Forensic Science International: Animals and Environments 1 (2021) 100035

Contents lists available at ScienceDirect

Forensic Science International: Animals and Environments

journal homepage: www.sciencedirect.com/journal/forensic-science-international-animals-and-environments

Identification of Gangetic turtles based on species-specific variations on mitochondrial cyt *b* and nuclear Cmos genes

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ARTICLE INFO

Keywords:
Gangetic turtles
DNA forensics
Phylogenetic mtDNA
Nuclear
Ganga river system

ABSTRACT

The freshwater turtles of the iconic Ganga river system are one such group of vulnerable turtles. Despite common knowledge of the severity of turtle trade in the region, Gangetic turtles continue being poached in large volume, evident from the numerous and extensive seizures across the Gangetic belt. The intensive wildlife trade in Gangetic turtles warrants immediate conservation and management attention. The genetic resource is a vital forensic tool to monitor the Gangetic turtle species to understand the pattern of illegal wildlife trade. We collected 64 softshell and hardshell turtles samples from the Ganga river and report species-specific variations among turtle species based on mitochondrial cytochrome *b* gene (1140 bp) and nuclear Cmos gene (602 bp). This genetic information will help augment the molecular database to identify Gangetic turtle species and lineages effectively. We identified unique species-specific variable sites, haplotypes, and Single Nucleotide Polymorphisms (SNPs) and analyzed genetic differentiation and phylogenetic relationships. The unique mitochondrial and nuclear signatures exhibited in this study will add to baseline information on the genetic relationship of turtles of river Ganga. It will be helpful in wildlife forensics characterization of the endangered turtles. It will also help in formulating *in-situ* and *ex-situ* conservation and management plan to improve the rescue and rehabilitation strategies.

1. Introduction

Globally, freshwater turtles are one of the most endangered vertebrates, with half of all species facing the risk of extinction [1]. Out of the 356 recognized Chelonian species, around 2% have already gone extinct and about 82% of species are 'Threatened' or 'Critically Endangered' [1,2]. In the South Asian region, the Ganga river passes through diverse biogeographic zones of India and sustains rich freshwater turtle diversity [3,4]. The Ganga river is a critical biodiversity hotspot and a significant socio-economic and cultural center [4–6]. It harbors 14 freshwater turtle species making the region a Global Turtle Priority Area [6]. The turtle species of river Ganga belong to the two families: Geoemydidae and Trionychidae, comprising nine hardshell and five softshell turtle species, respectively (Table 1). Out of 14 freshwater turtles, the northern river terrapin *Batagur baska* (Gray, 1830) is a large riverine and estuarine turtle species. Of these, *Batagur kachuga*, *Batagur baska*, *Batagur dhongoka* and *Chitra indica* are 'Critically Endangered'; *Geoclemys hamiltonii*, *Hardella thurjii*, *Melanochelys tricarinata*, *Nilssonia gangetica* and *Nilssonia hurum* are 'Endangered', and *Pangshura tecta* and *Lissemys punctata* are classified as 'Vulnerable' in the International Union for Conservation of Nature (IUCN) Red List. Taking cognizance of their large-scale trade, six species are listed in Appendix-*I* and seven in Appendix-*II* of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Of the 14 species, ten are listed in Schedule-*I* of the Indian Wild Life (Protection) Act 1972 (WPA), which prohibits hunting, trade, or utilization in any form of the turtle body parts and their derivatives (Table 1).

Natural populations of freshwater turtles depend on the flow of river undercurrents, river islands, sand and silt depositions, and growing floodplains that provide habitat for survival and sustenance [7]. Habitat degradation and poaching for wildlife trade are the primary threats to dwindling turtle populations that are even more vulnerable to the fluctuations due to climate change [8]. Turtle biodiversity hotspots coinciding with severe anthropogenic pressure areas pose challenges for

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https://doi.org/10.1016/j.fsiae.2021.100035
Received 16 February 2021; Received in revised form 14 September 2021; Accepted 27 October 2021
Available online 3 November 2021

efficient conservation and management of the species.

Despite several national and international conservation efforts, the Asian turtle trade grew unabated and has taken shape into a highly networked transnational syndicate [9]. A study by TRAFFIC (Trade Records Analysis of Flora and Fauna in Commerce) spanning a decade of turtle trade reported an estimate of at least 1,11,312 Indian turtles and tortoises traded illegally [10]. The Ganga river system passes through several towns and major cities, forming a critical part of the illegal turtle trade network. According to the seizure data, Uttar Pradesh and West Bengal states are two critical hotspots with confiscations in numbers representing more than half of all reported seizures from 10 States and two Union Territories of India, signifying the extensive reach of the illegal market [10]. Despite the seizure reports indicating crucial information about the trade network, many important resources remain unutilized. Over half of the confiscation reports fail to identify the turtle species in seizures, ranging from live or dead individuals, meat, body parts, and derivatives. Lack of information and resources available with enforcement agencies to reliably identify species is a major challenge for efficient conservation and management of these highly traded endangered turtles and effective enforcement of the wildlife protection laws [11,12].

Wildlife forensics is a vital tool in conservation science, which identifies unknown species samples using morphological identification of carcass or body parts such as hairs, bones and feathers [13,14], and DNA analysis of ivory, tissue and blood samples were used for identification of species and examination of trade networks to solve poaching cases [15,16]. Besides, generation of a reference mitochondrial and nuclear DNA database will also aid in the identification of species and origin [17–19]. Nuclear markers have proved resourceful for species delimitation in cryptic species and closely related subspecies [20,21]. The regions of mitochondrial DNA (mtDNA) serve as important tools for species identification and phylogenetic studies [22–25]. It has been widely applied in wildlife forensic science due to conserve nature [26,27]. Generating the species-specific variations incorporate DNA sequencing will help to identify seized samples based on changes in nucleotide sequences [11,28,29]. Therefore, we identified species-specific nucleotides variations in mitochondrial (cytochrome *b*) cyt *b* and nuclear Oocyte maturation factor (Cmos) genes and established a genetic database to generate a reliable forensic resource for efficient species and lineages identification of illegally traded turtles.

2. Materials and methods

2.1. Sample collection

A total of 64 samples comprising 13 softshell and hardshell turtles were collected between 2017 and 2019, covering nine different sites along the Ganga river (Fig. 1, Supplementary Table: ST1). Samples were collected by mouth swabbing and clipping a small piece of the toe pad, after which individuals were released in their natural habitat. The turtles were identified based on the morphological characteristics and also confirmed with the help of herpetologist. The Institutional Animal Ethics Committee approved the method of the sample collection from the live animal. Opportunistically, samples were also collected from carcasses from the sites mentioned above. The samples were preserved in 95% ethanol and stored at −20 °C until further processing.

2.2. DNA extraction, amplification and sequencing

ptDNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions in 80 μl elution volume. QIAxpert (Qiagen, Hilden, Germany) was used for determinating the DNA concentration at OD 260/280 ratio and gel electrophoresis was performed using 1% agarose gel (UltraPure™ Agarose, Thermo Fisher Scientific) containing green stain (Cyanagen) for DNA detection. The complete cyt *b* gene was amplified using the primers: cyt *b* G: 5′-AACCATCGTTGTWATCAACTAC-3′ [30] and H-15909: 5′-AGGGTGGAGTCTTCAGTTTTTGGTTTACAAGACCAATG-3′ [31]. For nuclear gene amplification, we used a part of the coding region (~ 649 bp) for the Cmos gene:

Table 1
Status of Gangetic turtles under IUCN, CITES and Indian Wild Life (Protection) Act, 1972 (WPA).

Family	Species	Common name	Shell	IUCN (Categ.)	CITES (App.)	WPA (Sch.)	Distribution
Geoemydidae	*Batagur dhongoka*	Three striped roofed turtle	Hardshell	CR	II	I	Bangladesh, Nepal, India (Assam, Bihar, Madhya Pradesh, Rajasthan, Uttar Pradesh, West Bengal)
	Batagur baska	Northern river terrapin	Hardshell	CR	I	I	Bangladesh (in the Sundarbans), Cambodia, India (parts- West Bengal & Odisha), Indonesia and Malaysia
	Batagur kachuga	Red-crowned roofed turtle	Hardshell	CR	II	I	Nepal (Central), Myanmar (North-West), Bangladesh, India (North-East)
	Geoclemys hamilitonii	Black spotted turtle	Hardshell	EN	I	I	Pakistan (Indus & Ganga drainage in South), Sri Lanka, Bangladesh, India (North-east: Assam)
	Hardella thurjii	Crowned river turtle	Hardshell	EN	II	IV	Pakistan, Bangladesh, India (watershed of Ganges, Brahmaputra & Indus)
	Pangshura tecta	Indian roofed turtle	Hardshell	VU	I	I	Pakistan, Bangladesh, India (Ganges, Brahmaputra, Indus & Mahanadi)
	Pangshura smithii	Brown roofed turtle	Hardshell	NT	II	IV	Nepal, Pakistan, Bangladesh, India (Ganges, Brahmaputra & Indus)
	Pangshura tentoria	Indian tent turtle	Hardshell	LC	II	I	Bangladesh, India (Mahanadi, Godavari & Krishna)
	Melanochelys trijuga	Indian black turtle	Hardshell	LC	Not listed	IV	India, Bangladesh, Myanmar, Sri Lanka, Maldives, Nepal
	Melanochelys tricarinata	Three-Keeled Land Turtle	Hardshell	EN	I	I	India (North-East), Bangladesh, Nepal
Trionychidae	*Chitra indica*	Narrow-headed turtle	Softshell	CR	II	IV	Pakistan, India (Ganges, Godavari & Mahanadi), Nepal, Bangladesh
	Lissemys punctata	Indian Flapshell turtle	Softshell	VU	II	I	Pakistan, India, Bangladesh, Myanmar, Sri Lanka, Nepal
	Nilssonia gangetica	Indian softshell turtle	Softshell	EN	I	I	India (Ganges, Indus & Mahanadi)
	Nilssonia hurum	Indian peacock turtle	Softshell	EN	I	I	Bangladesh, India (Mizoram, Assam, Bihar, Madhya Pradesh, Odisha, Rajasthan, Uttar Pradesh, West Bengal), Nepal, Pakistan

Note: IUCN: International Union for Conservation of Nature, CITES: Convention on International Trade in Endangered Species of Wild Fauna and Flora, WPA: Wild Life (Protection) Act, 1972 of India; Categ.: Category, App.: Appendix & Sch.: Schedule.CR, Critically Endangered; EN, Endangered; VU, Vulnerable, NT, Near Threatened; LC, Least Concern.

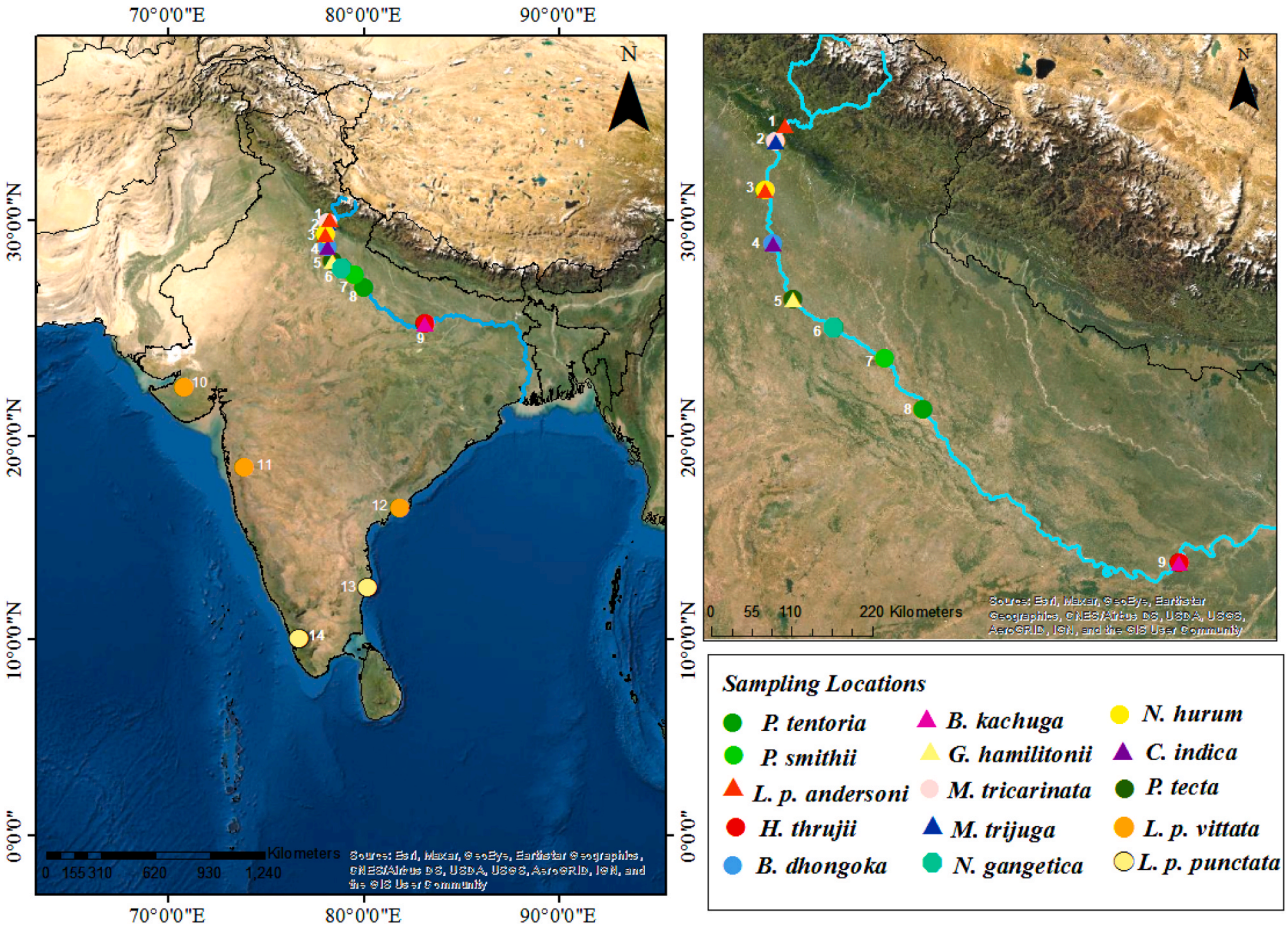

Fig. 1. Map showing the sample collection sites of Gangetic turtles (S. No. 1–9) along with the sequences used from other localities (S. No. 10–14).

Cmos1: 5′-GCCTGGTGCTCCATCGACTGGGATCA-3′ and Cmos3: 5′-GTAGATGTCTGCTTTGGGGGTGA-3′ [32]. PCR reactions were performed in Veriti™ Thermal Cycler, 96 well (Applied Biosystems) machine in a 20 μl reaction volumes with PCR buffer (10 mM Tri–HCl, pH 8.3, and 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 2 pmol of each primer, 5.0 U of Taq DNA polymerase (Thermo Fisher Scientific) and 1 μl (~ 50 ng) template DNA. All reactions were run along with negative controls. The PCR conditions were 95 °C for 5 min followed by 35 cycles at 95 °C for 45 s, annealing 54 °C (cyt *b* gene) 56 °C (Cmos gene) for 45 s and extension 72 °C for 1 min, with a final extension of 72 °C for 10 min. To monitor successfulness and possible contamination of the PCR, positive and negative controls were included. The amplified PCR amplicons were visualized in UV light on 2% agarose gel (UltraPure™ Agarose, Thermo Fisher Scientific) stained with green stain (Cyanagen) dye. Exonuclease I (EXO-*I*) and shrimp alkaline phosphatase (FastAP) (both Thermo Fisher Scientific) treatments were given to the amplified PCR products for 15 min each at 37 °C and 80 °C, respectively, to eliminate any residual primer. The amplified PCR products were sequenced from both directions using BigDye® Terminator cycle sequencing Kit v3.1 (Thermo Fisher Scientific) in the ABI 3500XL Genetic Analyzer (Applied Biosystems). The quality of generated sequences was visualized in SeqA v6 (Applied Biosystems).

2.3. DNA polymorphism analysis

The mitochondrial cyt *b* and nuclear Cmos genes were sequenced for 64 samples. We also included 14 sequences of cyt *b* gene of *Lissemys punctata vittata* (n = 5), *Lissemys punctata punctata* (n = 5) and *Batagur baska* (n = 4); and two sequences of Cmos gene of *Batagur baska* from the GenBank (Supplementary Table ST1) [33–36]. The raw sequences were aligned using CLUSTAL X multiple sequence alignment [37] and edited with SEQUENCHER® v4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). We used the Indian tent turtle's complete cyt *b* gene sequence of a voucher specimen (*Pangshura tentoria*, Accession number: MF432852) for mtDNA cyt *b* gene and Cmos sequence of Indian roofed turtle (*Kachuga tecta*, Accession number: EU030230) from GenBank to confirm the positions of SNPs [36]. The numbers of polymorphic sites (S) and haplotypes (h) were computed using the software DNASP v5.0 [38].

2.4. Genetic differentiation, phylogenetic tree and network analysis

For estimating genetic distance, we measured p-distance using a discrete Gamma distribution (TN92 + G, Tamura 3-parameter model with gamma distribution) with the lowest Bayesian information criterion (BIC) value using MEGA X [39]. MEGA X was used to estimate within-group genetic distance and between-group mean distance among the turtle species [40]. Phylogenetic analyses were conducted in BEAST v1.8 [40]. The Bayesian inference analysis was performed with MCMC chains for 10 million generations, sampling one tree at every 1000 generations. The first 10% per run was discarded as burn-in. The resultant phylogenetic trees were visualized in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). The spatial distribution of haplotypes was visualized by a median-joining (MJ) network created with the PopART software v1.7 [41].

3. Results

The complete cyt *b* and a partial fragment of the nuclear Cmos gene

were generated from all the samples. After sequencing, the exact lengths of cyt *b* and Cmos genes were 1140 bp, and 602 bp, respectively. Newly generated sequences were deposited in GenBank accession numbers (MT939525-MT939583, MT465671-MT465675 for mtDNA cyt *b* and MT939584-MT939647 for nuclear Cmos gene) (Supplementary Table: ST1).

3.1. Species-specific SNPs in cyt b and Cmos genes

The highest numbers of unique sites were found in *C. indica* followed by *G. hamiltoni*, *L. punctata* , *M. tricarinata*. We mapped four unique sites in *B. dhongoka, H. thurjii, M. trijuga, N. hurum* and *N. gangetica*, and three unique sites in *B. baska*. In the genus *Batagur*, most SNPs were observed in *B. dhongoka,* followed by *B. baska* and *B. kachuga*. *P. tentoria*, *P. tecta* and *B. kachuga* each had a singular, unique identifying site. In Trionychidae, a maximum number of unique SNPs was found in *L. p. andersoni,* followed by *L. p. vittata* and *L. p. punctata* (Table 2 and Supplementary Table ST2). In contrast to the mitochondrial gene in identifying species, the nuclear Cmos gene also showed unique SNPs in *C. indica*, *B. kachuga*, *H. thurjii*, *L. p. andersoni* and *N. hurum*; and a single variable site was present in *B. dhongoka*, *B. baska*, *G. hamiltonii* and *M. trijuga*. However, *P. smithii*, *P. tecta* and *P. tentoria* could not be differentiated using the Cmos gene, and genus *Pangshura* showed only one characteristic SNP (Table 2 and Supplementary Table: ST3).

3.2. Phylogenetic relationship and network analysis

The cyt *b* based phylogenetic tree and haplotype distribution network showed two significant clades: Geoemydidae and Trionychidae. The clades discriminated hardshell from softshell turtles forming distinct clusters with their respective species with high posterior probability (PP). In clade Geoemydidae, genus *Batagur* was clustered with genus *Pangshura* (PP ~ 0.60) followed by *Hardella* (PP ~ 1) while *Geoclemys* and *Melanochelys* formed the paraphyletic clade. In genus *Pangshura*, *P. smithii* and *P. tentoria* clustered together; similarly, *B. kachuga* and *B. baska* showed a close relationship in genus *Batagur*. In clade Trionychidae, the genus *Nilssonia* clustered with *Chitra*, followed by genus *Lissemys* (PP ~ 1). Among the three sub-species of *Lissemys punctata*, *L. p. andersoni* (spotted northern Indian flapshell turtle) and *L. p. vittata* (unspotted, central Indian flapshell turtle) were closer than *L. p. punctata* (unspotted, southern Indian flapshell turtle) (Fig. 2).

The phylogenetic tree based on the Cmos gene also indicated that among Geoemydidae clade, *Batagur* was close to *Hardella* (PP ~ 0.99) and clustered with *Pangshura* genus with no clear structuring to differentiate among the *P. smithii*, *P. tecta* and *P. tentoria* (PP ~ 1) clades. In contrast to cyt *b* based phylogeny, *B. baska* showed a close relationship with *B. dhongoka* and *H. thurjii* formed sister clade. Consistently, *G. hamilitonii* and *Melanochelys* formed the paraphyletic clade. In clade Trionychidae, genus *Nilssonia* clustered with genus *Chitra* (PP PP ~ 1), followed by *Lissemys* clade (PP ~ 1) (Fig. 3). The haplotypes distribution network also showed the same structuring pattern within the Geoemydidae and Trionychidae (Figs. 4 and 5).

Table 2
Signature SNPs of Gangetic turtles based on cyt *b* gene with reference to *Pangshura tentoria* (Accession number MF432852) and Cmos gene with reference to *Kachuga tecta* (Accession number EU030230).

Species	Signature SNPs cyt *b*	Signature SNPs Cmos
P. tentoria	0081	564
P. smithii	0096, 0504	
P. tecta	0375	
H. thurjii	0345, 0378, 0411, 0474	111, 175
B. dhongoka	0342, 0360,0369, 0390	222
B. kachuga	0537	079, 246, 324, 441, 526,
B.baska	0429, 0492, 0852	367
G.hamiltonii	0013, 0018, 0030, 0105, 0130, 0174, 0186, 0231, 0300, 0321, 0465, 0528, 0531, 0552	384
M. tricarinata	0222, 0228, 0235, 0399, 0479, 0561	042, 135, 308, 325, 355, 399[a]
M. trijuga	0014, 0050, 0334, 0339, 0358, 0549	
N. gangetica	0213, 0501, 0504, 0556	041[b], 063,072, 186, 210, 294,
N. hurum	0053, 0066, 0327, 0411, 0489, 0553	510[b]
C. indica	0007, 0024, 00120, 00130, 0132, 0144, 0213, 0247, 0249, 0268, 0279, 0309, 0318, 0327, 0393, 0414, 0510	057, 269, 255, 420, 456
L.punctata	0023, 0033, 0087, 0123, 0179,0181, 0182, 0235, 0247, 0279, 0289, 0324, 0381, 0525, 0530	–
L. p. andersoni	0022, 0351, 0360, 0420, 0552	042, 072, 081,086, 141, 182, 227, 302,307, 311, 340, 351, 357, 364, 390, 405, 408, 424, 457, 473, 522, 535, 537, 538, 544, 567
L. p. vittata	0054, 0339, 0565	–
L. p. punctata	0126, 0294	–

[a] Differentiating SNP between *M. tricarinata* and *M. Trijuga*.
[b] Differentiating SNP between *N. gangetica* and *N. hurum*.

3.3. Genetic differentiation

The mean pairwise genetic differentiation based on cyt *b* gene supported the clade pattern where values of Geoemydidae ranged from 5.7% for *P. tentoria* and *P. smithii* to 17.6% for *P. tecta* and *M.trijuga.* The genetic distance between *B. dhongoka* and *B. kachuga* was 9%, and between *B. dhongoka* and *B. baska* was 8.2%, while it was less between *B. kachuga* and *B. baska* 4.8%. In addition, Trionychidae's values were 4.2% for *L. p. andersoni* and *L. p. punctata,* to 19.8% for *L. p. punctata -N. gangetica*, and *C. indica-L. p. vittata* (Supplementary Table ST4). The genetic distance based on the Cmos gene also indicated significant differentiation within Geoemydidae. Though *P. tentoria* and *P. smithii* showed no differentiation, *B. kachuga* and *M. trijuga* indicated a differentiation estimate of 3.7%. In contrast to cyt *b* based genetic differentiation, nuclear gene showed higher genetic distance (1.4%) between *B. kachuga* and *B. baska*; however, it was low (0.5%) between *B. dhongoka* and *B. baska.* In Trionychidae, *N. gangetica* and *N. hurum* differed by 0.6%, whereas *N. hurum* and *L. P. andersoni* differentiated by 7.0% (Supplementary Table: ST5).

4. Discussion

To estimate and address the true extent of the illegal trade in the turtles species, lineage identification is of fundamental importance. Hence, we generated the complete cyt *b* gene and partial sequences of Cmos gene for the Gangetic turtles. We report for the first time Cmos gene sequences for *P. tentoria*, *M. tricarinata*, *C. indica* and *L. punctata* ; and cyt *b* gene sequence of *M. tricarinata*. Based on the phylogenetic trees, we assessed the phylogenetic relationships among the Gangetic turtles. Both the cyt *b* and Cmos genes indicated the close genetic relationship of *Batagur* genus with *Pangshura* and *Hardella* in the hardshell Geoemydidae turtles. Similarly, both the mitochondrial and nuclear genes attested to close affinity of *Nilssonia* with *Chitra* followed by *Lissemys* genus in the softshell Trionychidae. Interestingly, while the cyt *b* gene differentiated among *P. smithii*, *P. tecta* and *P. tentoria,* the Cmos gene could not, thus highlighting the conserved mitochondrial marker's utility over the nuclear gene for efficient species-level resolution. It is noteworthy that although the Cmos gene could not differentiate among the *Pangshura* species based on phylogeny, the species-specific signature database identified haplotypes unique to the genus. Moreover, both the genes showed unique signature SNPs and differentiated among in *B. dhongoka B. baska* and *B. kachuga*. Further, cyt *b* based genetic distance and phylogenetic tree analysis showed *B. baska* was close to

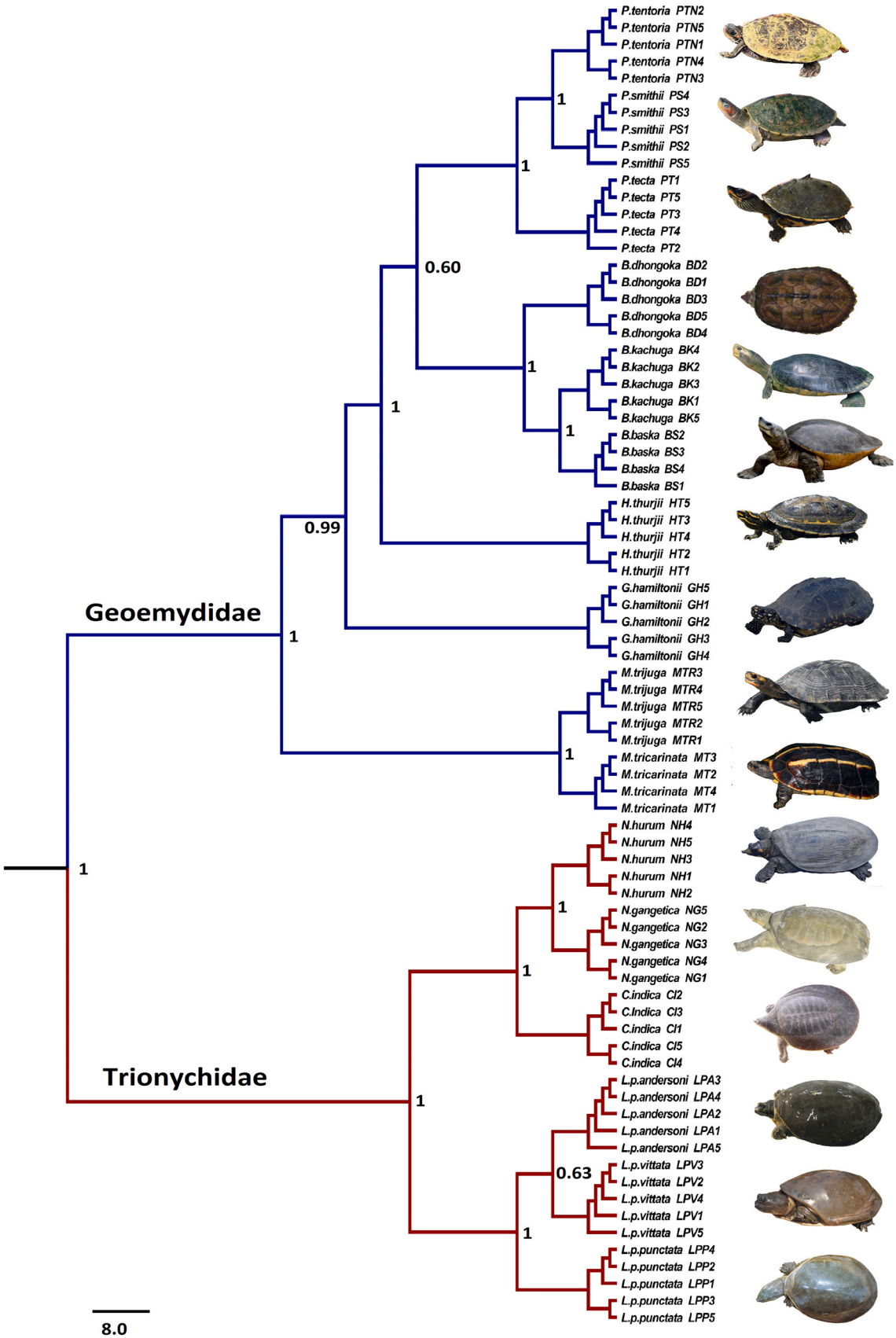

Fig. 2. Phylogenetic tree of hardshell and softshell turtles of Ganga river based on mtDNA cyt *b* gene (Images were obtained from the published literature/online sources as *Melanochelys tricarinata* by Harikrishnan S.; *Lissemys punctata vittata* by Shailendra Singh; *Lissemys punctata* punctate by Peter Praschag).

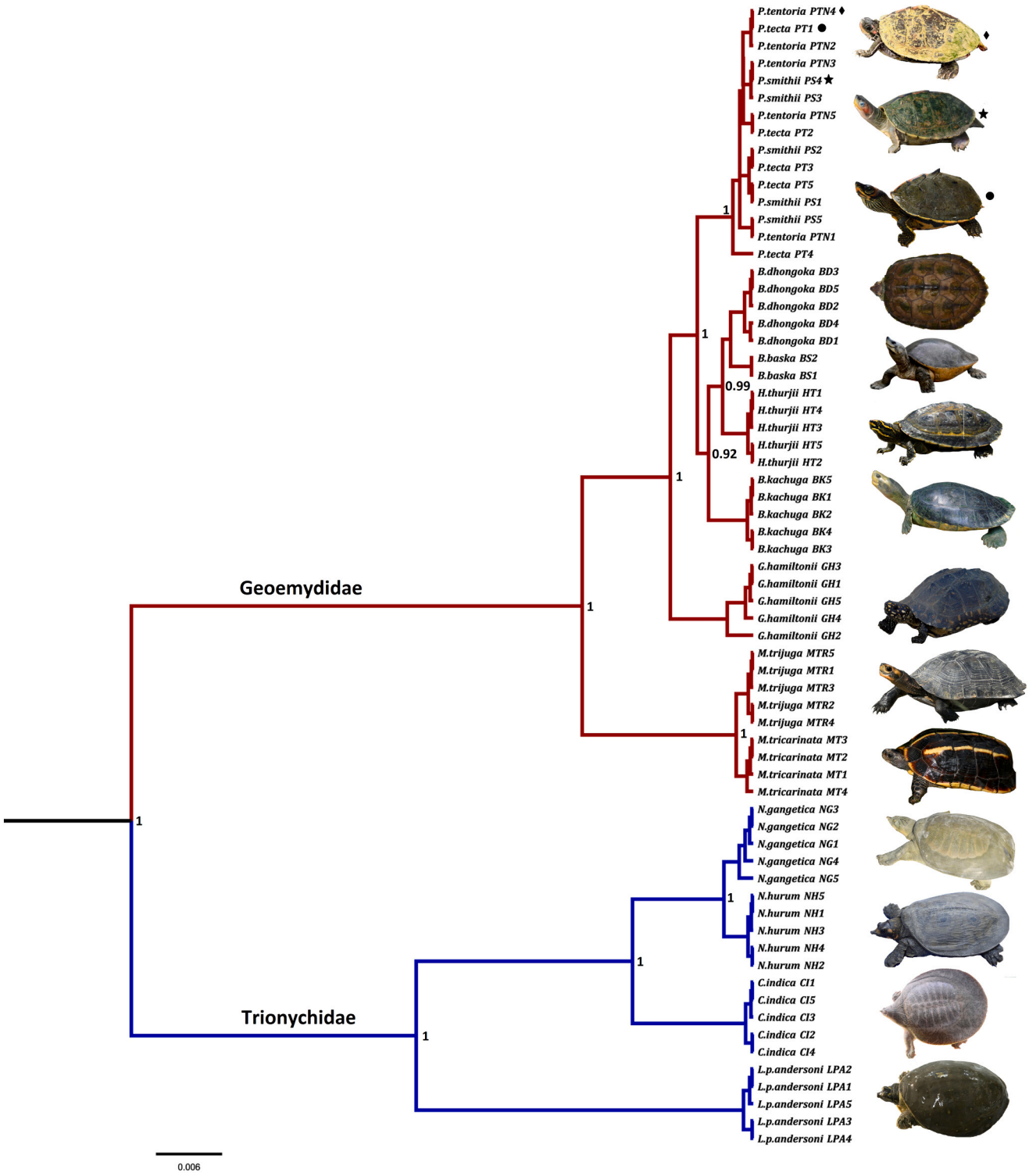

Fig. 3. Phylogenetic tree of hardshell and softshell turtles of Ganga river based on nuclear Cmos gene (Image of *Melanochelys tricarinata* was obtained from the published literature/online source by Harikrishnan S.).

B. kachuga, while results of Cmos gene showed *B. baska* was close to *B. dhongoka*. The genetic database elucidated numerous informative sites in cyt *b* and Cmos genes to identify the Gangetic turtle's species distinctly. Both the genes demonstrated multiple singleton sites, parsimony informative sites and separated the hardshell from softshell clades.

The SNP database's characterization indicates the robustness of mitochondrial cyt *b* and nuclear Cmos genes to identify threatened Gangetic turtle species. However, cyt *b* gene showed a higher number of signature SNPs and was more efficient than Cmos gene for the identification and differentiation of species. Our data also showed that the available GenBank sequences (FR850621- FR850636) of *L. p. punctata* and *L. p. vittata* do not correspond to their geographic distribution ranges. The distribution of *L. p. punctata* limits the southernmost part of India, only in the state of Kerala and Tamil Nadu, while *L. p. vittata* widely distributed in central to peninsular India. The sequences of *L. p.*

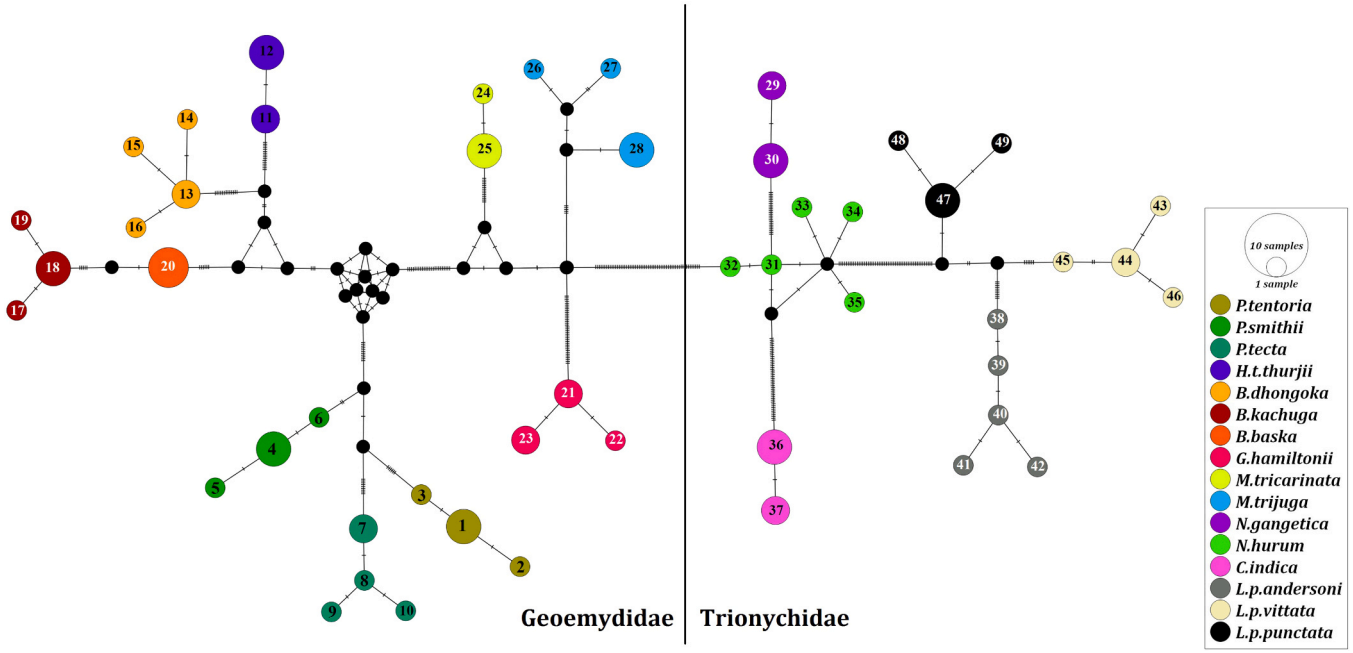

Fig. 4. Haplotype distribution among hardshell and softshell turtles of Ganga river based on mtDNA cyt *b* gene.

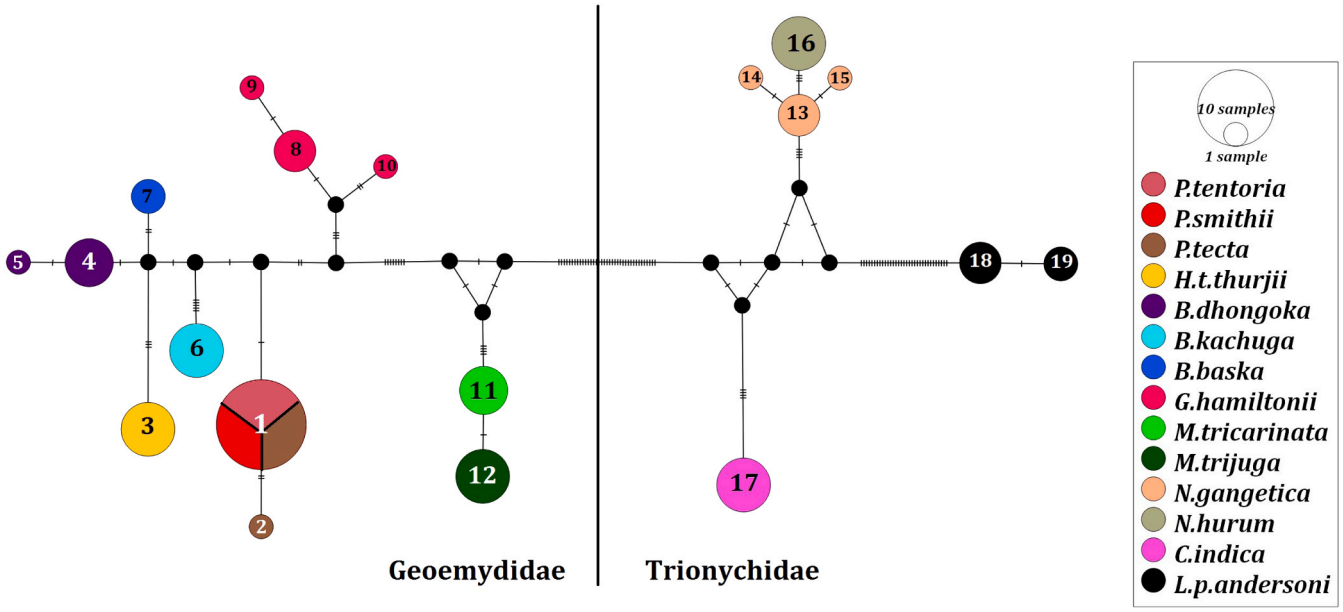

Fig. 5. Haplotype distribution among the hardshell and softshell turtles of the Ganga river based on the Cmos gene.

punctata from Kerala and Tamil Nadu may have been wrongly submitted as *L. p. vittata* (FR850632- FR850634, FR850642- FR850644), whereas sequences of the *L. p. vittata* may have been mentioned as *L. p. punctata* (FR850621-FR850631, FR850635-FR850636) in NCBI GenBank [33]. Hence, validated species-specific data are helpful in differentiating the subspecies of *Lissemys punctata.*

Moreover, our data suggest that *Melanochelys tricarinata* (JN202622) sequence was inaccurately deposited in GenBank, which matched with our generated sequence of *Melanochelys trijuga.* Therefore, caution needs to be taken while using these sequences with proper confirmation of the source of sample origin. The homologous sequences available at GenBank must be downloaded with care to avoid invalid results. Thus, after evaluating the sample in the query in GenBank for homologous sequences, it should be further reconfirmed with the in-house database generated by the user using an authentic sample source [12,28,42]. It can eliminate the probability of invalid results and identify the questioning sample's species with absolute assurance, which is an absolute requirement for forensic validation and reporting. Identification of unknown evidence samples with the help of SNPs analysis can be executed only when the gene sequences of the unidentified sample were introduced among a set of the reference sequence [28,42].

5. Conclusion

DNA-based methods hold promise in evaluating and analyzing the relatedness of threatened species such as turtles and their conservation. Our study highlighted the significance of species-specific variations to correctly identify the Gangetic turtles using mtDNA cyt *b* and nuclear

Cmos genes. We report unique haplotypes, nucleotide sites, genetic differentiation and phylogenetic relationships among the Gangetic turtles. We observed higher mtDNA cyt *b* nucleotide variations compared to the nuclear *Cmos* gene among studied Gangetic turtles. The identified signatures of mitochondrial and nuclear genes can play a pivotal role in wildlife forensic to identify the highly poached species. A robust database will help to aid conservation and management strategies and even guide release and rehabilitation protocols.

Financial disclosure

This study was funded by the National Mission for Clean Ganga (NMCG), Department of Water Resources, River Development & Ganga Rejuvenation, Ministry of Jal Shakti, Government of India through Grant number B/02/2015-16/1259/NMCG. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CRediT authorship contribution statement

SKG conceptualized the methodological framework of the genetic component of the project and designed the experiment. SAH developed the project and acquired funds and permission for conducting the study. PY, and AK collected the biological samples. PY, AK, and NY performed wet lab work and prepared maps. PY, AK, SS and SKG performed the statistical analysis and wrote the manuscript. All authors approved the final version of the paper.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by the Ministry of Jal Shakti, Government of India. We acknowledge the support provided by the Dr. Dhananjai Mohan, Director and Dr. Y.V. Jhala, Dean, WII, and the entire project team of NMCG. We thank the State Forest Departments of Uttarakhand and Uttar Pradesh for their support. We also acknowledge Dr. Abhijit Das for providing photographs of *Pangshura smithii* and Ms. Anuja Mital for providing photographs of *Batagur Kachuga*, *Hardella thurjii*, *Melanochelys trijuga* and *Geoclemys hamiltonii.*

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsiae.2021.100035.

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