MARINE MAMMAL SCIENCE, 23(2): 358–386 (April 2007)

DOI: 10.1111/j.1748-7692.2007.00110.x

TAXONOMIC STATUS OF THE GENUS *SOTALIA*: SPECIES LEVEL RANKING FOR "TUCUXI" (*SOTALIA FLUVIATILIS*) AND "COSTERO" (*SOTALIA GUIANENSIS*) DOLPHINS

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ABSTRACT

Dolphins of the genus *Sotalia* are found along the Caribbean and Atlantic coasts of Central and South America and in the Amazon River and most of its tributaries. At present, the taxonomy of these dolphins remains unresolved. Although five species were described in the late 1800s, only one species is recognized currently (*Sotalia fluviatilis*) with two ecotypes or subspecies, the coastal subspecies (*Sotalia fluviatilis guianensis*) and the riverine subspecies (*Sotalia fluviatilis fluviatilis*). Recent morphometric analyses, as well as mitochondrial DNA analysis, suggested recognition of each subspecies as separate species. Here we review the history of the classification of this genus and present new genetic evidence from ten nuclear and three mitochondrial genes supporting the elevation of each subspecies to the species level under the Genealogical/Lineage Concordance Species Concept and the criterion of irreversible divergence. We also review additional evidence for this taxonomic revision from previously published and unpublished genetic, morphological, and ecological studies. We propose the common name "costero" for the coastal species, *Sotalia guianensis*

(Van Bénéden 1864), and accept the previously proposed "tucuxi" dolphin, *Sotalia fluviatilis* (Gervais, 1853), for the riverine species.

Key words: tucuxi, mtDNA, nuclear DNA, taxonomy, *Sotalia guianensis*, *Sotalia fluviatilis*.

Dolphins of the genus *Sotalia* 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 inhabiting the Amazon River and most of its tributaries (Borobia *et al*. 1991, da Silva and Best 1996).

Alexander von Humboldt was probably the first naturalist to document the presence of coastal dolphins that ascended the mouths of rivers in Venezuela. During his travels in northern South America, between 1799 and 1804, he noted the presence of relatively small dolphins with prominent dorsal fins about 130 km up from the mouth of the Orinoco River, in San Fernando de Apure, Venezuela (Hershkovitz 1962). From these first descriptions, it is safe to assume that the dolphins described by Humboldt belonged to the genus later recognized as *Sotalia* (Hershkovitz 1962).

The taxonomy of this genus has been controversial. In the late 1800s, five species were described, three from riverine specimens and two from coastal specimens (Rice 1998). The first riverine species was described by Gervais in 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). In 1855 Gervais also described *Delphinus pallidus* from a specimen collected near Nauta in the Peruvian Amazon. However, *Delphinus pallidus* is now considered to be a coloration variant of the original *Delphinus fluviatilis* (Hershkovitz 1966, Robineau 1990). In 1856 Gray described a third riverine species, originally named *Steno tucuxi*, from a specimen collected in the Brazilian Amazon near Santarém (Hershkovitz 1966, da Silva and Best 1994). This species was later classified in the genus *Sotalia* by Flower (1883), but it is now recognized as a synonym of *Sotalia fluviatilis* (da Silva and Best 1994).

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 reclassified by Gray as a member of the genus *Sotalia*. In 1866 (Hershkovitz 1966, da Silva and Best 1994) Van Bénéden described a second coastal species in 1875, from a specimen collected in Rio de Janeiro Bay, Brazil. This species was designated as *Sotalia brasiliensis* and it is now considered a synonym of *Sotalia guianensis* (Hershkovitz 1966, 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). Furthermore, Cunha *et al*. (2005) reported differences between the two subspecies along their Brazilian distribution

based on the phylogenetic analysis of mitochondrial (mt) DNA (control region and cytochrome *b*).

Although cetacean species have generally been recognized under some interpretation of the Biological Species Concept (BSC) (Mayr 1963), the criterion of reproductive isolation is difficult to apply to allopatric forms or populations (Endler 1977, Rice 1998). An alternative is the use of the Genealogical/Lineage Concordance Species Concept (GCC) (Avise and Ball 1990, Avise and Wollenberg 1997, Avise 2000). This approach was used in defining a new species of beaked whale (*Mesoplodon perrini*) (Dalebout *et al*. 2002, 2004) and reviewed by a specialized workshop on cetacean taxonomy (Reeves *et al*. 2004). This concept attempts to reconcile elements from both the BSC and the Phylogenetic Species Concept (PSC), stating that phylogenetic diagnoses should be based on multiple independent genetic traits such as information contained in multiple loci (Avise and Ball 1990, Avise and Wollenberg 1997).

Here we present a formal proposal to recognize each *Sotalia* subspecies as full species under the GCC, based on diagnostic genetic characters consistent with the criterion of "irreversible divergence" (Reeves *et al*. 2004). We include analysis of DNA sequences from ten nuclear introns, three of which have fixed-site differences between coastal and riverine *Sotalia*, and three gene fragments of the mitochondrial genome. Our geographic sampling extends that of Cunha *et al*. (2005), with independent samples from populations along most of the distribution range of the two subspecies, including locations along the Amazon River and some of its tributaries as well as coastal locations in Nicaragua, Colombia, Venezuela, French Guiana, and Brazil. In support of our proposal, we also review unpublished morphometric data (Borobia 1989), as well as published biogeographical data (Borobia *et al*. 1991, da Silva and Best 1996) and ecological information for each *Sotalia* subspecies (Best and da Silva 1984, Borobia and Barrios 1989, da Silva and Best 1996, Santos *et al*. 2001, Rosas and Monteiro-Filho 2002).

METHODS

Sample Collection and DNA Extraction

A total of seventy-six samples of skin, liver, bone, or teeth samples were obtained from coastal and riverine *Sotalia* in twenty-one locations grouped into nine geographic regions throughout its range (Fig. 1, Table 1, Appendix 1). Tissue samples were obtained from dolphins found stranded dead or dolphins drowned in fishing nets ($n = 55$). Bones and teeth were obtained from skeletal remains found in the field ($n = 8$) and from museum specimens ($n = 4$). Skin samples from the Colombian Caribbean were obtained from captive ($n = 4$) as well as free ranging ($n = 2$) dolphins. Samples from captive dolphins were obtained by removing a small piece of skin from the tail. Skin from free-ranging dolphins was collected using a small biopsy dart deployed from a modified veterinary capture rifle (Krützen *et al*. 2002). Skin and liver samples were stored in 70% ethanol at $-20°C$. Bones and teeth samples were stored at room temperature in individual sealed bags. Two DNA samples were obtained from the DNA and Tissue Archive at the Southwest Fisheries Science Center (NOAA, La Jolla, CA). 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*a*). Samples collected in Brazil ($n = 30$) were analyzed at Universidade Federal de Minas Gerais (UFMG) in Belo Horizonte, Brazil. Other samples ($n = 46$) were analyzed at the University of Auckland.

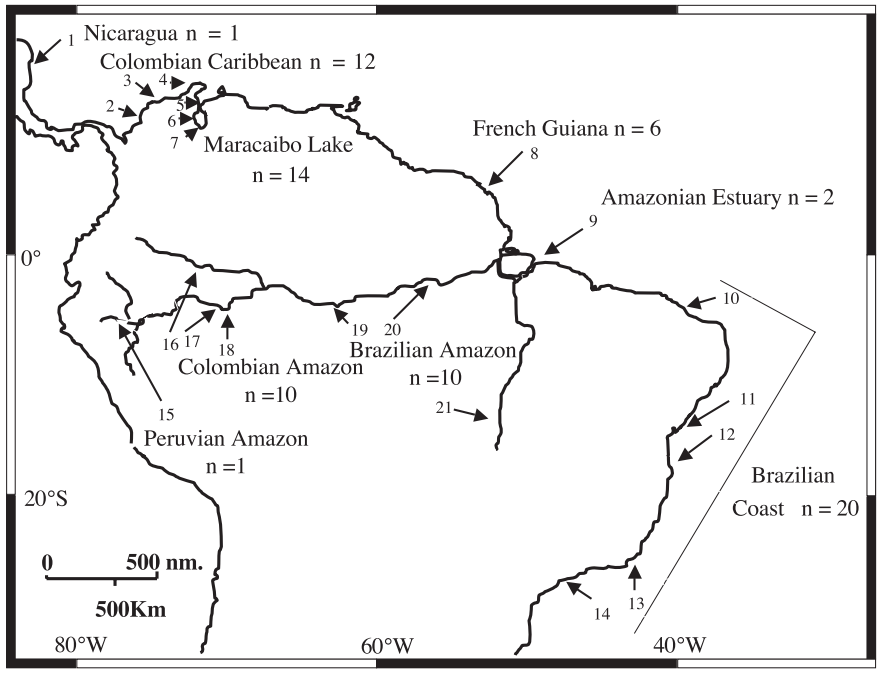

Figure 1. Distribution of coastal and riverine *Sotalia* showing geographic regions, sampling locations (numbers refer to Table 1), and sample sizes included in this study.

PCR Amplification and Sequencing

Three mitochondrial genetic markers were amplified and sequenced from all available samples (Table 2): a 627 base pair (bp) portion of the mitochondrial DNA control region (CR), a 425 bp fragment of the cytochrome *b* gene (Cyt-*b*), and a 1,044 bp fragment of the NADH dehydrogenase subunit 2 gene (ND2).

Ten nuclear introns (4,312 bp in total) were screened for ten *Sotalia* (five coastal and five riverine) as well as two other delphinid species: *Sousa chinensis* and *Steno bredanensis* (Table 2). Seven introns (including all Y chromosome introns) showed no variation between coastal and riverine *Sotalia* samples. The other three, including 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), showed fixed nucleotide differences between the initial ten samples of coastal and riverine *Sotalia* so were sequenced for the remaining eighteen specimens. Thus, a total of 28 *Sotalia* samples with high quality DNA (21 coastal and 7 riverine) from seven geographic locations (Table 1) were sequenced for these three introns.

For samples sequenced at the University of Auckland, free nucleotides and primers were removed from the PCR products using SAP (shrimp alkaline phosphatase) and ExoI (exonuclease I) (USB) and directly sequenced in both directions using the standard protocols of Big Dye terminator sequencing chemistry on an ABI 3100 automated capillary sequencer. Samples sequenced at Universidade Federal de Minas Gerais (UFMG) were amplified following the previously described protocol, cleaned using 20% PEG (Polyethyleneglycol), and sequenced using an ETDye terminator kit on a MegaBACE automated capillary sequencer (Amersham Biosciences, Piscataway, NJ).

Table 1. Summary of sampling locations, tissue type, and number of mitochondrial and nuclear sequences obtained for coastal and riverine *Sotalia* (see Appendix 1). 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
Nicaragua	(1) Mouth of the Layasiksa River, Waunta Lagoon	Coastal	1 tooth[a]	1	-
Colombian Caribbean	(2) Morrosquillo Gulf (Córdoba province)	Coastal	4 skin 1 tooth	5	4
	(3) Santa Marta (Magdalena province)	Coastal	3 skin	3	1
	(4) La Guajira province	Coastal	4 skin	4	3
Maracaibo	(5) Zapara Island	Coastal	11 skin	14	1
Lake	(6) Barranquitas	Coastal	2 skin		
	(7) Mouth of the Catatumbo River	Coastal	1 bone		
French Guiana	(8) Cayenne	Coastal	6 skin	6	4
Amazonian Estuary	(9) Belém (Pará state)	Coastal	2 skin	2	-
Brazilian Coast	(10) Ceará state	Coastal	1 liver	1	-
	(11) Bahía state	Coastal	2 skin	2	1
	(12) Espírito Santo state	Coastal	2 skin	2	1
	(13) Rio de Janeiro state	Coastal	2 skin 1 DNA[b]	3	-
	(14) Cananéia estuary (São Paulo state)	Coastal	12 skin	12	6
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[b]	1	1
	(19) Tefé (Amazonas state)	Riverine	7 skin	7	4
	(20) Santarém (Pará state)	Riverine	1 bone	1	-
	(21) Formoso Araguaia River	Riverine	1 bone	1	-

[a] Sample donated by the USNM: United States National Museum Smithsonian Institution, Washington, DC.

[b] Sample donated by the SWFSC: Southwest Fisheries Science Center, La Jolla, CA.

Table 2. Summary of loci and amplification conditions used in study of *Sotalia* 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)
Cyt- *b*	Tglu CB2	55°C	464 bp	Palumbi (1996)
ND2	ILP5100R BatH4823 tRNA-metF	55°C	1,050 bp	T. Mclenachan (Alan Wilson Centre, Massey University)
ND2	BatL4235 BatH4461	55°C	Sequencing	T. Mclenachan (Alan Wilson Centre, Massey University)
nuDNA (autosomal introns)				
Act-1	Act-3 Act-1385	55°C	980 bp	Palumbi and Baker (1994) and Conway (2005)
Lac-1	LacIR LacIIF	54°C	600 bp	Milinkovitch *et al*. (1998)
GBA	GBA-F GBA-R	55°C[a]	310 bp	Roca *et al*. (2001)
CHRNA1	CHRNA1-F CHRNA1-R	55°C[a]	360 bp	Roca *et al*. (2001)
CAT	CAT-F CAT-R	55°C[a]	520 bp	Lyons *et al*. (1997)
IFN	IFN-F IFN-R	55°C[a]	340 bp	Lyons *et al*. (1997)
nuDNA (Y chromosome introns)				
DBY7	DBY7-F DBY7-R	55°C[a]	400 bp	Hellborg and Ellegren (2003)
DBY8	DBY8-F DBY8-R	55°C[a]	200 bp	Hellborg and Ellegren (2003)
SMCY7	SMCY7-F SMCY7-R	55°C[a]	500 bp	Hellborg and Ellegren (2003)
UBE1Y7	UBE1Y7-F UBE1Y7-R	50°C[a]	500 bp	Hellborg and Ellegren (2003)

[a]Amplification using *Taq*GOLD.

DNA extracted from bones, teeth, or degraded skin proved unsuitable for amplification of nuclear genes. However, most of these samples were suitable for amplification of mtDNA. To protect against contamination, samples were run in at least two separate PCR reactions, including extraction blanks. To improve the confidence and accuracy of our results, PCR products from the two independent amplifications were sequenced in both forward and reverse directions separately and then compared.

Data Analyses

Sequence quality was evaluated using the program *Phred* v.020425 (Ewing and Green 1998, Ewing *et al*. 1998). Sequences with *Phred* scores $\leq$20 (a base call having a probability of more than 1/100 of being incorrectly called) were resequenced. Sequences with *Phred* score values between 20 and 40 (a probability between 1/100 and 1/10,000 of being incorrectly called) were checked by eye to confirm polymorphic sites. A polymorphic site was indicated by a secondary peak with a height $\geq$30% of the height of the primary peak and by a slight decline in the *Phred* score. All sequences were edited manually and aligned using Sequencher 4.1 software (Genes Code Corporation, Ann Arbor, MI). For the combined mitochondrial data set (2,096 bp), as well as for the combined nuclear data set (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). Sequences were submitted to GenBank as accession numbers EF027006 to EF027092.

Using the program MEGA2 (Kumar *et al*. 2001), 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). The model of nucleotide substitution for the two combined data sets was tested in Modeltest v3.06 (Posada and Crandall 1998), and the settings for this model were used in the Neighbor-Joining and Maximum-Likelihood phylogenetic reconstructions with bootstrap resampling performed in PAUP version 4.0b1 (Swofford 2002). A Maximum Parsimony reconstruction with bootstrap resampling was also performed with PAUP. *Steno bredanensis* (rough-toothed dolphin) and *Sousa chinensis* (Indo-Pacific humpbacked dolphin) were used as outgroups for these analyses. A Partitioning of Homogeneity Test was conducted with PAUP to determine if phylogenies reconstructed from combined mitochondrial genes (mtDNA) and combined nuclear genes (nuDNA) differed significantly from the total combined data set (mtDNA + nuDNA).

To estimate the time of divergence between the coastal and riverine *Sotalia*, we constructed a molecular clock using mtDNA CR sequences from sixteen coastal and thirteen riverine *Sotalia* and one harbor porpoise (*Phocoena phocoena*) sequence (GenBank accession number Y13875). These sequences were compared to estimate the rate of nucleotide substitution per lineage per year (λ) using the equation $\lambda = d_1/2T_1$, where d_1 is the adjusted divergence and T_1 is the time since divergence (Li 1997) between the Phocoenidae and Delphinidae lineages (Harlin *et al*. 2003). This date was assumed to be between 10 and 11 mya based on the fossil record (Barnes 1985) and from molecular data (Waddell *et al*. 2000). Due to software limitations, we used the Tamura–Nei model of nucleotide substitution (Nei and Kumar 2000) in MEGA2 to estimate d_1 with gamma-corrected Tamura–Nei pairwise distance ($\alpha = 0.5$ from the Modeltest output). Net nucleotide divergence (d_2) between and within *Sotalia* subspecies was calculated for the combined mitochondrial data set (2,096 bp) as well as for the initial 450 bp of the mitochondrial CR. We used the Tamura–Nei model (Nei and Kumar 2000) in MEGA2 to estimate d_2 with a pairwise gamma-corrected Tamura–Nei distance ($\alpha = 0.5$ from the Modeltest output). The previously calculated rate of nucleotide substitution per lineage per year (λ) was then used to calculate the divergence time between riverine and coastal *Sotalia* (T_2) using the equation $T_2 = d_2/2\lambda$.

Net nucleotide divergence between coastal and riverine *Sotalia* was also calculated from the total nuDNA combined data set (4,312 bp) using MEGA2 with a

gamma-corrected Tamura–Nei pairwise distance and the parameters from the Modeltest output.

RESULTS

DNA Sequence Data

mtDNA—A total of 2,096 bp of the mitochondrial genome (CR, ND2, and Cyt-*b*) was amplified and sequenced from 51 *Sotalia* specimens representing coastal and riverine subspecies. An additional 25 samples of poorer quality were sequenced for only a short fragment of the mtDNA CR (350–400 bp). A total of 31 different haplotypes were identified within these 76 samples (Table 3), 29 of which were distinguished by substitutions in the CR, and 2 that were distinguished by additional variable sites in the Cyt-*b* gene. A total of 85 variable sites were found along the 2,096 bp of the combined mtDNA genes data set (Table 3). For the CR, 37 variable sites were found, 11 of which represented fixed-site differences between the haplotypes corresponding to coastal and riverine *Sotalia*. For Cyt-*b*, 12 variable sites were found, 5 of which represented fixed-site differences between the 2 subspecies. For the ND2 gene, 36 variable sites were found, 20 of which were fixed between subspecies. Four of these fixed-site differences corresponded to non synonymous substitutions (Table 3).

nuDNA—Initial screening showed one fixed-site difference between coastal and riverine *Sotalia* in each of three introns, a 592 bp fragment of Lac-1, 950 bp of Act-1, and 308 bp of GBA. The presence of the three fixed sites was confirmed in an additional sample of coastal ($n = 16$) and riverine ($n = 2$) *Sotalia* for a total of twenty-eight specimens compared. All were transitions (A to G or G to A, Fig. 2). One variable site (position 32) was found in Act-1 in one individual from the Colombian Caribbean (a heterozygote) as evidenced by double peaks. Otherwise, no variable sites or heterozygotes were found in the screening.

Across the total of ten introns used in the initial screening, only one fixed-site difference in the Y chromosome intron UBE1Y7 was found to be diagnostic for *Sotalia* when compared to *Sousa chinensis*. Four additional fixed-site differences in the Y chromosome intron DBY8 and in the autosomal introns GBA, CAT, and Lac-1 were shared between *Sotalia* and *Sousa chinensis* relative to *Steno bredanensis* (Fig. 2).

Phylogenetic Analyses

For the three 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 data set. Phylogenetic reconstructions by Maximum Parsimony, Maximum Likelihood, and Neighbor Joining showed clear reciprocal monophyly for individual and combined genes between haplotypes of the two *Sotalia* subspecies (data for the Maximum Parsimony reconstruction based on the combined mitochondrial data set shown only, Fig. 2).

For the three nuclear loci, the best-fit model of nucleotide substitution was the HKY. A Partitioning of Homogeneity Test showed no conflicting phylogenies ($P = 0.96$) for the combined nuDNA data set or the total combined data set (mtDNA + nuDNA, $P = 0.99$). Phylogenetic reconstructions by Maximum Parsimony, Neighbor

Table 3. The 85 variable sites over 2,096 bp of the combined mitochondrial data set resolving 31 *Sotalia* haplotypes. A star (*) denotes fixed-site differences and (&) indicates non synonymous substitutions coding for a different amino acid in coastal and riverine *Sotalia*. (#) designates a haplotype defined by nucleotide substitutions in the Cyt-*b* gene. Haplotypes A to R are found in coastal *Sotalia* and haplotypes S to EE in riverine *Sotalia*. Short fragments derived from teeth or bone are labeled.

Haplotype	Variable sites: Control region (627 bp)	Variable sites: ND2 (1044 bp)	Cytochrome *b* (425 bp)
	1111222222222333333344444445556666	1113444455566666677788899999000	111122333
	6990469044666889146799901334775770012	1245228213791662345881281880168902 3	467012899099
	3045862447248123599201280356075471312	9332136325555047462778790959983163 74	968674447006
	** ** ** * ** **	& *** & * **** * *** & **** &	* ** * *
A	GCCACTATCAAACTTTAACCCTTTGTTCTTTATGGCA	AGCTATTGCATTAAAGCCGCATTTCTTATCGCGACT	GCCCCATCATTC
B	.....CC....T..C.....	GA.CG.....T.C....C.....	.....G.....T
C	.....CC....T..C.....	GA.CG.....T......C.....?	.....GC....T
D	.....C.....C.....	G..CG.....	.....
E	A...T.....C.....C.....	G..CG.....	.....
F	.....G....C.....C.....	G..CG.....	.....
G	.....C....T..CC.....AA..	G..CG.....G....T..C.....C.....	..T.....
H	A...T..C.....C.....C.....	G..CG.....	.....
I	A...T..C.....C.....C.....	G..CG.....	.....C..
J	A...T..C.G....C.....C.....	G..CG.....	.....C..
K	.....CC.G.TT..CC.....	GA.CG.....T......C.....C	.....G.....T
L	.....CC....T...C.....	G..CG.....	A.....

Table 3. Continued.

Haplotype	Variable sites		
	Control region (627 bp)	ND2 (1044 bp)	Cytochrome b (425 bp)
M	?.....TC.....????	.....?	.....
N	.....T.....????	.....?	.....
O	A...T.....C..T		
P	....T..C.....C..?	Bone samples	
Q	.....T.C.....C.ACT		
R	.....T.C.....C..CT		
S	.TTGTCG..GGGT.CC...T....A.....CG.AATG	..TCGCCATG..GG.ATTTTG..CTCCG.TATAGT.	.T.TA...G.C.
T	.TT.TCG..GGG..CC...T....A.....CG.AATG	..TCGCCATG..GG.ATTTTG..CTCCG.TATAGT?	.T.TA...G.C.
U	.TTGTCG..GGG..CC.T		
V	.TTGTCG..GGGT.CC.T	Bone samples	
W	.TTGTCG..GGG..CC...T....A.....CGCAATG	??TCGCCATG..GG.ATTTTG..CTCCG.TATAGT.	.T.TA...G.C.
X	.TTGTCG..GGG..CC...T....A.....CG.AATG	..TCGCCATGC.GG.ATTTTG..CTCCG.TATAG??	.T.TA...G.C.
Y	.TTGTCG..GGG..CC...T....A....C???????	..TCGCCATG..GG.ATTTTG..CTCCG.TATAGT.	.T.TA...G.C.
Z	.TTGTCG..GGGT.CC...T....A.....CG.AATG	..TCGCCATG..GG.ATTTTG..CTCCG.TATAGT.	.T.TA...G.C.
AA	.TTGTCG..GGG..C...		
BB	.TT.TCG..GGG..C....T....A...A.	Bone samples	
CC	.TTGTCG..GGG..CC...T....A.....CG.AATG	..TCGCCATG..GG.ATTTTG..CTCCG.TATAGT.	.T.TA...G.C.
DD	.TT.TCGC.GGG..C...TT.C..A.....CG.AATG	..TCGCCA.G.CG..ATTTTG..CTCCG.TATA.??	.T.TA..TG.C.
EE	.TT.TCGCTGGG..C...TT.C..A....C???????	..TCGCCA.G.CG..ATTTTG..CTCCG.TATA.T.	.T.TA...G.C.

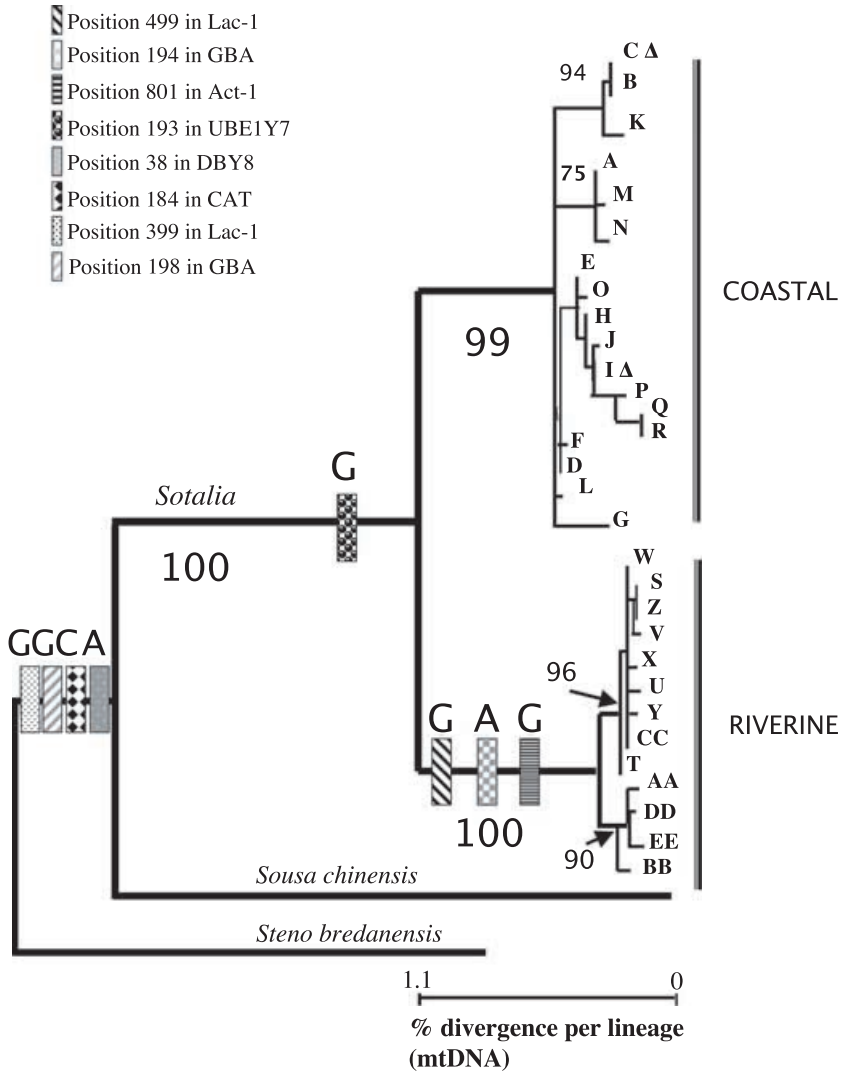

Figure 2. Maximum Parsimony phylogenetic reconstruction of the combined mitochondrial haplotypes (2,096 bp) of *Sotalia* and outgroups with bootstrap values from 1,000 replicates. Letters at the end of the branches represent haplotype codes. (Δ) indicates haplotypes distinguished on the basis of the Cyt-*b* gene. The percent divergence was calculated in MEGA2 using the Tamura–Nei distance option and the settings for the α and T_i/T_v output from Modeltest. Vertical bars on tree represent nuclear fixed-site differences between coastal and riverine *Sotalia* as cladistic characters, showing their derived state in riverine *Sotalia*, and fixed differences shared between *Sotalia* and *Sousa chinensis* relative to *Steno bredanensis.*

Joining, and Maximum Likelihood showed clear reciprocal monophyly for individual and combined gene fragments (including the combined mtDNA + nuDNA) between haplotypes and genotypes of the two subspecies with high bootstrap support (data not shown). The small number of fixed-site differences found between both subspecies

at the nuclear level (synapomorphies) were mapped as cladistic characters onto the phylogenetic reconstruction for the combined mitochondrial haplotypes (Fig. 2).

Genetic Divergence Within and Between Coastal and Riverine Sotalia

After controlling for within-species differences, net nucleotide divergence between the coastal and riverine subspecies was 2.2% for the combined mtDNA data set, 2.5% when considering only 450 bp of the CR, and 1.1% considering only 425 bp of Cty-*b*. Average difference within the riverine subspecies was estimated as 0.2% for the combined mtDNA gene fragment, 0.6% for the CR only, and 0.1% for Cty-*b*, compared to 0.4%, 1%, and 0.1% respectively for the coastal subspecies. For comparison, we calculated the net divergence between coastal *Sotalia* and *Steno bredanensis* to be 10.6% and between *Sotalia* and *Sousa chinensis* to be 10.9% for the CR only. Net divergence between coastal and riverine *Sotalia* at the nuclear level was 0.07% calculated from the 4,312 bp of the total data set of ten introns, and less than 0.001% within each subspecies. For comparison, net divergence at the nuclear level was 0.09% between *Sotalia* and *Sousa chinensis* and 0.16% between *Sotalia* and *Steno bredanensis*.

Divergence Time Between Coastal and Riverine Sotalia

Given the assumed divergence time of 10–11 mya and the net divergence of 24% between *Sotalia* and *Phocoena phocoena* (harbor porpoise) calculated for the mtDNA CR with the Tamura–Nei model, the rate of nucleotide substitution was estimated to range from 1.11×10^{-8} to 1.22×10^{-8} bp^{-1} yr^{-1}. Given the net divergence of 2.5% for the CR, this suggests that coastal and riverine *Sotalia* diverged approximately 1–1.2 mya.

DESCRIPTION

Our genetic evidence supports recognition of the two *Sotalia* subspecies as full species based on the four criteria of the GCC (Avise and Ball 1990, Avise and Wollenberg 1997, Avise 2000).

(1) Concordance across sequence characters within genetic locus leading to conclusive exclusion—In the combined mitochondrial data set, thirty-six fixed-site differences were found between coastal and riverine *Sotalia*. Four of these fixed-site differences corresponded to non synonymous substitutions in the mitochondrial gene ND2, suggesting some functional as well as neutral divergence. An additional three fixed-site differences were found among the combined nuclear gene fragments. We consider that these sites represent the primary molecular diagnostic characters distinguishing the two species. The 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).

(2) Concordance in genealogical patterns across multiple loci, both mitochondrial and nuclear—Phylogenies constructed from mtDNA lineages (*i.e.*, haplotypes) as well as from nuclear intron sequences 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 that are separated and distinct from other such clades by a single branch that contains character-state changes leading to fixed differences (Sites and Marshall 2003).

(3) Concordance with biogeographical patterns—The distribution of *Sotalia* showed complete concordance with the phylogenetic patterns observed in our analysis. Coastal and riverine populations occur in physical isolation (allopatry) with little overlap possible only at the mouth of the Amazon River and the Amazonian Estuary (Borobia *et al*. 1991, da Silva and Best 1996). With the number of samples analyzed to date, we cannot exclude the possibility of some hybridization of *Sotalia* in this area of overlap, as observed between the Antillean manatee (*Trichechus manatus*) and the Amazonian manatee (*T. inunguis*) (Vianna *et al*. 2006). Outside this area, however, we have found no evidence of either nuclear or mitochondrial gene flow or introgression between the two proposed species (Caballero *et al*. 2003, Cunha *et al*. 2005).

(4) 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. However, these are average differences and not discontinuities. Nevertheless, considered together, these average differences suggest some "morphological divergence." Borobia (1989) recorded and compared using paired *t*-tests a total of 37 cranial character measurements from 58 *Sotalia* skulls including 21 riverine and 38 coastal specimens. Out of the 37 cranial characters compared, 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 were found to provide best discrimination between riverine and coastal specimens (Borobia 1989). Overall, coastal specimens tended to have larger skulls than riverine specimens, as well as larger body sizes.

Monteiro-Filho *et al*. (2002) conducted tridimensional morphometric analysis of 22 landmarks from skulls of 92 coastal and 13 riverine specimens 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, and in coastal specimens these were aligned along this axis.

Taxonomic Treatment

Order Cetacea (Brisson, 1762)
Family Delphinidae (Gray, 1821)
Genus *Sotalia* (Gray, 1866)
Sotalia fluviatilis (Gervais, 1853)

Holotype and Type Locality

Rostrum and mandibles held at the Laboratory of Comparative Anatomy at the Natural History Museum (Laboratoire d'Anatomie Comparée du Muséum d'Histoire Naturelle), Paris, France. Collection number JAC: 1880–550 (Robineau 1990). Collected in Pebas (3°19′8″S, 71°49′02″W), Marañon River, upper Amazon, Peru.

Synonyms

Sotalia pallidus (Gervais, 1855) and *Sotalia tucuxi* (Gray, 1856).

Specimens Examined and Referred Specimen

DNA was examined from twenty-one specimens (Appendix 1). No analysis of skull morphology or external morphometrics of these specimens has been included in this study. As we have not examined the DNA of the holotype, we refer to specimen BA02 as representative of the genetic characters described for *Sotalia fluviatilis* (see Appendix 1). The skull of this specimen (Fig. 4A, C, E, G) is accessioned at Sociedade Civil Mamirauá (Belém, Brazilian Amazon) and a skin sample and DNA are accessioned at Universidade Federal de Minas Gerais (Belo Horizonte, Brazil) for future reference.

Morphological Description

Sotalia fluviatilis is a small delphinid with a moderately long, slender beak (Fig. 3A). The dorsal fin is triangular, short, and high, sometimes hooked at the peak (da Silva and Best 1996). Coloration is dark gray on the dorsum and rosy pink to white or light gray on the ventral side. A lateral area of light gray occurs behind the pectoral fin and another extends from mid body to the level of the anus (da Silva and Best 1996). Pectoral fins and flukes are dark gray underneath (da Silva and Best 1996). Mean body length is 1.4 m, with the largest recorded adults being a 1.49-m male and a 1.52-m female ($n = 17$, in Best and da Silva 1984; da Silva and Best 1994, 1996). Mean body measurements are from tip of jaw to the blowhole, 26.4 cm; from tip of jaw to the insertion of flippers, 39.0 cm; from tip of jaw to angle of gape of mouth, 22.7 cm; maximum length of flippers, 24.8 cm; and length of flukes tip to tip, 39.4 cm ($n = 8$, *in* da Silva and Best 1996). The number of upper teeth ranges from 28 to 35 ($n = 38$, in Borobia 1989). Mean cranial measurements are condylobasal length = 334.3 mm (range 288–369); greatest preorbital width = 126.9 mm (range 112.3–135.1); greatest postorbital width = 139.4 mm (range 126–148); least supraorbital width = 125.4 mm (range 110.9–133.8) ($n = 21$, in Borobia 1989).

Distribution

Sotalia fluviatilis is found throughout the Amazon River drainage, including some of its most important tributaries, like the Putumayo and Caquetá rivers (Colombia) (Trujillo *et al*. 2000), the Ucayali and Marañon rivers (Peru), Negro, Madeira, and Tapajos rivers (Brazil), and the Napo and Cuyabeno rivers (Ecuador) (Borobia *et al*. 1991; da Silva and Best 1994, 1996). Although the distribution of *Sotalia fluviatilis* seems to be continuous along the Amazon River and most of its tributaries, the number of distinct populations that might exist is unknown (Caballero 2006).

Etymology

We accept the previously proposed "tucuxi," "tucuchi-una" from the Tupi language of the Mayana indigenous group in the Amazon Region (da Silva and Best 1996) as the common name for the riverine species *Sotalia fluviatilis*.

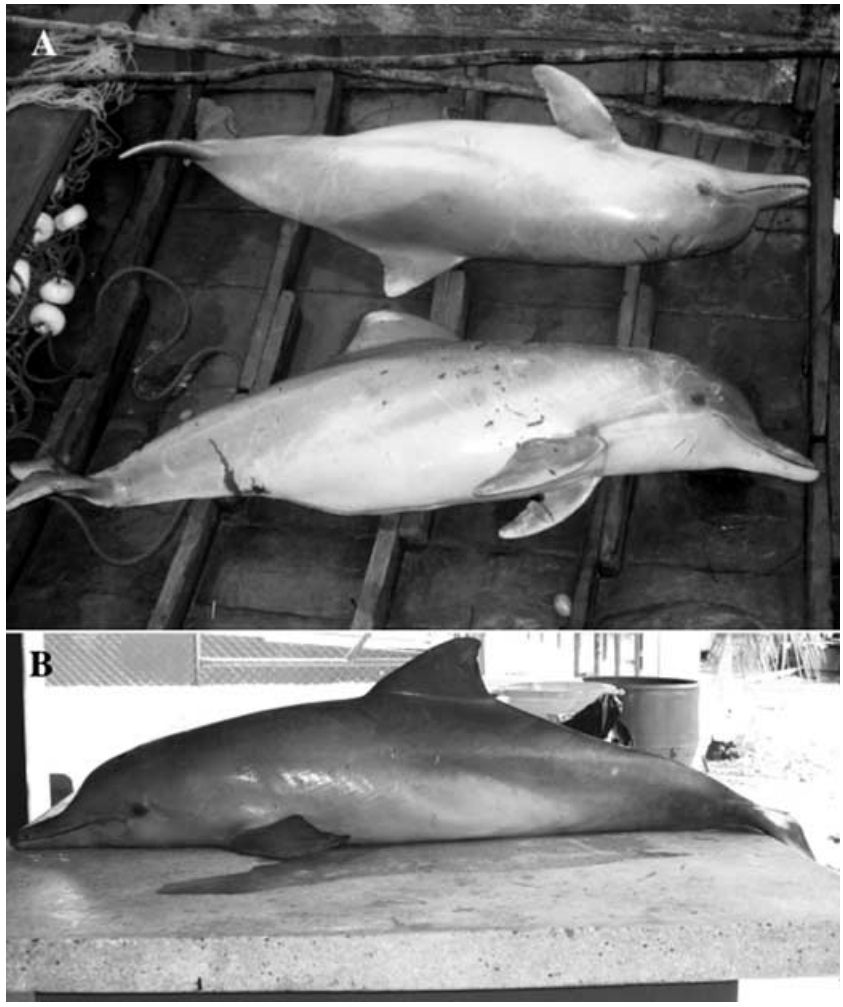

Figure 3. External morphology and coloration. (A) *Sotalia fluviatilis* (two stranded individuals, Lago Tefé, Brazilian Amazon [Photo: Miriam Marmontel, SCM]); (B) *Sotalia guianensis* (stranded individual, Cispatá Bay, Colombian Caribbean. [Photo: Salomé Dussan, CVS]).

Taxonomic Treatment

Order Cetacea (Brisson, 1762)
Family Delphinidae (Gray, 1821)
Genus *Sotalia* (Gray, 1866)
Sotalia guianensis (Van Bénéden, 1864) (survive)

Holotype and Type Locality

Skeleton and skull, 152 cm total length, held at the Institut Royal des Sciences Naturelles de Belgique, Brussels, Belgium (Van Bénéden 1864, Williams 1928, Husson

1978). (Previously held at the Stuttgart Museum.) Collection number IRSNB 1516 (Borobia *et al*. 1991). Collected at the mouth of the Maroni River (5°30′00″N, 54°01′98″W), on the border between French Guiana and Surinam. Husson (1978) refers to the type locality as Marowijne River. Considering the largely (if not completely) allopatric distribution of *Sotalia fluviatilis* and *Sotalia guianensis*, we assume this to be the correct holotype for *Sotalia guianensis*.

Paratypes

Two additional specimens were obtained with the holotype (co-types). One of these is damaged, lacking the rostrum (Husson 1978). They are held at the collection of Louvain University, Belgium (Husson 1978). No further information on the collection number of these specimens is available at present.

Synonyms

Sotalia brasiliensis (Van Bénéden, 1875)

Specimens Examined and Referred Specimen

DNA was examined from fifty-five specimens (Appendix 1). No analysis of skull morphology or external morphometrics of these specimens has been included in this study. As we have not examined the DNA of the holotype, we refer to specimen CCBC 0103 as representative of the genetic characters described for *Sotalia guianensis* (see Appendix 1). The skull of this specimen (Fig. 4B, D, F, H) is accessioned at Corporación Autónoma Regional de los Valles del Rio Sinú y del Rio San Jorge (Montería, Colombia), and a skin sample and DNA are accessioned at the University of Auckland Molecular Archive (Auckland, New Zealand) for future reference.

Diagnosis

Molecular—Based on 2,096 bp segment of the MtDNA genome and 1,850 bp segment of nuDNA (three introns), there are 39 fixed-site differences (diagnostic sites) that distinguish *Sotalia guianensis* from *Sotalia fluviatilis*.

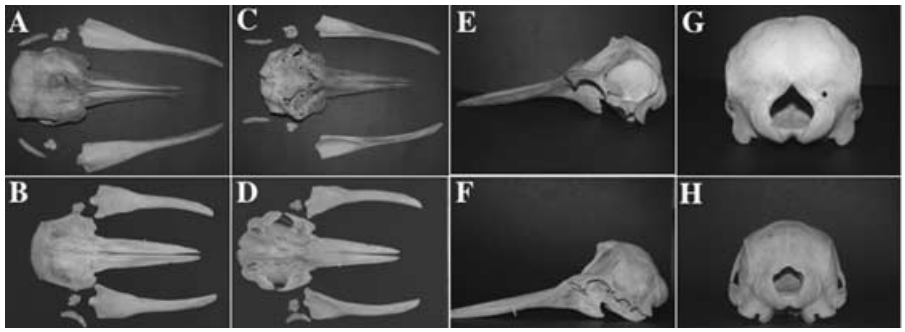

Figure 4. Views of the skull of *Sotalia fluviatilis* (referred specimen BA 02, above) and *Sotalia guianensis* (referred specimen CCBC 0103, below). Dorsal (A, B); ventral (C, D); lateral (left, E, F) and posterior (G, H).

Morphological—Although a large number of average morphological differences (not discontinuities) have been found between *Sotalia fluviatilis* and *Sotalia guianensis* (Borobia 1989), the best separation of the two species is based on four cranial measurements: (1) condylobasal length, (2) greatest preorbital width, (3) greatest postorbital width, and (4) least supraorbital width (Borobia 1989). In all cases, averages and ranges of these measures were significantly greater for *Sotalia guianensis*. Mean body length is also significantly greater for *Sotalia guianensis* (da Silva and Best 1996).

Morphological Description

The coloration of *Sotalia guianensis* is gray on the dorsum and rosy pink light gray on the ventral side (da Silva and Best 1996) (Fig. 3B). The pectoral fins and flukes are gray underneath (da Silva and Best 1996). In some individuals, there is a light streak of gray that slopes anteriorly and ventrally from the upper edge of the caudal peduncle for 10–15 cm (da Silva and Best 1996). Mean body length is 1.7 m ($n = 17$, in Husson 1978) with the largest recorded adults being a 1.87-m male and a 2.06-m female (Barros 1991). Mean body measurements are from tip of jaw to the blowhole, 25.9 cm; from tip of jaw to the insertion of flippers, 41.2 cm; from tip of jaw to angle of gape of mouth, 22.9 cm; maximum length of flippers, 29.2 cm; and length of flukes tip to tip, 42.2 cm ($n = 4$ or 5, in da Silva and Best 1996). The number of upper teeth ranges from 30 to 36 ($n = 38$, in Borobia 1989). Mean cranial measurements are condylobasal length = 375.2 mm (range 337–400); greatest preorbital width = 142.6 cm (range 130.1–152.2); greatest postorbital width = 159.1 mm (range 135.7–173); least supraorbital width = 139.8 mm (range 124.4–158) ($n = 38$, in Borobia 1989).

Distribution

Sotalia guianensis is distributed along the Caribbean and Atlantic coasts of South America, from Nicaragua (13°N) (Carr and Bonde 2000) and possibly Honduras (up to 15°N) (Edwards and Schnell 2001) to Florianopolis in southern Brazil (27°S) (Geise and Borobia 1987, Borobia *et al*. 1991). It has also been reported in some Caribbean islands including Trinidad and Tobago (da Silva and Best 1996) and the Abrolhos Archipelago of Brazil (Borobia *et al*. 1991). One population has also been described in Maracaibo Lake (Hershkovitz 1962), a large estuarine system located in northwestern Venezuela. Individuals from this population seem to have a smaller body size than individuals from other coastal populations (Casinos *et al*. 1981, Rice 1998), as well as some differences in the measures of a few cranial features (Casinos *et al*. 1981).

Sotalia dolphins reported in the lower Orinoco River, 300 km up river, close to Ciudad Bolívar (Venezuela), appear to be *Sotalia guianensis* (Borobia *et al*. 1991, Boher *et al*. 1995, Trujillo *et al*. 2000), but additional data are required for confirmation. Evidence for overlap in the distribution (sympatry) of *Sotalia fluviatilis* and *Sotalia guianensis* in the mouth of the Amazon River is uncertain (da Silva and Martin 2000). Surveys and additional collection of genetic samples are needed in order to clarify boundaries in the distribution of each species in this region. Although the distribution of *Sotalia guianensis* seems to be continuous along the coast, the number of distinct populations that might exist is unknown (Caballero 2006).

Etymology

We propose the common name "costero" (Spanish for coastal) for the coastal species *Sotalia guianensis*. The common name "estuarine dolphin" has been proposed previously (Rosas and Monteiro-Filho 2002), but we consider that this name is misleading, as the distribution of these dolphins is not restricted to estuaries and other dolphins are also estuarine (*e.g.*, *Orcaella*). These dolphins are known locally with a variety of names, such as tonina (Venezuela), lam (Nicaragua), Guyanese dolphin or Surinam dolphin (French Guiana and Surinam), and boto or boto-cinza in Brazil (da Silva and Best 1996). The proposed "costero" is applicable across the entire range, as this species is rarely, if ever, sighted in pelagic waters.

DISCUSSION

Evidence of "Irreversible Divergence"

"Irreversible divergence" was considered important for delimiting cetacean species by a recent workshop on cetacean taxonomy (Reeves *et al*. 2004). The workshop considered that the criterion of irreversible divergence required at least two lines of evidence. Multiple morphological characters were considered likely to be correlated and, thus, to represent only a single line of evidence. However, genetic characters from unlinked loci were considered to represent multiple lines of evidence. Based on these guidelines, the proposed species-level ranking of coastal and riverine *Sotalia* is supported by at least three lines of evidence: morphology, as reviewed here; mtDNA, as presented here and by Cunha *et al*. (2005); and single-copy nuclear DNA, as represented by three introns. No evidence presented or reviewed was in conflict with the proposal (*i.e.*, all genetic loci were either in agreement or were non informative).

Although the workshop did not offer guidelines on the degree of genetic divergence required to recognize species, we employed a comparative approach, using the divergence between other accepted cetacean sister-taxa (assuming similar rates of molecular evolution in other cetacean species), to evaluate the proposed species of *Sotalia* (Table 4). For the mtDNA CR, this comparison showed that nucleotide divergence of the two *Sotalia* was within the range of other accepted species. The net divergence of 2.5% for *Sotalia guianensis*/*Sotalia fluviatilis* was identical to that of the Chilean and Commerson's dolphins (*Cephalorynchus commersoni* and *C. eutropia*) (Pichler *et al*. 2001*b*), greater than that of the shortbeaked and longbeaked common dolphins (*Delpinus delphis* and *D. capensis*) (Rosel *et al*. 1994) and the dusky and Pacific white-sided dolphins (*Laagenorhynchus obscurus* and *L. obliquidens*) (Hare *et al*. 2002), but less than that of Indian Ocean bottlenose and bottlenose dolphins (*Tursiops aduncus* and *T. truncatus*) as reported by Wang *et al*. (1999) and that of the recently proposed snubfin and Irrawaddy dolphins (*Orcaella heinsohni* and *O. brevirostris*) (Beasley *et al*. 2005).

For nuclear introns, net divergence between *Sotalia guianensis* and *Sotalia fluviatilis* was consistent with divergence between dusky and Pacific white-sided dolphins (*Laagenorhynchus obscurus* and *L. obliquidens*) as reported by Hare *et al*. (2002). Although no value for divergence was calculated for the Southern right and North Atlantic right whales (*Eubalaena australis* and *E. glacialis*) (Gaines *et al*. 2005), the proportion of fixed sites observed in nuclear introns between these whale species was similar to the proportion of fixed sites in nuclear introns of coastal and riverine *Sotalia* (Table 4).

Table 4. Summary of comparative nucleotide divergence (%) and fixed-site differences in mtDNA and nuDNA for selected sister species of cetaceans.

Species and sample size	Number of base pairs (bp) analyzed		% Divergence		Fixed differences		Reference
	mtDNA	nuDNA	mtDNA	nuDNA	mtDNA	nuDNA	
Tucuxi dolphin (*S. fluviatilis*) ($n = 21$) *vs.* "costero" dolphin (*S. guianensis*) (n = 55)	450 bp (CR only) or 425 bp (Cyt-*b* only)	4,312 bp (introns)	2.5 % (450 bp CR only) 1.1% (425 bp Cyt-*b* only)	0.07% (net)	36 (CR, Cyt-*b*, ND2) 11 (CR only)	3 (≅1/1,400 bp)	This study
Commerson's dolphin (*C. commersonii*) ($n = 47$) *vs.* Chilean dolphin (*C. eutropia*) ($n = 20$)	485 bp (CR)	n.r.	2.5 %	n.r.	3	n.r.	Pichler *et al.* (2001*b*)
Shortbeaked common dolphin (*D. delphis*) ($n = 11$) *vs.* longbeaked common dolphin (*D. capensis*) ($n = 18$)	404 bp (CR)	n.r.	%	n.r.	1 4[a]	n.r.	Rosel *et al.* (1994)
Bottlenose dolphin (*T. truncatus*) ($n = 21$) *vs.* Indian Ocean bottlenose dolphin (*T. aduncus*) ($n = 19$)	397 bp (CR)	n.r.	4.4 %	n.r.	7	n.r.	Wang *et al.* (1999)

Table 4. Continued.

Species and sample size	Number of base pairs (bp) analyzed		% Divergence		Fixed differences		Reference
	mtDNA	nuDNA	mtDNA	nuDNA	mtDNA	nuDNA	
Irrawaddy dolphin (*O. brevirostris*) ($n = 24$) *vs.* Snubfin dolphin (*O. heinsohni*) ($n = 4$)	403 bp (CR)	n.r.	5.9 %	n.r.	16	n.r.	Beasley *et al.* (2005)
Southern right whale (*E. australis*) ($n = 45$) *vs.* North Atlantic right whale (*E. glacialis*) ($n = 2$)	289 bp (CR)	n.r.	3%–7%	n.r.	n.r.	n.r.	Baker *et al.* (1999)
Southern right whale (*E. australis*) ($n = 11$) *vs.* North Atlantic right whale (*E. glacialis*) ($n = 8$)	292 bp (CR)	14,823 bp (introns)	n.r.	n.r.	3	14 (≅1/1,000 bp) (Monophyly)	Gaines *et al.* (2005)
Dusky dolphin (*L. obscurus*) ($n = 5$) *vs.* Pacific whitesided dolphin (*L. obliquidens*) ($n = 6$)	496 bp (Cyt-*b*)	7,118 bp (introns)	1.22 %	0.03%–0.21% (Net) (Average: 0.086%)	5	1[a]	Hare *et al.* (2002)

n.r.: not reported.
[a]Close to fixation.

We further considered that ecological adaptation provides evidence of irreversible divergence. This ecological adaptation would be directed by divergent natural selection, which would be expected when considering the distinct environments where the two proposed sister species are found (Schluter 2001). The pronounced differences in salinity between the riverine and coastal-estuarine waters, as well as the changes in the water level at different times of the year, represent different selective pressures. Salinity ranges from less than 0.05 ppm (parts per million) in the Amazon River to over 35 ppm on the Atlantic coast of Brazil with a steep cline in the last 200 km of the mouth of the river (Tundisi *et al*. 1999). For example, although both coastal and riverine *Sotalia* prey mostly on pelagic fish from the same families (*i.e.*, *Sciaenidae* and *Clupeidae*), they prefer different species found exclusively either in freshwater or saltwater, and no overlap in prey species has been observed in diet studies (Best 1984, Borobia and Barrios 1989, Santos *et al*. 2002).

Borobia (1989) proposed that the differences found in the skull measurements around the eye orbit between riverine and coastal *Sotalia* could be related to the relative importance of vision in coastal and freshwater environments. Monteiro-Filho *et al*. (2002) proposed that differences in cranial shape between coastal and riverine *Sotalia* could reflect functional distinctions between both subspecies and, ultimately, adaptation to two very different environments and selective pressures (Schluter 2001). Dissimilarities in echolocation clicks have also been attributed to ecological differences (Kamminga *et al*. 1993). The dominant echolocation frequency of coastal *Sotalia* (55–65 kHz) is higher than that of riverine *Sotalia* (40–45 kHz), and the signal of the latter is more similar to that of the sympatric Amazon River dolphin (*Inia geoffrensis*) (36–46 kHz, Kamminga *et al*. 1993).

Finally, adaptation to seasonal fluctuation in the water levels of the Amazon River and its tributaries has influenced the seasonality of reproduction in riverine *Sotalia*, perhaps contributing to an incipient prezygotic isolating mechanism. A high proportion of births in riverine *Sotalia* occur during the dry season (October–November) when water levels are low (Best and da Silva 1984). Births in coastal *Sotalia* occur year round (Santos *et al*. 2001, Rosas and Monteiro-Filho 2002) with a peak in the rainy season (May–November) in some localities (Bössenecker 1978). Duration of gestation is also longer in coastal individuals, 11.6–11.7 mo (Rosas and Monteiro-Filho 2002), compared to 10.0–10.3 mo in riverine *Sotalia* (Best and da Silva 1984). Other differences in reproductive parameters include ovarian activity restricted to the left ovary in riverine *Sotalia* and activity in both ovaries in coastal *Sotalia* (Rosas and Monteiro-Filho 2002). Furthermore, seasonal testicular activity has been suggested in riverine *Sotalia* (Best and da Silva 1984), whereas it has not been detected in coastal *Sotalia* (Rosas and Monteiro-Filho 2002).

Timeframe for Speciation of Sotalia fluviatilis

The proposed divergence time of 1.0–1.2 mya for coastal and riverine *Sotalia* is consistent with the values reported by Avise *et al*. (1998) for time of initial divergence between sister species in many mammalian groups. These results are also consistent with the prominent role of Pleistocene events in the differentiation of extant mammalian sister species (Avise *et al*. 1998, Knowlton 2000). By comparison Cunha *et al*. (2005) proposed an older time of divergence between coastal and riverine *Sotalia*

(between 2.5 and 5.0 mya) based on a slower molecular clock calculated by Hoelzel *et al*. (1991). Further confirmation of the proposed dates is currently not possible given the lack of fossil record for *Sotalia* (da Silva and Best 1994).

The time frame of 1.0–1.2 mya proposed in our study coincides with a series of marine transgressions and regressions that occurred in the Amazon basin at the end of the Pliocene and during the Pleistocene (Plio-Pleistocene 3.4 mya–125,000 B.P.; Marroig and Cerqueira 1997, Gorring *et al*. 2003). Based on geological evidence (Marroig and Cerqueira 1997), it has been estimated that about 2.5 mya, the ocean level was 180 m above the present and since then a general trend of descending sea level has occurred. From 2.5 mya to the present, it is thought that the entire Amazon basin underwent alternating periods of ponding with sedimentation as well as regional erosion (Putzer 1984, Müller *et al*. 1995). This series of ponding events, accompanied by tectonic activity in the western Amazon, has been proposed as the "Amazon Lagoon" (Lago Amazonas) hypothesis (Frailey *et al*. 1988, Campbell 1990). According to this hypothesis, the Amazonian lowland could have been covered by water until approximately 750,000 B.P. (Marroig and Cerqueira 1997). Tectonic events, climatic change (cooling) and environmental change have been proposed as the cause for the transition of the Amazon Basin from a large lake to a braided fluvial system in the early Pleistocene (Brooks *et al*. 1981, Räsänen *et al*. 1987, Rosseti *et al*. 2005). We suggest that these transitional events isolated the riverine *Sotalia* from the coastal populations, maintaining a possible connection only in the mouth of the Amazon River, and promoting the divergence of these two species closer to the date of 750,000 B.P.

The relatively recent divergence of *Sotalia* is also suggested by comparison with the wider distribution of *Inia*. Although the family Iniidae is ancient, having occupied the Amazon drainage for around 15 million yr (Hamilton *et al*. 2001), divergence of the Bolivian Amazon subspecies (*Inia geoffrensis boliviensis*) is more recent, having occurred between 5 and 6 mya (Banguera-Hinestroza *et al*. 2002) by the formation of the Madeira-Mamoré rapids. As *Sotalia* is not found in the Bolivian Amazon above the rapids, this seems to suggest an upper time limit of 5 mya for its occurrence in the Amazon.

A similar upper bound is suggested by the divergence of the Amazon and Orinoco subspecies (*Inia geoffrensis geoffrensis* and *Inia geoffrensis humboldtiana*). Connectivity between the Amazon and Orinoco *Inia* subspecies is suspected through the Casiquiari Channel, which connects the Upper Orinoco and the upper Rio Negro, one of the main tributaries of the Amazon River (Banguera-Hinestroza *et al*. 2002). This is, at present, the only possible point of contact between the Amazon and Orinoco river basins after isolation of the Orinoco drainage from the main Amazon drainage initiated with the uplift of the Eastern Andean Cordillera in the Late Middle Miocene (*ca*. 12 mya) and continued with the uplift of the Mérida Cordillera in the Late Pliocene (5–3.4 mya) (Díaz de Gamero 1996, Audemard and Audemard 2002). If *Sotalia* entered the Amazon and expanded toward other tributaries, and the divergence of the coastal and riverine proposed sister species occurred between 5.0 and 2.5 mya as suggested by Cunha *et al*. (2005), we would expect *Sotalia* to be found in the upper Orinoco, with a distribution similar to that of *I. g. humboldtiana* and similar connectivity between Orinocoan and Amazonian *Sotalia* populations. To date, presence of *Sotalia* in this region has not been confirmed and sightings of *Sotalia* (presumably coastal transients) are restricted to the lower Orinoco and its mouth (Borobia *et al*. 1991, Boher *et al*. 1995, Rice 1998).

ACKNOWLEDGMENTS

We are grateful to all the people and institutions who gave us access to samples for this study: J. G. Mead and C. Potter (Smithsonian Institution National Museum of Natural History), R. L Brownell, students and researchers at Fundación Omacha (Colombia), R. Vieira (Oceanario Islas del Rosario, CEINER, Colombia), M. Ruíz-García (Universidad Javeriana, Colombia), F. Ospina-Navia (Acuario de Santa Marta, Colombia), M. C. Rosso and N. Jiménez (UJTL, Colombia), S. Dussan (CVS), B. de Thoisy (KWATA, French Guiana), AQUASIS (Ceará, Brazil), L. Flach (PUC-MG, Brazil), IBAMA (Brazil), the DNA Archive (*S. fluviatilis* and *S. bredanensis* samples), and T. Jefferson from the NMFS South West Fisheries Science Center, and to the Agriculture, Fisheries and Conservation Department of Hong Kong (access to *S. chinensis* samples). All Brazilian samples were collected under the government permit IBAMA 131/2004. This research was developed according to the special authorization for access to genetic resources in Brazil #03/2004 issued by IBAMA/CGEN. In Colombia, authorization was granted by Ministerio del Medio Ambiente, Vivienda y Desarrollo Territorial (Contrato de Acceso a Recursos Genéticos No. 001). Thanks especially to P. Lara (UFMG, Brazil) for help with the laboratory work in Brazil, M. Oremus for his help with analyses, and D. Steel and K. Nutt for their comments on an earlier version of the manuscript. ND2 primer sequences were provided by T. Mclenachan from the Alan Wilson Centre, Massey University, Auckland, New Zealand. Thanks to M. Borobia for allowing access to data from her unpublished M.Sc. dissertation. Thanks to S. Dussan for help with photographs of the referred specimen of *Sotalia guianensis*. Funding for fieldwork and laboratory analysis was provided by the New Zealand Marsden Fund (to C. S. Baker), a University of Auckland International PhD Scholarship (to S. Caballero), Colciencias-LASPAU (to S. Caballero), a Cetacean Society International Grant-In-Aid (to S. Caballero and J. A. Vianna), Universidad de los Andes (Colombia), Universidad Javeriana (Colombia), Conselho Nacional de Pesquisas (CNPq-Brazil), the University of Auckland Graduate Research Fund, and private resources. Special thanks to W. Perrin, A. Berta, and an anonymous reviewer for comments on an earlier version of the manuscript, and to W. Perrin and J. Mead for guidance on taxonomic convention.

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Received: 23 March 2006
Accepted: 25 October 2006

SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online:
Supplementary Appendix 1