Indian J. Fish., **54**(3) : 339-343, 2007

Note

Species of a whale and an unknown fish sample identified using molecular taxonomy

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ABSTRACT

Molecular genetics provides a powerful tool for conservation of species protected by international regulations or threatened by overexploitation. The present communication is the first report from India on the application of molecular tools for the accurate identification of a stranded whale in putrefied condition as it was impossible to identify the species status using conventional taxonomy and the carcass of an unknown animal devoid of its head and tail, collected from a fish market. Partial sequences of mtDNA control region and cytochrome b gene of the whale were generated and tested with BLAST search and *DNA surveillance* for molecular identification. It was identified as Bryde's whale (*Balaenoptera edeni*). Partial sequence of mtDNA cytochrome b gene of the unknown fish from the market was generated, tested with BLAST search and was identified as sword fish *Xiphias gladius.*

Accurate identification of species is fundamental to conservation efforts of biodiversity. Conventional morphology-based taxonomy often fails to offer unambiguous identification of groups showing little evolutionary differentiation, cryptic members of species complexes, members of closely related species that can only be identified at a particular life stage, fry, fingerlings or juveniles of cultured species, inter-species hybrids and also in the verification of illegal fishing as well as marketing of endangered species. Molecular techniques have become a major tool for systematic biologists at the species level and above.

DNA sequence analysis is a powerful tool for conservation - identifying the source of samples thought to be derived from threatened or endangered species. The technique could be effectively used in the forensic identification of commercial products and verification of trade records (Baker *et al.*, 1996). The DNA-based approach would help the conservationist to identify the species even from a small piece of tissue sample, such as skin from the marketed product. Besides marine mammals, molecular based taxonomic identification has great application in validation of marketed species, such as sea turtles, sharks and sturgeons (Dizon *et al.*, 2000).

The present paper describes two cases of species identification carried out by molecular taxonomic approach. One was a baleen whale of about 12m in total length stranded on the beach at Kundugal near Mandapam (Gulf of Mannar), Tamil Nadu on 8th August 2006. It was in a decayed condition making it impossible to identify the species status using morphological characters (Fig 1). The skin tissue in a portion of the caudal fluke was almost intact and about 3 g of skin tissue was sampled from this region and preserved in 70% ethanol. Total genomic DNA was extracted using the standard phenol-chloroform method

(Sambrook *et al.*, 1989) with slight modifications as follows: about 25 mg of skin tissue was finely chopped and placed in extraction buffer (containing 0.1M NaCl, 10 mM Tris HCl, 1mM EDTA), with 10% SDS and digested by proteinase K (20 µl) at 65ºC for 3 h. This was followed by the routine phenol, chloroform:isoamyl alcohol extractions and the extracted DNA was resuspended in TE and stored at - 20ºC until further use.

PCR was performed on PTC100 (MJ Research) thermocycler in a total volume of 25 µl containing 10-100 ng of extracted genomic DNA template, 10 mM of Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 240 µM each of dATP, dTTP, dCTP and dGTP, 1 U of Taq DNA polymerase and 10-25 pM each of forward and reverse primers. Table 1 shows the particulars of the primers used in this study to amplify mtDNA control region and cytochrome b gene. The temperature profile for the amplifications was an initial denaturation at 94ºC for 2 min followed by 35 cycles of 94ºC for 1 min, 54ºC - 57ºC for 2 min and 72ºC for 1 min and a final extension at 72ºC for 7 min. Quality of the PCR products was checked on 1.5% agarose gel and the products were purified using the KT 62 Genei quick PCR purification kit.

Fig. 1. Beach-stranded baleen whale (later identified as Bryde's whale) in a highly putrefied condition

Cycle sequencing reaction was performed with the purified PCR product based on ABI AmpliTaq FS dye terminator cycle sequencing chemistry on a ABI 3100 PE automated capillary sequencer in presence of the forward primer, reaction buffer and the fluorescently labeled dye terminators for the required number of cycles at specific temperature. The sequences were first edited using Bio Edit ver 7.0.5.3 (Hall, 1999), aligned using the computer software Clustal W multiple alignments (Thompson *et al*., 1994) with gap opening 10, gap extension 0.2 and bootstrap 1000 and corrected manually. The primer and other ambiguous sequences were deleted. Nucleotide sequences of cyt b gene were translated to amino acid sequences using the software Primer Premier ver 5.00.

Molecular identification of the whale sample was done in two steps: initially sequence similarity search of the edited user sequence was done in BLAST (Basic Local Alignment Search Tool) contained in a comprehensive database, GenBank (www.ncbi.nlm.nih.gov)

TABLE 1: *Particulars of the mtDNA primers used in the study*

Locus	Primer sequence	Annealing temp (^{0}C)	PCR product size (bp)	Source
Cytochrome b	GLUDG-L (5'-TGACTTGAARAACCAYCGTTG-3') CB2-H (5'-CCCTCAGAATGATATTTGTCCTCA-3')	54	432	Palumbi (1996)
Control region	M13-Dlp1.5-L (5'-TGTAAAACGGCCAGTTCACCCAAAGCTGRARTTCTA-3') Dlp5-H (5'-CCATCGWGATGTCTTATTTAAGRGGAA-3')	54	527	Dalebout *et al*. (1998)

(Benson *et al*., 2007). Once it was confirmed that the tissue sample was from a cetacean (dolphin, porpoise or whale), the species identity was searched with the database of *DNA surveillance* (www.cebl. auckland.ac.nz: 9000) (Baker *et al*., 2003, Ross *et al*., 2003). The sequences, after their confirmation, were submitted in GenBank using a standalone multi-platform submission program called Sequin (www.ncbi.nlm.nih.gov/Sequin/index.html).

Partial sequences of rapidly evolving mtDNA D-loop (control region) and cytochrome b gene were generated and tested to identify the whale sample. The BLAST search showed 99-100% sequence similarity with Bryde's whale (*Balaenoptera edeni*). The *DNA surveillance* search also unambiguously showed the species of the whale as Bryde's whale (Figs. 2 and 3). The sequences were deposited in the GenBank under accession

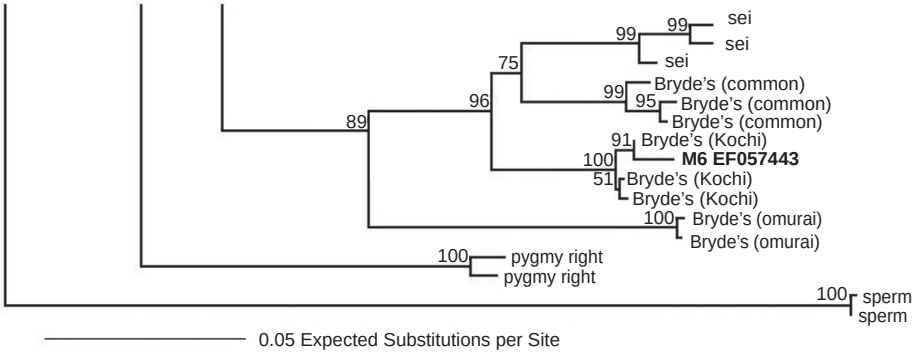

Fig. 2. Neighbour joining tree of mtDNA control region partial sequence of *Balaenoptera edeni* (M6, GenBank accession # EF057443) based on reference sequences in *DNA surveillance*. The numbers on the tree branches indicate bootstrap values

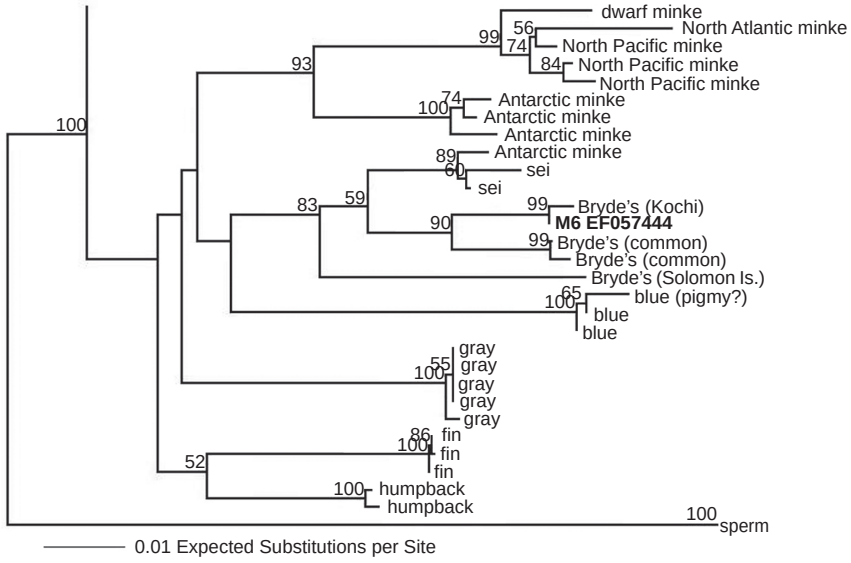

Fig. 3. Neighbour joining tree of mtDNA cytochrome b gene partial sequence of *Balaenoptera edeni* (M6, GenBank accession # EF057444) based on reference sequences in *DNA surveillance*. The numbers on the tree branches indicate bootstrap values

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         10         20         30         40         50         60         70
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
GGATCTGCTG CAAGATATCA TTCTGATGGG CTCCGACTAA ACCCACCCCC TCCTAAAAAT CGCAAACGAC  70

         80         90        100        110        120        130        140
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
GCACTAGTGG ATCTCCCAAC TCCCTCCAAC ATTTCAGTCT GATGAAACTT CGGCTCCCTC CTCGGCCTCT  140

        150        160        170        180        190        200        210
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
GTTTAGCTGC CCAAGTCCTC ACAGGCCTGT TTCTCGCTAT ACACTATACG TCAGACATCG CGACGGCCTT  210

        220        230        240        250        260        270        280
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
TACATCCGTA GCACATATCT GCCGAGATGT AAATTATGGA TGACTCATCC GAAACATGCA CGCCAACGGC  280

        290        300        310        320        330        340        350
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
GCATCCTTCT TCTTCATCTG CATTTACCTC CACATTGGCC GAGGTCTTTA CTATGGCTCC TATCTATACA  350

        360        370        380        390        400        410        420
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
AAGAAACATG GAATATTGGA GTTGTTCTCC TTCTCCTAGT AATGATGACC GCTTTCGTAG GTTATGTATT  420

        430        440        450
....|....| ....|....| ....|....|
ACCCTGAGGA CAAATTCCAA ATTCTGAGGG  450
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Fig. 4. Cytochrome b partial sequence of the fish sample (later identified as sword fish, *Xiphias gladius*)

numbers EF057443 (mtDNA control region) and EF057444 (mtDNA cytochrome b gene).

The second case was a headless and tailless animal carcass observed on 14th October 2006 in the Ernakulam fish market. The carcass looked much like a dolphin with thick black skin. A piece of skin tissue was collected from its dorsal fin and proceeded with the protocols described above for molecular-based identification of the species. BLAST search of the sequence (Fig. 4) from the GenBank (www.ncbi.nlm.nih.gov) produced an unexpected result. The mtDNA cytochrome b gene sequence of the source 'animal' showed 99% sequence similarity with that of sword fish, *Xiphias gladius*.

These two case studies proved the utility of molecular techniques to identify the species of stranded marine mammal accurately even in the absence of whole intact animal to examine the morphological characters as well as in the identification of unknown samples. It is even more significant that it could be achieved from a small piece of skin tissue of the dead animal.

Acknowledgments

The authors are grateful to the Ministry of Earth Sciences for financial assistance to carry out this work. Thanks are also due to the Director, CMFRI, Cochin for the facilities provided and to Dr. R. Paul Raj, Principal Scientist, for critically going through the manuscript.

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Date of Receipt : 20-06-07
Date of Acceptance : 31-08-07