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Indian Efforts on the Inventorization of Marine Mammal Species for their Conservation and Management

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Abstract

The present study is the first attempt to use molecular tools for identification of marine mammals in India. The objective was to develop a database of genetic sequences for future marine mammal research in addition to confirming the species identity of cetaceans and dugongs using a molecular approach. Partial sequencing of mitochondrial DNA loci was carried out in accidentally caught/stranded specimens of Spinner dolphin (*Stenella longirostris*), Pantropical spotted dolphin/bridled dolphin (*Stenella attenuata*), Bottlenose dolphin (*Tursiops aduncus*), Long-beaked common dolphin (*Delphinus capensis*), Indopacific humpbacked dolphin (*Sousa chinensis*), Risso's dolphin (*Grampus griseus*), Finless porpoise (*Neophocaena phocaenoides*), Sperm whale (*Physeter macrocephalus*), Blue whale (*Balaenoptera musculus*), Bryde's whale (*Balaenoptera edeni*) and Dugong (*Dugong dugon*). Molecular identification of species was done by phylogenetic reconstruction of the sequences using portals GenBank and *DNA Surveillance*. Apart from ratifying their morphological identification, the analysis was able to distinguish specimens that otherwise, could not have been identified using conventional approaches. Phylogenetic analysis of the *Sousa-Stenella-Tursiops-Delphinus* group indicated more or less robust monophyly for all species in this complex, except *Delphinus capensis*. A sister-group relationship for Sperm whales and Baleen whales was evident, that would place the former closer to the latter than to any other group of toothed whales.

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Introduction

In cetaceans, morphological differences within and among species are often subtle and difficult to compare because specimens are rare or their distributions are wide spread (Baker et al. 2004). Identifying the geographical variants of recognized species of cetaceans is even more cumbersome using conventional approaches, while molecular genetics can provide significant advantages to develop a better taxonomic understanding of inter and intra-specific variation for conservation and management purposes (Rosel et al. 1999; LeDuc et al. 1999; Dizon et al. 2000; Reeves et al. 2004; Amaral et al. 2007). DNA sequence analysis has become a powerful tool for conservation – particularly for identifying the source of samples thought to be derived from threatened or endangered species. Only minute amounts of DNA are required, allowing for remote sampling. PCR-based techniques technically are simple and rapid, making them practical for conservation and population studies. In cetaceans, the technique can also be used effectively for forensic identification of commercial products and verification of trade records (Baker et al. 1996) and for identifying ambiguous beach-cast specimens (Reeves et al. 2004). Illegal trade in animal/plant products is a common practice in some Asian countries, where some endangered species are marketed in the guise of common ones approved by authorized bodies such as, the International Whaling Commission (Dizon et al. 2000).

In the Indian Ocean, it is still unclear how many species of cetaceans exist there, due currently to the absence of any dedicated survey that has assessed their relative abundances (Sathasivam 2004). Though extant cetacean species in Indian seas are estimated to be 25, the number may be higher: further, lack of adequate field keys and reliable inventory has resulted in several cases of misidentification (Kumaran 2002). About 50% of stranded baleen whales have not even been identified to the species level (Sathasivam 2004) while about 25% of reports on baleen whales were misidentified (Kumaran 2002). For example, flipper to body length ratio, a trait used commonly for identification of baleen whales can often lead to misidentification as in the case of Fin whale and Sei whale, where it overlaps. Better resolution of taxonomic status will require more discrete information to confirm species identification (Kumaran 2002). Conventional approach, such as dependence on skeletal material to answer questions about taxonomic status is often cumbersome or destructive. To date molecular tools have not been used for identification of marine cetaceans and dugong in Indian seas. The dugong (*Dugong dugon*) is endangered (Sathasivam 2004; Ilangakoon and Tun 2007) and to devise adequate conservation and management strategies for the species of concern, it will be essential to study the population genetic characteristics of this species across its natural distribution range (Moritz 1995).

Against this background, the present study was undertaken with a view to generating species-diagnostic mitochondrial DNA (mtDNA) sequences for molecular identification of cetaceans and Dugong from the Indian seas. A large number of mtDNA sequences for cetaceans are available in two databases, GenBank (NCBI) and *DNA Surveillance*. Molecular taxonomic identification of species is possible from the carcasses

of ambiguous stranded specimens or even from tissues from unknown samples. The present study will help designate any given sample to a species level, ultimately leading to development of a robust inventory of these vulnerable/endangered groups of animals in the Indian seas.

Materials and Methods

The locations of sample collection are furnished in Fig. 1 and particulars of the samples including accession numbers of mtDNA partial sequences deposited in the

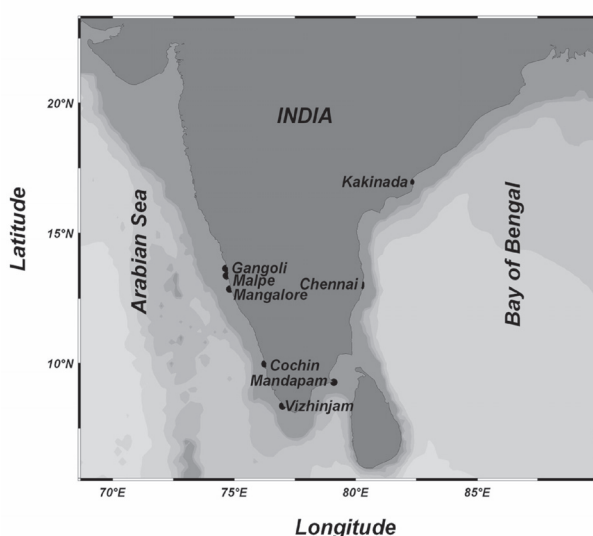


Figure 1. Locations of sample collection.

GenBank are given Table 1. Only tissues collected opportunistically were available for the study here. Skin samples were obtained from incidental fishery kills (*Stenella longirostris*, n=16; *S. attenuata*, n=1; *Tursiops aduncus*, n=3; *Delphinus capensis*, n=2; *Sousa chinensis*, n=2; *Grampus griseus*, n=1; *Neophocaena phocaenoides*, n=12; *Dugong dugon*, n=1) and from stranding (*Physeter macrocephalus*, n=1; *Balaenoptera musculus* n=1; *B. edeni*, n=1). Tissue samples were taken either from the dorsal fin or caudal fluke and stored in 70% ethanol for subsequent genetic analysis.

Total genomic DNA was extracted using a standard phenol-chloroform method (Sambrook et al. 1989) with slight modification. PCR amplification reactions were performed in a PTC100 (MJ Research) thermocycler in a total volume of 25 ml containing 10-100 ng of extracted genomic DNA template, 10 mM of Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 240 mM each of dNTPs, 1 U of *Taq* DNA polymerase and 10-25 pM each of forward and reverse primers. The temperature profile for amplifications were; an initial denaturation (94°C) for 2 min followed by 35 cycles of 94°C for 1 min, 54°C - 57°C for 2 min and 72°C for 1 min and a final extension of 72°C for 7 min. Initially, 4 primers designed for the mitochondrial DNA cytochrome b region and 6 primers for the mtDNA control region (D-loop) were trialed (Table 2). Based on their PCR consistency and robustness, the primers GLUDG-L/CB2-H and M13-Dlp1.5-L/Dlp5-H were used for majority of samples in subsequent amplifications for sequencing. Due to inconsistency of results, sequence data of mtDNA control region were not further used for analyses.

Table 1. Particulars of marine mammal samples examined during the present study

Sl. No	Species	Place & Date of sample collection	Sample code	GenBank (NCBI) accession numbers	
				Control region	Cytochrome b (CYB)
1	<i>Tursiops aduncus</i>	Vizhinjam (5.11.04)	Viz1	—	DQ232769
2	<i>T. aduncus</i>	Chennai (4.10.04)	CHO4	—	DQ270184
3	<i>T. aduncus</i>	Chennai (12.10.04)	CHO8	—	EF203434
4	<i>Stenella longirostris</i>	Kakinada (20.09.04)	VRC/Dol/05	—	DQ270182
5	<i>S. longirostris</i>	Kakinada (20.09.04)	VRC/Dol/04	EF203451	EF203445
6	<i>S. longirostris</i>	Kakinada (20.09.04)	VRC/Dol/06	—	EF057433
7	<i>S. longirostris</i>	Chennai (4.10.04)	CHO2	EF203452	EF203446
8	<i>S. longirostris</i>	Chennai (4.10.04)	CHO3	EF438307	EF203447
9	<i>S. longirostris</i>	Mangalore (8.9.04)	MNG 3	—	EF203448
10	<i>S. longirostris</i>	Chennai (4.10.04)	CH6	—	EF057434
11	<i>S. longirostris</i>	Chennai (4.10.04)	CHO7	EF057435	DQ232770
12	<i>S. longirostris</i>	Chennai (26.10.04)	CH9	EF438306	EF057436
13	<i>S. longirostris</i>	Chennai (26.10.04)	CH10	—	EF203449
14	<i>S. longirostris</i>	Chennai (26.10.04)	CH11	—	EF203450
15	<i>S. longirostris</i>	Chennai (26.10.04)	CH13	—	EF446614
16	<i>S. longirostris</i>	Chennai (26.10.04)	CH17	EF438309	EF057437
17	<i>S. longirostris</i>	Chennai (26.10.04)	CH18	—	EF057438
18	<i>S. longirostris</i>	Chennai (26.10.04)	CH19	EF438303	EF446613
19	<i>S. longirostris</i>	Cochin (15.9.07)	COK1	—	EU204619
20	<i>Stenella attenuata</i>	Chennai (12.10.04)	CH5	EF438305	EF438304
21	<i>Delphinus capensis</i> (?)	Kakinada (23.08.04)	VRC/Dol/03	—	DQ320765
22	<i>D. capensis tropicalis</i>	Malpe (24.02.06)	MNG18	—	EF061405
23	<i>Sousa chinensis</i>	Gangoli (24.11.05)	MNG 4	—	DQ364689
24	<i>S. chinensis</i>	Mangalore (24.12.05)	MNG16	EF061406	EF057445
25	<i>G. griseus</i>	Chennai (26.10.04)	CH15	EF438308	DQ270178
26	<i>Neophocaena phocaenoides</i>	Gangoli (25.11.05)	MNG 5	—	EF203435
27	<i>N. phocaenoides</i>	Gangoli (25.11.05)	MNG6	—	EF203436
28	<i>N. phocaenoides</i>	Gangoli (25.11.05)	MNG 7	DQ364690	DQ364692
29	<i>N. phocaenoides</i>	Gangoli (25.11.05)	MNG 8	DQ364694	DQ364691
30	<i>N. phocaenoides</i>	Gangoli (25.11.05)	MNG 9	—	EF203437
31	<i>N. phocaenoides</i>	Gangoli (25.11.05)	MNG 10	—	EF203438
32	<i>N. phocaenoides</i>	Gangoli (25.11.05)	MNG 11	—	EF203439
33	<i>N. phocaenoides</i>	Gangoli (25.11.05)	MNG 12	—	EF203440
34	<i>N. phocaenoides</i>	Malpe (17.11.05)	MNG 13	—	EF203441
35	<i>N. phocaenoides</i>	Malpe (5.11.05)	MNG 14	—	EF203442
36	<i>N. phocaenoides</i>	Mangalore (1.12.05)	MNG 15	—	EF203443
37	<i>N. phocaenoides</i>	Mangalore (2.1.06)	MNG 17	—	EF203444
38	<i>Physeter macrocephalus</i>	Chennai (26.10.04)	CHWI	—	DQ270180
39	<i>Balaenoptera musculus</i>	Mandapam (17.7.06)	M5	EF057441	EF057442
40	<i>B. edeni</i>	Mandapam (8.8.06)	M6	EF057443	EF057444
41	<i>Dugong dugon</i>	Mandapam (29.3.06)	M4	EF057439	EF057440

Table 2. MtDNA primers used in the present study

Locus	Primer sequence	Annealing temp (°C)	PCR product size range (bp)	Source of primer sequence
Control region	M13-Dlp1.5-L (5'-TGTAACCGCCAGTTCACCCAAAGCTGRATTCTA-3') Dlp5-H (5'-CCATCGWGATGTCTTATTTAAGRGGAA-3')	54	395-527	Dalebout et al. (1998)
Control region	M13-Dlp1.5-L (5'-TGTAACCGCCAGTTCACCCAAAGCTGRATTCTA-3') Dlp4-H (5'-GCG GGW TRY TGR TTT CAC G-3')	57	390-404	Dalebout
Control region	Dlp4-H (5'-GCG GGW TRY TGR TTT CAC G-3') Dlp10-L (5'-CCA CAG TAC TAT GTC CGT ATT-3')	57	290-309	Dalebout (2002)
Cytochrome b	GLUDG-L (5'-TGACTTGAARAACCAACGTTG-3') CB2-H (5'-CCCTCAGAATGATATTTGTCCTCA-3')	54	421-530	Palumbi (1996)
Cytochrome b	CYBMF-L (5'-GAACTATAAGAACTAATGACCAA-3') CYBMR-H (5'-GATTCAGCCATAGTTAACGTCTCGAC-3')	54	200-238	Dalebout (2002)

Purified PCR products were sequenced in an ABI 3100 PE automated capillary sequencer and sequences were edited using Bio Edit ver 7.0.5.3 (Hall 1999), aligned using the computer software Clustal W multiple alignment (Thompson et al. 1994) and corrected by eye.

Morphology-based identification of individual marine mammals were undertaken as per Rice (1998). Molecular identification of the sample was undertaken in two steps: Initially a sequence similarity search of the edited user sequence was done in BLAST (Basic Local Alignment Search Tool) contained in GenBank (www.ncbi.nlm.nih.gov). Once it was confirmed that the tissue sample was from a particular type of cetacean, species identity was searched with the database in *DNA Surveillance* (www.cebl.auckland.ac.nz:9000/), that contains a comprehensive database of mitochondrial DNA sequences from curated and mostly validated (by taxonomic experts) species (Ross et al. 2003). Checking the higher systematic level (genus) of an unknown sample first with BLAST search was important, because if a sample does not belong to the order Cetacea, results of the phylogenetic identification may be misleading. All sequences, after confirmation, were deposited in GenBank.

The input sequence data for phylogenetic relationships consisted of (a) all individuals belonging to the "SSTD" complex (*Sousa-Stenella-Tursiops-Delphinus*) (Reeves et al. 2004) and (b) all individuals of all species in the present study. Outgroups

(i.e., more distantly related species) were used for rooting the trees to protect against a mis-classification error. Both parsimony and genetic-distance based methods were used to reconstruct inter-specific and intra-specific relationships. Maximum likelihood (ML), maximum parsimony (MP) and neighbor joining (NJ) methods were all undertaken for comparative purposes. NJ analysis was performed in Mega ver 3.1 (Kumar et al. 2004) with distance matrix generated according to Tamura-Nei Gamma distance and with 500 bootstraps. ML and MP analyses were performed using the Phylogenetic Inference Package (PHYML) ver 3.65 (Felsenstein 2005) with global rearrangement and outgroup options (for 'all individuals of all species' the outgroup was a single cytochrome b sequence of *Dugong dugon* generated in the present study and for SSTD cytochrome b comparisons, one sequence of *Grampus griseus* and 2 sequences of *Globicephala melas*).

Results

Molecular identification versus conventional approach

Except in four cases, species identification using molecular techniques conformed to that made using conventional morphological-based taxonomy. In one case, the tissue sample (sample code MNG18) was identified to be from *Sousa chinensis*, but both the BLAST search and *DNA Surveillance* searches identified the species as *Delphinus capensis* unambiguously. In a second case, a specimen collected from Chennai (sample code CH5), was earlier field-identified as Bottlenose dolphin (*Tursiops aduncus*), but molecular taxonomic techniques clearly showed the species to be a Pantropical spotted dolphin, also known as Bridled dolphin (*Stenella attenuata*). In another case, we had a stranded baleen whale (sample code M6), that was putrefied beyond recognition. Genomic DNA yielded from its fairly well preserved caudal fluke portion lead to its species identification as *Balaenoptera edeni* (Jayasankar et al. 2007). Lastly, an unidentified dolphin (sample code COK1) was marketed in a market at Cochin. The species was recognized unambiguously as spinner dolphin (*Stenella longirostris*). Without molecular approach, identification would not have been possible given the condition of the specimen.

Molecular identification

Primers GLUDG-L/CB2-H generated robust PCR product of mtDNA CYB gene in all the five species with readable sequences ranging from 421 to 530bp. Particulars of search results of sample sequences in the two databases are summarized in table 3. Sequences of all the 10 species of cetaceans showed good bootstrap values in their phylogenetic reconstruction clusters.

Spinner dolphin was the most common species in the present collections, with 14 specimens coming from the east coast (Kakinada and Chennai) and 2 from the west coast (Mangalore and Cochin), followed by Finless porpoise with 100% of

individuals collected from the west coast of India. Between the 2 specimens of Long-beaked common dolphin, the sequence divergence was as great as 5.9% (data not shown).

Phylogenetic analyses

A perusal of the three phylogenetic algorithms of the *Sousa-Stenella-Tursiops-Delphinus* complex (Fig. 2) indicates more or less robust monophyly with high bootstrap values for all species in this complex except *D. capensis*, which appears to form paraphyletic cluster with *T. aduncus*. When the CYB sequences of all 40 individuals of 10 cetacean species were analyzed using the lone dugong sequence as the outgroup, in all three phylogenetic trees, all species except *Delphinus capensis* formed monophyletic clusters (Fig. 3). Both specimens of the long-beaked common dolphin were placed close in the tree to the bottlenose dolphin cluster. The monophyletic cluster of *N. phocaenoides* was clearly separated from the delphinid group comprising; *S. longirostris*, *S. attenuata*, *T. aduncus*, *D. capensis* and *S. chinensis*. Sperm whale, though belonging to Odontoceti, appeared close to the two baleen whales, *Balaenoptera musculus* and *B. edeni*.

Table 3. Summary of molecular identification of marine mammals

Species	sequence homology in GenBank (%)	Distance values closest reference Sequence or in DNA Surveillance	Remarks
<i>Stenella longirostris</i>	99-100	0.0025-0.0450	High bootstrap values
<i>Stenella attenuata</i>	99-100	0.0049	High bootstrap values
<i>Tursiops aduncus</i>	98-100	0.0010-0.0028	Good bootstrap support
<i>Delphinus capensis</i>	94-100	0-0.0573	In GenBank, 100% sequence homology with <i>D. tropicalis</i> and 94-98% with <i>D.</i> <i>capensis</i> ¹
<i>Sousa chinensis</i>	98-100	0.010-0.015	Good bootstrap support
<i>Grampus griseus</i>	97-99	0.005	High bootstrap values
<i>Neophocaena phocaenoides</i>	99-100	0.008-0.011	High bootstrap values
<i>Physeter macrocephalus</i>	99	0.005	High bootstrap values
<i>Balaenoptera musculus</i>	98-100	0.003	Pygmy blue whale accessions present in the cluster
<i>Balaenoptera edeni</i>	98-100	0.0084	High bootstrap values
<i>Dugong dugon</i>	97-100	--	--

¹See Discussion

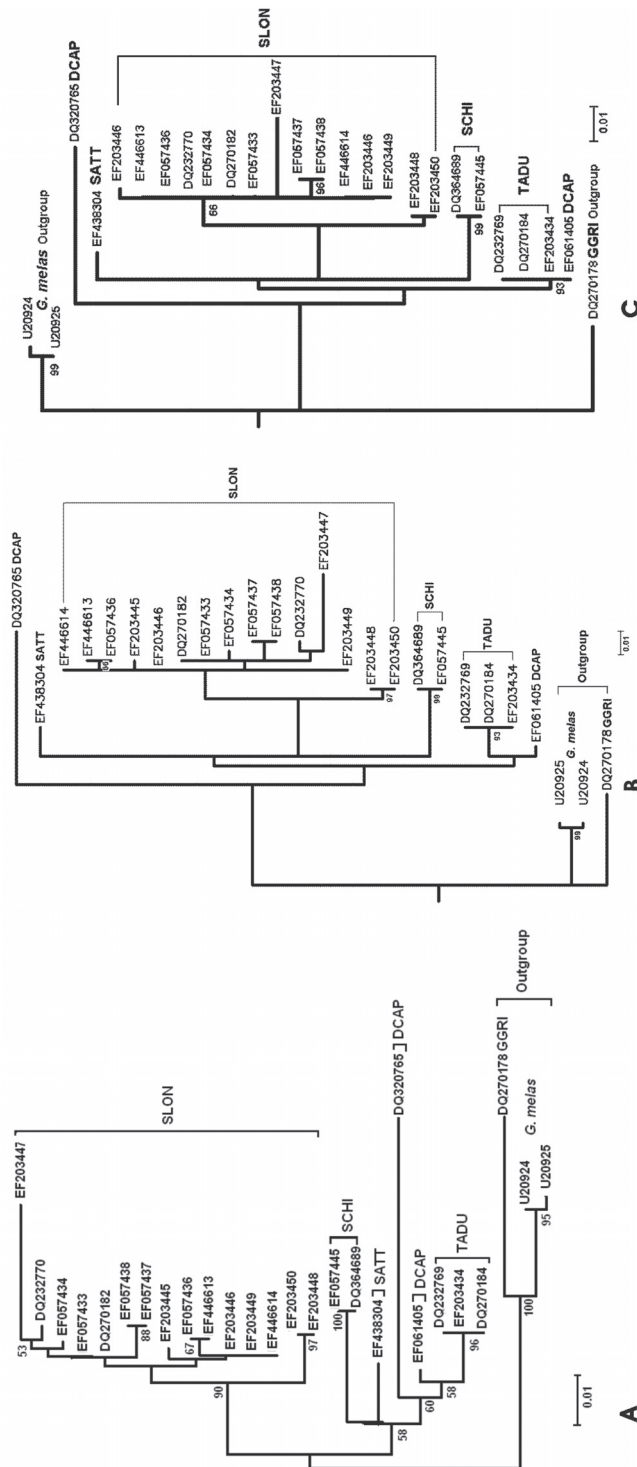


Figure 2. Three phylogenetic algorithms obtained using Maximum likelihood (ML), maximum parsimony (MP) and neighbor joining (NJ) methods.



Discussion

Though accurate estimates are not available, it appears that a few thousand dolphins and porpoise may die of non-targeted fishing every year in India (Yousuf *et al.*, 2008). For addressing all issues impacting the cetaceans around India, their unambiguous identification, inventory and cataloguing are essential. Kumaran (2002) has pointed out several cases of misidentification of cetaceans committed by earlier Indian workers who solely depended on conventional tool of taxonomy – molecular approach can help address species identity through standardized comparisons.

Of the eleven species of marine mammals identified using molecular taxonomy in the present study, ten were recorded by earlier workers from Indian seas, except *Delphinus capensis*, which was reported previously as *D. delphis* (Kumaran 2002). The specimen of *S. attenuata* collected in the present study was initially misidentified as Bottlenose dolphin in the field. From the photograph and body measurements, the specimen of this species was confirmed as *S. attenuata* (William Perrin, Patricia Rosel, Susana Caballero, Richard LeDuc, personal communications). Molecular analysis confirmed its species status.

Intra-specific sequence variability of spinner dolphin examined in the present study was very high (data not shown). The taxonomy of *Stenella* is a matter of ongoing debate and presence of multiple subspecies of *S. longirostris* (Perrin 1990, Perrin *et al* 1999) could further complicate the scenario. *DNA Surveillance* itself recommends caution on phylogeny-based molecular identification.

The earlier published studies from India have mentioned the bottlenose dolphin species as *Tursiops truncatus* (Sathasivam 2004). However, it is now evident that the species of bottlenose dolphin which is often killed accidentally in the coastal gillnet fisheries is likely to be *T. aduncus*. We have sited *T. truncatus* in the oceanic waters off Indian coasts while undertaking many cruises (data not shown). *T. truncatus* is larger than *T. aduncus* and has a shorter beak. All the three specimens collected in the present study showed closest genetic proximity to *T. aduncus*.

All the earlier workers have mentioned the species of common dolphin from Indian seas as *Delphinus delphis* (Sathasivam 2004). But the species encountered in the present study had a fairly long beak and based on the morphological features as well as mtDNA sequencing, is identified here as either *Delphinus capensis* or *D. tropicalis*. Jefferson & Van Waerebeek (2002) concluded on the basis of morphological comparisons that the *tropicalis* form should be regarded as a subspecies of *D. capensis* and suggested that the present species is most likely to be *Delphinus capensis tropicalis* (T. A. Jefferson, personal communication). While one of the specimens in the present study had absolute

genetic similarity with the one reported earlier by [Leduc et al. \(1999\)](#), the other one was extremely divergent (long branch) and in *DNA Surveillance* was placed in a cluster grouping two short-beaked common dolphins as well as one *tropicalis* form. We named this specimen as *Delphinus capensis* with an interrogation mark (Table 1, sample code VRC/Dol/03). Although the possibility of contamination of this sample is unlikely, sequencing of a nuclear pseudogene, which came about as a replication of cytochrome *b* cannot be ruled out ([Mirol et al. 2000](#)).

The present samples of Indopacific humpbacked dolphins were all from the West coast of India, hence we could not verify the possible genetic differences between the West and East coastal forms of this species. Populations along the two coasts are reported to differ markedly in their body color and size of the dorsal hump ([Sutaria and Jefferson 2004](#)).

Species in the *Sousa-Stenella-Tursiops-Delphinus* complex are recently evolved, closely related, and often confused ([Reeves et al. 2004](#)). Based on the cytochrome *b* sequences, [LeDuc et al. \(1999\)](#) attempted a reclassification of this complex and observed that a “comprehensive taxonomic revision of this group awaits further study”. The two specimens of *D. capensis* examined here were widely separated in the MP and ML trees and this result suggests that greater sampling of all species in this group will be required before arriving at any conclusion on their relative phylogenetic positions. A sister-group relationship for Sperm whales and Baleen whales is suggested by the results here and this would place the former closer to the latter than to any other group of toothed whales ([Milinkovitch et al. 1994](#)).

The relative small numbers of individuals analyzed in most instances here means that the study cannot resolve the species identity issues of cetaceans. This study is expected however, to instigate more investigations in the future. This first attempt on molecular identification of cetaceans and dugong in Indian seas has clearly shown the need for more studies of the phylogenetic relationships of those organisms to better understand their evolution; and genetic variation *vis-à-vis* geographic distribution of different species for the biodiversity conservation plans of these vulnerable/endangered animals.

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