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Abstract

The taxonomy of the genus *Sousa* has been contentious due to the uncertainty in the number of species it includes. Currently, four species are recognized: *S. teuszii* occurring along the eastern Atlantic Ocean; *S. plumbea* occurring in the western Indian Ocean; *S. chinensis* occurring in the Eastern Indian and Western Pacific Oceans and *S. sahulensis* occurring in Northern Australia. In the Bay of Bengal, situated in the presumed but uncertain distributional split between *S. plumbea* and *S. chinensis*, both forms have been observed. In this study we clarify the population and taxonomic identity of humpback dolphins occurring off Bangladesh, northern Bay of Bengal. We sequenced the mitochondrial DNA control region and two nuclear introns and compared them to previously published sequences encompassing the different described species within *Sousa*. We found high levels of genetic differentiation separating Bangladesh dolphins from other *Sousa* in a separate cluster. Similar levels of differentiation have been found to differentiate *S. plumbea* from *S. chinensis*. Phylogenetically, based on mtDNA control region sequences, humpback dolphins from Bangladesh seem to be more closely related to *S. sahulensis* than to other species. Our results suggest that these dolphins constitute a separate phylogenetic and management unit within *Sousa*.

Introduction

Humpback dolphins of the genus *Sousa* (subfamily Delphininae) are distributed discontinuously in coastal waters of the Eastern Atlantic, Indian and Western Pacific Oceans (Parra and Ross 2009). The taxonomy of the genus has been contentious due to the uncertainty about the number of species it includes (Frère *et al.* 2008; Jefferson and Rosenbaum 2014). Historically, some views based only on morphology recognized a single species, *Sousa chinensis*, while others considered three nominal species: *S. chinensis* in the Eastern Indian and Western Pacific Oceans, *S. plumbea* in the Western Indian Ocean, and *S. teuszii* in the Eastern Atlantic Ocean (Parra and Ross 2009).

While a fourth species was later proposed in Northern Australia based on a phylogenetic analysis of mtDNA sequences (Frère et al. 2008), it was not substantiated with additonal evidence. The most comprehensive genetic study conducted to date, which included samples throughout the range of the genus, found diagnostic characters in both mitochondrial and nuclear DNA markers and high levels of divergence separating dolphins from West Africa (*S. teuszii*), Southeast Africa, Arabia-Oman and the Indian subcontinent (*S. plumbea*), Thailand and China (*S. chinensis*) and Australia (*S. sahulensis*) (Mendez et al. 2013). One sample from Bangladesh showed remarkable differences in the mitochondrial DNA, with its placement in phylogenetic trees as distant as those from Australia (Mendez et al. 2013). Subsequently, an exhaustive review of multiple lines of evidence from skeletal morphology, external morphology, coloration, molecular genetics, and biogeography provided strong support for the recognition of four species of *Sousa* (Jefferson and Rosenbaum 2014). This scenario has been accepted by the SMM Committee on Taxonomy (2014).

In terms of external appearance, *S. chinensis* has light adult coloration, often with black spotting and lacks the prominent dorsal hump; *S. plumbea* has a darker coloration with little spotting and a prominent hump; *S. teuszii* has a similar appearance to that of *S. plumbea* but with significantly shorter rostra and lower tooth counts; *S. sahulensis*, the newly recognized species in Northern Australia has no visible dorsal hump and the dorsal fin is low and triangular, with adults having a dark grey back and a lighter belly (Jefferson and Rosenbaum 2014).

Phylogenetically, *S. teuszii* seems to be more closely related to *S. plumbea* and *S. chinensis* to *S. sahulensis* (Mendez et al. 2013). *S. plumbea* appears to be polyphyletic with one mtDNA haplotype from Oman clustering with Southeast Africa haplotypes (Mendez et al. 2013).

Analyses conducted to date suggest the existence of strong genetic population structure within *S. plumbea*. Populations from Oman and Tanzania show a remarkable level of differentiation when compared with populations from South Africa and Mozambique (Mendez et al. 2011; Mendez et al. 2013). Oceanographic features such as sea surface temperature and primary productivity were found to be among the drivers leading to this population differentiation. *S. chinensis* populations in China also show a high degree of divergence (Chen et al. 2008; Chen et al. 2010), and many populations have yet to be sampled.

Humpback dolphins are associated with coastal shallow waters. This makes them highly vulnerable to fatal entanglements in the densely distributed fisheries as well as increasing degradation that characterize much of their habitat. Prior to the May 2015 Red List Assessment/Re-Assessment workshop, *S. chinensis* is considered as 'near threatened' in the IUCN Red List but both are likely "vulnerable" if *S. chinensis* and *S. plumbea* are evaluated separately. A sub-population in the Eastern Taiwan Strait is considered 'critically endangered.' Humpback dolphin populations are becoming increasingly fragmented which makes them even more susceptible to extirpation due to anthropogenic

factors. A clear understanding of demographically isolated populations and their taxonomy is vitally important for determining biologically relevant conservation units.

The presumed but uncertain distributional split between *S. plumbea* and *S. chinensis* is centered around the Indian sub-continent. Humpback dolphins observed along the west coast of India (Arabian Sea) show a large hump and appear dark grey, thus resembling *S. plumbea*, while those observed along the east coast of India in the Bay of Bengal do not have an hump and are much lighter in color, thus resembling *S. chinensis* (Sutaria and Jefferson 2004). However, animals exhibiting the '*plumbea*-type' coloration, but without an obvious hump, have been observed as far east as the Mergui Archipelago, Myanmar (Smith and Tun 2008). Given the genetic distinctiveness of the one animal sampled off Bangladesh (Mendez et al. 2013), it is particularly important to clarify the phylogenetic position of humpback dolphins in this region.

The aim of this study is to clarify the genetic and taxonomic identity of Indo-Pacific humpback dolphins occurring in the northern Bay of Bengal, Bangladesh, based on an analysis of the mitochondrial control region and two nuclear introns, and a broader range-wide sampling and phylogenetic analysis including all currently described species in the *Sousa* genus.

Material and Methods

<u>Sampling</u>

In total, 251 humpback dolphin samples were included in this study (Table 1). From these, 235 samples were from West Africa (WA, *n*=6), Southeast Africa (SEA, *n*=38), Oman (OM, *n*=58), Thailand (TH, *n*=8), India (*n*=3), China (CH, *n*=92), Australia (AUS, *n*=28) and one from Bangladesh (BAN) that had already been analyzed by Mendez *et al.* (2013). Fifteen additional samples were obtained from the northern Bay of Bengal, Bangladesh, 14 from biopsy darting in coastal waters offshore the Sundarbans mangrove forest and one from a stranding in Cox's Bazaar in the far south of the country close to the border with Myanmar (Figure 1).

A minimally invasive darting system was used. A precautionary approach was taken. If there was any uncertainty about the safety of the target animal no shot was taken. Biopsy attempts were abandoned if strong reactions were observed to either vessel approach or after a shot was taken. Dolphin groups were approached from an angle and the research vessel attempted to match their travel direction and speed. Special care was taken with groups with calves, and biopsy attempts were immediately stopped if it appeared that a group was tiring out or if there was any possibility of calves getting separated from the mothers. Mothers or calves were not targeted. Dolphins were targeted in the shoulder area before or just behind the dorsal fin. To avoid injury to the dolphin from the impact of the dart, a minimum firing distance of 5 m was observed.
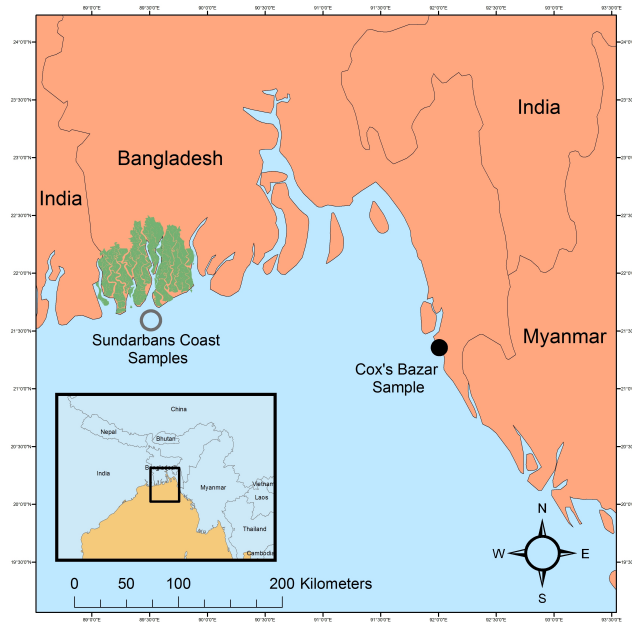
Figure 1. Map showing the coastal waters of Bangladesh where 14 tissue samples of humpback dolphins were collected by biopsy darting offshore the Sundarbans mangrove forest and where one sample was collected from a stranding of a single humpback dolphin in Cox's Bazaar.

Laboratory procedures

Genomic DNA was extracted from tissue samples using the QIAamp Tissue Kit (QIAGEN, Valencia, CA, USA). A fragment of the mitochondrial DNA control region was amplified and sequenced (Baker et al. 1993). The PCR profile consisted of an initial denaturation for 3 min at 94ºC followed by 32 amplification cycles (30s at 94ºC, 30s at 52º, 1 min at 72ºC) and a final 5 min of extension at 72ºC. Two nuclear introns, PLP and PTH, were also amplified and sequenced as in Mendez et al. (2013). Both strands were directly sequenced (BigDye Terminator CycleSequencing; Applied Biosystems) on an ABI 3730 automated sequencer (Applied Biosystems).

Statistical analyses

DNA sequences were inspected, edited and aligned by eye in Sequencher 5.0.1 (Gene Codes, Corp.). Sequences were collapsed into haplotypes using DNAsp v. 5.10 (Librado and Rozas 2009). Diversity measures (nucleotide and haplotype diversities and the average number of nucleotide differences) were also estimated in DNAsp. Population differentiation was tested by calculation pairwise F_{ST} (using haplotype frequencies) and ϕ_{ST} (using genetic distance) in Arlequin v. 3.5. (Excoffier and Lischer 2010).

A median-joining network of haplotypes was constructed in NETWORK v. (Bandelt *et al.* 1999). A Bayesian phylogenetic tree was obtained in MrBayes v. 3.1.2. (Huelsenbeck & Ronquist 2001) by running four simultaneous MCMC chains for 2 million generations, with trees sampled at intervals of 100 generations. The first 3,000 trees were discarded as "burn-in". A sequence of *Steno bredanensis* was used as outgroup.

For the nuclear intron data, only 10 individuals from Bangladesh were successfully sequenced for PLP and 11 for PTH. These sequences were concatenated with the control region sequences and a phylogenetic tree was estimated using MrBayes as described above.

Results

A fragment of 456 bp of the mitochondrial DNA control region was sequenced for 15 humpback dolphins from Bangladesh. These sequences were aligned with an already existent dataset comprising most of the genus distribution (Mendez et al. 2013). Samples from Bangladesh grouped into 9 haplotypes and showed the highest levels of genetic diversity when compared to the other geographical regions analysed when haplotypic diversity and average number of nucleotide differences are considered (Table 1). There were no shared haplotypes between samples from Bangladesh and the other regions.

A fragment of 878 bp of the nuclear intron PLP and a fragment of 242 bp of the nuclear intron PTH were sequenced for 10 and 11 humpback dolphin samples from Bangladesh, respectively. These sequences were aligned with an already existent dataset (Mendez et al. 2013). For PLP, only two polymorphisms were detected, corresponding to two mutations in a sample from Oman. For PTH, only one polymorphism was detected, corresponding to a fixed character separating samples from China and Thailand from all others.

Table 1. Genetic diversity measures for regional humpback dolphin samples (WA – West Africa; SEA – Southeast Africa; OM – Oman; BAN – Bangladesh; TH – Thailand; CH – China; AUS – Australia).

Region	N	h (SD)	Hd (SD)	π (SD)	k (SD)
WA	6	1	0 (0)	0 (0)	0 (0)
SEA	39	8	0.82 (0.03)	0.04 (0.02)	2.79 (1.51)
OM	58	10	0.79 (0.03)	0.11 (0.06)	7.29 (3.46)
BAN	**16**	**9**	**0.88 (0.06)**	**0.02 (0.00)**	**7.71 (1.80)**
TH	8	3	0.61 (0.16)	0.01 (0.01)	0.93 (0.71)
CH	91	8	0.34 (0.06)	0.02 (0.01)	1.41 (0.87)
AUS	23	4	0.55 (0.04)	0.05 (0.03)	3.73 (1.94)

N, number of individuals; h, number of haplotypes; Hd – Haplotype diversity; π, nucleotide diversity; k – average number of nucleotide differences; SD – standard deviation.

Pairwise F_{ST} and ϕ_{ST} values show highly significant levels of genetic differentiation among all geographical regions as described for the single sample analyzed by Mendez et al. (2013). Samples from Bangladesh showed F_{ST} values ranging from 0.195 to 0.531, with the lowest values observed in comparisons with regions from the Western Indian Ocean (SEA and OM) and the highest values observed in comparisons with the Eastern Atlantic and Indo-West Pacific.

Table 2. Pairwise F_{ST} (below diagonal) and ϕ_{ST} (above diagonal) values for the different geographical regions studied. (WA – West Africa; SEA – Southeast Africa; OM – Oman; BAN – Bangladesh; TH – Thailand; CH – China; AUS – Australia). All values were statistically significant ($P < 0.001$).

	WA	SEA	OM	BAN	TH	CH	AUS
WA		0.793	0.483	**0.470**	0.700	0.744	0.835
SEA	0.445		0.496	**0.744**	0.859	0.861	0.803
OM	0.479	0.255		**0.544**	0.623	0.689	0.660
BAN	**0.470**	**0.195**	**0.242**		**0.263**	**0.531**	**0.326**
TH	0.700	0.278	0.326	**0.263**		0.632	0.432
CH	0.744	0.494	0.508	**0.531**	0.632		0.604
AUS	0.595	0.325	0.360	**0.326**	0.432	0.604	

The haplotype network obtained showed 6 distinct haplogroups, each corresponding to a different geographical region (Figure 2). The Bangladesh dolphins appear clearly distinct from all the other regions, with the exception of a single haplotype from the sample collected in Cox's Bazaar in the far south of Bangladesh close to the border with Myanmar that clustered with samples from Thailand. Of interest is also the distinct clustering of the assemblage from Southeast Africa and from Oman.
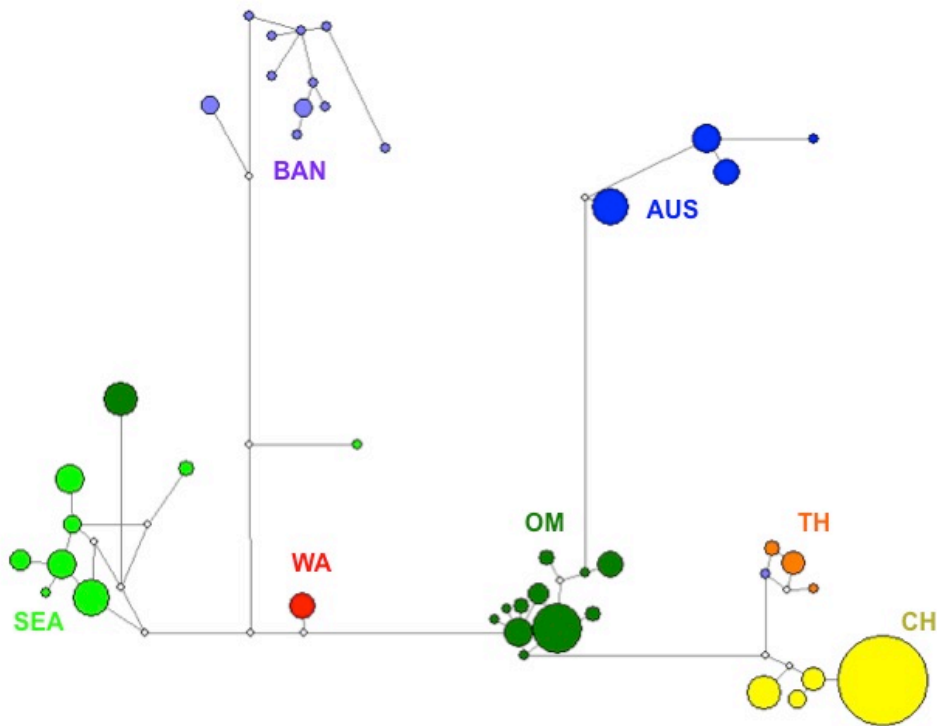

Figure 2. Median-joining haplotype network of the mitochondrial control region. Circle size is proportional to the number of individuals exhibiting the corresponding haplotype and proportional of each population within each haplotype is coloured according to the legend. Length of lines is proportional to the number of mutational steps separating haplotypes. White circles indicate missing intermediate haplotypes.

The relationships obtained in the Bayesian phylogenetic tree estimated with the mitochondrial control region sequences also showed distinct clusters that correspond to different geographical regions (Figure 3). Samples from Bangladesh appear as a distinct cluster, with a high posterior probability value (0.97) and as sister group to the Australian samples (*S. sahulensis*). The remaining geographical regions appear in separate distinct groups in a different clade, which is not well supported (posterior probability 0.53), suggesting that phylogenetic relationships among these assemblages is not fully resolved.
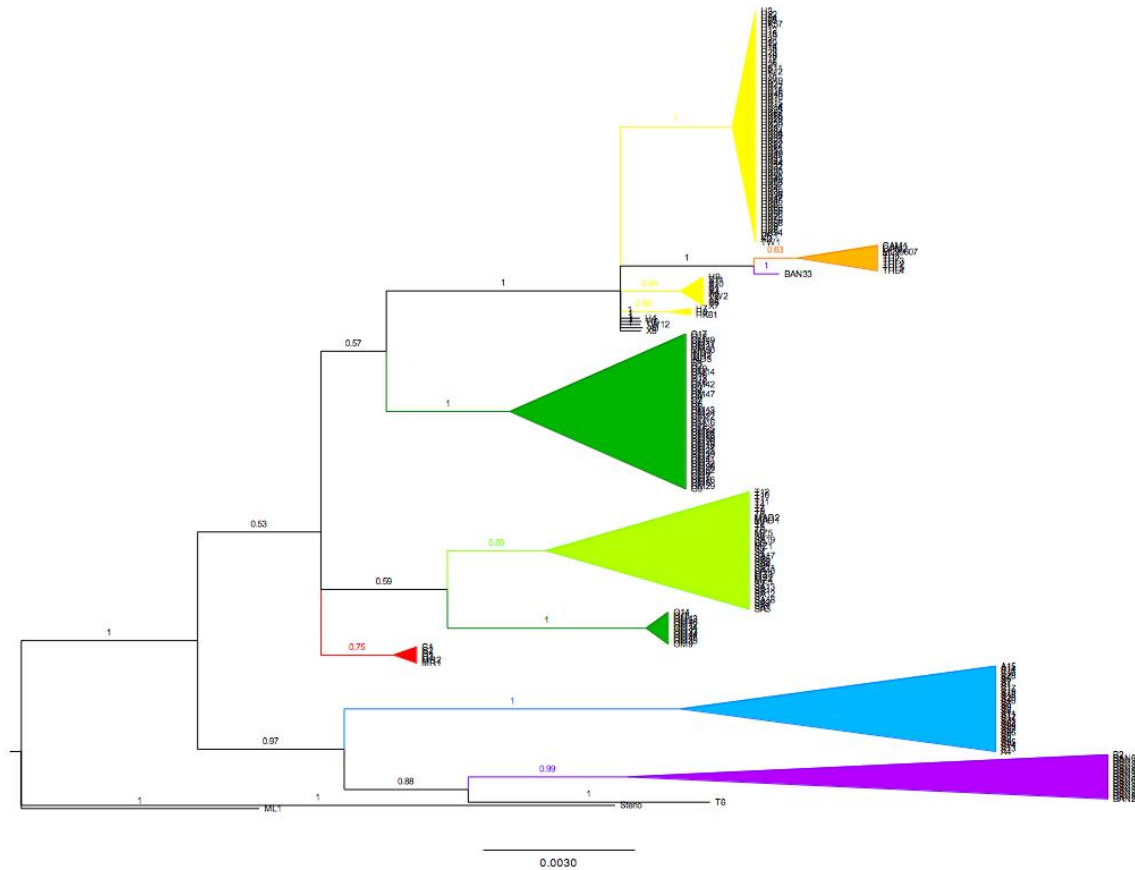

Figure 3. Bayesian phylogenetic tree obtained for the humpback dolphin mitochondrial control region sequences. Values above branches represent the posterior probability values. The different geographical assemblages are represented in different colours: purple – Bangladesh; Blue – Australia; Red – West Africa; light green – Southeast Africa; dark green – Oman; yellow – China; orange – Thailand.

The character matrix of the fixed nucleotide positions that define the different haplotypes obtained for the mitochondrial DNA control region clearly shows marked differences among the different assemblages (Figure 4). There is one fixed diagnostic site (position 393G) that allows the diagnosis of all the Bangladesh samples. In addition, three character sites (positions 93G, 94A and 135T) set apart the Bangladesh samples and from all others if the sample that clusters with Thailand is excluded.
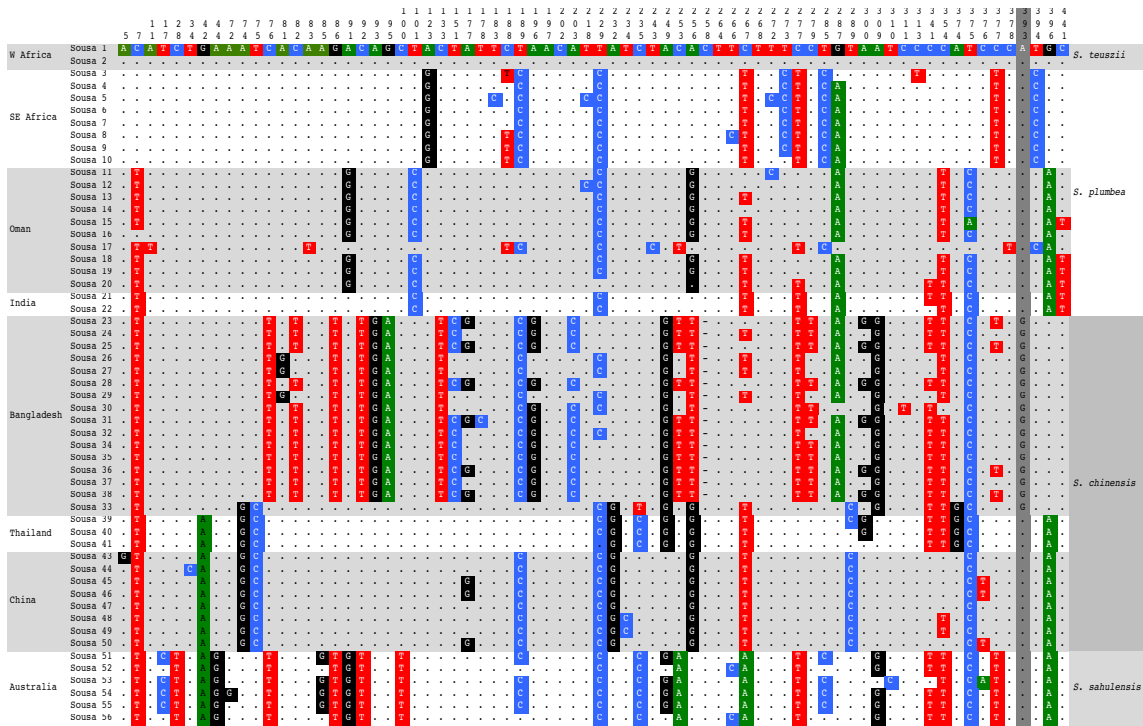
Figure 4. Character matrix depicting the mitochondrial control region polymorphisms that define the different *Sousa* spp. haplotypes. The fixed character that diagnoses all Bangladesh sequences is highlighted in dark grey.

The Bayesian phylogenetic tree estimated for the concatenated mitochondrial and nuclear DNA datasets did not resolve relationships between the different *Sousa* geographical assemblages (Figure 5). Although there are different clusters grouping samples from Bangladesh, from Australia and from China, these are supported by low levels of posterior probability. This may be attributed to the lower level of polymorphism obtained for the nuclear introns when compared to the mitochondrial DNA, as well as the high number of missing characters that had to be included in the dataset in order to concatenate the sequences.
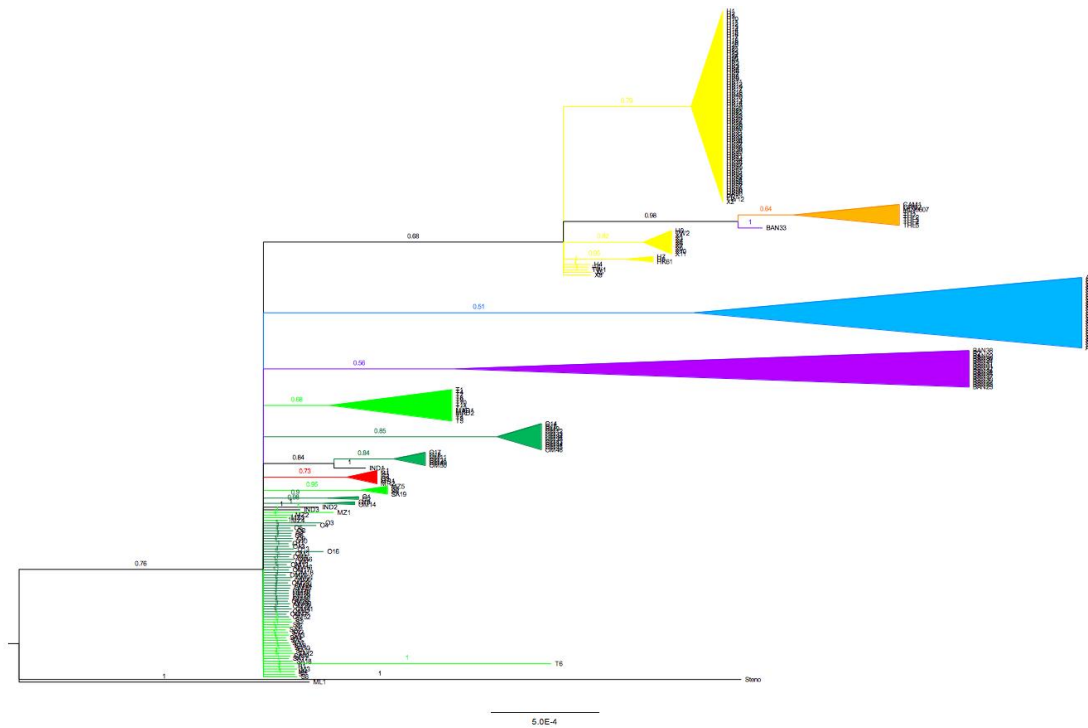
Figure 5. Bayesian phylogenetic tree obtained for the mitochondrial control region and nuclear introns concatenated sequences. Values above branches represent the posterior probability values. The different geographical assemblages are represented in different colours: purple – Bangladesh; Blue – Australia; Red – West Africa; light green – Southeast Africa; dark green – Oman; yellow – China; orange – Thailand.

Discussion

In this study we analysed humpback dolphin samples from Bangladesh, a part of the range of these dolphins that has not been extensively included in earlier genetic studies even though these waters appear to constitute a transition point in the distribution of *S. plumbea* and *S. chinensis*. The results indicate that humpback dolphins in Bangladesh are genetically distinct from other members of the genus in the Atlantic Ocean (*S. teuszii*); Southeast Africa and Arabia (*S. plumbea*); Thailand and China (*S. chinensis*); and in Australia (*S. sahulensis*). No shared haplotypes (Bangladesh n= 15) were found between any of these groups. Levels of genetic divergence indicate that humpback dolphins from Bangladesh are similarly as differentiated from the other putative species as *S. sahulensis*. One fixed site in the mitochondrial control region diagnoses all Bangladesh samples from all others. However, if the sequence that clustered with Thailand sequences is not considered, three additional fixed sites diagnose Bangladesh samples from all others. Phylogenetically, the results from mitochondrial DNA analysis suggest that these dolphins are more closely related to *S. sahulensis* than to the other putative species, which group into a separate clade. The existence of a single sample from southern Bangladesh that was closely related to *S. chinensis* in Thailand implies that the range of the phylogenetically unique humpback dolphin population in Bangladesh may be limited to areas affected by freshwater input from the Ganges-Brahmaputra-Meghna River (see

9

below). The two nuclear introns sequenced were not informative enough to resolve the phylogenetic position of Bangladesh sequences within the genus. Additional loci are needed in order to obtain a phylogeny that more closely represents the species tree.

The discrete, fixed character of the mitochondrial control region, as well as the distinct, highly supported cluster in the phylogenetic tree indicate that humpback dolphins in Bangladesh are a distinct evolutionary unit when considering the mitochondrial DNA, with important implications for conservation management. However, additional molecular markers need to be analysed before a formal distinction at the species level can be considered.

In addition to molecular character information, there are indications of behavioral differences between humpback dolphins in the Northern Bay of Bengal and other members of the *Sousa* genus. Throughout their range, humpback dolphins are generally found in groups of less than 10 with a maximum group size of 30 individuals (Parra and Ross 2009). From dorsal fin photographs, 205 non-calf individuals were identified in a single group in Bangladesh. The actual group size was undoubtedly greater considering the estimated proportion of unmarked non-calf individuals (26%) plus the estimated proportion of calves (12%) suggesting that the actual group size was around 330 which is more than an order of magnitude greater than the maximum group sizes reported elsewhere for the species. Other large groups were also occasional observed including two with 95 and 110 individuals estimated in the field of which 27 and 81 individuals were photoidentified, respectively (Mansur et al., in prep.). The ecological and/or social reason(s) for these sporadic sightings of large groups are unknown but they appear to be related to the clumped nature of estuarine prey driven by the complex dynamics of freshwater flow, and marine currents and tides.

A mark resight analysis under a Pollock's robust design applied to 468 photo-identified humpback dolphins during winter seasons of 2010-2013 estimated their abundance to be 132 (SE=10, 95% CI = 115-153), 131 (SE=3, 95% CI = 124-137), and 636 (SE=58, 95% CI = 531-761) individuals, respectively. The substantial jump during the winter of 2012/2013 can be explained by the large group size mentioned above combined with a relatively low resighting rate during this year despite similar survey effort. The estimated probability of remaining in an unobservable state in the next survey when in an unobservable state during the previous survey was of 55%. Together these findings indicate that the humpback dolphin population offshore of the Sundarbans in the Bangladesh, where the tissue samples were collected, is likely part of a superpopulation that occupies more extensive coastal waters across the border in India and in the mouth of the Meghna (Mansur *et al.* in prep).

The ecology of the Northern Bay of Bengal is strongly influenced by freshwater flow from the third-largest river system in the world: the Ganges-Brahmaputra-Meghna, circulated by a seasonally reversing, wind-driven, basin-scale gyre. These conditions combine to produce a highly stratified and productive sea-surface layer supporting the humpback dolphin population described above as well as the world's largest population of Irrawaddy dolphins *Orcaella brevirostris* and relatively large populations Indo-Pacific

finless porpoise *Neophocaena phocaenoides* and Indo-Pacific bottlenose dolphins *Tursiops aduncus* (Smith *et al.* 2008; Mansur *et al.* 2012). The rare global occurrence of freshwater inputs of this scale combined with the patchy distribution of even small sources of freshwater input south of the Meghna River mouth implies a high potential for endemism. This hypothesis is supported by an analysis of the mitochondrial DNA control region of bottlenose dolphins inhabiting waters at the head of the Swatch-of-No-Ground submarine canyon located just offshore of the range of humpback dolphins in Bangladesh which obtained similarly results of strong genetic distinctiveness and clustering in separate clades from other members of *T. aduncus* occurring to the east and west (Amaral *et al.* 2015).

The genetic distinctiveness of the humpback dolphin population in Bangladesh implies that it should be treated a separate evolutionary unit and therefore a separate management unit. The relatively large estimates of population size summarized above, combined with information that these estimates are only for a portion of a larger metapopulation extending to the east and west of waters offshore the Sundarbans in Bangladesh are encouraging. However, the common occurrence of scars and mutilations in photo-identified dolphins that were almost certainly associated with entanglements in fishing gears (15%) and the extensive spatial overlap between entangling fishing gears and the preferred habitat of humpback dolphins indicate that the current favorable status of humpback dolphins in Bangladesh may be threatened by intensifying fisheries, particularly gillnetting (Mansur *et al*. in prep).

Acknowledgments

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