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Molecular taxonomy of marine mammals stranded along Kerala coast, India

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Application of molecular tools for the identification of threatened marine mammals has gained importance in recent years. Though live and dead strandings of cetaceans are common along the Indian coasts, the specimens are often not properly identified due to the lack of local taxonomic expertise and poor quality of the specimens. Two marine mammals washed ashore in a putrefied condition at Edayar (08°25′N lat., 76°57′E long.), Thiruvananthapuram District, southwest coast of India, were identified by sequencing of 16S rRNA and COI genes. Sequence and phylogenetic similarity search done with all entries in the DNA sequence database, GenBank using BLAST identified the stranded mammals as Bryde's whale (*Balaenoptera edeni*) and finless porpoise (*Neophocaena phocaenoides*). The present report is the second record of *B. edeni* from the southwest coast of India.

Keywords: Cetaceans, molecular taxonomy, phylogeny, stranded mammals.

THE marine mammal diversity of Indian seas, represented by around 30 recorded species, forms almost one-fourth of the world's marine mammals, and almost 8% of all mammalian fauna recorded in India[1]. The qualitative deficiency of data on marine mammals in India notwithstanding, the shortcomings of marine mammal research in India include geographic disproportionateness in records, non-reporting of mortality due to fishing operations, lack of peer review, incorrect identification of species, incorrect geographic information, inaccuracy in measurements, repeated citation of incorrect records, misinterpretation of observations and lack of molecular data[2]. Though live and dead strandings of marine mammals, especially cetaceans (whales, dolphins and porpoises), are common along the Indian coasts, the specimens are often not properly identified due to the lack of local taxonomic expertise and poor condition of the specimens. Since all the cetaceans are of importance from the conservation point of view, documenting their presence in the ecosystem and precise taxonomy would provide valuable information on various aspects of distribution and migratory nature of different species in the seas around India.

The application of molecular techniques has given stimulating impulses to marine mammal identification. In particular, partial or complete sequences of mitochondrial rRNA genes have been evaluated for appropriate evolutionary rates to resolve some aspects of the higher groups, such as genus, family and order. Because they contain information from old splitting events in their conserved regions, fast-evolving parts should be useful to resolve more recent events, e.g. intraspecific or intrageneric[3]. In addition, DNA sequences of mitochondrial cytochrome oxidase subunit I (COI) gene have been used to estimate phylogenetic relationships among closely related species[4,5]. DNA sequencing technology has provided us the ability to determine the source of tissue samples believed to be derived from threatened or endangered species[6]. A phylogenetic approach to identifying marine mammal sequences from unknown sources has gained support in recent years[7], especially for identifying whale meat products in the open market[6,8]. Above all, sequencing of mitochondrial DNA has been used for describing new species of marine mammals[9–11] and for describing their phylogeny[12]. From India, initial efforts have been made by the Central Marine Fisheries Research Institute, Cochin towards molecular identification of marine mammals[13–15].

Two putrefied carcasses of marine mammals were washed ashore at Edayar (08°25′N lat., 76°57′E long.), Thiruvananthapuram District, southwest coast of India on 27 June 2009 (Figure 1 *a* and *b*). Based on field observations, though the presence of long ventral pleats on the lower profile of the head extending up to the navel confirmed the identity of one specimen as a whale belonging to the genus *Balaenoptera* (Bryde's whale, fin whale and blue whale in this group, recorded from India)[16], confusion prevailed in the identification because of the dented upper jaw. Further, detailed examination of the specimen (total length = 3.9 m) also was difficult since the body was rotten, emanating foul odour, and the demand from

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Figure 1. *Balaenoptera edeni* Anderson (***a***) and *Neophocaena phocaenoides* (Cuvier) (***b***) washed ashore at Edayar, Thiruvananthapuram.

Table 1. Primers used in the present study

Gene	Primer sequence	Annealing temperature (°C)	Product size (bp)	Source
16S rRNA	F – 5′-CGCCTGTTTATCAAAAACAT-3′ R – 5′-CCGGTCTGAACTCAGATCACGT-3′	55	540	Palumbi *et al.*[17]
COI	F – 5′-GGTCAACAAATCATAAAGATATTG-3′ R – 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	51	658	Folmer *et al.*[18]

the public (as the stranding was near a thickly inhabited area with many tourist resorts) was high to bury the carcass immediately. The second specimen (total length = 1.05 m) could not be identified precisely due to the collapsed head; blunt, laterally compressed teeth with expanded crowns and extremely short beak are the taxonomic characters distinguishing dolphins and porpoises[16]. Absence of pertinent taxonomic characters in the stranded marine mammals prompted us to opt for tools of molecular taxonomy.

The tissue samples collected from the marine mammals were processed for extraction of DNA using QIAGEN DNeasy Blood and Tissue kit, and amplified with 16S rRNA and COI genes in a 25 µl reaction volume with QIAGEN *Taq* PCR master mix kit using the thermal cycler Eppendorf. All the PCR products were visualized on 1% agarose gel and the most intense products were selected for sequencing. Primer details[17,18] are given in Table 1. Sequencing was performed directly using the corresponding PCR primers and products were labelled using the BigDye Terminator V.3.1 Cycle sequencing kit (Applied Biosystems, Inc.) and sequenced using an ABI 3730 capillary sequencer following the manufacturer's instructions.

Sequence similarity search was done to identify the source species of the tissue, with all entries in the DNA sequence database GenBank, using Basic Local Alignment Search Tool (BLAST). In the case of the first specimen, BLAST search of COI showed 100% sequence similarity with Bryde's whale (*Balaenoptera edeni* Anderson; family: Balaenopteridae), whereas in the case of the second specimen search of the 16S rRNA showed 97% sequence similarity with finless porpoise [*Neophocaena phocaenoides* (Cuvier); Family: Phocoenidae]. Though COI gene of *N. phocaenoides* was sequenced and the data were submitted to GenBank, further comparison was not possible due to absence of its COI sequence data in public domains and other publications.

Phylogenetic position of both the samples for 16S rRNA gene sequences was determined using neighbour-joining tree of Kimura-2-parameter distance model[19], and the resultant clustering patterns are given in Figures 2–4. The results unequivocally showed that the first specimen belongs to *B. edeni* and the second to *N. phocaenoides.* The sequences were deposited in GenBank under accession numbers GQ856368 (16S rRNA of *B. edeni*), GQ856369 (16S rRNA of *N. phocaenoides*), GQ856370 (COI of *B. edeni*) and HQ268824 (COI of *N. phocaenoides*).

B. edeni occurring in the tropical, subtropical and warm temperate waters around the world is the least known of large baleen whales[20]. The Bryde's whale is included in Appendix I of Conservation of International Trade of Endangered Species of Flora and Fauna (CITES) and listed in Appendix II of Convention on Migratory Species (CMS), and is thus offered protection at the international level. Among the whales, Bryde's whale is the least recorded from India, with only seven records from the country[2]. The present report is the second from the

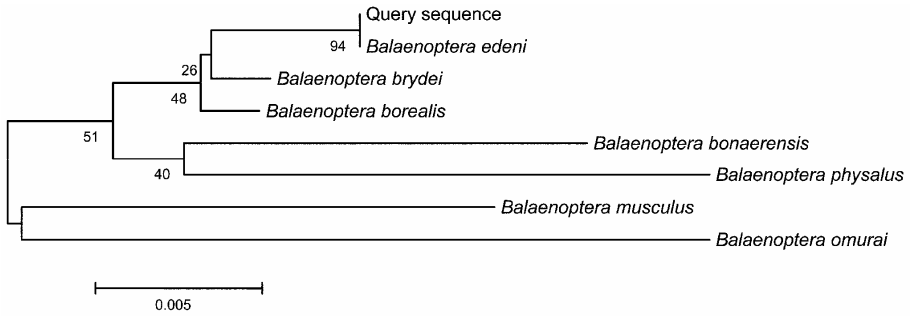

Figure 2. Neighbourhood joining tree of 16S mtDNA gene partial sequence of *B. edeni* based on reference sequences in GenBank. Numbers on the tree branches indicate bootstrap values.

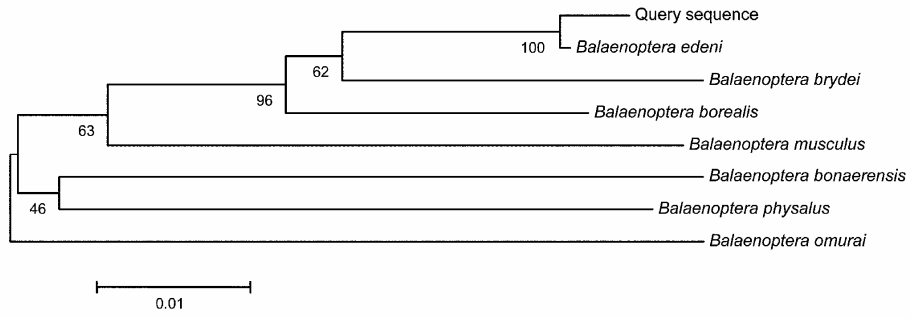

Figure 3. Neighbourhood joining tree of COI mtDNA gene partial sequence of *B. edeni* based on reference sequences in GenBank. Numbers on the tree branches indicate bootstrap values.

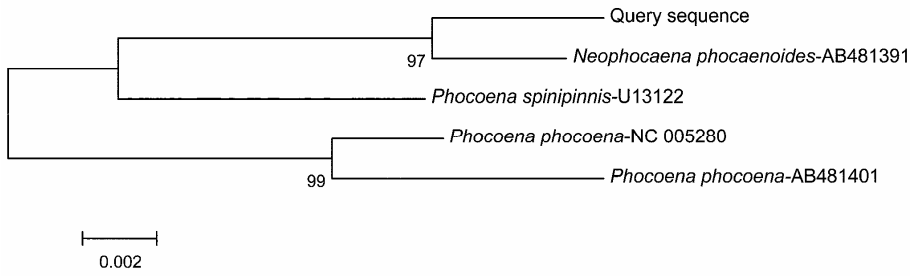

Figure 4. Neighbourhood joining tree of 16S mtDNA gene partial sequence of *N. phocaenoides* based on reference sequences in GenBank. Numbers on the tree branches indicate bootstrap values.

southwest coast of India. The earlier stranding that occurred along the southwest coast of India[21] was from Beypore near Calicut, Kerala in 1979.

Three species have been described in the 'Bryde's whale complex', including *B. edeni*, *Balaenoptera brydei*, and *Balaenoptera omurai*, and taxonomic refinement based on mtDNA studies separated *B. brydei* (Bryde's whale) and *B. edeni* (Eden's whale) into two distinct species[10,11]. Considering the earlier molecular taxonomic study of *B. edeni* from the east coast of India[13] and our study, it may be inferred that the populations inhabiting the seas around India could be the 'ordinary' form of species in the Bryde's whale complex. We propose that the common name of the whale inhabiting the seas around India could be considered as Eden's whale, rather than as Bryde's whale, pending taxonomic uncertainties of the species involved in the complex. Our phylogenetic studies also corroborate the contention of related studies[11] that *B. edeni* constitutes a sister taxon to *B. brydei*. Further investigation is warranted to ascertain the population genetics of Bryde's whale complex inhabiting the seas around India, since recent studies recorded that gene flow between Bryde's whale populations is low and that effective management actions should treat them as separate entities to ensure continued existence of the species[22].

Classified as vulnerable in IUCN red data book, *N. phocaenoides* inhabits shallow coastal waters and has the most interaction with humans and fishing gears[1,2]. There is need to identify the sub-species of *N. phocaenoides* and their molecular data collection from the Indian waters. Frequent monitoring of the stranded marine mammals and molecular taxonomic studies would throw more light on the lesser known marine mammal diversity of the Indian coast. The present study proposes that though

osteological details would provide clues for identification of marine mammals, mtDNA sequences can be used as a reliable method for assigning species status to marine mammals stranded in putrefied condition or even not as whole organism. Further, molecular taxonomy could also be used for documenting the unknown marine mammals that often form bycatch of modern fishing gears.

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