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.

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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 Delphinioidea, 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 Delphininae) 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, Orcaelinae, Lissodelphininae 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 tympanic periosteal 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. periosteal bones) (Heyning, 1997). These phenoetic classifications also fail to distinguish among shared derived characters (synapomorphies), shared ancestral characters (synaplesiomorphies) and characters subject to potential convergence (homoplasies).

However, cladistics have a strictly cladistic classification that 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 of the few cladistic studies of delphinoids, 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 Agrasson, 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 localities 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 monodentid 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 MgCl2 was used, as well as Perkin-Elmer AmpliTaq polymerase. For all other autosomal and Y chromosome introns, a 2.0 mM concentration of MgCl2 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 40 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...
directly sequenced in both directions using the standard protocols of BigDye™ terminator sequencing chemistry on a ABI 3100 Perkin-Elmer automated capillary sequencer.
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 \( \geq 20 \) (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 \( \geq 40 \) were checked by eye to confirm variable sites. A variable site or heterozygote 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. 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 tree topologies

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
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. 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 0.94, indicating a high level of homoplasy among 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 (\(\alpha\)) 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 *Orcella 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>
<thead>
<tr>
<th>Dataset</th>
<th>mtDNA</th>
<th>nuDNA</th>
<th>mtDNA+nuDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum of internal branch lengths</td>
<td>684.02</td>
<td>321.63</td>
<td>568.27</td>
</tr>
<tr>
<td>ME score</td>
<td>1373.36</td>
<td>477.55</td>
<td>1030.53</td>
</tr>
<tr>
<td>Stemminess value (%)</td>
<td>49.8</td>
<td>67.3</td>
<td>55.1</td>
</tr>
</tbody>
</table>

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)
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

<table>
<thead>
<tr>
<th>Node</th>
<th>mtDNA (884 bp)</th>
<th>nuDNA (4312 bp)</th>
<th>mtDNA+nuDNA (5196 bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI = 296</td>
<td>TL = 730, CI = 0.58, RI = 0.79</td>
<td>TL = 1100, CI = 0.66, RI = 0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BS = 72, PP = 1.00</td>
<td>BS = 72, PP = 1.00</td>
</tr>
<tr>
<td>Sotalia + Sousa (sister taxa) (node E)</td>
<td>MP = &lt;50</td>
<td>ML = 50</td>
<td>MP = &lt;50</td>
</tr>
<tr>
<td></td>
<td>ML = &lt;50</td>
<td>ML = 50</td>
<td>ML = &lt;50</td>
</tr>
<tr>
<td>Sotalia in Delphininae (node D)</td>
<td>MP = &lt;50</td>
<td>ML = 50</td>
<td>MP = &lt;50</td>
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<tr>
<td></td>
<td>ML = &lt;50</td>
<td>ML = 50</td>
<td>ML = &lt;50</td>
</tr>
<tr>
<td>Lissodelphininae (node A)</td>
<td>MP = 72</td>
<td>ML = 85</td>
<td>MP = 100</td>
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<tr>
<td></td>
<td>ML = 72</td>
<td>ML = 85</td>
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</tr>
<tr>
<td>Orcininae (node B)</td>
<td>MP = &lt;50</td>
<td>ML = 50</td>
<td>MP = &lt;50</td>
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<tr>
<td></td>
<td>ML = 50</td>
<td>ML = 50</td>
<td>ML = &lt;50</td>
</tr>
<tr>
<td>Grampus in Globicephalinae (node C)</td>
<td>MP = &lt;50</td>
<td>ML = 63</td>
<td>MP = &lt;50</td>
</tr>
<tr>
<td></td>
<td>ML = 63</td>
<td>ML = 63</td>
<td>ML = &lt;50</td>
</tr>
<tr>
<td>Orcaella, Steno and Grampus with Globicephalinae (node C')</td>
<td>MP = &lt;50</td>
<td>ML = &lt;50</td>
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<tr>
<td></td>
<td>ML = &lt;50</td>
<td>ML = &lt;50</td>
<td>ML = 94</td>
</tr>
<tr>
<td>Polypphy of Stenella (node H)</td>
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<td>ML = &lt;50</td>
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<tr>
<td></td>
<td>ML = &lt;50</td>
<td>ML = &lt;50</td>
<td>ML = 76</td>
</tr>
</tbody>
</table>

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.
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, contrasting 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 (\(z\)) 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

In \( L \) 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

<table>
<thead>
<tr>
<th>Dataset used in the ML analysis</th>
<th>Topology (constraint tree)</th>
<th>nuDNA</th>
<th>mtDNA+nuDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtDNA</td>
<td>(-\ln L) (constrained) = 4840.397</td>
<td>(-\ln L) (mtDNA) = 4802.876</td>
<td>(-\ln L) (mtDNA+nuDNA) = 4811.511</td>
</tr>
<tr>
<td></td>
<td>Difference = 37.52</td>
<td>Difference = 8.63</td>
<td>Difference = 15.6</td>
</tr>
<tr>
<td></td>
<td>( P = 0.10 )</td>
<td>( P = 0.16 )</td>
<td>( P = 0.02 )</td>
</tr>
<tr>
<td>NuDNA</td>
<td>(-\ln L) (constrained) = 6787.613</td>
<td>(-\ln L) (mtDNA) = 6761.333</td>
<td>(-\ln L) (mtDNA+nuDNA) = 6676.47</td>
</tr>
<tr>
<td></td>
<td>Difference = 26.28</td>
<td>Difference = 8.71</td>
<td>Difference = 6.71</td>
</tr>
<tr>
<td></td>
<td>( P &lt; 0.01 )</td>
<td>( P = 0.26 )</td>
<td>( P = 0.26 )</td>
</tr>
<tr>
<td>MtDNA + NuDNA</td>
<td>(-\ln L) (constrained) = 13838.947</td>
<td>(-\ln L) (mtDNA+nuDNA) = 13869.087</td>
<td>(-\ln L) (mtDNA+nuDNA) = 13869.087</td>
</tr>
<tr>
<td></td>
<td>Difference = 64.09</td>
<td>Difference = 30.14</td>
<td>Difference = 30.14</td>
</tr>
<tr>
<td></td>
<td>( P = 0.02 )</td>
<td>( P = 0.34 )</td>
<td>( P = 0.34 )</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Partition (locus)</th>
<th>Total char. (bp)</th>
<th>PI char.</th>
<th>(-\ln L) (unconstrained)</th>
<th>Diff (-\ln L)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lac-1</td>
<td>592</td>
<td>38</td>
<td>1213.795</td>
<td>30.898</td>
<td>0.001</td>
</tr>
<tr>
<td>CHRNA1</td>
<td>359</td>
<td>15</td>
<td>643.272</td>
<td>31.883</td>
<td>0.024</td>
</tr>
<tr>
<td>CAT</td>
<td>504</td>
<td>14</td>
<td>849.909</td>
<td>8.316</td>
<td>0.319</td>
</tr>
<tr>
<td>GBA</td>
<td>308</td>
<td>11</td>
<td>523.620</td>
<td>8.316</td>
<td>0.078</td>
</tr>
<tr>
<td>IFN</td>
<td>337</td>
<td>5</td>
<td>504.853</td>
<td>6.936</td>
<td>0.076</td>
</tr>
<tr>
<td>Act-1</td>
<td>963</td>
<td>60</td>
<td>1934.366</td>
<td>37.012</td>
<td>0.009</td>
</tr>
<tr>
<td>Y chromosome introns (DBY7, DBY8, SMYC7, UBE1Y7)</td>
<td>1248</td>
<td>64</td>
<td>2356.379</td>
<td>12.847</td>
<td>0.100</td>
</tr>
<tr>
<td>Cyt-b</td>
<td>425</td>
<td>124</td>
<td>1996.437</td>
<td>13.367</td>
<td>0.281</td>
</tr>
<tr>
<td>CR</td>
<td>459</td>
<td>172</td>
<td>2787.998</td>
<td>8.212</td>
<td>0.316</td>
</tr>
</tbody>
</table>

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 categorized into data partitions: CR and Cytb, mtDNA of Sotalia, and mtDNA of Delphininae. 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.

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.
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 Orcella 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 Orcella

Our results supported inclusion of Orcella as a member of Delphinidae (Gréatarsdóttir and Arnason, 1992; Arnold and Heinsohl, 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 Orcella, 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 Lcr-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 *Orcaldea* 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. albiventer*. 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 over-estimation of topological confidence based on very few sites). However, it can be difficult to distinguish between the effects of incomplete lineage sorting and homoplasies 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 (Armason et al., 1991; Carracher, 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-taxon 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-taxon 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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