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Record of the rough toothed dolphin *Steno bredanensis* (G. Cuvier in Lesson, 1828) in Indian seas after 19th century

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ABSTRACT

A specimen of the rare delphinid *Steno bredanensis*, was washed ashore on 25 August 2008 in Uttara Kannada, south-west coast of India. This report presents the first stranding record of *S. bredanensis* in Indian waters after more than 100 years as the previous confirmed record was only in 1891. There were a few stranding records of this species in 19th century, but thereafter no single record is available till now. The number of records between the years 1800 and 1900 were only 3. Earlier works from Indian seas depended on conventional taxonomic approaches which led to misidentification of species. Skin samples were collected for genetic analysis and the genes of control region (CR) and cytochrome b (cyt b) of MtDNA were PCR amplified and sequenced. Partial sequences of mtDNA control region and cytochrome b genes were generated and tested with the reference sequences available in GenBank (NCBI) and the web-based program DNA Surveillance, and the specimen was confirmed as *Steno bredanensis.* Amplification of sex Y chromosome gene SRY confirmed the visual identification of the specimen as a male.

Keywords**:** Cetacean, Delphinids, Mitochondrial DNA, Molecular taxonomy, Rough toothed dolphin

Introduction

Rough toothed dolphins (*Steno bredanensis*) belong to the family delphinidae, inhabiting deeper parts of tropical and warm temperate waters of Pacific, Atlantic and Indian Oceans. Although, occurrence of this species is worldwide, their deep water inhabitancy makes them inconspicuous and rare to observe in the wild and thus, they are one of the least known species among delphinids. Globally, reports on distribution and population status of this species is confined to few investigations in most of its distributional range, particularly from eastern tropical Pacific and north-eastern Pacific (Perrin and Walker, 1975; Carwadine, 1995). In Atlantic Ocean, more records are reported from the Scheldt Estuary along the north-eastern Atlantic, North Sea and the western coast of Africa from Mauritania to Namibia (Addink and Smeek, 2001; Perrin and Van Waerebeek, 2007). Unlike Pacific and Atlantic, in the Indian Ocean, their occurrence is known from few scattered records, mostly resultant of sporadic sighting surveys and stranding reports in the Gulf of Oman in the Arabian Sea to the Andaman Seas (Alling, 1986; Leatherwood and Reeves, 1989; Balance and Pitman, 1998; Parson, 1998; Anderson *et al.,* 1999; Van Waerebeek *et al.,* 1999). Few stranding records from south and east China and Indonesia are evidence for their distribution in Indian Ocean (Yang, 1976; Wenji, 1980; Corkeron *et al*., 2003).

The extent of occurrence of Rough toothed dolphin in India is known from few historical stranding records dated back to late 19th century (Kumaran, 2002), but thereafter not reported across the vast Indian coastline. This species has never been reported in any Indian fishery, probably due to absence of fishing in deeper waters where it occurs. However, there was no sighting observation in the recent ship-based oceanic cetacean surveys that spanned for 7 years between 2003 and 2010 in Indian waters (Afsal *et al*., 2008). In Sri Lanka and Pakistan waters, adjacent to Indian coast, they are barely reported (Alling, 1988; Kiani *et al*., 2013). Hence, rough toothed dolphins are believed to be uncommon throughout their range in these waters and every record is useful to add knowledge on this species. In this paper, we present a rare stranding event of *S. bredanensis* and results of morphometric and genetic analyses. The genetic analysis focused on the species confirmation and sex determination based on molecular markers.

Materials and methods

A dead dolphin specimen was washed ashore on 25 August 2008 at Malikarjun Point near Belekeri, in Uttara Kannada (14° 46' N; 74° 29' E), along the west coast of India (Fig.1). Based on morphological features and teeth pattern, the dead dolphin was identified as *Steno bredanensis*. A set of 21 morphometric parameters following Perrin (1975) were measured (Table 1). In order to substantiate visual identification, molecular level identification was attempted with skin sample of the specimen. A small piece of skin sample was crafted from the anterior part of the body right below the dorsal fin, for DNA extraction.

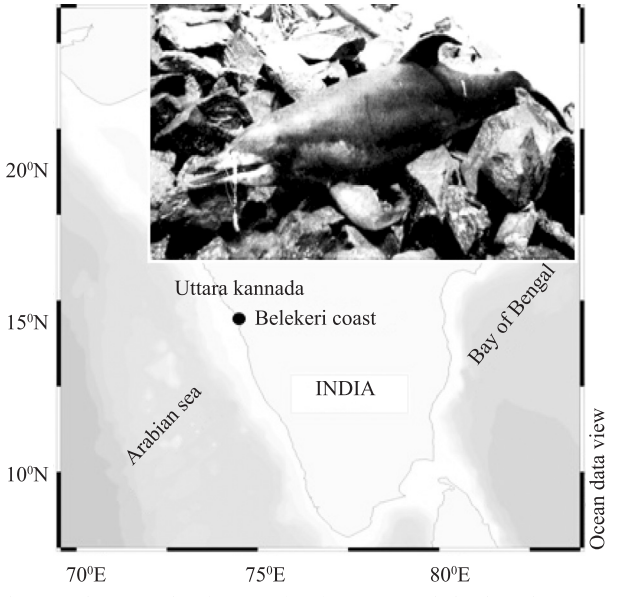

Fig. 1. Photograph of *Steno bredanensis* and the location map of stranding indicated by black spot

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 (0.1M NaCl, 10 mM Tris HCl, 1mM EDTA), with 10% SDS and digested by proteinase K (0.05 mg ml^{-1}) at 65ºC for 3 h. This was followed by the routine phenol: chloroform: isoamyl alcohol extractions and the extracted DNA was resuspended in TE Buffer and stored at -20ºC until further use. The partial sequences of mitochondrial DNA cytochrome b (cyt b) gene and control region (CR) were amplified using PCR. The amplifications were 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 and10-25 pM each of forward and reverse primers (Table 2). Nucleotide sequencing was performed in an AB 1 prism - DNA sequencer (Applied Bio systems, USA) using big dye terminator cycle sequencing ready reaction kit. The raw DNA sequences were edited using Bio-edit sequence alignment version 7.0.5.2 (Hall, 1999).

Molecular identification of the sample was done in two steps: initially sequence similarity search of the edited user sequence was done in the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov) (Benson *et al.,* 2007). The species identity was then searched with the database of DNA surveillance (www.cebl. auckland. ac.nz: 9000). The sequences, after confirmation, were submitted to NCBI GenBank.

Table 1. Morphometric measurements (cm) of *Steno bredanensis* washed ashore in Uttara Kannada

Morphometric parameters	Measurements (cm)
Total length (cm)	262
Length, tip of the upper jaw to center of eye	40
Length, tip of the upper jaw to apex of the melon (snout length)	33
Length of the gape (tip of the upper jaw to angle of the gape)	62
Center of eye to angle of gape	5.4
Center of eye to center of blowhole	14
Girth at axilla	67
Length, tip of upper jaw to blowhole along the mid line	60
Length, tip of upper jaw to anterior insertion of flipper	66.3
Length, tip of upper jaw to tip of dorsal fin	140
Length, tip of upper jaw to mid point of genital aperture	179
Length, tip of upper jaw to center of anus	197
Length, tip of upper jaw to base of fluke	225
Length, dorsal fin base to base of fluke	117
Length of flipper (anterior insertion of tip)	35.4
Length of flipper (axilla to tip)	90
Width of flipper (maximum)	16
Height of dorsal fin (fin tip to base)	32
Width of dorsal fin	25
Base of dorsal fin	73
Fluke span	41
Width of flukes	22

Table 2. Particulars of the mtDNA primers used in the present study

Gene	Primer sequence	Annealing temperature (^{0}C)	Product size (bp)	Source
Control region	5'TGTAAAACGGCCAGTTCACCCAAAGCTGRARTTCTA-3 *F* 5'CCATCGWGATGTCTTATTTAAGRGGAA-3' *R*	54	395-527	Dalebout *et al*. (1998)
Cyt b	5' -TGACTTGAARAACCAYCGTTG-3' *F* 5' -CCCTCAGAATGATATTTGTCCTCA-3' *R*	54	421-530	Palumbi (1996)

The same genomic DNA samples extracted for molecular taxonomy, population and phylogenetic analyses were used for amplification of sex specific markers by a duplex PCR reaction using two sets of primers to amplify *SRY* and *ZFY/ZFY*. A reaction volume of 20 ml 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 mM each of dATP, dTTP, dCTP and dGTP, 1 U of *Taq* DNA polymerase (Genei™ HotStart) and 25 pM each of forward and reverse primers for both the sets. The primer sequences for *SRY* were F 5' CCC ATG AAC GCA TTC ATT GTG TGG 3' & R 5' ATT TTA GCC TTC CGA CGA GGT CGA TA 3' (Gilson *et al*., 1998) and those for *ZFX/ZFY* were F 5' ATA ATC ACA TGG AGA GCC ACA AGC T 3' & R 5' GCA CTT CTT TGG TAT CTG AGA AAG T 3' (Aasen and Medrano, 1990). The temperature profile for the amplifications was an initial denaturation (94^{o}C) for 1 min followed by 35 cycles of 94^{0}C for 45 sec, 58^{o}C for 45 sec and 72^{o}C for 1 min and a final extension of 72^{o}C for 7 min. The amplification products were resolved in 1.2% agarose gel, stained with ethidium bromide and documented using Bioprofil; a charge coupled device (CCD) video camera imaging system (Vilber Lourmet, France). The sex was identified by amplification of sex specific markers following Gilson *et al.* (1998). The *SRY* gene had a size ranging from 220 to 224 bp, which is present only in males, whereas *ZFX/ZFY* locus was longer with a size range of 442-445 bp which appear in both sexes. Y-chromosome-specific *SRY* locus was amplified simultaneously with the homologous *ZFX/ZFY* genes on the X-chromosome of females (*ZFX*) and XY chromosomes of males (*ZFX/ZFY*) as positive control. Females lack Y-chromosome and the test is based on the absence of a *SRY* product in females.

Results and discussion

The present record of *S. bredanensis* is the stranding record of the species in Indian waters after more than 100 years. Earlier, this species was reported on three occasions between 1800 and 1900, and has not been reported in the 20th century. The present specimen measured 2.62 m in total length which may be considered as an adult (Miyazaki and Perrin, 1994; Jefferson *et al*., 2008). The carcass was fresh and did not show any signs of external injury though there was bleeding from the mouth and blowhole suggesting internal damage. On enquiry with the local fishermen, it was learnt that the mortality might have occurred during fishing activity in nearshore waters. *S. bredanensis* formed incidental catch from several locations around the world. These include coastal and offshore Japan (driftnet fishery), Sri Lanka (gillnet fishery), the eastern tropical Pacific (purse-seine fishery), Brazil (gillnet fishery), and the Mediterranean Sea (gillnet fishery) (Alling, 1986; Watkins *et al.*, 1987; Hobbs and Jones, 1993; Perrin *et al*., 1994).

Earlier works from Indian seas were dependent upon conventional taxonomy which led to misidentification of species (Kumaran, 2002). Owen described the specimen collected by Walter Elliot from Vishakhapatnam in east coast of India as *Delphinus maculiventer,* but there is no complete description of this species (Jerdon, 1867). Blanford (1891) described another specimen of similar type from Nicobar Island and renamed as *Steno frontatus.* Finn (1929) stated that the spot-bellied dolphin *Steno maculiventer* is found along the 'Madras coast'. These two reports probably refer to the rough toothed dolphin. However, Blanford (1891) and Finn (1929) have not given any morphometric details as per conventional taxonomy to record this species as *S. bredanensis.*

According to the phylogenetic studies (LeDuc *et al.,* 1999; Agnarsson and May-Collado, 2008), the taxonomic status of *Steno bredanensis* is not fully resolved. Mitochondrial cytochrome b sequences indicate that *S. bredanensis* differs considerably and demands for placement of this species in a separate genus. Hence, molecular methods like cyt b gene sequence analysis was opted to see whether the specimen shows the same unique sequence characteristics with considerable differences from the closely related species as reported earlier. Partial sequences of rapidly evolving mtDNA control region (CR) and cytochrome b (cyt b) gene were generated and tested to confirm species identity of the specimen. The sizes of the edited sequences were 506 bp for CR and 408 bp for cyt b. The BLAST search showed 99-100% sequence similarity with *S. bredanensis* deposits in the NCBI GenBank. The *DNA surveillance* search also unambiguously confirmed the species as *S. bredanensis*.

The mtDNA control region and cyt b gene sequence were compared with available sequences for *S. bredanensis* from NCBI database (Tables 3 - 5). The sequence comparison of control region revealed no similar haplotypes. Out of the 5 samples analysed using cyt b primers, three different haplotypes were obtained. The specimens from Atlantic Ocean (AFO84077; Leduc *et al.,* 1999) and Pacific Ocean (AFO84076; Leduc *et al.,* 1999) were found to be different haplotypes with very low genetic divergence values of 0.0024 with 100% bootstrap support. The values obtained fall within the expected genetic divergence levels. The PCR based gender identification confirmed the sex of *S. bredanensis* as male. The sequences generated in the present study were deposited in the GenBank under accession numbers FJ411044 (mtDNA control region) (Fig. 2) and GQ253567 (mtDNA cytochrome b gene) (Fig. 3).

The present specimen, considering rarity in Indian waters, could probably be a vagrant. Therefore, finding of such a specimen after an appreciable time gap underline the need to further explore the possible migratory path of this animal in Indian waters. Earlier records confirmed this species based on conventional taxonomy procedures, whereas during the present study, the species identity was confirmed through molecular methods, and hence form the first tangible evidence supported by molecular data. This can be taken as a baseline work to deal with future sightings of some of the rare cetaceans in Indian seas.

Table 3. Variable positions in the 506 bp of mtDNA CR sequences of *Steno bredanensis* determined in the present study in comparison with those of same species from GenBank database

mtDNA CR sequences of *S. bredanensis*	Nucleotide positions																		
	1	6	8	9	9	1	1	1	2	2	2	2	2	2	3	3	3	3	4
	1	6	8	9	9	1	2	4	3	5	6	6	8	9	3	8	8	8	0
	5	6	1	8	9	2	0	0	7	7	1	6	5	5	9	3	4	5	6
FJ411044 (Present study)	T	A	T	T	G	C	T	G	T	G	C	C	T	G	G	T	C	T	A
AY842471	.	G	C	C	A	T	C	A	.	.	.	T	C	.	.	C	T	C	G
EU121131	.	.	.	C	.	.	.	.	.	A	T	.	.	A	.	.	.	.	.
EF027007	C	.	.	C	.	.	C	.	C	.	T	.	C	A	A	C	.	C	.

Table 4. Variable positions in the 408-bp of mtDNA cyt b sequences of *Steno bredanensis* determined in the present study in comparison with those of same species from GenBank database

mtDNA cyt b sequences of *S. bredanensis*	Nucleotide positions	
		1
	4	8
	4	1
GQ253567 (Present study)	G	C
EU121107	.	.
EF027032	.	.
AFO84077	.	T
AF084076	A	.

Table 5. Details of Gene sequences of *Steno bredanensis* downloaded for the present study

NCBI GeneBank Accession no.	Mitochondrial gene	Location	Reference
EF027007	Control region	Unknown	Caballero *et al.* (2007)
EF027032	Cytochrome b	Unknown	Caballero *et al.* (2007
EU121131	Control region	Unknown	Caballero *et al.* (2008)
EU121107	Cytochrome b	Unknown	Caballero *et al.* (2008)
AFO84077	Cytochrome b	Atlantic Ocean	Leduc *et al*. (1999)
AF084076	Cytochrome b	Pacific Ocean	Leduc *et al.* (1999)

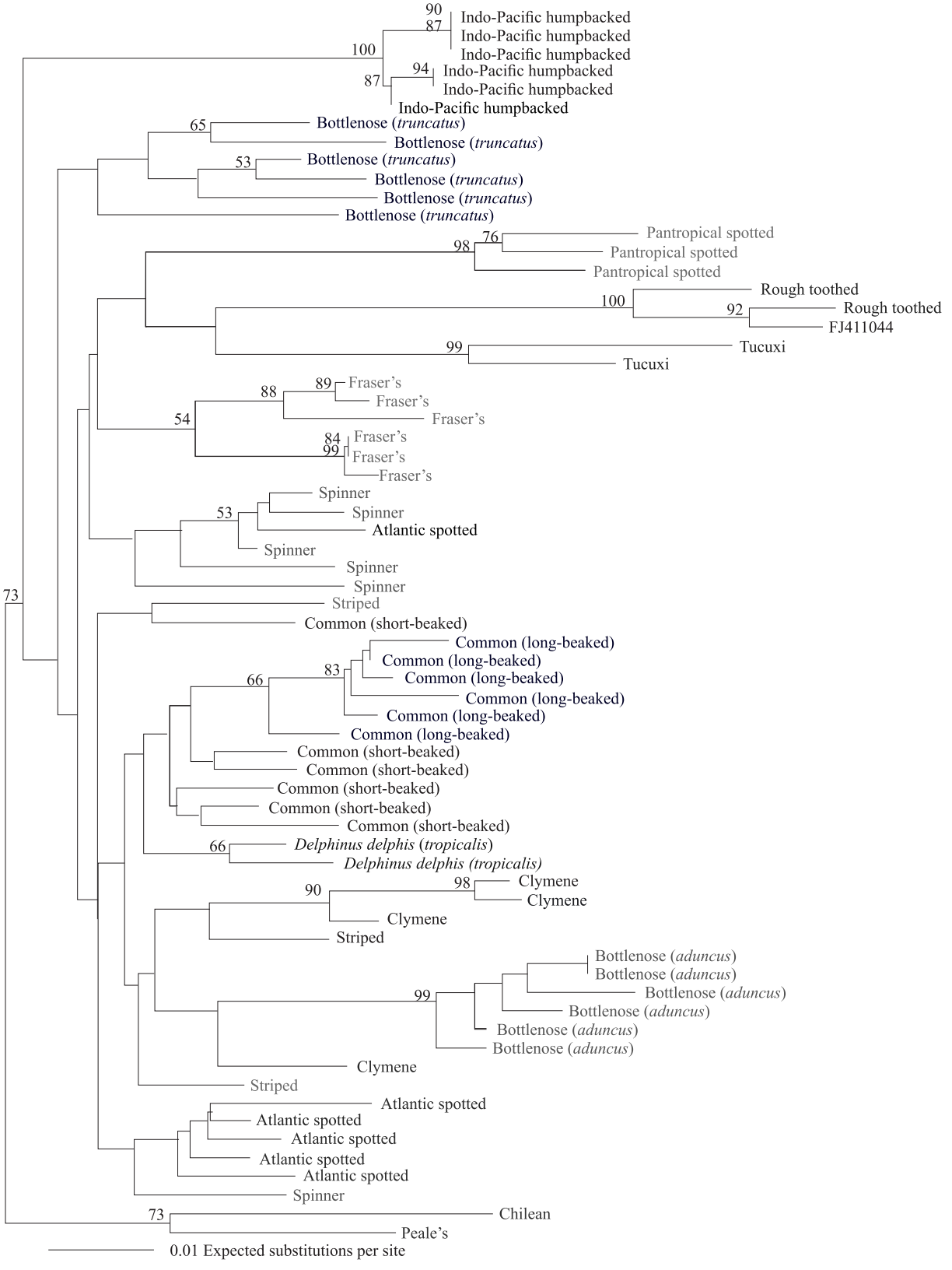

Fig. 2. Neighbor Joining tree of *Steno bredanensis* mtDNA control region (GenBank Acc. no. FJ411044) based on reference sequences in *DNA Surveillance*

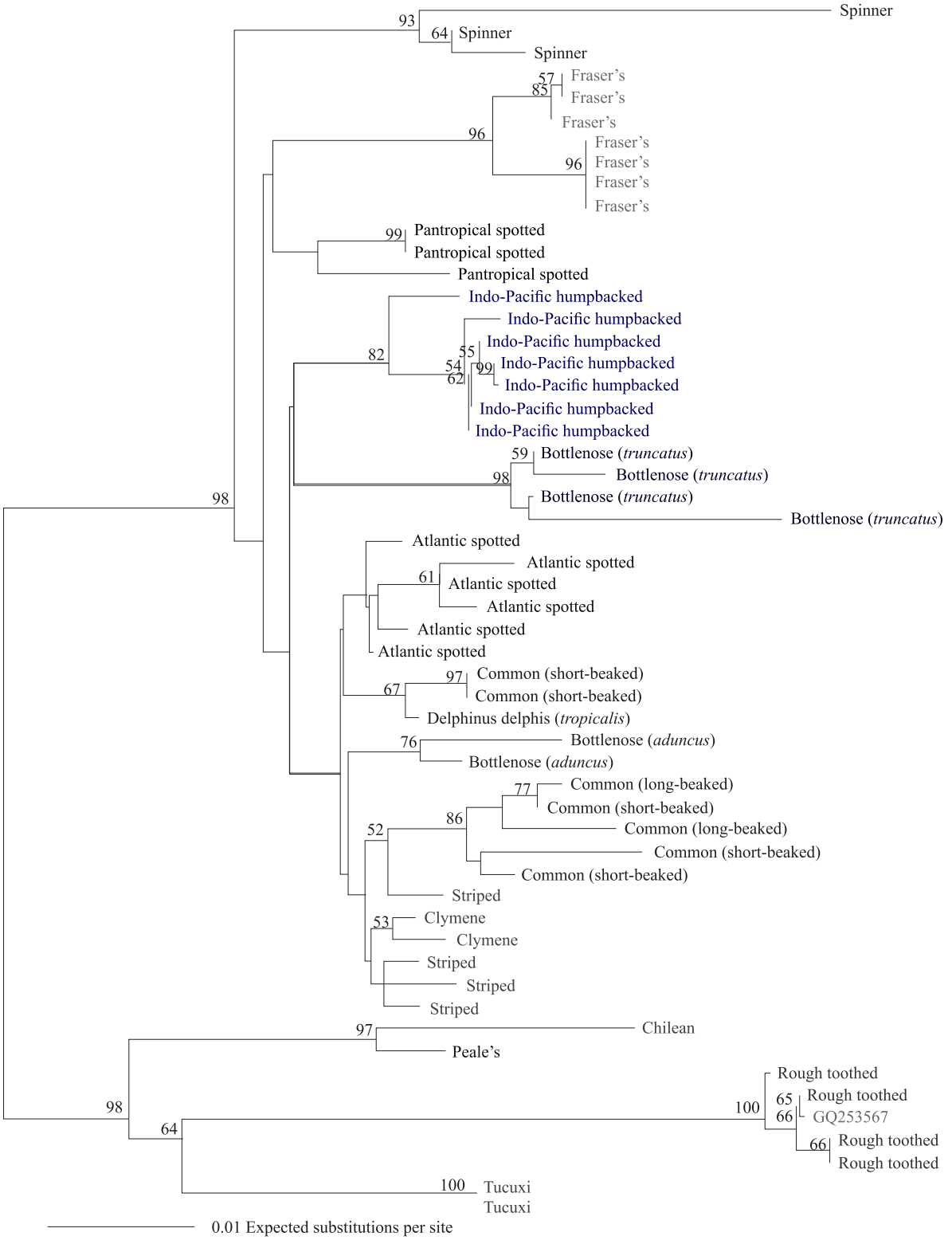

Fig. 3. Neighbor Joining tree of *Steno bredanensis* mtDNA cytochrome b (GenBank Acc. no. GQ253567) based on reference sequences in *DNA Surveillance*

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