



Complete mitogenome of Ganges river dolphin, *Platanista gangetica gangetica* and its phylogenetic relationship with other cetaceans

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Abstract

The Ganges river dolphin, *Platanista gangetica gangetica* is one of the endangered cetaceans. Due to increasing anthropogenic activities, it has faced a significant reduction in distribution range since the late 1800s and has even gone extinct from most of the early localities. The investigation of complete mitogenome holds significant relevance for identifying evolutionary relationships and monitoring the endangered species. Herein, we report and characterize for the first time the 16,319 bp complete mitochondrial genome of *P. g. gangetica*. It comprises 13 protein-coding genes, 22 transfer RNA (tRNA) genes, two ribosomal RNA genes, and one control region (CR). The genome composition was A + T biased (59.6%) and exhibited a positive AT-skew (0.104) and negative GC-skew (−0.384). All the genes were encoded on the heavy strand, except eight tRNAs and the ND6 gene. In the CR, an 18 bp tandem repeat sequence was observed. Our Bayesian Inference (BI) and Maximum Likelihood (ML) based phylogenetic analysis indicated that studied river dolphins were polyphyletic and the placement of *Platanista* was to be more basal than other river dolphins (*Lipotes*, *Inia* and *Pontoporia*). The pairwise genetic distance of *Platanista* with other cetaceans was varied, with an overall close affinity with whales. The model-based BI and ML phylogenetic analysis indicated that *Platanista* clustering with Ziphiidae with high to moderate supportive values (PP/BP = 98/68). The results of this study provide insights important for the conservation genetics and further evolutionary studies of the freshwater river dolphins.

Keywords Gangetic dolphin · Conservation · *Platanista* · Ganga river · Molecular phylogeny

Introduction

The South Asian river dolphins of the genus *Platanista* is a highly threatened mammal [1]. It is an iconic freshwater species and the only extant representative of the family Platanistidae. Currently, two subspecies of South Asian river dolphin are recognized: the Indus River dolphin (*P. gangetica minor*) confined to the Indus river system of India and Pakistan, and the Ganges River dolphin (*P. g. gangetica*)

inhabiting in Ganges, Brahmaputra, Meghna and Karnaphuli–Sangu of India, Bangladesh Nepal and Bhutan [1–3]. The Ganges is the lifeline and holy river of India, and the basin supports more than 600 million people [4]. Despite its iconic and religious status, high anthropogenic pressure threatens its biodiversity. As the large mammal of the river Ganges, the Gangetic dolphin experiences many risks such as pollution, dam, barrages, low water levels, and illegal catching for oil and meat. The extracted oils are used to catch catfish in the net fishery [5]. These threats are long-lasting and challenging to address and have led to a drastic decline in the distribution range as well as the population size of the Gangetic river dolphin [6]. Hence, the species is listed under IUCN Red List as “Endangered” [7] and protected in Schedule I of the Indian Wildlife (Protection) Act, 1972. It is also included in Appendix I of the Convention on International Trade in Endangered Species of Flora and Fauna (CITES) and Appendix II of the Convention on Migratory Species (CMS).

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Apart from its conservation status, the taxonomic placement of *Platanista* amongst cetaceans warrants attention and has remained controversial over decades due to its diverse evolutionary history [8–12]. Phylogenetic inference based on mtDNA is a widely accepted tool for delineating species due to the organelle's uniparental inheritance, presence of conserved genes, lack of recombination, and high evolutionary rate [13]. In some species, short fragments of the mtDNA genome can be uninformative for phylogeographic analysis [14, 15]. Hence, extensive coverage of mitogenome provides robustly resolves phylogenetic affinity and genetic associations [16, 17].

The complete mitogenome of *P. g. gangetica* is not available in the GenBank for comparison with the other river dolphins. Therefore, the study aimed to generate the complete mitochondrial genome of the Gangetic dolphin and compare the sequences with the subspecies *P. g. minor* and other three genera of the world's river dolphin: Amazon river dolphin or 'boto' (*Inia geoffrensis*), La Plata river dolphin or 'franciscana' (*Pontoporia blainvillei*), the Yangtze river dolphin or 'baiji' (*Lipotes vexillifer*). A comparative analysis of the mitogenome of *Platanista* with other cetaceans was performed to provide insight into the phylogenetics of cetaceans and support future studies on biogeography, conservation and evolutionary genetics of river dolphin..

Materials and methods

The tissue samples of four Gangetic dolphins (one each from Narora and Kanpur, Uttar Pradesh and two from Patna, Bihar) were collected from dead individuals during the ecological survey of the Ganges river conducted in the year 2017–2019 under the project "Biodiversity Conservation and Ganga Rejuvenation". Samples were stored in 70% ethanol till DNA extraction. We used DNeasy Blood Tissue Kit (QIAGEN, Germany) to extract genomic DNA in a final elution volume of 100 μ l. The extracted DNA was quantified on 0.8% agarose gel and diluted in a final concentration of 30 ng/ μ l for the Polymerase Chain Reaction (PCR) amplification.

PCR amplification and sequencing

PCR amplification of the complete mitogenome was performed using 22 different sets of primers [18] in 20 μ l reaction volume containing 1 \times standard PCR buffer, 1.5 mM MgCl₂, 0.25 mM of each dNTPs, 4 pmol of each primer, 0.5 units of AmpliTaq Gold DNA Polymerase (Invitrogen) and 1 μ l of template DNA. PCR conditions were: initial denaturation at 94 °C for 10 min, followed by 35 cycles at 95 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C

for 75 s. Finally, we extended the final extension at 72 °C for 10 min. PCR amplification was checked and visualized on 2% agarose gel under a UV transilluminator. The PCR amplicons were cleaned with exonuclease-*I* and shrimp alkaline phosphatase (Thermo) at 37 °C for 20 min, followed by inactivation of enzymes at 85 °C for 15 min. The chemically cleaned PCR fragments were sequenced in 3500XL ABI Genetic Analyzer from forward and reverse direction using BigDye Ver 3.1 Kit.

Sequence alignment and mitogenome annotation

The overlapping fragments of mitogenome sequences were aligned and analyzed using Sequencher® Ver 5.4.6 (Gene Codes Corporation). Mitochondrial DNA annotation was done using MitoS WebServer [19] and confirmed with MitoFish [20]. The complete mitogenome map of the Gangetic dolphin was generated using CGView Server [21]. Nucleotide compositions and genetic code were calculated in MEGA X [22]. For estimating the bias in nucleotide composition among the complete mitogenome, protein-coding genes (PCGs), transfer RNA (tRNA), ribosomal RNA (rRNA), and control region (CR), skew analysis was carried out using the following method: GC skew = (G – C)/(G + C), AT skew = (A – T)/(A + T) [23]. The overlapping regions and intergenic spacer among genes of complete mitogenome were manually estimated. The Open Reading Frame Finder [24] online web tool was used to check the start and stop codons of PCGs. In the control region (CR), tandem repeats were identified by the online Tandem Repeats Finder web tool [25].

Phylogenetic analysis and genetic differentiation

For phylogenetic analysis, 50 mitogenome sequences belonging to families Platanistidae, Delphinidae, Iniidae, Monodontidae, Phocoenidae, Lipotidae, Ziphiidae, Kogiidae, Physeteridae, Pontoporiidae and suborder Mysticeti (Balaenidae and Eschrichtiidae) were included (Supplementary Table ST 1). Additionally, the mitogenome of the hippopotamus (*Hippopotamus amphibious*, AJ010957) was downloaded from GenBank and used as an outgroup. Alignments of concatenated sequences of 12 PCGs excluding the ND6 gene were conducted in MUSCLE, implemented in MEGA X [22] and manually checked. We employed Bayesian Inference (BI) analyses to construct a phylogenetic tree among cetaceans using BEAST Ver 1.7 [26]. The general time-reversible model (GTR) with a gamma distribution (+G) and a proportion of invariable sites (+I) was selected

for the concatenated genes based on AIC criteria generated in jModelTest Ver 2.1.10 [27]. The Monte Carlo Markov Chain (MCMC) was run for 10 million steps with a random starting tree, birth–death default priors, and sampled one tree every 1000 steps. We used Tracer Ver 1.7 for estimating the convergence of values and effective sample sizes [28]. We discarded the first 25% steps as burn-in. Subsequently, to confirm the tree topology, we again ran the same molecular dataset using raxmlGUI Ver 2.0 beta (Maximum-likelihood method) and the node support values were calculated using 1000 replicates [29]. The model for the substitution for sequences was GTR + I + G. FigTree Ver 1.4.0 was used to visualize the resulting phylogenetic tree (<http://tree.bio.ed.ac.uk/software/figtree/>). We estimated the similarity of complete mitogenomes among river dolphins using MatGAT Ver 2.0 [30].

Results

Mitogenome feature and organization

We obtained a 16,319 bp complete circular mitochondrial genome from four Gangetic dolphins (Fig. 1) and deposited in GenBank (acc. no. MN896020–MN896023). The mitogenome was encoded by two rRNA genes, 22 tRNA genes, one origin of replication (O_L), 13 PCGs, and a non-coding CR. Among these, 28 genes (2rRNA, 14 tRNA and 12 PCGs) were located on the heavy strand (H-strand), except *ND6* gene and eight tRNA genes (*tRNA^{Gln}*, *tRNA^{Ala}*, *tRNA^{Asn}*, *tRNA^{Cys}*, *tRNA^{Tyr}*, *tRNA^{Ser}*, *tRNA^{Glu}*, *tRNA^{Pro}*) and O_L was located on the light strand (L-strand). The total nucleotide composition of the Gangetic dolphin mitochondrial genome was A (32.9%), T (26.7%), C (27.9%) and G (12.4%), and it was biased towards A + T (59.6%). The total length of all 13 PCGs was 11,400 bp; however, it was 1,519 bp for tRNAs and 2,548 bp for rRNA genes. The A + T composition of PCGs, tRNAs, rRNAs and CR were 58.9%, 63.5%, 60.7% and 60.5% respectively. A significant bias towards A/T was also observed in complete mitogenome and 13 PCGs of other cetaceans (Supplementary Figs. 1, 2). The nine pairs overlapping region in mitogenome were observed among *tRNA^{Ile}/tRNA^{Gln}*, *ND2/tRNA^{Trp}*, O_L /*tRNA^{Cys}*, *ATP8/ATP6*, *ATP6/COIII*, *COIII/tRNA^{Gly}*, *ND4L/ND4*, *ND5/ND6* and *tRNA^{Thr}/tRNA^{Pro}*. The overlapping regions were ranging from –1 to –39 bp. The longest overlap was located between *ATP8* and *ATP6* (39 bp), whereas it was smallest between *ATP6/COIII*, *COIII/tRNA^{Gly}* and *tRNA^{Thr}/tRNA^{Pro}* (1 bp). The O_L sequence was 35 bp in length and was located between the *tRNA^{Asn}* and *tRNA^{Cys}*; this region plays an important role in initiating DNA synthesis by recognizing the start site of DNA polymerase. The non-coding CR region was located between *tRNA^{Pro}* and *tRNA^{Phe}*. In

addition, 14 intergenic spacers were observed between the mitochondrial regions ranging from 1 to 32 bp length; the longest intergenic was present between *tRNA^{Asn}* and *tRNA^{Cys}* (Table 1). The AT and GC skewness was found to be 0.103 and –0.382, respectively, in the complete mitogenome of Gangetic dolphin. We also estimated base skews for all freshwater river dolphins (Table 2) and other cetaceans (Supplementary Fig. 3) for understanding the nucleotide distribution in the mitogenomes.

Protein coding genes (PCGs)

The total length of 13 PCGs in the mitogenome of the Gangetic dolphin was 11,400 bp that includes 61 bp overlapping fragments, which accounted for 69.85% of the complete mitogenome. The average base composition of PCGs was A = 30.8% T = 28.1%, G = 12.1 and C = 29.1. The abundance of AT% was much higher than the GC% (Table 2). Out of 13 PCGs, 12 were present on H-strand while *ND6* was located on L-strand as commonly observed in other vertebrate species (Fig. 1 and Table 1). The PCGs region of Gangetic dolphin consisted of seven NADH dehydrogenases subunits (*ND1*, *ND2*, *ND3*, *ND4*, *ND5*, *ND6* and *ND4L*), three cytochrome c oxidases (*COI*, *COII* and *COIII*), two ATP synthase (*ATP6* and *ATP8*) and one cytochrome *b* (*Cyt b*) gene. The size length ranged from 165 bp (*ATP8*) to 1821 bp (*ND5*). The average AT and GC skews values for Gangetic dolphin in PCGs were 0.069 and –0.465, respectively. In all cetaceans, AT skewness was positive, which indicated that the adenine was more frequent than thymine, whereas negative GC skewness indicated that the cytosine was more common than guanine base in PCGs (Supplementary Fig. 4). All PCGs of Gangetic dolphin started with an ATG initiation codon, except for *ND2*, *ND3* and *ND5* genes that started with ‘ATA’ and *ND4L* with ‘GTG’. Meanwhile, five PCGs had typical ‘TAA,’ three PCGs had ‘TAG,’ and *Cyt b* was found to possess ‘AGA’ as termination codon whereas incomplete termination codon TA or T– was seen in *ND3*, *ND4*, *ND5* and *ND6* (Table 1).

Relative synonymous codon usage (RSCU) for the 13 protein-coding genes of the Gangetic dolphin revealed that Leucine is the most frequently occurring amino acid, followed by Isoleucine and Threonine, whereas Methionine and Cysteine were relatively less abundant (Supplementary Fig. 5). The genetic relatedness among the river dolphin species was estimated using the 13 PCGs. We observed high genetic similarity between the Gangetic dolphin and the Indus river dolphin, with values ranging from 85.96% (*ND6*) to 100% (*ND4L*). However, these values varied for other river dolphins and ranged from 75.38% (*ND6*) to 83.62% (*COXI*); 75.12% (*ATP8*) to 84.64% (*COXII*); 74.62% (*ATP8*) to 84.53% (*COXI*) for Gangetic dolphin as compared to Amazon river dolphin, La Plata river

Table 1 The organization and characterization of the complete mitochondrial genome of the Gangetic dolphin (*P. g. gangetica*)

Gene	Start position	End position	Length (bp)	Initiation codons	Termination codons	Anti-codon	Strand	Space/overlap
tRNA-Phe	1	73	73	–	–	GAA	H	0
12S ribosomal RNA	74	1044	971	–	–	–	H	0
tRNA-Val	1045	1111	67	–	–	TAC	H	0
16S ribosomal RNA	1112	2688	1577	–	–	–	H	0
tRNA-Leu	2689	2763	75	–	–	TAA	H	0
ND1	2766	3722	957	ATG	TAA	–	H	+2
tRNA-Ile	3728	3796	69	–	–	GAT	H	+5
tRNA-Gln	3794	3866	73	–	–	TTG	L	–3
tRNA-Met	3868	3936	69	–	–	CAT	H	+1
ND2	3937	4980	1044	ATA	TAG	–	H	0
tRNA-Trp	4979	5046	68	–	–	TCA	H	–2
tRNA-Ala	5052	5120	69	–	–	TGC	L	+5
tRNA-Asn	5122	5195	74	–	–	GTT	L	+1
rep origin- <i>O_L</i>	5196	5230	35	–	–	–	L	0
tRNA-Cys	5228	5294	67	–	–	GCA	L	–3
tRNA-Tyr	5295	5360	66	–	–	GTA	L	0
COXI	5362	6906	1545	ATG	TAA	–	H	+1
tRNA-Ser	6908	6976	69	–	–	TGA	L	+1
tRNA-Asp	6984	7051	68	–	–	GTC	H	+7
COXII	7052	7735	684	ATG	TAA	–	H	0
tRNA-Lys	7739	7805	68	–	–	TTT	H	+3
ATP8	7807	8007	201	ATG	TAG	–	H	+4
ATP6	7968	8648	681	ATG	TAA	–	H	–39
COXIII	8648	9433	786	ATG	TAG	–	H	–1
tRNA-Gly	9433	9501	69	–	–	TCC	H	–1
ND3	9502	9848	346	ATA	TA–	–	H	0
tRNA-Arg	9849	9918	70	–	–	TCG	H	+1
ND4L	9919	10,215	297	GTG	TAA	–	H	0
ND4	10,209	11,586	1378	ATG	T–	–	H	–7
tRNA-His	11,587	11,655	69	–	–	GTG	H	0
tRNA-Ser	11,656	11,715	60	–	–	GCT	H	0
tRNA-Leu	11,717	11,786	70	–	–	TAG	H	+1
ND5	11,787	13,607	1821	ATA	T–	–	H	0
ND6	13,593	14,112	520	ATG	T–	–	L	–14
tRNA-Glu	14,113	14,181	69	–	–	TTC	L	0
CYT <i>b</i>	14,186	15,325	1140	ATG	AGA	–	H	+4
tRNA-Thr	15,326	15,396	71	–	–	TGT	H	0
tRNA-Pro	15,396	15,462	67	–	–	TGG	L	–1
Control region	15,463	16,319	857	–	–	–	H	0

The 22 tRNA genes were distributed along the mitogenome and were identical to other mammalian species. The length of tRNA varied from 60 (*tRNA^{Ser}*) to 75 bp (*tRNA^{Leu}*). Fourteen tRNA genes were encoded on the H-strand while the remaining eight were present on the L-strand (Fig. 1). The total tRNA length was 1519 bp, with the overall A + T and G + C content as 63.5 and 36.4%,

respectively. The average AT and GC skews values for tRNAs were 0.048 and 0.439, respectively. All the tRNA genes folded into the classic secondary cloverleaf structure, except *tRNA^{Ser}(GCT)* in which the dihydrouridine 'DHU' arm did not form a stable structure. The 'DHU' arm of *tRNA^{Ser}* was a large loop instead of a conserved stem-loop structure (Supplementary Fig. 6).

Table 2 Nucleotide composition indices in different regions of mitogenomes of the river dolphins

Species	Accession number	Whole mitogenome				Protein coding genes (PCGs)		Ribosomal RNA (rrnl)	
		Length (bp)	AT%	AT Skew	GC skew	Length (bp)	AT%	Length (bp)	AT%
<i>P.g.gangetica</i>	MN896020-MN896023 ^a	16,319	59.6	0.103	-0.382	11,400	58.9	2548	60.7
<i>P.g.minor</i>	NC005275 ^b	16,324	59.7	0.101	-0.378	11,406	59.1	2548	60.5
<i>Inia geoffrensis</i>	NC005276 ^b	16,588	58.7	0.114	-0.364	11,406	57.9	2554	59.5
<i>Pontoporia blainvillei</i>	NC005277 ^b	16,593	59.5	0.105	-0.333	11,401	58.6	2560	60.8
<i>Lipotes vexillifer</i>	NC007629 ^c	16,392	60.8	0.114	-0.369	11,406	60.2	2554	60.8

^aThis study^bArnason et al. [31]^cYan et al. [12]

Mitochondrial control region sequence

The length of CR in the Gangetic dolphin was 857 bp long and was located between *tRNA^{Pro}* and *tRNA^{Phe}*, which was less than most cetaceans. The base composition of CR was 28.4% A, 32.2%T, 15.1%G, and 24.4%C. The AT content was higher than the GC content. In particular, 18 bp repeat consensus (AATACTAATAACAAAAC) was found within 14,062–14,101 bp with a copy number 2.2 of CR. However, no such repeat sequence was detected in the mitogenome of *P. g. minor*, *Inia geoffrensis*, *Pontoporia blainvillei* and *Lipotes vexillifer*.

Phylogenetic analyses

The phylogenetic analysis was performed with BI and ML trees using 12 PCGs among cetaceans. The topological structure generated from both methods and analyses yielded similar results (Fig. 2, Supplementary Fig. 7). The BI and ML trees revealed that *Platanista* clustered with Ziphiidae clade with high posterior probability (PP) and bootstrap value (BP) (PP/BP = 0.98/68). Within cetaceans, the tree topology was (((Iniidae,Pontoporiidae),(Delphinidae,Phocoenidae,Monodontidae))),Lipotidae),(Platanistidae,Ziphiidae),(Kogiidae,Physeteridae),Mysticeti))). The two subspecies of *Platanista*, *gangetica* and *minor* clustered together with high PP/BP (~0.99/100).

Discussion

The distribution and arrangement of mitogenes of Gangetic dolphins were similar to other mammalian species [32, 33]. However, we found some notable differences in length and nucleotide position in a few genes. The highly conserved regions such as 12 s rRNA and 16 s rRNA regions have been widely acknowledged for species identification and

phylogenetic resolution [34]. Comparisons of the 12S rRNA and 16S rRNA genes between Gangetic and Indus river dolphins indicated a high genetic similarity > 99%, which may lead to misidentification in the absence of a reliable and comprehensive reference database. The 13 PCGs of Ganges river dolphin, like other vertebrates, showed A + T bias and C base preference. The hypervariable A + T rich non-coding control region regulated the transcription and replication of mtDNA [35]. Due to its higher mutation rate as compared to regions of mitogenome, it serves as a vital region to assess the intra-species variations [34]. In comparison to the other four river dolphin, the 18 bp repeat consensus sequence was only found in Ganges river dolphin, and the length of this region varied extensively between species and within species due to the presence of insertions and deletions (INDEL) as well as multiple tandem repeats [36, 37].

The phylogenetic position of *Platanista* holds scientific relevance. It is considered the evolutionary link with beaked whales rather than other existing river dolphins (*Pontoporia blainvillei*, *Lipotes vexillifer* and *Inia geoffrensis*). The clustering pattern of the *Platanista* with Ziphiidae was consistent with the previous studies [8, 12, 38]. Our phylogenetic analysis indicated that studied river dolphins were polyphyletic and the placement of *Platanista* was more basal and had no affinity with *Lipotes*, *Inia*, and *Pontoporia*. Based on this, it was clear that *Platanista* evolved independently and had a diverse lineage. Based on the partial mitochondrial Cyt *b* and nuclear interphotoreceptor retinoid-binding protein (IRBP) gene sequences, a sister genetic relationship between Platanistidae and Mysticeti was suggested [39]. The method used by Verma et al. [39] was conventional neighbor-joining analyses, which is just a clustering algorithm that clusters groups based on genetic distance. Therefore, to construct the phylogeny for such a highly diverse species, model-based approaches, like ML and BI, along with choosing the best fit model, provide better insight. The high fragility in the phylogenetic position of *Platanista* within cetaceans might be because the South Asian river dolphin, baleen, beaked,

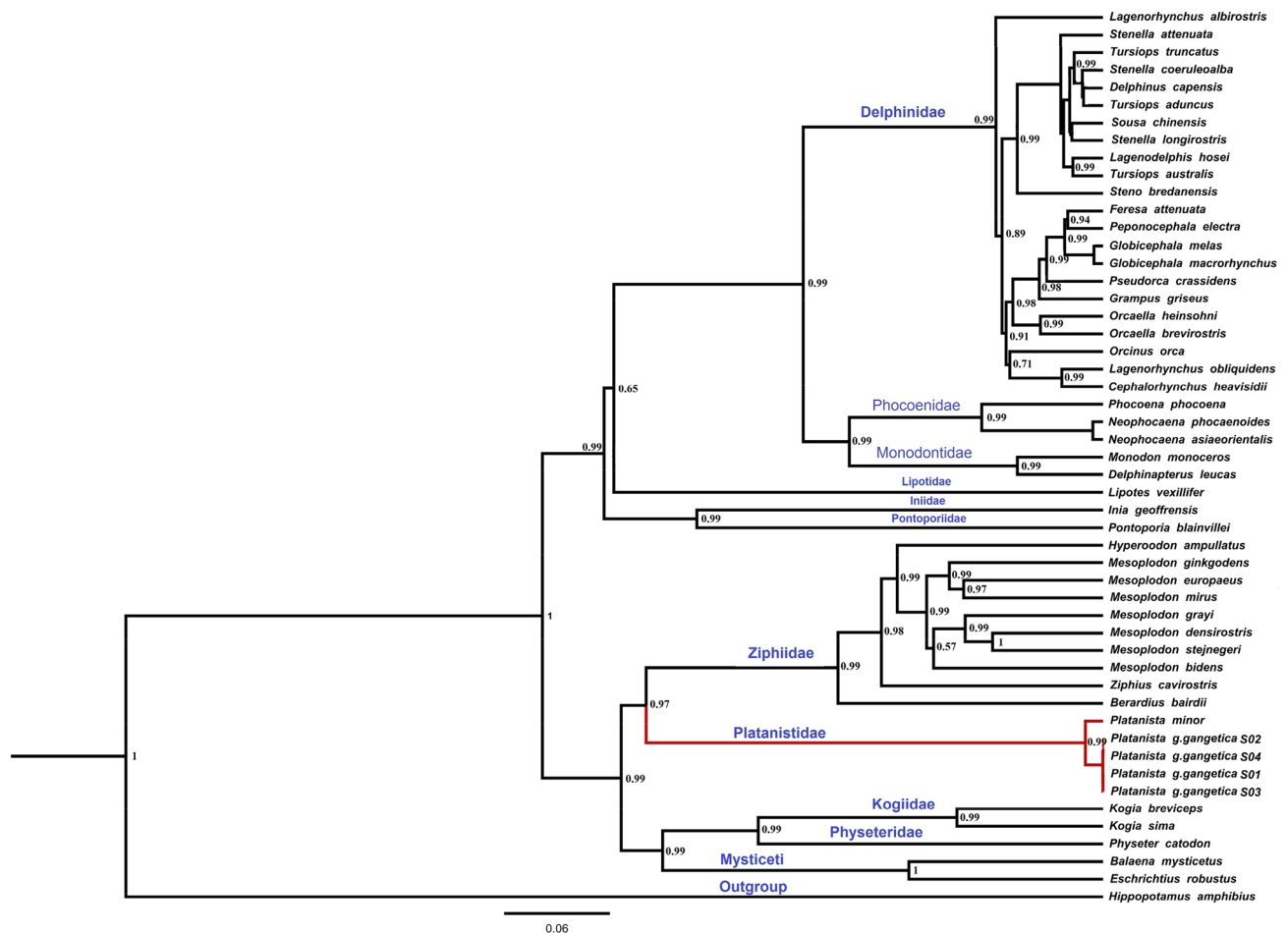


Fig. 2 Phylogenetic tree inferred from Bayesian inference (BI) analysis using 12 concatenated protein-encoding genes. Bayesian posterior probability (PP) values are shown at the node of the tree. *Hippopotamus amphibius* (AJ010957) was used as an outgroup. (Color figure online)

and sperm whale lineages have been evolved through a very rapid split event in the Eocene [8, 40].

The data generated in this study will serve as crucial baseline information for genetic monitoring, spatial distribution range, and evolutionary relationship analysis. The single nucleotide polymorphisms (SNPs) in different genes such as PCGs, rRNA, and CR will be helpful in wildlife forensic cases for tracking confiscate biological samples. This information would support further genetic investigation that will help formulating scientifically informed *in-situ* and *ex-situ* management strategies towards better conservation of the endangered species.

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Compliance with ethical standards

Conflict of interest The author(s) declare no competing interests.

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