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Molecular systematics of South American dolphins *Sotalia*: Sister taxa determination and phylogenetic relationships, with insights into a multi-locus phylogeny of the Delphinidae

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Abstract

The evolutionary relationships among members of the cetacean family Delphinidae, the dolphins, pilot whales and killer whales, are still not well understood. The genus *Sotalia* (coastal and riverine South American dolphins) is currently considered a member of the Stenoninae subfamily, along with the genera *Steno* (rough toothed dolphin) and *Sousa* (humpbacked dolphin). In recent years, a revision of this classification was proposed based on phylogenetic analysis of the mitochondrial gene cytochrome *b*, wherein *Sousa* was included in the Delphininae subfamily, keeping only *Steno* and *Sotalia* as members of the Stenoninae subfamily. Here we investigate the phylogenetic placement of *Sotalia* using two mitochondrial genes, six autosomal introns and four Y chromosome introns, providing a total of 5,196 base pairs (bp) for each taxon in the combined dataset. Sequences from these genomic regions were obtained for 17 delphinid species, including at least one species from each of five or six currently recognized subfamilies plus five odontocete outgroup species. Maximum Parsimony, Maximum Likelihood and Bayesian phylogenetic analysis of independent (each fragment) and combined datasets (mtDNA, nuDNA or mtDNA+nuDNA) showed that *Sotalia* and *Sousa* fall within a clade containing other members of Delphininae, exclusive of *Steno*. *Sousa* was resolved as the sister taxon to *Sotalia* according to analysis of the nuDNA dataset but not analysis of the mtDNA or combined mtDNA+nuDNA datasets. Based on the results from our multi-locus analysis, we offer several novel changes to the classification of Delphinidae, some of which are supported by previous morphological and molecular studies.

Keywords: *Sotalia*; Delphinidae; Nuclear DNA; Mitochondrial DNA; Autosomal introns; Y chromosome introns

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1. Introduction

The Delphinidae is the largest and most diverse family of cetaceans. Rice (1998) listed 36 species, while Mead and Brownell (2005) listed 35. Recently two additional species have been recognized (Beasley et al., 2005; Caballero et al., 2007) raising the current total to about 37. The Delphinidae are one of three extant families in the cetacean superfamily Delphinoidea, along with the families Monodontidae and Phocoenidae. It is believed that a rapid taxonomic and ecological radiation of cetaceans occurred during the Oligocene, with many lineages appearing and diversifying over about 5 MY (Nikaido et al., 2001). The explosive radiation of delphinoids (especially Delphinidae) seems to have happened later, in the mid to late Miocene (11–12 MYA) (Barnes et al., 1985; Nikaido et al., 2001). Growth and reproductive characteristics (Kasuya, 1995), as well as social structure (Gygax, 2002; Lusseau, 2003) and trophic diversification (Lipps and Mitchell, 1976) have been proposed as possible driving factors for their evolution and radiation. Other authors have proposed geographic barriers, changes in the sea level and climatic changes, e.g. the glacial periods of the Pleistocene (Gaskin, 1976), as the main factors delimiting the distribution of some delphinid groups (Davies, 1963).

Evolutionary relationships among Delphinidae are still not well understood. Different characters, ranging from morphological (Flower, 1883; True, 1883; Nishiwaki, 1963; Fraser, 1966; Gaskin, 1972; Kasuya, 1973; Mead, 1975; Muizon, 1988) to molecular (Shimura and Numachi, 1987; LeDuc et al., 1999), have been used in various revisions of the taxonomy and phylogeny of this family. In one proposed classification of Delphinidae, based on molecular data (LeDuc et al., 1999), the species are distributed among five subfamilies: Stenoninae, Delphininae, Orcaellinae, Lissodelphinae and Globicephalinae, with two species of *Lagenorhynchus* defined as *incertae sedis* (see Fig. 4).

Rice (1998) classified three genera as members of Stenoninae: *Steno, Sotalia* and *Sousa.* These were classified together in early taxonomical reviews (Flower, 1883), based on similarities of the skull morphology between *Sotalia* and *Steno*. It is important to note that members of the genus *Sousa* were classified as *Sotalia* until Kellogg (as reported by Fraser, 1966) divided this genus into *Sotalia*, for species found in South America, and *Sousa* for species found in the eastern Tropical Atlantic and the Indo-Pacific (Fraser, 1966). Flower (1883) noted further similarities between *Sotalia* and *Sousa* in comparison with *Steno*. These included differences in the shape of the pterygoid bone between *Steno* and *Sotalia* (*Sotalia* + *Sousa*); *Steno* lacks lateral grooves in this bone. He also noted the lower number of longer vertebrae in *Sotalia* (*Sotalia* + *Sousa*) when compared to *Steno*. Flower (1883) also observed that *Sotalia* (*Sotalia* + *Sousa*) skulls had a larger number of small teeth and that the outer digits of the pectoral fin bones (manus) were broader at the base and more developed that in other delphinids, including *Steno*. More recent classifications, like the one proposed by Gaskin (1972), as well as the classification by Mead (1975) based on the anatomy of the nasal passages and the facial structures and musculature, still considered *Steno, Sousa* and *Sotalia* as members of the same subfamily.

Kasuya (1973) considered *Steno* to be a member of Delphininae, with genera like *Tursiops, Stenella, Lagenorhynchus* and *Delphinus,* and included *Sotalia, Sousa* and *Cephalorhynchus* in the subfamily Sotaliinae, based on the morphology of the tympano periotic bones. The characters of these bones shared by *Sotalia, Sousa* and *Cephalorhynchus* included the closure of the elliptical foramen, a weak ventral keel, and no bilateral compression of the tympanic bulla, all features considered to be primitive. Of these three genera, *Sousa* and *Sotalia* showed a greater resemblance to each other in the morphology of these structures.

The majority of the early studies were based on overall similarities and often considered highly correlated characters as independent units, rather than as parts of functional units (e.g. periotic bones) (Heyning, 1997). These phenetic classifications also fail to distinguish among shared derived characters (synapomorphies), shared ancestral characters (symplesiomorphies) and characters subject to potential convergence (homoplasies).

However, cladistics have a strictly cladistic classification has proven difficult to apply to cetaceans due to the lack of clear diagnostic morphological characters (Messenger and McGuire, 1998; Heyning and Lento, 2002; Geisler and Sanders, 2003). Muizon (1988), in one on the few cladistic studies specific of delphinids, classified *Sotalia, Sousa* and *Steno* in Delphininae, based on the expansion of the posterior lobe of the pterygoid sinus as a synapomorphy. In another cladistic study, a more derived condition in the cranial and skeletal morphology of the genus *Sousa* was noted with respect to both *Sotalia* and *Steno* (Arnold and Heinsohn, 1996).

Mitochondrial genes (mtDNA) have been commonly used in cetacean systematics and phylogenetics (Arnason et al., 1993; Messenger and McGuire, 1998; LeDuc et al., 1999; Hamilton et al., 2001; May-Collado and Agnarsson, 2006). The rate of evolution of mitochondrial genes has been estimated to be between three and ten times faster than the rate of evolution of nuclear genes (Hoelzel et al., 1991; Ballard and Whitlock, 2004; Lin and Danforth, 2004). This can be advantageous when studying closely related taxa (sister-species, subspecies), but may lead to high levels of homoplasy, obscuring phylogenetic signal for taxonomic divergences occurring more than 5–10 million years ago (Springer et al., 2001; Lin and Danforth, 2004).

The only comprehensive molecular study of the Delphinidae is that of LeDuc et al. (1999). This study, based on mitochondrial cytochrome *b* gene sequences, placed *Sousa* within Delphininae while *Sotalia* and *Steno* were retained as the only members of Stenoninae, with low bootstrap support for this grouping (refer to Fig. 4b for guidance).

Nuclear markers are being used with increasing frequency in cetacean phylogenetics (Cassens et al., 2000; Waddell et al., 2000; Dalebout et al., 2004; Kingston and Rosel, 2004; Gaines et al., 2005; Harlin-Cognato and Honeycutt, 2006; Caballero et al., 2007) and in cetacean population structure (Palumbi and Baker, 1994; Baker et al., 1998) and demographic studies (Hare et al., 2002). Nuclear genes (nuDNA) can be more challenging to work with for several reasons: fewer conserved primers, potential gene duplication and difficulties for resolving alleles as well as the need for better quality DNA to carry out amplification (Zhang and Hewitt, 2003). Additionally, one has to include information from a large number of independent nuclear loci to obtain a useful number of phylogenetically informative characters (Hare, 2001). This is needed because nuclear alleles will take longer to reach monophyly than mitochondrial genes (Palumbi et al., 2001) increasing the chance of shared ancestral polymorphisms (incomplete lineage sorting). Introns are a common source of nuDNA data since they are generally more variable than protein coding nuDNA (exons) (Zhang and Hewitt, 2003) and because they appear to be under less structural and evolutionary constraints than coding sequences (Hare, 2001; Hare and Palumbi, 2003). Exon-anchor primers, referred to as EPICS (Palumbi and Baker, 1994) or CATS (Lyons et al., 1997) for amplification of introns are becoming increasingly available (Lyons et al., 1997; Hare et al., 2002; Hellborg and Ellegren, 2003; Aitken et al., 2004).

Here we evaluate the phylogenetic relationships among *Steno*, *Sousa* and *Sotalia,* investigate their systematic position within the subfamilies of Delphinidae; and assess the support of multi-locus genetic data, including two mitochondrial markers, four Y chromosome and six autosomal introns, to the delphinid relationships suggested by previous morphological and molecular analyses.

2. Materials and methods

2.1. Sample acquisition

Thirty-two individual samples, representing 17 delphinid species, were included in this study. At least one species representing each subfamily (*sensu* Perrin, 1989) was included (Table 1). Seven species were represented by multiple specimens from different ocean basins or different locations within the geographic range of the species. For *Sotalia* both coastal (costero, *Sotalia guianensis*) and riverine (tucuxi, *Sotalia fluviatilis*) samples were included (Caballero et al., 2007). One sample of the monodontid *Delphinapterus leucas* (beluga whale), one sample of *Inia geoffrensis boliviensis* (Bolivian Amazon River dolphin) and one of *I. g. geoffrensis* (Amazon River dolphin) as well as four samples from the two phocoenid species *Phocoena phocoena* (harbor porpoise) and *Phocoenoides dalli* (Dall's porpoise) were included as outgroups in all phylogenetic analyses. Samples were obtained as skin tissue from dead stranded animals, from animals kept in captivity or from free-ranging animals using a small biopsy dart deployed from a crossbow or modified veterinary capture rifle (Krützen et al., 2002). Some samples were sent from the Southwest Fisheries Science Center (SWFSC-NOAA, La Jolla, CA) as extracted DNA. Species identifications were made in the field by the collector or by experienced researchers independent of the genetic analysis.

2.2. DNA extraction, amplification and sequencing

DNA extraction from skin samples followed the protocol of Sambrook et al. (1989) as modified by Baker et al. (1994). The Polymerase Chain Reaction was used to amplify two fragments of mtDNA, and nine introns; four from the Y chromosome, and five from autosomal regions (Table 2). Reactions were carried out in a 25 μl final volume. A master mix of 2.5 μl of 10× Perkin-Elmer *Taq* buffer, 0.3 μl of a 200 μM dNTPs mix, 1 μl of each 10 μM primer was used.

For the first intron of the Actin gene (Act-1) and the first intron of the α-Lactalbumin gene (Lac-1), as well as for the mitochondrial gene fragments, a 1.5 mM concentration of $MgCl_2$ was used, as well as Perkin-Elmer Ampli*Taq* polymerase. For all other autosomal and Y chromosome introns, a 2.0 mM concentration of $MgCl_2$ was used as well as Perkin-Elmer *Taq* GOLD polymerase. BSA (Bovine Serum Albumin) was added to all reactions to decrease inhibition of the PCR. The temperature profile for the first intron of the Actin gene (Act-1), first intron of the α-Lactalbumin gene (Lac-1), CAT, GBA, IFN and for the mitochondrial genes was an initial denaturation at 94 °C for 2 min (12 min if using TaqGOLD), followed by 35 cycles at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 30 s. A final extension at 72 °C for 10 min was performed. For CHRNA1, a touchdown PCR was performed, with an initial denaturation at 94 °C for 12 min, followed by 10 cycles at 94 °C for 20 s, 64–55 °C (decrease of 0.9 °C per cycle) for 20 s, 72 °C for 40 s. This touchdown was followed by 30 cycles at 94 °C for 20 s, 55 °C for 20 s and 72 °C for 40 s. A final extension at 72 °C for 10 min was performed. For all Y chromosome introns, touchdown PCR was also used. For DBY7, DBY8 and SMCY7, the PCR profile started with an initial denaturation at 94 °C for 12 min, followed by 20 cycles at 94 °C for 30 s, 60-50 °C (decrease of 0.5 °C per cycle) for 1 min and 72 °C for 1.5 min. This touchdown was followed by 20 cycles at 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1.5 min. For UBE1Y7, the touchdown decreased from 55 to 45 °C and the annealing temperature for the posterior cycles was 45 °C. A final extension at 72 °C was performed in all cases.

As a negative control for introns of the Y chromosome, two female samples were used in the initial PCRs. Female samples showed no amplification products on 1.6% agarose gel electrophoresis when compared with male samples.

Free nucleotides and primers were removed from PCR products using SAP and Exo1 (shrimp alkaline phosphatase and exonuclease 1) (USB) and the products were

Table 1
Samples included in this study with family designations following Rice (1998) for Iniidae, Monodontidae and Phocoenidae, and family and subfamily designations following Perrin (1989) for Delphinidae

Species	Sample code	Geographic location	Sex	Collector/Institute[b]
Family Iniidae				
Amazon River dolphin *(Inia geoffrensis geoffrensis)*	Igeo01	Peru	Male	M. Ruíz, PUJ
Bolivian Amazon River dolphin *(I. g. boliviensis)*	Ibol03	Bolivia	Male	M. Ruíz, PUJ
Family Monodontidae				
Beluga whale *(Delphinapterus leucas)*	z35280	Canada	Male	SWFSC
Family Phocoenidae				
Dall's porpoise *(Phocoenoides dalli)*	z1881	West Coast, USA	Male	SWFSC
	z1692	West Coast, USA	Male	SWFSC
Harbor porpoise *(Phocoena phocoena)*	z13627	West Coast, USA	Male	SWFSC
	z13628	West Coast, USA	Male	SWFSC
Family Delphinidae				
Subfamily Globicephalinae				
Long-finned pilot whale *(Globicephala melas)*	Gme096	New Zealand	Male	M. Oremus, UAMA
Short-finned pilot whale *(G. macrorhynchus)*	Gma013	French Polynesia	Male	M. Oremus, UAMA
Melon-headed whale *(Peponocephala electra)*	Pelec223	Puerto Rico	Male	A. Mignucci, CSN
Killer whale *(Orcinus orca)*	Pelec848	Puerto Rico	Male	A. Mignucci, CSN
	OorRS01	Ross Sea, Antarctica	Male	C. Olavarría, UAMA
	OorCP01	Pacific Coast, Colombia	Female	FY
Subfamily Orcaellinae				
Irrawaddy dolphin *(Orcaella brevirostris)*	z23971	Thailand	Male	SWFSC
Subfamily Cephalorhynchinae				
Commerson's dolphin *(Cepahlorhynchus commersonii)*	Cco0302	Chile	Male	C. Olavarría, UAMA
	CcoPA01	Chile	Female	C. Olavarría, UAMA
Chilean dolphin *(C. eutropia)*	CeuCO01	Chile	Male	C. Olavarría, UAMA
Subfamily Delphininae				
Rissos's dolphin *(Grampus griseus)*	Ggri173	Puerto Rico	Male	A. Mignucci, CSN
Short-beaked common dolphin *(Delphinus delphis)*	Dde029	New Zealand	Male	UAMA
Peale's dolphin *(Lagenorhynchus australis)*	Lau1302	Chile	Male	C. Olavarría, UAMA
Bottlenose dolphin *(Tursiops truncatus)*	TtruSWANI	Caribbean Coast, Colombia	Female	CEINER
	TtruBoI08	New Zealand	Male	G. de Tezanos Pinto, UAMA
Spinner dolphin *(Stenella longirostris)*	SloFP12	French Polynesia	Male	M. Oremus, UAMA
Atlantic spotted dolphin *(S. frontalis)*	Sfront366	Puerto Rico	Male	A. Mignucci, CSN
Fraser's dolphin *(Lagenodelphis hosei)*	Lhosei571	Puerto Rico	Male	A. Mignucci, CSN
	Lhosei615	Puerto Rico	Male	A. Mignucci, CSN
	Lhosei842	Puerto Rico	Male	A. Mignucci, CSN
Subfamily Stenoninae				
Rough-toothed dolphin *(Steno bredanensis)*	z18126	Eastern Tropical Pacific	Male	SWFSC
	z138	West Coast, USA	Male	SWFSC
	z9838	Brazil	Female	SWFSC
	SbreFP01	French Polynesia	Female	M. Oremus, UAMA
Pacific humpback dolphin *(Sousa chinensis)*	z8929	Hong Kong	Female	T. Jefferson, SWFSC
	z7893	Hong Kong	Male	T. Jefferson, SWFSC
"Costero"[a] dolphin (*Sotalia guianensis*)	SflML0202	Maracaibo Lake, Venezuela	Male	H. Barrios M. G. Montiel, UZV
	SflCCSM0203	Caribbean Coast, Colombia	Male	N. Jimenez, M. C. Rosso, UJTL
	SflCCMO0103	Caribbean Coast, Colombia	Male	S. Caballero, UAMA
	SflFG0501	French Guiana	Male	S. Beltrán, CULM
Tucuxi dolphin (Sotalia fluviatilis)	SflPA0104	Peruvian Amazon	Male	M. Ruíz, PUJ
	SflCA0104	Colombian Amazon	Male	M. Ruíz, PUJ

[a] Suggested common name for *Sotalia guianensis* (Caballero et al., 2007).
[b] Institutional abbreviations: PUJ: Pontificia Universidad Javeriana (Bogotá, Colombia), SWFSC: Southwest Fisheries Science Center (La Jolla, CA, U.S.A), UAMA: Molecular Archive, The University of Auckland (Auckland, New Zealand), FY: Fundación Yubarta (Cali, Colombia), CEINER: Oceanario Islas del Rosario (Caribbean Coast, Colombia), CSN: Caribbean Stranding Network (San Juan, Puerto Rico), UZV: Universidad del Zulia (Maracaibo, Venezuela), UJTL: Universidad Jorge Tadeo Lozano (Santa Marta, Colombia), CULM: Centro Universitario Luterano de Manaus (Manaus, Brazil).

directly sequenced in both directions using the standard protocols of BigDye™ terminator sequencing chemistry on a ABI 3100 Perkin-Elmer automated capillary sequencer.

Table 2
Molecular markers used in this study

Locus	Approximate product size (bp)	Primer pair	Location[a]	Reference
Act-1	980	Act-3 Act-1385	Nuclear	Palumbi and Baker (1994) Conway (2005)
Lac-1	600	LacIR LacIIF	Nuclear	Milinkovitch et al. (1998)
CHRNA1	360	CHRNA1-F CHRNA1-R	Nuclear (Chromosome 2)	Roca et al. (2001)
CAT	520	CAT-F CAT-R	Nuclear (Chromosome 11)	Lyons et al. (1997)
GBA	310	GBA-F GBA-R	Nuclear (Chromosome 1)	Roca et al. (2001)
IFN	340	IFN-F IFN-R	Nuclear (Chromosome 9)	Lyons et al. (1997)
DBY7	400	DBY7-F DBY7-R	Nuclear (Y chromosome)	Hellborg and Ellegren (2003)
DBY8	200	DBY8-F DBY8-R	Nuclear (Y chromosome)	Hellborg and Ellegren (2003)
SMCY7	500	SMCY7-F SMCY7-R	Nuclear (Y chromosome)	Hellborg and Ellegren (2003)
UBE1Y7	500	UBE1Y7-F UBE1Y7-R	Nuclear (Y chromosome)	Hellborg and Ellegren (2003)
Cyt-*b*	464	Tglu CB2	Mitochondrial	Palumbi (1996)
CR (Dloop)	800	t-Pro-whale M13Dlp1.5 Dlp8	Mitochondrial	Dalebout et al. (2004)

[a] Chromosomal locations assigned in human, cow, mouse and cat (Lyons et al., 1997).

2.3. Data analysis

2.3.1. Sequence quality

Sequence quality was evaluated using *Phred* v.020425 (Ewing and Green, 1998; Ewing et al., 1998). Sequences with *Phred* scores $\leqslant 20$ (a probability of more than 1/100 of being incorrectly called) were excluded from the analysis or re-sequenced. Sequences with *Phred* scores values between 20 and 40 (a probability between 1/100 and 1/10,000 of being incorrectly called) were edited manually, and sequences with *Phred* scores values $\geqslant 40$ were checked by eye to confirm variable sites. A variable site or heterozygote was indicated by a secondary peak with a height $\geqslant 30\%$ of the height of the primary peak and by a slight decline in the *Phred* score. Sequences were manually edited and aligned using Sequencher 4.1 software (Genes Code Corporation). Species represented by multiple specimens were manually examined for variable sites in order to control for intraspecific variation, both at the mitochondrial and nuclear levels. Identical sequences were collapsed into a single OTU (operational taxonomic unit) for the final phylogenetic analyses.

2.3.2. Dataset construction and combination of loci

A Partitioning of Homogeneity Test was used to evaluate overall congruence in phylogenetic signal among the loci (PAUP version 4.0b10, (Swofford, 2002). Since this test indicated no significant overall conflict ($P = 0.96$) for the individual partitions (loci), three datasets were built in MacClade (Maddison and Maddison, 2000), combining the sequences of every gene fragment for each individual. These datasets corresponded to "nuDNA", for the dataset combining all autosomal and Y chromosome introns (10 introns, 4312 bp), "mtDNA", for the dataset combining the partial sequences of the control region (CR) and the cytochrome *b* (Cyt-*b*) gene (2 gene fragments, 884 bp), and the "mtDNA+nuDNA" dataset, combining both mitochondrial and nuclear sequences (12 fragments, 5196 bp). In addition, Maximum Parsimony (MP) reconstructions were performed independently for combined autosomal introns (six introns, 3064 bp) and for combined Y chromosome introns (four introns, 1248 bp). This was done to evaluate possible incongruence in phylogenetic signal between autosomal and Y chromosome intron datasets due to differences in their mode of inheritance (data not shown).

2.3.3. Phylogenetic analysis and measures of support

Each dataset was analysed using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian analysis. MP and ML phylogenetic reconstructions were implemented in PAUP 4.0b10. For the MP analysis (unweighted), heuristic searches with 1000 bootstrap

replicates were used. Tree bisection-reconnection (TBR) was chosen as the branch-swapping algorithm. The number of phylogenetically informative sites was calculated for each gene fragment and for the overall datasets (Tables 4, 6 and 7). The most parsimonious tree, with the shortest tree length (TL), was transferred to MacClade for further analysis and was used to calculate a consistency index (CI) and a retention index (RI) as indicators of homoplasy, synapomorphies and the degree of phylogenetic signal in the different datasets (Farris, 1989). The GTR+G+I substitution model (with base frequencies estimated from the dataset) was chosen for the ML and Bayesian analysis of each dataset (mtDNA, nuDNA and mtDNA+nuDNA). These parameters were estimated in PAUP for the ML analysis, using a distance-corrected Neighbour-Joining (NJ) tree built from each dataset. A Maximum Likelihood bootstrap (100 replications) was also performed.

For the Bayesian Analysis, MrBayes v. 3.0 (Huelsenbeck and Ronquist, 2001) was used. Each dataset (mtDNA, nuDNA and combined mtDNA+nuDNA) was analysed using the GTR+G+I model of substitution with base frequencies estimated from the dataset. Metropolis-coupled Markov-chain Monte Carlo sampling (MCMCMC) was performed with six incrementally heated chains that were simultaneously run for 8,000,000 generations (mtDNA and combined mtDNA+nuDNA datasets) or 10,000,000 generations (nuDNA dataset) using the program default priors as starting values for the model. Trees were sampled every 1000 generations during the analysis. Bayesian posterior probabilities were obtained from the 50% majority-rule consensus of all trees sampled after trees from the initial "burn-in" stage had been removed. Burn-in was set at 10% of the final number of generations. Posterior probability values, provided by Bayesian analysis are a valid yet more liberal measure of support than ML bootstrap support values (Suzuki et al., 2002; Erixon et al., 2003; Simmons et al., 2004).

A "stemminess" analysis was performed to calculate the amount of phylogenetic signal contributed by the internal branches to the structure of the different phylogenetic reconstructions obtained with each dataset (Lanyon, 1988; Phillips et al., 2001). This analysis was used to describe the contribution of internal branches to total branch lengths in a tree built from uncorrected distances. A higher stemminess value reflects proportionally longer internal branches, indicating a greater degree of phylogenetic information in the tree. PAUP 4.0b10 was used to build NJ trees with uncorrected distance estimates (minimum evolution, ME). Internal branch lengths were summed and stemminess was determined as the contribution of internal branch lengths to the total minimum-evolution score (sum of internal branch lengths/ME score).

Branch Support (BS) (Bremer, 1994) measures the number of additional steps in tree-length required to obtain a tree without a particular node. BS values can be positive, negative or zero. The overall BS was calculated in a parsimonious framework for the most parsimonious tree for each dataset (mtDNA, nuDNA, combined mtDNA+nuDNA) using AutoDecay (Eriksson, 2001).

2.3.4. Statistical Tests of alternative tree topologies

The Shimodaira-Hasegawa test (SH test) (Shimodaira and Hasegawa, 1999) was performed in PAUP 4.0b10 to test the level of agreement provided by each of the three datasets for the evolutionary hypotheses (tree topologies) generated by the ML analysis. The best ML tree topology obtained with each dataset was used as a constraint topology and likelihood scores under this constraint were determined for each of the other datasets in turn.

2.3.5. Contribution of particular partitions (loci) to the combined mtDNA+nuDNA tree

Although the Partitioning of Homogeneity Test suggested no overall conflict in the combined datasets, two approaches were used to evaluate the contribution of each partition (locus), both quantitatively and in terms of overall support or conflict, to the combined mtDNA+nuDNA tree. First, as a Maximum Likelihood approach, SH tests were performed to test agreement or conflict provided by each partition (locus) to the evolutionary hypotheses (tree topology) generated by the ML analysis of the combined mtDNA+nuDNA dataset. The best ML tree topology obtained with the combined mtDNA+nuDNA dataset was used as a constraint topology, and likelihood scores under this constraint were determined for each of the partitions in turn.

Second, a parsimony approach, employing Partitioned Branch Support (PBS) values, referred to by others as Partitioned Bremer Support values (Lin and Danforth, 2004), was used to evaluate quantitatively the contribution of a given partition (locus) to the overall support of the combined MP mtDNA+nuDNA tree and to estimate the amount of support or conflict that a particular partition (locus) provides to a particular node (Table 4) (Baker et al., 2001; Gaines et al., 2005). Positive PBS values indicate that a partition (locus) supports a particular node, negative values indicate that a partition provides conflict at a particular node and PBS values of zero indicate that a particular partition provides neither support nor conflict for a particular node (Remsen and O'Grady, 2002). PBS values were calculated using *TreeRot* v.2 (Sorenson, 1999). The PBS was then standardized for each partition (locus) by dividing it by the minimum number of steps contributed by that partition (locus). This controls for differences in size in data partitions (Baker et al., 2001). The measure (PBS/min. steps), has been previously used, as it provides a quantitative measure of each locus' relative contribution to tree resolution (Baker et al., 2001; Lin and Danforth, 2004).

3. Results

3.1. mtDNA phylogeny

Partial Cyt-*b* (425 bp) and partial CR sequences (459 bp) were generated for 17 delphinid species and 5

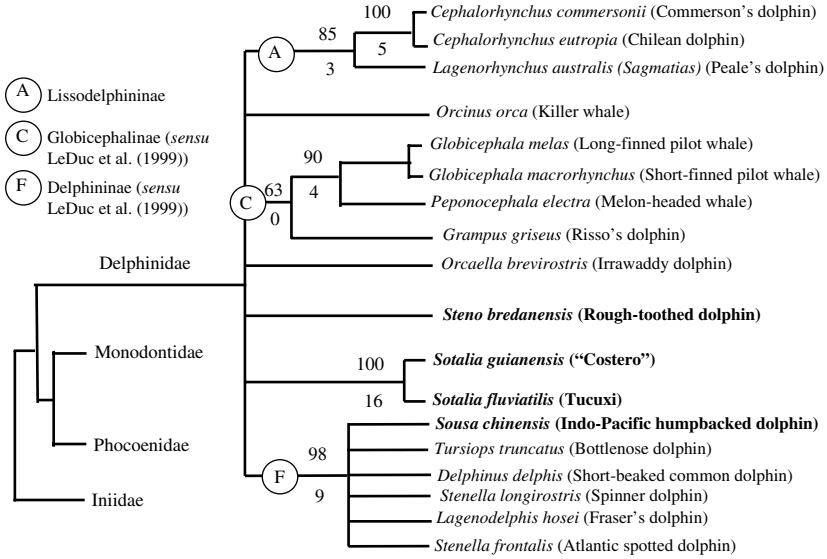

Fig. 1. Consensus tree obtained from the Maximum Likelihood, Maximum Parsimony and Bayesian analysis of 884 bp of mitochondrial DNA (Cyt-*b*+CR), for 17 delphinid species and 5 outgroups from three odontocete families. Circled letters in branches indicate nodes relevant to delphinid subfamily definitions. Support for each of these nodes is represented by ML bootstrap support values (above the branch, 100 replicates) and branch support values (below the branch). Members of the Stenoninae subfamily (sensu Perrin, 1989) are shown in bold.

outgroups. We were unable to sequence the partial CR in *I. g. geoffrensis*, *I. g. boliviensis* and *P. dalli* because we obtained double bands in every amplification attempt. Therefore, we used CR sequences from these species and subspecies previously deposited in GenBank identified by the Accession Nos.AF521126.1 (*I. g. geoffrensis*), AF521124.1 (*I. g. boliviensis*) and AY239119.1 and AY239116.1 for *P. dalli*. Sequences (mitochondrial and nuclear) generated in this study were submitted to GenBank under the Accession Nos. EF02741–EF02762 and EU120949–EU121229.

For the dataset of the combined mtDNA, 296 characters were parsimony-informative. The topologies of the mtDNA tree were very similar using the three analysis methods, with minor discrepancies overall, and were characterized by a low level of resolution for most nodes. Given the similarity of results we present here a consensus tree of the ML, MP and Bayesian analysis of mtDNA (Fig. 1). TL of the most parsimonious tree was 730. The CI was 0.58, indicating a high level of homoplasy in this dataset. The RI was 0.79, indicating that the phylogenetic signal is concentrated along the terminal branches of the tree. Bootstrap support values <50% were obtained for half of the nodes in the mtDNA phylogenies and BS values of nodal support were zero for the majority of the nodes or low positive numbers for a couple of well defined nodes, A and C (Table 4). The shape parameter of the gamma distribution (α) was 0.71. The stemminess analysis indicated that in the mtDNA phylogeny there is an approximate contribution of 50% from the internal branches to the structure of the tree (Table 3). Relationships among *Steno*, *Sotalia* and the delphinines (including *Sousa*) were unresolved.

3.2. nuDNA phylogeny

A total of 4312 bp of nuclear DNA were obtained for 17 delphinid species and five outgroups. Amplification of the Y chromosome introns DBY7 and DBY8 was unsuccessful for *Orcaella brevirostris*.

In the nuDNA dataset, 284 characters were parsimony-informative. The topologies of the nuDNA tree were, again, very similar using the three analysis methods, with minor discrepancies overall and the nuDNA phylogenies being characterized by more internal structure. We present here a consensus tree of the ML, MP and Bayesian analysis of nuDNA (Fig. 2). TL of the most parsimonious tree was 333; CI was 0.88 indicating a low level of homoplasy in this dataset. RI was 0.94, indicating a high number of informative shared-characters (synapomorphies) concentrated on internal nodes. Bootstrap support values were higher than those obtained in the mtDNA phylogenetic reconstruction for most nodes, especially in the ML analysis. Posterior probability support values from the Bayesian analysis were also high for this dataset (Table 4). BS values of nodal support were positive (two for the lowest (E), 16 for the highest

Table 3
Sum of internal branch lengths, minimum-evolution scores and stemminess values (sum of internal branch lengths/ME score, in percentage), calculated from Neighbor-joining trees (NJ) with uncorrected distance estimates reconstructed from each dataset (mtDNA, nuDNA and mtDNA+nuDNA)

Dataset:	mtDNA	nuDNA	mtDNA+nuDNA
Sum of internal branch lengths	684.02	321.63	568.27
ME score	1373.36	477.55	1030.53
Stemminess value (%)	49.8	67.3	55.1

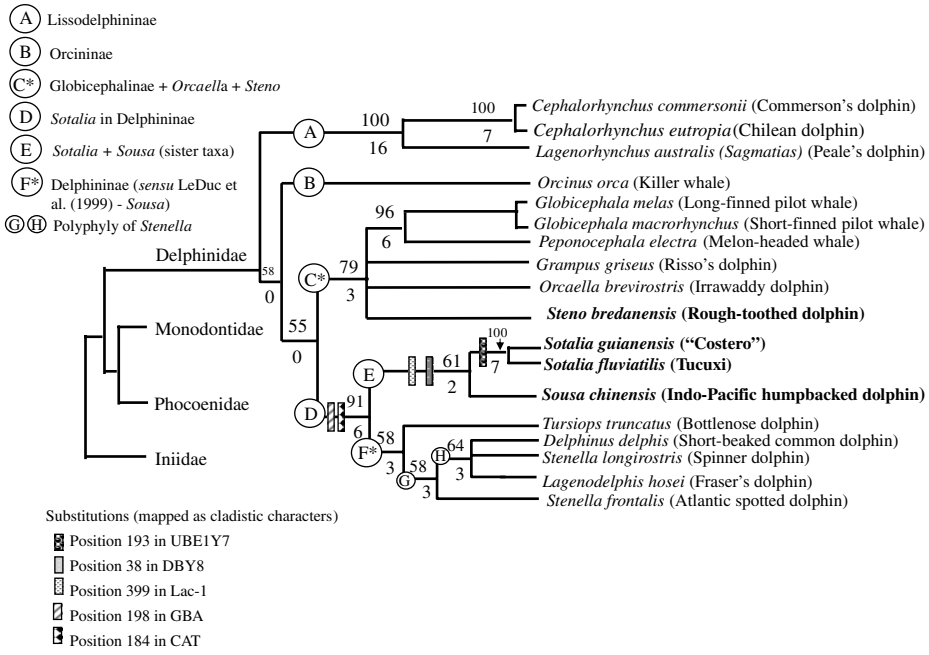

Fig. 2. Consensus tree obtained from the Maximum Likelihood, Maximum Parsimony and Bayesian analysis of 4312 bp of nuclear DNA, for 17 delphinid species and 5 outgroups from three odontocete subfamilies. Circled letters in branches indicate nodes related with delphinid subfamily definitions or polyphyletic groups within subfamilies. Support for each of these nodes is represented by ML bootstrap support values (above the branch, 100 replicates) and branch support values (below the branch). Vertical bars represent shared substitutions (synapomorphies) uniting *Sotalia*, *Sotalia*/*Sousa* and Delphininae. Members of the Stenoninae subfamily (sensu Perrin, 1989) are shown in bold.

(A)) and only one node received a value of zero (B) (Table 4). The shape parameter of the gamma distribution (α) was 0.85, indicating less heterogeneity across sites than in the mtDNA dataset. The stemminess analysis indicated that in the nuDNA phylogeny, 67% of the structure of the tree is contributed by internal branches (Table 3).

Table 4
Bootstrap support values (BP) obtained for the specified nodes in phylogenetic reconstructions by Maximum Parsimony (MP) and Maximum Likelihood (ML) as well as posterior probability support values (PP) from the Bayesian analyses using the three datasets, mtDNA, nuDNA and mtDNA+nuDNA

Node	mtDNA (884 bp)			nuDNA (4312 bp)			mtDNA+nuDNA (5196 bp)		
	PI = 296 TL = 730, CI = 0.58, RI = 0.79			PI = 284 TL = 333, CI = 0.88, RI = 0.94			PI = 493 TL = 1100, CI = 0.66, RI = 0.81		
	BP	BS	PP	BP	BS	PP	BP	BS	PP
Sotalia + *Sousa* (sister taxa) (node E)	MP = <50 ML = <50	0	<0.5	MP = 54 ML = 61	2	1.00	MP = <50 ML = <50	0	<0.5
Sotalia in Delphininae (node D)	MP = <50 ML = <50 B = <50	0	<0.5	MP = 87 ML = 91	6	1.00	MP = 64 ML = 83	2	1.00
Lissodelphininae (node A)	MP = 72 ML = 85	3	1.00	MP = 100 ML = 100	16	1.00	MP = 100 ML = 100	19	1.00
Orcininae (node B)	MP = <50 ML = <50	0	<0.5	MP = <50 ML = 58	0	0.89	MP = <50 ML = <50	0	<0.5
Grampus in Globicephalinae (node C)	MP = <50 ML = 63	4	0.96	MP = 73 ML = 79	3	0.96	MP = 91 ML = 98	8	1.00
Orcaella, Steno and*Grampus* with Globicephalinae (node C*)	MP = <50 ML = <50	0	<0.5	MP = 73 ML = 79	3	0.98	MP = 90 ML = 94	3	1.00
Polyphyly of *Stenella* (node H)	MP = <50 ML = <50	0	<0.5	MP = 64 ML = 64	3	0.91	MP = <50 ML = 76	2	0.71

Branch support values (BS), tree length (TL), consistency index (CI) and retention index (RI) and parsimony-informative characters (PI) were calculated for the best tree found in the MP analysis for each dataset.

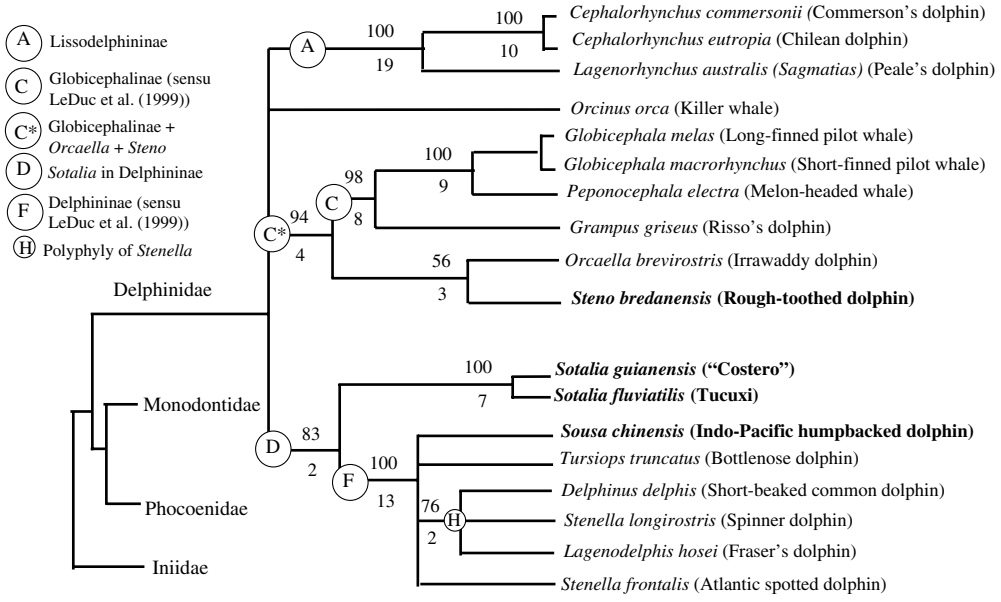

Fig. 3. Consensus tree obtained from the Maximum Likelihood, Maximum Parsimony and Bayesian analysis of 5196 bp of combined mtDNA+nuDNA, for 17 delphinid species and 5 outgroups from three odontocete subfamilies. Circled letters in branches indicate nodes related with delphinid subfamily definitions. Support for each of these nodes is represented by ML bootstrap support values (above the branch, 100 replicates) and branch support values (below the branch). Members of the Stenoninae subfamily (sensu Perrin, 1989) are shown in bold.

One transversion in the Y chromosome intron UBE1Y7 (C to G, position 193) was diagnostic for the genus *Sotalia* when compared to all other delphinids. Two substitutions were shared between *Sotalia* and *Sousa chinensis*, one in the Y chromosome intron DBY8 and the second one in the autosomal intron Lac-1. Of these, the first was a transversion (C to A) and the second was a transition (A to G). Two substitutions were shared between *Sotalia, Sousa chinensis*, *Tursiops truncatus* and all other members of the subfamily Delphininae included in these analyses (*Delphinus, Stenella* and *Lagenodelphis*). These substitutions were detected in the autosomal intron CAT (position 184) and in the autosomal intron GBA (position 198). These were one transversion and one transition, respectively (A to C or A to G). Shared substitutions (synapomorphies) were represented by vertical bars in Fig. 2. Relationships among *Steno*, *Sotalia*, *Sousa* and the rest of the delphinines were resolved.

3.3. Combined mtDNA+nuDNA phylogeny

The combined mtDNA+nuDNA dataset was 5196 bp, of which 493 characters were parsimony-informative. The topologies of the mtDNA+nuDNA trees were very similar using the three analysis methods, with minor discrepancies overall. We present here a consensus tree of the ML, MP and Bayesian analysis of combined mtDNA+nuDNA (Fig. 3). TL of the most parsimonious tree was 1100; CI was 0.66, indicating a higher level of homoplasy in this dataset when compared to the nuDNA, but less homoplasy when compared to the mtDNA dataset. The RI was 0.81, indicating a medium to high number of informative shared-characters (synapomorphies) in this dataset, concentrating the phylogenetic signal mostly on the internal nodes of the tree. An increase in bootstrap values in the MP, ML and posterior probability support values from Bayesian analysis was observed in one node (C*) when compared to the values obtained for the same nodes in the individual mtDNA and nuDNA phylogenies (Table 4). BS values of nodal support were positive for the majority of the nodes (ranging from two (D) to 19 (A)) (Table 3). The shape parameter of the gamma distribution (α) was 0.62, indicating a higher rate of heterogeneity across sites than in the individual mtDNA and nuDNA datasets. The stemminess analysis indicated that in the combined mtDNA+nuDNA phylogeny, internal branches contribute approximately 55% of the total structure of the tree (Table 3).

Overall, branching patterns differed little between the nuDNA and combined mtDNA+nuDNA trees, although the bootstrap support values for one node (D) together with the overall CI and RI were reduced in the latter (Table 4). This was likely due to higher levels of homoplasy and possible saturation in the mtDNA component of the combined dataset. The combined mtDNA+nuDNA offered less resolution than the nuDNA dataset in some cases (e.g. position of *Orcinus*, node B in Fig. 2, and relationships between *Sotalia*, *Sousa* and the rest of the delphinines). However, two nodes (C* and H) obtained higher bootstrap support values (but lower Bremer (PBS) support in the case of the latter).

3.4. Alternative tree topologies

The S-H test was used to test support for the alternative topologies provided by each of the datasets (mtDNA and

nuDNA) and for the combined mtDNA+nuDNA dataset (Table 5). It is important to note that the topology of the mtDNA dataset presented here was the same as that of the cytochrome *b* MP phylogeny consensus tree presented by LeDuc et al. (1999). Therefore, we can test the support of these datasets for LeDuc's topology and compare this support value with that given for the topology of our nuDNA and mtDNA+nuDNA trees. Out of the six tests, two were significant: the ML topology generated by the mtDNA dataset was a poor fit to both the nuDNA and combined mtDNA+nuDNA datasets (rejected at $p < 0.05$ in both cases).

3.5. Agreement and relative contribution of particular partitions (loci) to the combined mtDNA+nuDNA tree

The relative contribution of each locus to the consensus tree generated using the combined mtDNA+nuDNA dataset was assessed by using an overall support or conflict approach as well as a quantitative approach. The S-H tests revealed that out of twelve partitions (loci), three showed significant conflict when they were constrained to the combined mtDNA+nuDNA topology (Table 6). These three loci were Lac-1, Act-1 and CHRNA1.

Table 5
$\ln L$ scores, differences in $-\ln L$ scores between ML trees and constrained tree topologies and probability values at a significance level of $p < 0.05$, obtained in the Shimodaira-Hasegawa test of alternative topologies

Dataset used in the ML analysis	Topology (constraint tree)		
	mtDNA	nuDNA	mtDNA+nuDNA
mtDNA		$-\ln L_{(\text{constrained})} = 4840.397$ $-\ln L_{(\text{mtDNA})} = 4802.876$ Difference = 37.52 $P = 0.10$	$-\ln L_{(\text{constrained})} = 4811.511$ $-\ln L_{(\text{mtDNA})} = 4802.876$ Difference = 8.63 $P = 0.16$
nuDNA	$-\ln L_{(\text{constrained})} = 6787.613$ $-\ln L_{(\text{nuDNA})} = 6761.333$ Difference = 26.28 $P <$ **0.01**		$-\ln L_{(\text{constrained})} = 6676.47$ $-\ln L_{(\text{nuDNA})} = 6667.76$ Difference = 8.71 $P = 0.26$
mtDNA+nuDNA	$-\ln L_{(\text{constrained})} = 13838.947$ $-\ln L_{(\text{mtDNA+nuDNA})} = 13903.037$ Difference = 64.09 **$P = 0.02$**	$-\ln L_{(\text{constrained})} = 13838.947$ $-\ln L_{(\text{mtDNA+nuDNA})} = 13869.087$ Difference = 30.14 $P = 0.34$	

Statistically significant values are shown in bold.

Table 6
Shimodaira-Hasegawa tests to evaluate agreement provided by each partition (locus) unconstrained and constrained by the ML combined mtDNA+nuDNA tree topology

Partition (locus)	Total char. (bp)	PI char.	$-\ln L$	Diff $-\ln L$	Probability
Lac-1	592	38	1213.795 (unconstrained) 1244.693 (constrained)	30.898	**0.001**
CHRNA1	359	15	643.272 (unconstrained) 675.156 (constrained)	31.883	**0.024**
CAT	504	14	849.909 (unconstrained) 852.782 (constrained)	2.872	0.319
GBA	308	11	523.620 (unconstrained) 531.937 (constrained)	8.316	0.078
IFN	337	5	504.853 (unconstrained) 511.790 (constrained)	6.936	0.076
Act-1	963	60	1934.366 (unconstrained) 1971.379 (constrained)	37.012	**0.009**
Y chromosome introns (DBY7, DBY8, SMCY7, UBE1Y7)	1248	64	2356.379 (unconstrained) 2369.226 (constrained)	12.847	0.100
Cyt-*b*	425	124	1996.437 (unconstrained) 2009.805 (constrained)	13.367	0.281
CR	459	172	2787.998 (unconstrained) 2796.211 (constrained)	8.212	0.316

Probability values indicating rejection of the hypothesis of the combined mtDNA+nuDNA topology (at a significance level of $p < 0.05$) are indicated in bold. Total number of characters analyzed and parsimony informative characters (PI) are included.

Table 7
Total number of characters analyzed, parsimony informative characters (PI), tree length (TL), consistency index (CI) and retention index (RI) for the best Maximum Parsimony tree obtained with each separate partition and partitioned branch support values (PBS) obtained in the analysis of data partitions supporting specified nodes when considering the combined mtDNA+nuDNA dataset

Data partition	Lac-1*	CHRNA1*	GBA	IFN	CAT	Act-1*	DBY7	DBY8	SMCY7	UBE1Y7	Cyt-*b*	CR
Total char. (bp)	592	359	308	337	504	963	273	119	443	413	425	459
PI char.	38	15	11	5	14	60	19	3	21	21	124	172
TL	63	23	15	6	23	92	26	5	25	29	333	326
CI	0.89	1	1	1	1	0.96	0.96	1	1	0.93	0.88	0.50
RI	0.96	1	1	1	1	0.98	0.98	1	1	0.97	0.94	0.74
Sotalia + *Sousa* (sister taxa) (node E)	0.26	0	0	0	0	0	0	0.26	0	0	0	0
Sotalia in Delphininae (node D)	0	0	1	0	1	0	0	0	0	0	0	0
Lissodelphininae (node A)	0	1	1	1	0	5	0	0	2	2	2	5
Grampus in Globicephalinae (node C)	1.5	0.33	−1	0	−0.16	1	0	0	0	0	2.83	3.5
Expanded Globicephalinae (node C*) excluding *Steno*	2	0	1	0	0	−2	0	0	0	0	−2	4
Expanded Globicephalinae (node C*) excluding *Orcaella*	−0.54	−0.15	0.77	0.54	0.7	−2.77	2.6	0	0.46	0.15	−0.54	1.77
Polyphyly of *Stenella* (node H)	−0.14	0	0	0	0	0.86	0	0	0	1.42	−1	0.85
Summed PBS	3.08	1.18	2.77	1.54	1.54	2.09	2.6	0	2.47	3.57	1.29	15.12
Min. steps per partition	72	28	17	7	24	101	27	6	30	30	341	516
PBS/min.steps	**0.042**	**0.042**	**0.162**	**0.220**	**0.064**	**0.020**	**0.096**	**0.043**	**0.082**	**0.119**	**0.003**	**0.029**

PBS values for each data partition were summed across all the specified nodes on the combined analysis tree and standardized by the minimum possible number of steps for each partition to investigate the amount of phylogenetic support provided by each data partition to the combined tree (data in bold) (Baker et al., 2001). A star (*) indicates partitions rejecting the hypothesis of the combined mtDNA+nuDNA topology in the Shimodaira-Hasegawa test (refer to Table 6).

We calculated Partitioned Branch Support (PBS) divided by the minimum number of steps for each partition (locus) (Table 7). The highest PBS/minimum steps values, (reflecting high relative support for the combined mtDNA+nuDNA tree topology), were obtained for five out of the ten nuclear introns analyzed: IFN, followed by UBE1Y7, GBA, DBY7 and SMCY7. The mitochondrial gene fragments CR and Cyt-*b* obtained relatively low values, especially the cytochrome *b* gene fragment, even though its CI and RI were high in the MP analysis by itself. Lac-1, Act-1 and CHRNA1 showed conflict at one or two nodes present in the combined mtDNA+nuDNA tree (negative PBS values) and some of the partitions were characterized by low PBS/minimum steps values, indicating low relative support for the combined mtDNA+nuDNA tree.

4. Discussion

4.1. Delphinid phylogeny: primary findings, novel agreement and suggested taxonomic changes

The consensus tree obtained from the analysis of the combined mtDNA+nuDNA is arguably the "best" single tree. This tree includes the largest number of PI sites and was not rejected by either dataset independently (mtDNA or nuDNA). This tree offers a view of delphinid evolutionary relationships based on the consensus among several molecular markers with different patterns of inheritance; from mitochondrial (one locus), Y chromosome (four introns) and autosomal (three introns) within the nuclear genome. These can be differentially affected by population and demographic trends (e.g. effective population size, reproductive strategies, etc). Each is subject to potentially different evolutionary histories and biases but, overall, they should provide the most robust estimate of organismal relationships. Based on this consensus tree, we suggest a series of novel taxonomic changes to Delphinidae. We also refer to some of the findings that were strongly supported by the nuDNA dataset. Some of our results provide additional support for systematic changes suggested by LeDuc et al. (1999). We summarize these suggestions and compare the proposed taxonomic changes with the previous classifications of Delphinidae by Perrin (1989) based on morphology and by LeDuc et al. (1999) based on mtDNA cytochrome *b* (shown in Fig. 4).

4.1.1. Sotalia and Delphininae

Our results suggest that *Sotalia* should be included as a member of Delphininae (node D in our trees) as well as *Sousa*, as suggested by LeDuc et al. (1999). Node D (grouping *Sotalia* with *Sousa*, *Tursiops* and other members of Delphininae included in these analyses) has high bootstrap support and branch support values in all phylogenetic analysis using both the nuDNA and the combined mtDNA+nuDNA datasets. Kasuya (1973) also suggested a close relationship of *Sotalia* and *Sousa* with other members of Delphininae based on morphology.

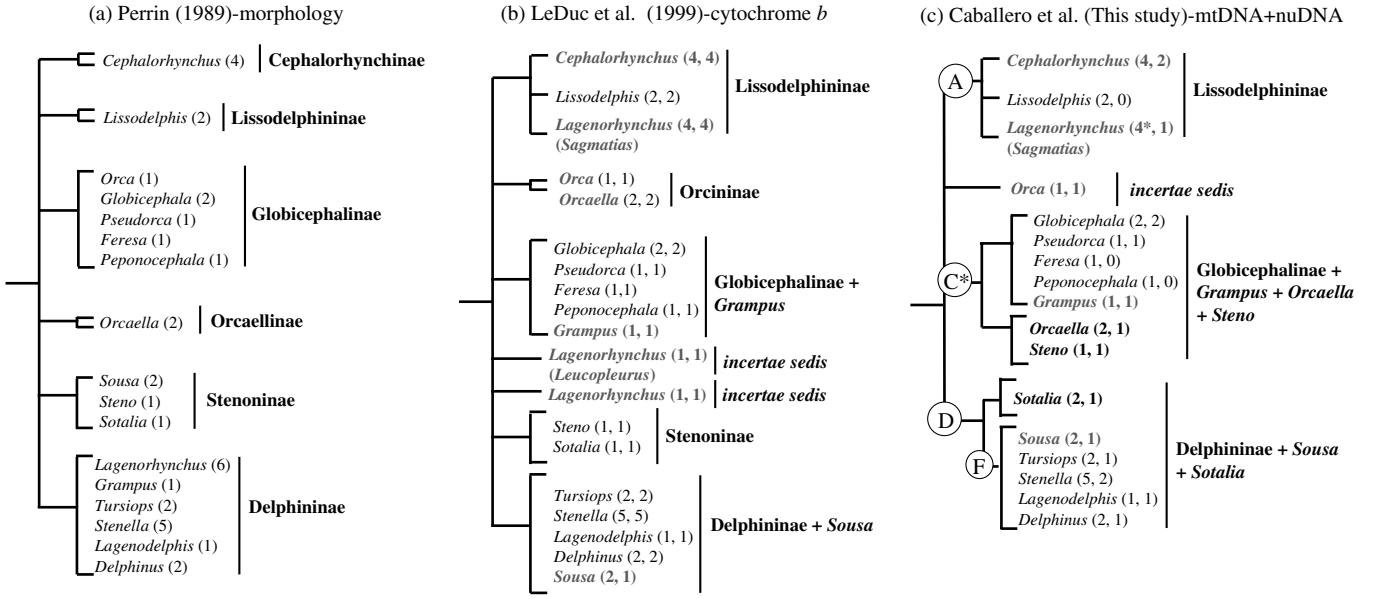

Fig. 4. Classifications of the family Delphinidae: (a) Classification by Perrin (1989). Numbers in parentheses represent the number of species recognized in each genus. (b) Proposed classification suggested by LeDuc et al. (1999), based on cytochrome-*b* analyses. Suggested taxonomic changes to Perrin's classification are shown in gray. Numbers in parentheses represent the number of species recognized in each genus, followed by the number of species per genus included in LeDuc et al. (1999). (c) Proposed classification suggested by this study, based on the nuDNA and combined mtDNA+nuDNA analyses. Suggested taxonomic changes in agreement with LeDuc's classification are shown in gray, novel taxonomic changes suggested by this study are shown in bold. Numbers in parentheses represent the number of species recognized in each genus, followed by the number of species per genus included in this study. (*) indicates lack of information regarding two species recognized by Perrin (1989) in the genus *Lagenorhynchus* (*Lagenorhynchus albirostris* and *Lagenorhynchus acutus*), whose systematic position was uncertain in LeDuc et al. (1999).

4.1.2. Stenoninae

Sousa, Steno and *Sotalia* did not group together in any analyses, suggesting that Stenoninae (*sensu* Perrin, 1989) is an artificial grouping, perhaps created based on ancestral morphological characters (symplesiomorphies). In the mtDNA phylogenies *Steno* tended to form an unresolved node by itself. In the nuDNA and combined mtDNA+nuDNA phylogenies, it grouped with *Orcaella* and *Grampus* and with members of Globicephalinae (node C*). Making *Sotalia-Sousa-Steno* a monophyletic group, exclusive of Delphininae in the combined mtDNA+nuDNA MP reconstruction, would require an additional 56 steps, increasing the tree length from 1110 to 1166 and decreasing the CI from 0.66 to 0.63 and the RI from 0.81 to 0.79. In the nuDNA MP reconstruction, it would require an additional 17 steps, increasing the tree length (TL) from 333 steps to 350 steps. In this case, the CI decreased from 0.88 to 0.83 and the RI decreased from 0.94 to 0.92.

4.1.3. Position of Orcaella

Our results supported inclusion of *Orcaella* as a member of Delphinidae (Grétarsdóttir and Árnason, 1992; Arnold and Heinsohn, 1996; LeDuc et al., 1999) and suggested positioning with the Globicephalinae (node C* in our trees). Morphological similarities in the anatomy of the nasal passages and facial structures and musculature between *Orcaella, Globicephala, Pseudorca, Feresa* and *Orcinus* were observed by Mead (1975) supporting this suggestion. Muizon (1988) previously classified this genus as a member of Globicephalinae on the basis of expansion of the premaxillae at the apex of the rostrum.

4.1.4. Position of Steno

The inclusion of *Steno* in a node grouping the members of Globicephalinae (C*), supported by high bootstrap and branch support values in both the nuDNA and combined mtDNA+nuDNA phylogenies, was surprising. To our knowledge, this is the first time that it has been suggested that *Steno* should be allied with this subfamily, since morphological studies considered this genus more closely related to *Sotalia* and *Sousa* (Fraser, 1966; Gaskin, 1972; Mead, 1975; Muizon, 1988) or even to *Tursiops* (Barnes, 1990) and sometimes placed it in Delphininae (Kasuya, 1973; Muizon, 1988). Additional morphological, nuDNA and mtDNA datasets are needed to determine if *Steno* belongs in fact to Globicephalinae or if it should be maintained as the sole member of Stenoninae.

4.1.5. Unresolved systematic relationships

A sister-taxa relationship between *Sousa* and *Sotalia* was supported by moderate bootstrap support in the nuDNA tree (node E) as well as by two substitutions shared by *Sotalia* and *Sousa* (position 38 of the Y chromosome intron DBY8 and position 399 of the autosomal intron Lac-1). This is in agreement with morphology as interpreted by Perrin (1989). Grouping *Sousa* with *Tursiops truncatus* and *Stenella frontalis*, separated from *Sotalia*, would require two additional steps in the nuDNA MP reconstruc-

tion, increasing the TL from 333 steps to 335 steps. The CI decreased from 0.88 to 0.86 and the RI decreased from 0.94 to 0.93. However, this apparent sister-taxa relationship was obscured in the combined mtDNA+nuDNA phylogenies, as *Sousa* tended to group in node F with *Tursiops truncatus* and *Stenella frontalis*. Grouping *Sousa* with *Sotalia* in the combined mtDNA+nuDNA MP reconstruction would require 13 additional steps, increasing the TL from 1100 to 1113. The CI decreased from 0.66 to 0.60 and the RI decreased from 0.81 to 0.76.

In all our analyses, *Orcinus* was excluded from the node that groups members of Globicephalinae (nodes C and C*). This result is partly consistent with LeDuc et al. (1999) and his proposal for the exclusion of *Orcinus* from Globicephalinae and the creation of the subfamily Orcininae, but not for the grouping of the genus *Orcaella* and *Orcinus*. Because of the low ML analysis bootstrap support for this node (B) (58%) in the nuDNA phylogeny, and the unresolved position of *Orcinus* in the combined mtDNA+nuDNA phylogeny, we suggest *Orcinus* to be considered *incertae sedis*.

4.1.6. Support to systematic changes previously proposed by LeDuc et al. (1999)

A number of results of the present analyses were congruent with current morphology-based classification and with some of the taxonomic changes suggested previously by LeDuc et al. (1999) to the classification of Delphinidae.

Our results support the designation of Lissodelphininae (node A in our trees), grouping the members of the genus *Cephalorhynchus* and presumably at least four of the six currently accepted *Lagenorhynchus* species, represented in our analysis by only one species, *Lagenorhynchus australis*. These four *Lagenorhynchus* species (*L. obscurus, L. australis*, *L. cruciger* and *L. obliquidens*) have been proposed as the genus *Sagmatias* (Cope 1866), resurrected by LeDuc et al. (1999) to the exclusion of *L. acutus* and *L. albirostris*. These results have been also confirmed by others using mitochondrial markers (Cipriano, 1997; Pichler et al., 2001) and recently, monophyly of Lissodelphininae and polyphyly of the *Lagenorhynchus* was confirmed by Harlin-Cognato and Honeycutt (2006) using combined analyses of two mitochondrial markers (Cyt-*b* and CR) and two nuclear markers (Actin and recombination activation gene 2, RAG2). Paraphyly of *Lagenorhynchus* (*L. obscurus, L. australis*, *L. cruciger* and *L. obliquidens)* within Lissodelphininae was also supported in the later study.

Our results showed high bootstrap and branch support values for the inclusion of *Grampus* in Globicephalinae (node C in our trees), as suggested by LeDuc et al. (1999). This result was also supported by initial molecular studies of cetacean phylogeny using restriction mapping of mitochondrial DNA (Ohland et al., 1995).

Our results also supported the polyphyly, or, at least, do not support monophyly of the genus *Stenella* (nodes G and H in our trees), as suggested by LeDuc et al. (1999) and others (Perrin et al., 1981; Perrin et al., 1987). Although only two species belonging to this genus were included in these analyses (*S. frontalis* and *S. longirostris*), polyphyly of this genus was supported in all phylogenetic reconstructions (mtDNA, nuDNA and mtDNA+nuDNA), with high bootstrap support values in the nuDNA and mtDNA+nuDNA reconstructions. Rendering *Stenella* monophyletic required three additional steps in the nuDNA MP reconstruction, increasing the tree length from 333 to 336 and decreasing the CI from 0.88 to 0.87. The RI remained constant (0.94). In the combined mtDNA+nuDNA MP reconstruction, it required two additional steps, increasing the tree length from 1110 to 1112. The CI and RI remained constant at 0.66 and 0.81 respectively.

4.2. Combining datasets

In general, when combinations of mtDNA and nuDNA datasets have been used in phylogenetic analysis, it has been observed that the nuclear loci have greater resolving power at deeper systematic levels as a result of lower levels of homoplasy, and provide greater bootstrap support values (Springer et al., 2001; Lin and Danforth, 2004). This was also observed in the analysis of our nuDNA dataset. However, mitochondrial markers provide important phylogenetic information for resolving terminal branches, even if some of these (e.g. control region) are a source of character conflict, as suggested by Harlin-Cognato and Honeycutt (2006). Further, they provide more species specific information and help to resolve subspecies level questions (Rosenbaum et al., 1997; Pichler et al., 2001; Dalebout et al., 2004).

There is ongoing debate about "combining" versus "not combining" different types of data for phylogenetic analyses (Bull et al., 1993; Huelsenbeck et al., 1996). The Partitioning of Homogeneity Test performs a statistical test of the null hypothesis of data homogeneity and is often used as the basis for deciding to combine or not to combine data partitions (Bull et al., 1993). In our study, the partitioning of homogeneity test found no significant differences in the total length of trees from the individual partitions (loci) compared to the tree from the combined partitions. Given that we did not reject homogeneity, we considered it appropriate to combine all data in an effort to provide the most comprehensive single estimate of phylogeny. However, when each data partition (locus) was constrained independently to the topology of the best ML tree obtained from the combined mtDNA+nuDNA dataset, three autosomal introns (Lac-1, Act-1 and CHRNA1) showed significant disagreement (conflict) with this topology. For each of these introns, polyphyly was observed between members of different subfamilies, suggesting allele sharing in some of these loci (data not shown). Less allele sharing was observed for the Y chromosome introns analyzed to date (data not shown).

Shared ancestral polymorphism and incomplete lineage sorting together can result in the incongruence of topology

from individual genes (Pamilo and Nei, 1988; Nichols, 2001; Gadagkar et al., 2005) and, in this case, can be the result of rapid radiation within the delphinids (Palumbi et al., 2001). This result suggested, again, advantages of analyses combining partitions (concatenated approach) over analyses considering partitions separately (consensus approach) (Gadagkar et al., 2005). The presumably slow mutation rates of nuDNA partitions result in a small number of informative changes across each tree, so concatenation of multiple nuDNA partitions increases the total number of informative sites and, in this way, increases the total phylogenetic signal in these datasets (Gadagkar et al., 2005). Additionally, slower mutation rates in nuDNA could also lead to the generation of homoplasies by chance, due to "too little mutation" (and consequent overestimation of topological confidence based on very few sites). However, it can be difficult to distinguish between the effects of incomplete lineage sorting and homoplasy in nuDNA partitions (Harris and Disotell, 1998; McCracken and Sorenson, 2005).

The CI and PBS provided further insight into differences among partitions. Our results suggested that five of the nuDNA data partitions (loci), including GBA, IFN, UBE1Y7, DBY7 and SMCY7, had the highest PBS/min. steps values as well as high CI values, when compared to the remaining five nuDNA partitions (loci) (Act-1, Lac-1, DBY8, CHRNA1 and CAT) and the two mtDNA data partitions (Cyt-*b* and CR). It is especially interesting that out of five nuclear introns with high PBS/min. steps, three were Y chromosome introns, suggesting that these markers can provide consistent phylogenetic signal for deeper divergences in the Delphinidae tree (5-8 MY). However, no variability was detected in the Y chromosome introns analyzed between closely related species, thought to have diverged between 1-3 MYA (e.g. *Stenella-Tursiops-Delphinus* complex). The absence of signal at the intrageneric and specific level in cetaceans contrasts with their utility in other mammalian groups, for example primates (Tosi et al., 2000; Tosi et al., 2003) and felids (Pecon-Slattery and O'Brien, 1998; Pecon-Slattery et al., 2000).

4.3. Limitations of this study

This study presents a powerful and taxonomically broad nuDNA dataset for representative species of delphinid subfamilies. Further corroboration of our proposed changes with a more exhaustive taxonomic dataset would help to fully confirm these findings. One obvious approach would be to compile a similarly powerful dataset of mtDNA protein-coding genes (Arnason et al., 1991; Carraher, 2004). This would allow development of combined mtDNA+nuDNA datasets where greater phylogenetic signal comes from the mtDNA partitions, as shown by comparative studies of other mammalian phylogenies based on both whole mitochondrial genomes and nuclear genes (Reyes et al., 2004). A combined phylogenetic approach of mtDNA protein-coding genes and nuclear introns could also provide additional evidence to clarify the potential sister-taxa relationship between *Sotalia* and *Sousa*. Mitochondrial protein coding genes evolve faster than most of the nuclear introns considered in this analysis, but more slowly than non-coding mitochondrial DNA (e.g. control region; Moore, 1995; Zardoya and Meyer, 1996). A combined approach looking at both nuclear loci and whole mitochondrial genomes has improved resolution of sister-taxa and sister-group relationships in birds and mammals in which apparently rapid diversification obscures phylogeny (Mindell et al., 1999; Phillips et al., 2001; McCracken and Sorenson, 2005).

Further, it would be useful to include representatives of the "phylogenetically challenging" delphinid species (*L. australis, L. acutus, T. aduncus*, *Stenella clymene*, etc) as well as at least one representative of each delphinid species in further nuDNA, protein-coding mtDNA and combined mtDNA+nuDNA analyses to evaluate the phylogenetic hypotheses presented here with an even more complete dataset.

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