DNA barcoding of the Bryde's Whale *Balaenoptera edeni* Anderson (Cetacea: Balaenopteridae) washed ashore along Kerala coast, India

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Abstract: Three whales washed ashore along Kerala coast of southwest India were identified as Bryde's Whale *Balaenoptera edeni* Anderson based on sequencing of mitochondrial cytochrome c oxidase subunit 1 and cytochrome b genes. The results of mtDNA sequencing in the present study confirm the presence of *B. edeni* species of 'Bryde's Whale complex' in the coastal waters of India.

Keywords: *Balaenoptera,* Bryde's Whale complex, cytochrome b, cytochrome *c* oxidase subunit 1, morphometry, mitochondrial DNA.

Malayalam Abstract: ഇന്ത്യയുടെ തെക്കു പടിഞ്ഞാറൻ ഭാഗത്തെ കേരളതീരത്തടിഞ്ഞു കയറിയ മൂന്ന് തിമിംഗലങ്ങൾ ബ്രൈഡസ് തിമിംഗല വിഭാഗത്തിൽപ്പെട്ട ബലീനോപ്റ്റീറ എഡേനി ആൻഡേഴ്സൻ ജാതിയാണെന്ന് മൈറ്റോകോൻഡ്രിയയിലെ സൈറ്റോക്രോം സി ഓക്സിഡേസ് സബ് യൂണിറ്റ് 1, സൈറ്റോക്രോം ബി എന്നീ ജീനുകളുടെ ശൃംഖലാപഠനം വഴി വെളിവായി. ഈ പഠനം, ഇന്ത്യൻ കടലിലെ ബ്രൈഡസ് തിമിംഗലങ്ങളുടെ സാന്നിധ്യത്തെ അടിവരയിട്ടുറപ്പിക്കുന്നു.

INTRODUCTION

Though an integral component of marine ecosystems, marine mammals, particularly whales, are given little attention by conservation biologists and taxonomists in India. Baleen whales are included in the suborder Mysticeti (Ceteacea: Balaenopteriidae) and are characterised by the presence of a filtering structure in the mouth called Baleen or Whalebone, flippers representing the forelimbs, a tail with horizontal flukes and nasal openings (blowholes) on top of the head (Jefferson et al. 1993). In Indian coastal waters this suborder includes the Blue Whale *Balaenoptera musculus*, Fin Whale *B. physalus,* Sei Whale *B. borealis*, Bryde's Whale *B. edeni*, Mink Whale *B. acutorostrata* and the Humpback Whale *Megaptera novaeangliae* (Kumaran 2002; Sathasivam 2004; Jayasankar & Anoop 2010).

Stranding of marine mammals occurs frequently in India, yet precise identification is not done in many cases due to lack of local taxonomic expertise and poor condition of specimens (George et al. 2011). Since all cetaceans are important from the conservation point of view, precise documenting of their presence would provide valuable information regarding the distribution and migratory nature of different species in the seas around India. Of late, DNA barcoding or sequencing of mitochondrial genes, particularly cytochrome c oxidase subunit 1 (cox1) (Amaral et al. 2007; George et al. 2011) and cytochrome b (cyt b) (Ross et al. 2003; Dalebout et al. 2004; Herath 2007; Sholl et al. 2008; Jayasankar et al.

Image 1. *Balaenoptera edeni* Anderson washed ashore Thanni Beach at Kollam, Kerala

Image 2. *Balaenoptera edeni* Anderson washed ashore Muthalapozhi Beach at Anchuthengu, Kerala

Image 3. *Balaenoptera edeni* Anderson washed ashore Cheriyathura Beach at Poonthura, Kerala

2007, 2008; Viricel & Rosel 2011), has been used to successfully identify cetaceans.

Three whales were stranded at Kollam (Thanni Beach; 08^{0}49'44.4"N & 76^{0}33.3'14.3"E; 24 May 2011; Image 1), Anchuthengu (Muthalapozhi Beach; 08^{0}40'23"N & 76^{0}45'23"E; 04 June 2011; Image 2) and Poonthura (Cheriyathura Beach; 08^{0}26'36.21"N & 76^{0}56'32.70"E; 10 June 2011; Image 3) along southern Kerala. The precise identity of the specimens at Anchuthengu and Poonthura could not be made since the specimens were putrefied. The whale stranded at Kollam measured 960cm (total length) and was identified as Bryde's Whale *Balaenoptera edeni*, Anderson, based on morphological features and morphometry (Table 1). The Bryde's Whale can be distinguished from other baleen whales by the presence of three conspicuous ridges on the snout, 40–70 throat pleats extending to the navel and a tall and falcate dorsal fin that generally rises abruptly out of the back (Jefferson et al. 1993).

Tissue samples were collected from all the whales to confirm identification by the sequencing of two mitochondrial genes, cox1 and cyt b. The samples in absolute ethanol were processed for the extraction of DNA using QIAGEN DNeasy Blood and Tissue Kit (cat No.69506) and cox1 and cyt-b genes were amplified using universal primers [cox 1: Forward primer- 5'-GGTCA ACAAATCATAAAGATATTGG-3', Reverse primer- 5'-TAAACTTCAGGGTGACCAAAAAATCA-3', Tm value : 45–51 ^{0}C (Folmer et al. 1994); cyt b: Forward primer- 5'-TACCATGAGGAC AAATATCATTCTG-3', Reverse primer-5'-CCTCCTAGTTTGTTAGGGATTGATCG-3', Tm value: 46^{0}C (Verma & Singh 2003)] in a 25µl reaction

Table 1. Morphometry of Bryde's Whale *Balaenoptera edeni* Anderson washed ashore at Thanni Beach, Kerala

	Measurement	cm
1	Length, total (tip of the upper jaw to the deepest part of notch between flukes)	960
2	Length, tip of the upper jaw to centre of eye	270
3	Length of gape (tip of the upper jaw to angle of gape)	378
4	Length, tip of upper jaw to blowhole along midline	330
5	Length, tip of upper jaw to anterior insertion of flipper	424
6	Length, tip of upper jaw to tip of dorsal fin	670
7	Length of flipper (anterior insertion of tip)	122
8	Width, flipper (maximum)	32
9	Height of dorsal fin (fin tip to base)	96
10	Fluke span	282
11	Width of flukes (distance from nearest point on anterior border of fluke notch)	88

volume with QIAGEN *Taq* PCR master mix kit in GenAmp PCR System 9700 (Applied Biosystems). The following thermal cycling conditions were used for amplifications: 95^{0}C for 5 min, followed by 10 cycles of 95^{0}C for 30s, 45^{0}C for 40s, 72^{0}C for 90s, followed by 30 cycles of 95^{0}C for 30s, 51^{0}C for 40s, 72^{0}C for 90s, and a final extension step at 72^{0}C for 5 min (for cox 1) and 95^{0}C for 5 min, followed by 40 cycles of 95^{0}C for 30s, 46^{0}C for 30 s, 72^{0}C for 30s, and a final extension step at 72^{0}C for 7 min (for cytb).

All the PCR products were visualized on 1% agarose gels and the most intense products were selected for sequencing. 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 manufacturer's instructions. Sequence similarity search was done to identify the species of the tissue, with all entries in the DNA sequence database GenBank using Basic Local Alignment Search Tool (BLAST, Altschul et al. 1990). Twenty-six cytb sequences and 28 cox1 sequences were used for the phylogenetic analysis and after final alignment the lengths were 400bp for cytb and 513bp for cox1. Phylogenetic position of the query sequences was determined using the maximum likelihood and maximum parsimony methods using MEGA Ver. 5 (Tamura et al. 2007; Kumar et al. 2008) and the branch support was evaluated using 1000 bootstrap replicates

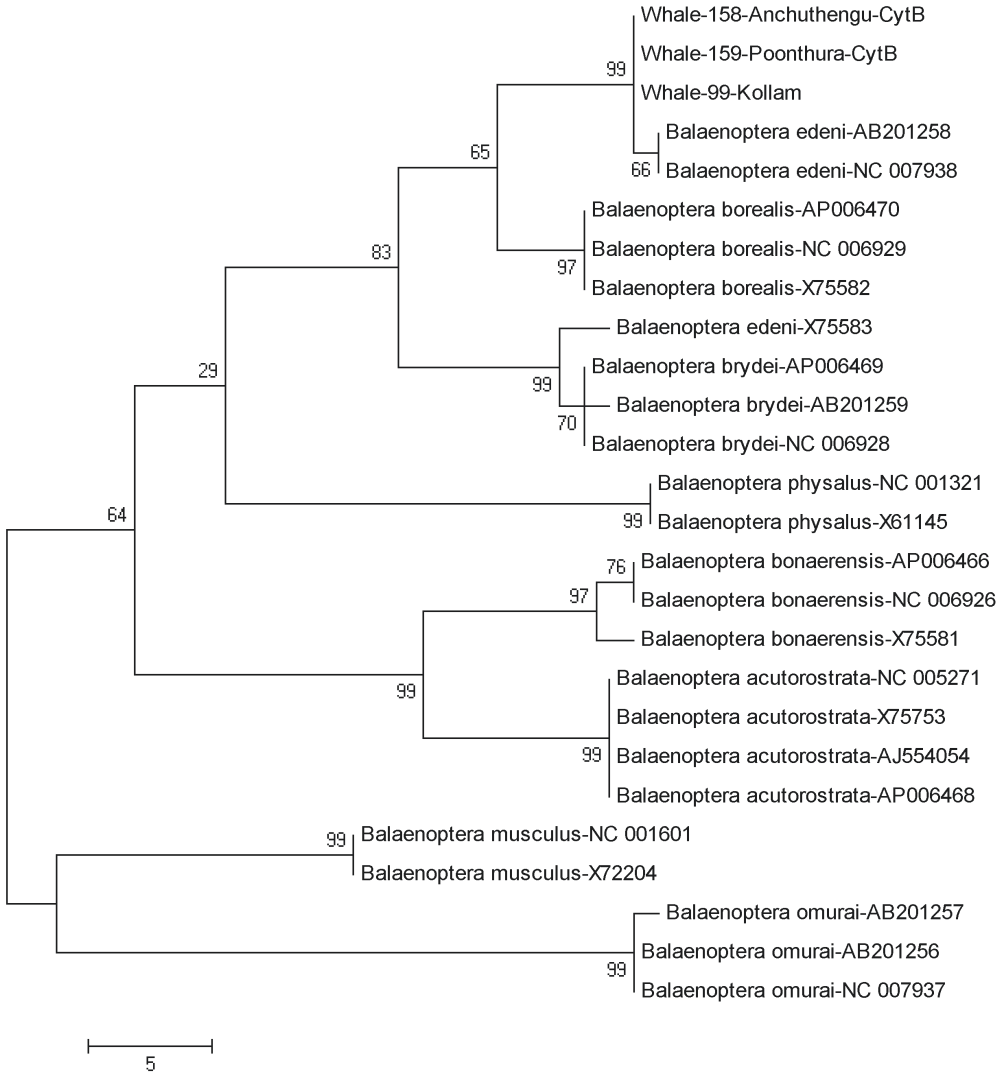

Figure 1. Maximum Parsimony phylogram using cytb partial sequences of the samples compared with other reference sequences of *Balaenoptera* spp. in GenBank. The numbers on the tree branches indicate bootstrap values

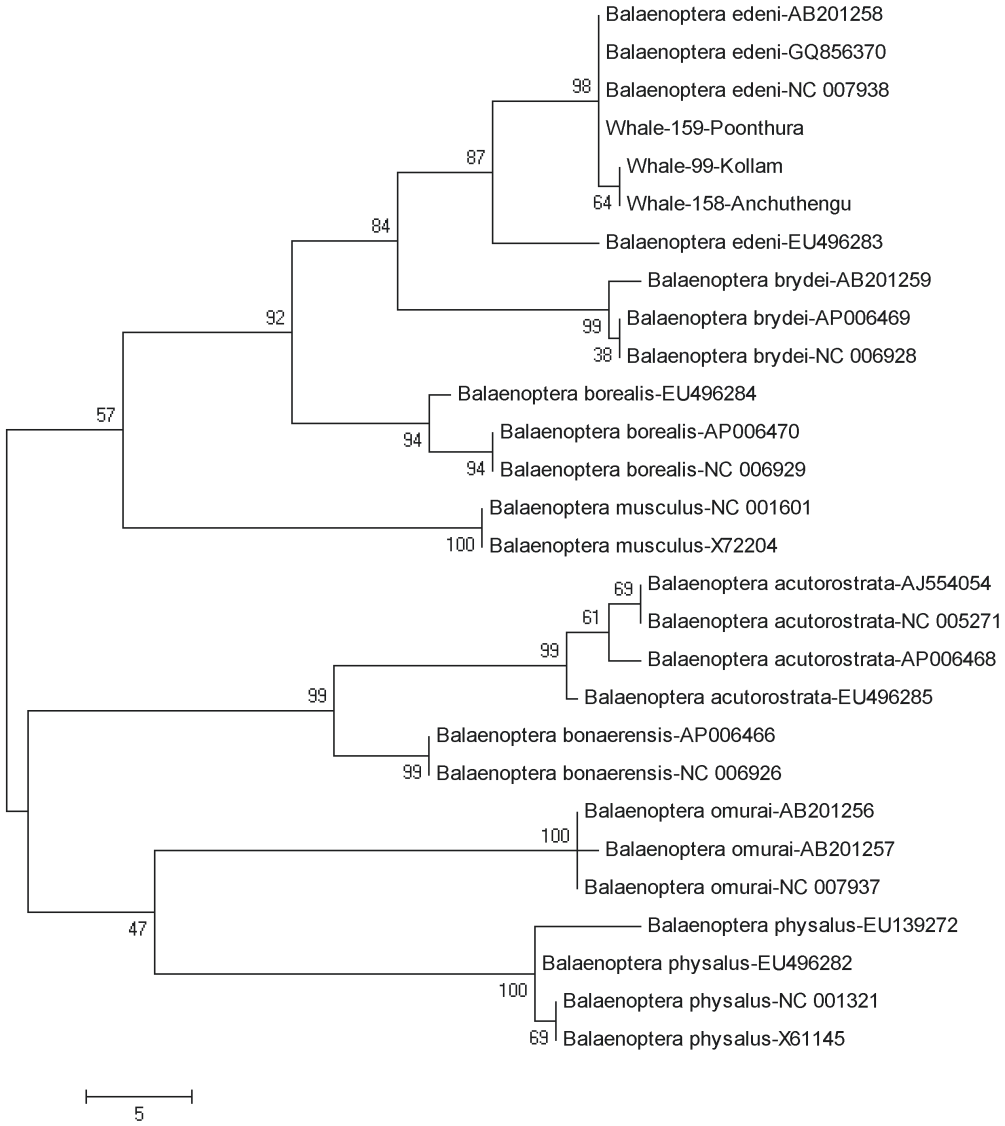

Figure 2. Maximum Parsimony phylogram using cox1 partial sequences of the samples compared with other reference sequences of *Balaenoptera* spp. in GenBank. The numbers on the tree branches indicate bootstrap values

(Felsenstein 1985) (Figs. 1–4). The best fit nuclear substitution model was selected as HKV+I for cytb and HKY+G for cox1 using model test, implemented in MEGA Ver. 5.

The BLAST search of cox1 and cytb showed 99.8% sequence identity with Bryde's Whale *Balaenoptera edeni*. The phylogenetic trees obtained with maximum likelihood and maximum parsimony were very similar by clustering all the three stranded whales with other *B. edeni* sequences except Acc. No.X75583 (cytb) of the GenBank, which was confirmed as *B. brydei* after BLAST search. The GenBank accession numbers of the cox1 and cytb sequence data generated in the study is given in Table 2.

Table 2. GenBank accession numbers of the cox1 and cytb sequences of Bryde's whale *Balaenoptera edeni* Anderson samples collected from Kerala

Whale code number with locality	GenBank accession number	
	cox1	cytb
Whale-159-Poonthura	JN190945	JN190949
Whale-99-Kollam	JN190946	JN190947
Whale-158-Anchuthengu	JN190944	JN190948

Bryde's Whales are the least known of the large baleen whales and are reported from warm temperate, subtropical, and tropical oceans between 40^{0}N and 40^{0}S (Kato 2002). In India presence of this species has been reported only through occasional stranding

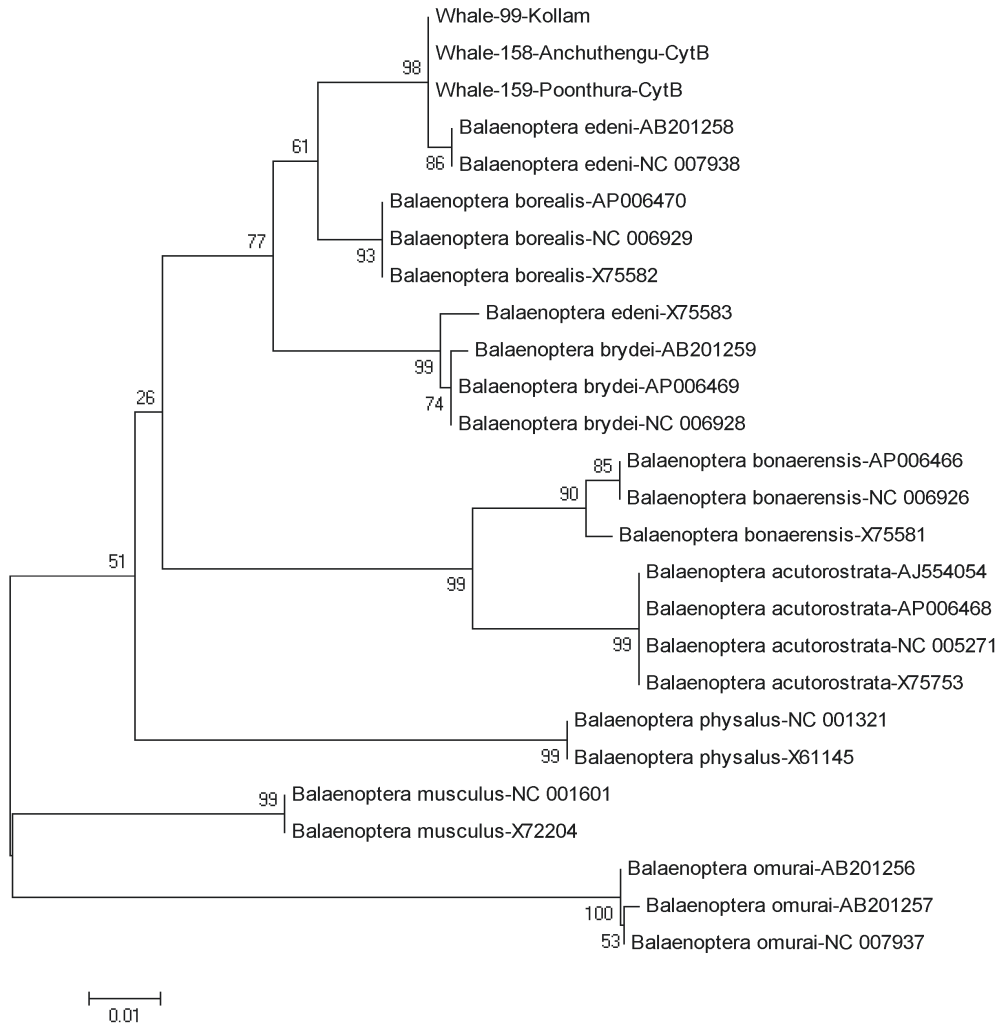

Figure 3. Maximum Likelihood phylogram using cytb partial sequences of the samples compared with other reference sequences of *Balaenoptera* spp. in GenBank. The numbers on the tree branches indicate bootstrap values

data (Table 3) and behaviour, seasonal occurrence and abundance in our coastal waters remains to be documented.

Balaenoptera edeni was first described by Anderson (1879) from a stranded specimen in Burma and was named Eden's Whale, after Sir Ashley Eden, the British High Commissioner to Burma at the time. In 1912, Olsen described a new species of mysticete whale from South Africa, and named this new species *Balaenoptera brydei* after Johan Bryde, the Norwegian consul to South Africa, who set up the first whaling station in Durban (Olsen 1913). *Balaenoptera edeni* and *B. brydei* were subsequently synonymised based on skeletal comparisons (Junge 1950) and *B. edeni*

Table 3. Stranding records of Bryde's Whale *Balaenoptera edeni* Anderson along the Indian coast

	Date	Place	Total length (m)	Reference	Remarks
1	02.vii.1979	Beypore, Calicut, Kerala	13	Lal Mohan (1992)	
2	20.ii.1983	Dhanushkodi Island in Gulf of Mannar, Tamil Nadu	13.52	Lal Mohan (1992)	
3	14.xi.2000	Point Calimere, Tamil Nadu	12	Sathasivam (2002)	
4	08.viii.2006	Kundugal near Mandapam, Tamil Nadu	12	Jayasankar et al. (2007)	DNA barcoding using Cytb
5	27.vi.2009	Edayar, Thiruvananthapuram District, Kerala	3.9	George et al. (2011)	DNA barcoding using CO1 and 16S mtDNA

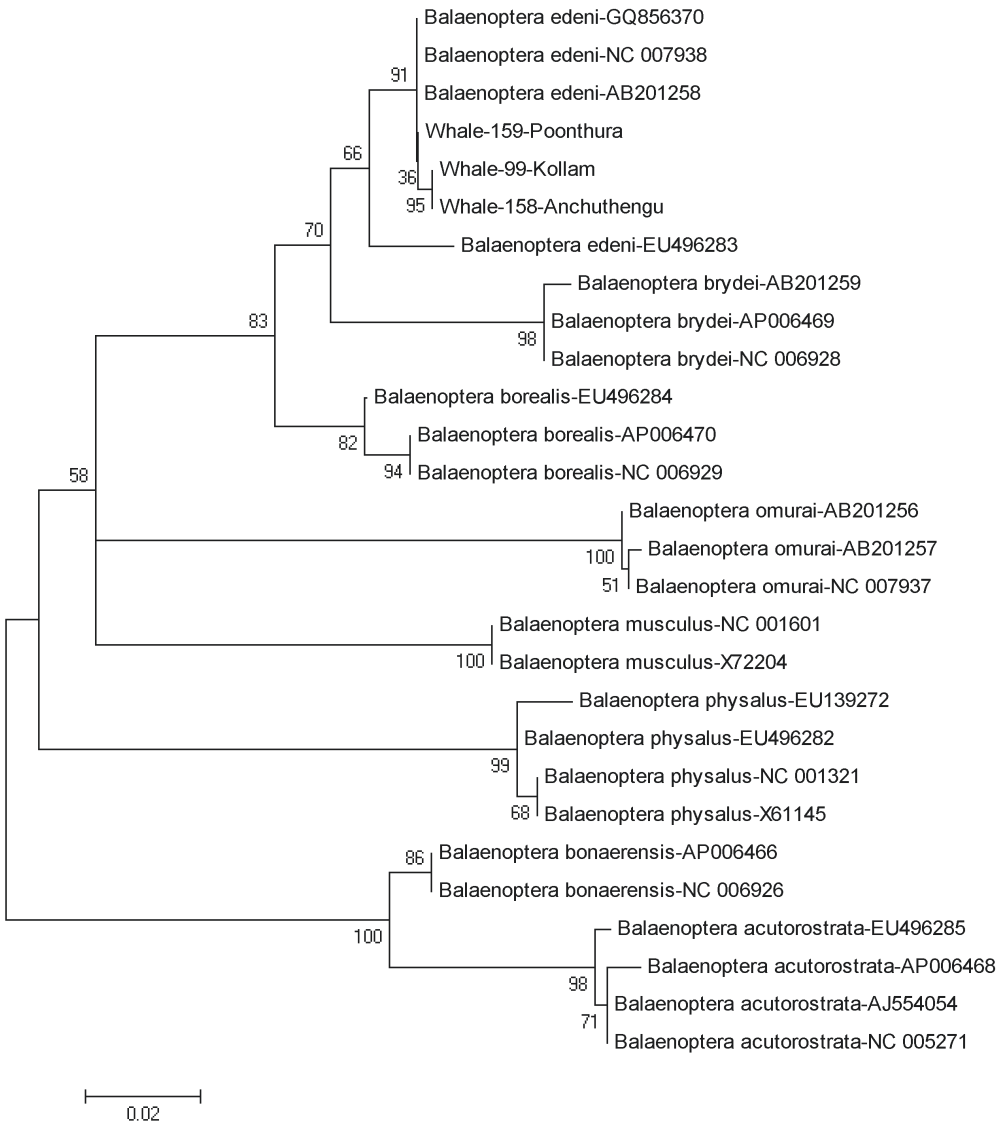

Figure 4. Maximum Likelihood phylogram using cox 1 partial sequences of the samples compared with other reference sequences of *Balaenoptera* spp. in GenBank. The numbers on the tree branches indicate bootstrap values

was used as the scientific name and Bryde's Whale as the common name. This synonymisation was not accepted by many taxonomists and molecular analysis of mtDNA from all nominal species of 'Bryde's whale complex' has separated *brydei* from *edeni* and resulted in a third species called *B. omurai* described from specimens collected mostly in tropical waters of the western Pacific and eastern Indian oceans (Wada et al. 2003). The studies by Wada et al. (2003) demonstrated that *B. edeni* forms a sister taxon to *B. brydei* (Sasaki et al. 2006).

Although the recent findings outlined above support that *B .edeni* and *B. brydei* may be separate species, and that genetic differentiation is high among different oceanic regions, further molecular studies are required to identify which populations of Bryde's Whales belong to each species, and consensus on a type specimen for *brydei* is required. Eden's Whale and Bryde's Whale may be used as the common name of *B. edeni* and *B. brydei* respectively as suggested by Wada et al. (2003) and George et al. (2011). The results of mt DNA sequencing in the present study confirms the presence of *B. edeni* species of 'Bryde's Whale complex' in the coastal waters of India.

According to the recent International Union for Conservation of Nature (IUCN) assessment, Bryde's whale taxonomy is unresolved and they are classified as 'Data Deficient' (Reilly et al. 2008). They are

currently listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and in Appendix II of the Convention on the Conservation of Migratory Species of Wild Animals (CMS), under the United Nations.

Marine mammal strandings may be attributed to natural or anthropogenic factors and the stranding data can provide insight on spatial distribution, seasonal movements, and mortality factors pertaining to marine mammal populations (Woodhouse 1991). A deep injury was noticed on the back of the whale washed ashore Thanni beach which could be due to a ship collision. Vessel collisions are considered an important source of mortality for Bryde's Whale in New Zealand waters (Stockin et al. 2008). In many cases the causes of death in stranded marine mammals are not properly investigated, and detailed necropsy studies and post-mortem examination would help in evaluating the impact of anthropogenic interactions.

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U. Suresh Kumar, holds MPhil degree in bioinformatics and is currently working as the DNA examiner of the Regional Facility for DNA Fingerprinting at Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum. His major research interests are DNA barcoding and DNA fingerprinting. Currently conducting training programmes on molecular markers, human DNA fingerprinting and DNA barcoding. Sequence analysis of the work was done by him in the work.

S.S. Jijith possesses Masters in Biotechnology and is currently working as a project fellow in Regional facility for DNA fingerprinting at RGCB. His current project addresses the development of a reference DNA barcoding database of selected mammals of Kerala Forest. For this work DNA isolation and amplification was done by him.

Dr. S. George is currently working as a Scientist in the chemical biology laboratory of Rajiv Gandhi Centre for Biotechnology, Trivandrum. His main area of research is centered around amphibians of Western Ghats with particular interest in DNA barcoding and bioprospecting. At present he is the Principal Investigator of DNA barcoding projects on amphibians, mammals and molluscs of India. In this paper he has contributed towards the writing of molecular taxonomy and interpretation of data.