y7	UBE1Y7-F UBE1Y7-R	50°C*	500 bp	Hellborg and Ellegren (2003)

* *Taq*GOLD

Table 3. 46 polymorphic sites over 1052 bp of the combined mitochondrial dataset (Cyt-b +CR) determining 38 *Sotalia* spp. haplotypes. A star (*) denotes fixed site differences and (Δ) designates a haplotype defined by nucleotide substitutions in the Cyt-*b* gene. (?) indicates missing data. Haplotype designation follows Cunha *et al*. (2005) and Caballero *et al*. (in review).

Haplotypes	Variable Sites	
	Cytochrome-*b* (425 bp) 111122333 467012899099 968674347006 * ** * *	Control Region (627 bp) 1111222222222333333444555566666 6999046904466688916799901755770012 3048586244724812359201280745471312 ** * ** ** **
Coastal		
A	GCCCCATCATTC	GCCCACTATCAAACTTTACCCTTTGTTTATGGCA
B	.....G.....T	..............CC...T..C..........
C •	.....GC....T	..............CC...T..C..........
D	....	..............C.......C..........
E	....	A....T.........C.......C..........
F	....	..........G....C.......C..........
G	..T.........	..............C...T..CC......AA..
H	....	A....T..C......C.......C..........
I •	.........C..	A....T..C......C.......C..........
J	.........C..	A....T..C.G....C.......C..........
K	.....G.....T	..............CC.GTT..CC..........
L	A...........	..............CC...T...C..........
M	....	?...................TC........????
N	....	....................T.........????
PA08	....	..............CC..TT..C...????????
PA11	....	..............CC...T..C...????????
PA13	....	..............CC...T...C..A???????
PA14	....	..............C...T...C..????????
PA21	....	........C...................????????
PA24	....	........C....T.....T......????????
PA25	....	..............C...T..C...????????
PA27	....	..............C..TT...C..????????
PA29	....	...T...........C...T...C..????????
S/SE01	....	........................????????
RN04	....	......................C..????????
Riverine		
S	.T.TA...G.C.	.TT.GTCG..GGGT.CC..T....A..CG.AATG
T	.T.TA...G.C.	.TT..TCG..GGG..CC..T....A..CG.AATG
W	.T.TA...G.C.	.TT.GTCG..GGG..CC..T....A..CGCAATG
X	.T.TA...G.C.	.TT.GTCG..GGG..CC..T....A..CG.AATG
Y	.T.TA...G.C.	.TT.GTCG..GGG..CC..T....AC????????
Z	.T.TA...G.C.	.TT.GTCG..GGGT.CC..T....A..CG.AATG
CC+AM75	.T.TA...G.C.	.TT.GTCG..GGG..CC..T....A..CG.AATG
DD	.T.TA..TG.C.	.TT..TCGC.GGG..C..TT.C..A..CG.AATG
EE	.T.TA...G.C.	.TT..TCGCTGGG..C..TT.C..AC????????
AM109	.T.TA...G.C.	.TT..T.G..GGG..CC..T......????????
AM113	.T.TA...G.C.	.TT..TCG..GGG..CC..T....A.????????
AM802	.T.TA...G.C.	.TT.GTCG..GGG..C...T....A.????????
AM805	.T.TA...G.C.	.TT..TCG.TGGG..C..TT.C..A.????????

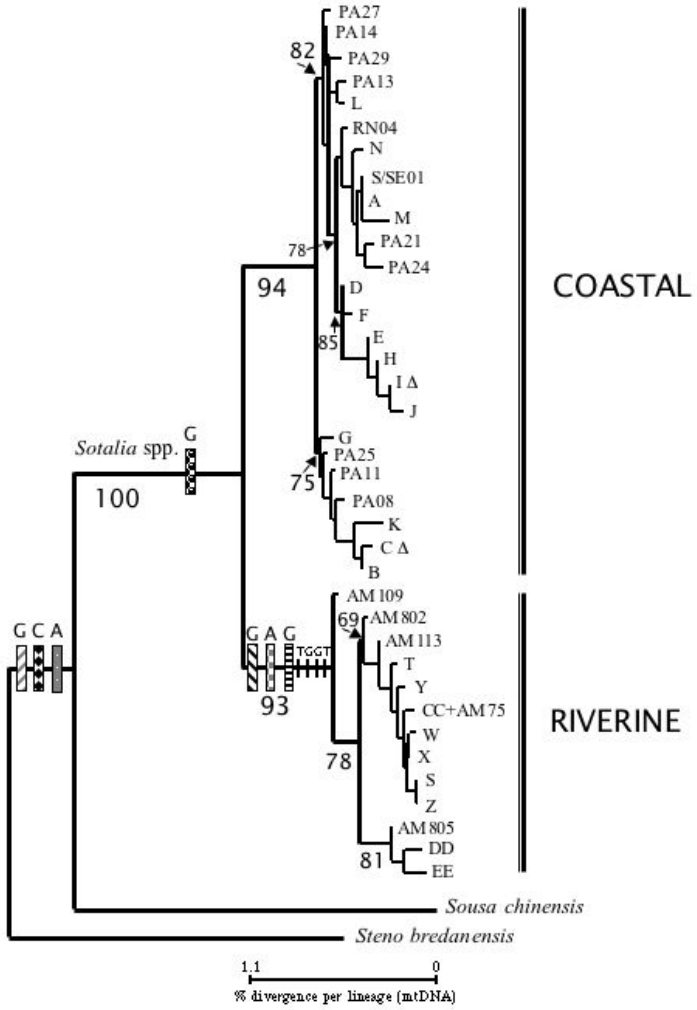

Figure 2. Neighbour-joining phylogenetic reconstruction of the combined mitochondrial haplotypes (CR+Cyt-*b*, 1,052 bp) of *Sotalia* spp. and outgroups with bootstrap values from 1,000 replicates. Haplotype codes follow Cunha *et al*. (2005) and Caballero *et al*. (in review). (Δ) indicates haplotypes distinguished on the basis of the Cyt-*b* gene. % divergence was calculated using the Tamura-Nei distance. Vertical bars on the tree represent nuclear fixed sites as cladistic characters, showing their derived state in riverine *Sotalia*. Represents position 498 in Lac-1, position 193 in GBA and position 800 in Act-1. Four black vertical bars represent non-synonymous substitutions in the mitochondrial coding gene ND2 (positions 23, 415, 889 and 1027). The vertical bar represents one fixed site in the Y chromosome intron UBE1Y7 (position 193) distinguishing both *Sotalia* spp. from the outgroups. Fixed differences shared between both *Sotalia* spp. and *Sousa chinensis* relative to *Steno bredanensis*: represents position 38 in the Y chromosome intron DBY8, position 198 in GBA and position 184 in CAT