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# Taxonomic rank of Indian tortoise: revisit with DNA barcoding perspective

## Abstract

Taxonomic rank of terrestrial tortoise inhabiting in India and adjacent countries has been an unresolved issue. Wild living species of *Manouria* and *Indotestudo* genera have been classified through conventional taxonomy, but not adequately discriminated by mitochondrial cytochrome *b* gene. Cytochrome oxidase (COI) marker has been quite successful to achieve the exact species level information. There needs of an accurate sequence based effort for the extant northeast (NE) Indian tortoises to identify them accurately and to provide locality information. To estimate the Kimura-2-Parameter (K2P) divergences and to construct the Neighbour-Joining (NJ) phylogeny, we generated six partial COI sequences derived from expert-identified tortoise specimens. Both BLASTn and Bold-IDs revealed the definitive identity of *Manouria*, whereas the congeners of *Indotestudo* remain inconclusive by only a two-fold interspecific divergence gap with the other named Indian tortoise species. The NJ phylogeny readily differentiated the three Indian tortoise genera with their respective conspecifics but depicted a nonsynonomous group of *Indotestudo* congeners. This study shows that the molecular identification of *Manouria emys phayrei*, *Indotestudo elongata* and an allopatric population of *Indotestudo forstenii* in NE India corresponds correctly with existing morphological key support.

## Keywords

Testudinidae • Northeast India • Morphology • Mitochondrial cytochrome oxidase (COI) • Distribution

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## Introduction

Turtles and tortoises are the oldest living animals on the earth and play important roles in the food chains and life cycles within their environment [1,2]. Most threatened tortoise species occur in Asia [3-6]. India is reportedly inhabited by four tortoises, among them *Indotestudo elongata* and *Manouria emys* in the northeastern regions [7,8]. These land tortoises are the subject of many recurrent taxonomic controversies about their sporadic distributions. Also, all the land tortoises inhabiting in the Northeast (NE) India have been listed in Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), Indian Wildlife (Protection) Act, 1972 (IWPA) and International Union for Conservation of Nature (IUCN), yet they are still poached [9], despite being the subject of lists e.g., CITES drawn up to protect wildlife against overexploitation and to prevent international trade from threatening species [10]. Therefore, it is a burning issue to protect the land tortoises of NE India before they reach the verge of extinction.

The Asian brown tortoise, *Manouria emys* is classified into two subspecies based on morphological key characters and distinct geographical distribution [11,12]. The other terrestrial elongated

tortoise, *Indotestudo elongata* was selected as type species of the new subgenus *Indotestudo* [13]. Later on, cladistic analyses promoted *Indotestudo* to full generic rank, and it was reported as comprising of three species after re-examining the morphological key characters [14-16]. Furthermore, morphological as well as molecular data depict the presence of *Indotestudo elongata* in Arakan and Western Myanmar, *Indotestudo travancorica* in Western Ghats of South-Western India, and *Indotestudo forstenii* in Sulawesi and the adjacent island of Halmahera, so the species can be regarded as “Endemic in Indonesia” [17,18]. However, species identification and locality information obtained through the traditional taxonomy and limited molecular markers still appears intuitive.

Accurate species-level identification is strictly essential prior to the development of conservation application [19]. The identification helps to understand the species-specific life history and distribution patterns [20-22]. DNA barcoding of world's biodiversity has received momentum with the objective to enrich the Barcode of Life Data System (BOLD), a global reference library [23]. The barcoding technique utilizes a partial Cytochrome Oxidase c subunit I (COI) gene as a molecular marker that has been proven instrumental in identification at the species level

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[24-28]. Distance based cladistic approach has furnished better resolution in species discrimination where the similarity search failed to identify the species correctly [29,30]. This study was intended to i) examine the taxonomic key character of *Manouria emys*, *Indotestudo elongata* and *Indotestudo forstenii* ii) analyze the species-level discrimination ability of partial COI within *Manouria* and *Indotestudo* genera.

## Methods

The land tortoises inhabiting in the wild habitat of NE India were identified through previously reported morphological key characteristics [7,8,12,16-18]. Morphological characters along with photographs were recorded, and the required amount of blood samples were collected from the right hind limb of different individuals and subsequently stored in the buffer at 4°C. The investigators acquired permission to conduct the research under section 28 of the Wildlife (Protection) Act, 1972 (as amended up to date) from the Chief Conservator of Forest (Wildlife) Assam. The population of the target group of animals is very scarce in the nature, so this study relied solely on judicious sampling.

Genomic DNA was extracted from each of the blood samples using standard phenol-chloroform extraction method and subsequently precipitated with a double volume of chilled ethanol [31]. Thereafter, the extracted DNA was dissolved in 200µl nuclease-free water and stored at (-80)°C. The barcode segment was amplified using the published primer pair [32]. The 25 µl PCR reaction mixture contains 10 pmol of each primer, 25-50 ng of DNA template, 1x PCR buffer, 1.0-1.5 mM of MgCl<sub>2</sub>, 0.25 mM of each dNTPs, and 0.25 U of high-fidelity *Taq*DNA polymerase (Applied Biosystems Inc., CA, USA). Amplification was performed using a Veriti® Thermal Cycler (Applied Biosystems Inc., CA, USA). The PCR products were extracted using QIA quick<sup>®</sup> Gel extraction kit (QIAGEN, Cat. No. 28704, USA) and sequenced bi-directionally using automated DNA sequencer (ABI3500, Applied Biosystems Inc., CA, USA).

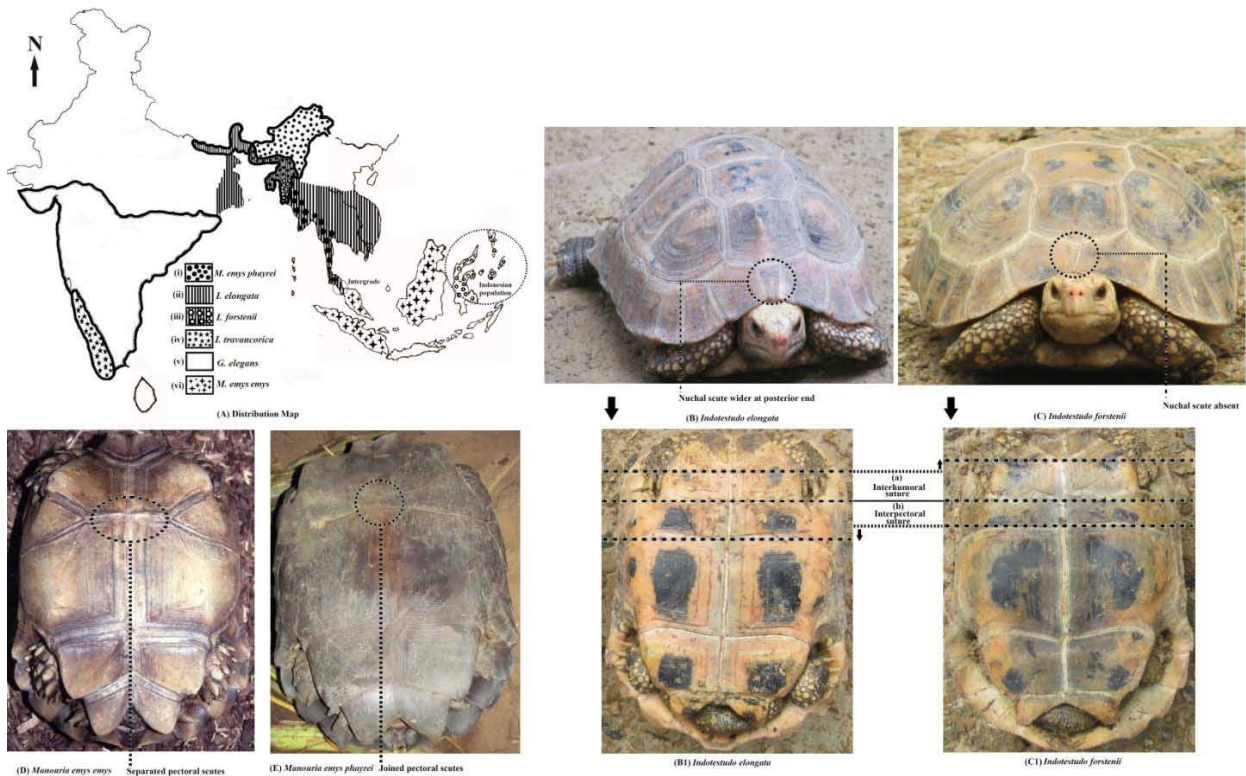
The sequences were screened and retrieved from each of the bidirectional sequences by using the Sequence Scanner Software v1.0. Applied Biosystem and ~2% overlap bases were discarded. The developed nucleotide sequences were checked by BLASTn searches and Bold-IDs, and correct amino acid sequences were determined by checking the open reading frames (ORF) using ORF prediction by online software and subsequently submitted to GenBank. Six self developed sequences and 12 database sequences were incorporated for the comparison of congeneric and conspecific divergences of the Indian tortoise taxa. All the sequences were aligned to form combined data set of 18 sequences using the ClustalX program [33]. In order to follow the recommended criteria of distance based phylogeny, the Kimura-2-Parameter (K2P) models for nucleotide substitutions of the studied species was evaluated using MEGA 5 [34,35]. Pairwise nucleotide divergences were calculated using the K2P model and the Neighbour- Joining (NJ) tree on ~627 bp was constructed using the *defacto* K2P model with 1000 replicates.

## Results

The collected wild living individuals having the joined pectoral scute were taxonomically depicted as *Manouria emys phayrei*, a subspecies of *Manouria emys* (Figures 1D,E). The three individuals of *Indotestudo* from NE India (23.36 N 93.00 E) were identified as *Indotestudo elongata*, with the presence of nuchal scute and the ratio of interhumeral suture to interpectoral suture being 0.87 (Figures 1B,B1). One individual of *Indotestudo* collected from the same site appeared to be *Indotestudo forstenii*, with the absence of nuchal scute and the ratio of interhumeral suture to interpectoral suture being 1.66 (Figures 1C,C1).

Table 1 shows the comprehensive identification results based on GenBank and BOLD databases as well as the existing status of the Indian tortoise species. The developed sequences of *Manouria emys* showed 90% - 93% identity with their singlet congeners, *Manouria impressa* and 90% maximum identity with nearest neighbor (NN) genus *Chelonoidis* in both GenBank and BOLD databases. The genus *Indotestudo* and its two congeners remained inconclusive in both BLASTn and Bold-IDs identity results. The three developed sequences of *Indotestudo elongata* showed 96% - 99% identity with the first NN congeners and 94% - 97% with the second NN congeners in both databases. However, the acquired sequences of *Indotestudo elongata* from GenBank showed 96% - 99% identity with the first NN congeners in both databases but showed 97% identity with the second NN congeners only in Bold-IDs. Furthermore, the developed sequence of *Indotestudo forstenii* showed 96% - 97% identity with the first NN congeners and 97% with the second NN congeners in both databases. The acquired sequence of *Indotestudo forstenii* found 96% identical with the second NN congeners only in Bold-IDs but no match in GenBank. The two studied species of *Indotestudo* also showed 92% identity with nearest neighbor (NN) genus *Chelonoidis* in both GenBank and BOLD databases.

In the dataset, the interspecific K2P divergences were in the range of 1.43% ± 0.004 to 15.75% ± 0.017 within all the named Indian tortoise species (Table 2). The *Indotestudo* and *Manouria* genera showed 1.43% ± 0.004 to 5.31% ± 0.009 and 10.05% ± 0.013 congeneric divergence with their relevant respective congeners. *Geochelone elegans*, one extant species of India shows 12.92% ± 0.016 to 14.85% ± 0.017 and 15.24% ± 0.017 divergences from *Indotestudo* and *Manouria* genera respectively. The intraspecific K2P divergences were 1.86% ± 0.004 for *Manouria emys*, 0.44% ± 0.001 for *Manouria impressa* and 1.12% ± 0.002 for *Indotestudo elongata* yet, the intraspecific divergences were not calculated for *Indotestudo travancorica*, *Indotestudo forstenii* and NE Indian *Indotestudo forstenii* due to the lack of barcode sequences in databases as well as in the current study. The named Indian species *Geochelone elegans*, *Manouria emys* and its congeners *Manouria impressa* showed a minimum of 10.05 ± 0.013 interspecific divergences, that holds two fold gap with the interspecific divergences generated by the congeners of *Indotestudo* genus.



**Figure 1.** A distribution map of *Manouria* and *Indotestudo* genus, figure not to scale (A). Key character distinction between *Indotestudo elongata* (with Nuchal Scute) (B) and *Indotestudo forstenii* (without Nuchal Scute) (C). The ratio of Interhumeral to Interpectoral Suture is 0.87 in *Indotestudo elongata* (B1) and 1.66 in *Indotestudo forstenii* (C1). Morphological differences between *M. emys* having separated pectoral scutes (Photograph was collected from [www.dght.de/ag\\_schildkroeten/aktropland/bilder/084.jpg](http://www.dght.de/ag_schildkroeten/aktropland/bilder/084.jpg)) (D) and *M. emys phayrei* having joined pectoral scutes (E).

The two developed sequences of *Manouria emys* clustered separately from the GenBank *Manouria emys* sequences with 100 bootstrap supports. The NJ phylogeny readily discriminated the three studied genera of Indian tortoises into distinct clusters by their respective congeners and conspecifics with significant bootstrap supports. However, both the developed and database sequences of *Indotestudo elongata*, *Indotestudo travancorica* and *Indotestudo forstenii* showed nonsynonymous clustering (Figure 2).

## Discussion

The NE India region has been hitherto reportedly inhabited by two land tortoises viz., *Manouria emys* and *Indotestudo elongata*; while the Indonesian *Indotestudo forstenii* and south Indian *Indotestudo travancorica* were claimed both to be endemic through conventional taxonomy, supported also by molecular approaches [36]. Based on morphometric analysis, two distinct geographical populations of *Manouria emys* were given the rank of subspecies, viz., *Manouria emys phayrei* and *Manouria emys emys* (Figure 1D,E) [12]. The collected wild living individuals of *Manouria emys* from NE India exhibited morphological characters similar to those defined for the northern Asiatic subspecies *Manouria emys phayrei* (Figure 1E). BLASTn and Bold-IDs results for our query sequences found similarity with the complete

mitochondrial sequences of *Manouria emys* (Accession No. NC007693 and DQ080040; Specimen Voucher MVZ238129 of the Museum of Vertebrate Zoology and California Academy of Sciences). Accordingly, the generated barcode sequences have been submitted to GenBank with the name *Manouria emys*. The developed barcode sequences of *Manouria emys* ( $n=2$ ), however, exhibited no difference between themselves but exhibited  $1.86\% \pm 0.004$  intraspecific K2P divergence with database sequences ( $n=2$ ). Moreover, NJ tree with 100 bootstrap repetitions among the two data sets (developed and GenBank sequences) depicted a separate clade of *Manouria emys*. The observed genetic divergence between the two datasets of *Manouria emys* may perhaps relate to subspecies status as proposed previously through morphological studies [12]. Had the locality information of the database sequences of *Manouria emys* been given, it would have been easier to speculate about subspecies status with molecular evidence.

Furthermore, the congeners of *Indotestudo* reportedly inhabit distant geographical locations; among the congeners, *Indotestudo forstenii* [11] is the oldest named species and is believed to be restricted in Sulawesi, Indonesia; *Indotestudo elongata* [37] is distributed through East and North East India, Myanmar, LAOs, Thailand, Cambodia to Vietnam; and *Indotestudo travancorica* [38] is restricted in South India. As stated above, *Indotestudo travancorica* is difficult to

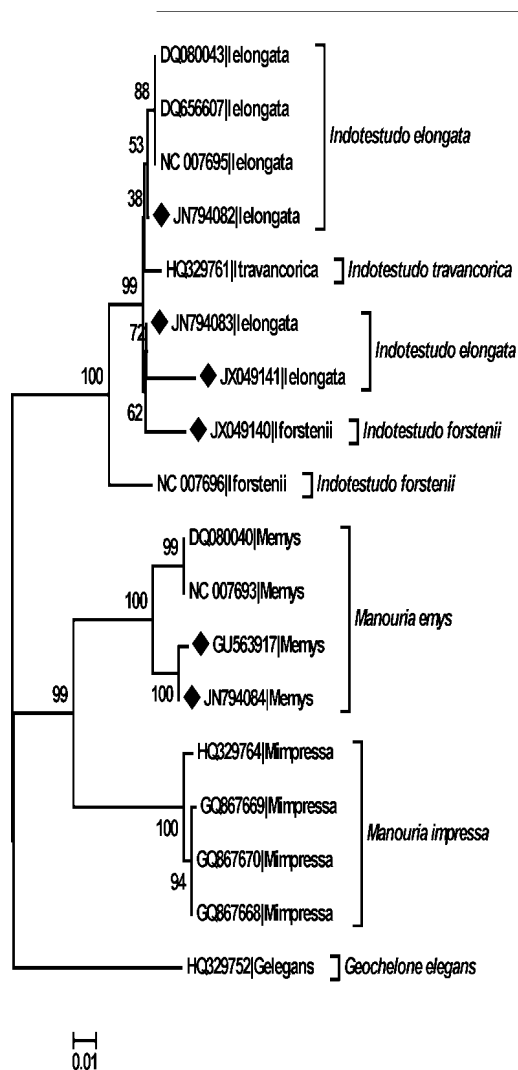
Table 1. Common names, species status, scientific name, accession number and database comparison of the Northeast Indian tortoise species.

Common Name	Status			Scientific Name	Accession No.	NN Congeners1 (% identity)				NN Congeners2 (% identity)				NN Different genus (% identity)																	
	CITES	IWPA	IUCN			90		95		100	90		95		100	85		90		95											
Asian forest tortoise or Asian brown tortoise	Appendix II	Schedule IV	Endangered	<i>Manouria emys</i>	GU563917	■	■										■	■	■	■											
					JN794084	■	■														■	■	■	■							
					NC_007693	■	■	■														■	■	■	■						
					DQ080040	■	■	■														■	■	■	■						
Elongated Tortoise	Appendix II	Schedule IV	Endangered	<i>Indotestudo elongata</i>	JX049141	■	■	■	■	■	■	■	■	■	■																
					JN794083	■	■	■	■	■	■	■	■	■	■	■	■	■	■												
					JN794082	■	■	■	■	■	■	■	■	■	■	■	■	■	■												
					*NC_007695	■	■	■	■	■	■	■	■	■	■	■	■	■	■												
					*DQ656607	■	■	■	■	■	■	■	■	■	■	■	■	■	■												
					*DQ080043	■	■	■	■	■	■	■	■	■	■	■	■	■	■												
						■	■	■	■	■	■	■	■	■	■	■	■	■	■												
Forsten's Tortoise	Appendix II	Schedule IV	Endangered	<i>Indotestudo forstenii</i>	*JX049140	■	■	■	■	■	■	■	■	■	■																
					*NC_007696	■	■	■	■	■	■	■	■	■	■	■	■	■													

Asterisk (\*) indicates the ambiguous identification for *I. elongata* and *I. forstenii* sequences from both developed and GenBank database. Blue bar indicates the BLASTn result and Gray bar indicates the Bold-IDs result. CITES=Convention on International Trade in Endangered Species of Wild Fauna and Flora, Appendix I= Most threatened species (No trade allowed), Appendix II=Species likely to be threatened due to unregulated trade (Trade is allowed through licensing). IWPA= Indian Wildlife Protection Act, 1972, IUCN=International Union for Conservation of Nature and Natural Resources, NN= Nearest Neighbor

**Table 2.** K2P divergence of Indian tortoise species. The bold letter indicates the intraspecific divergences within the studied species. n/c denoted for not calculated intraspecific data due to the lack sequences of the particular taxa in databases.

Indian Tortoises	<i>I. elongata</i>	(NE) <i>I. forstenii</i>	<i>I. travancorica</i>	<i>I. forstenii</i>	<i>M. emys</i>	<i>M. impressa</i>	<i>G. elegans</i>
<i>Indotestudo elongata</i>	1.12 ± 0.002						
(NE) <i>Indotestudo forstenii</i>	2.57 ± 0.005						
<i>Indotestudo travancorica</i>	1.43 ± 0.004	2.69 ± 0.006	n/c				
<i>Indotestudo forstenii</i>	4.06 ± 0.007	5.31 ± 0.009	3.99 ± 0.008				
<i>Manouria emys</i>	13.77 ± 0.015	15.75 ± 0.017	14.25 ± 0.016	14.48 ± 0.017	<b>1.86 ± 0.004</b>		
<i>Manouria impressa</i>	14.39 ± 0.016	15.35 ± 0.017	14.75 ± 0.016	15.00 ± 0.017	10.05 ± 0.013	<b>0.44 ± 0.001</b>	
<i>Geochelone elegans</i>	14.02 ± 0.016	14.85 ± 0.017	14.71 ± 0.017	12.92 ± 0.016	15.24 ± 0.017	15.24 ± 0.017	n/c



**Figure 2.** NJ Phylogeny of Indian tortoise species. Numbers along branches indicate the bootstrap values. Black diamonds indicate the developed sequences for the current study.

distinguish, so it was once considered synonymise species of *Indotestudo forstenii* [39]. Further, the previous hypothesis that recognized Indonesian *Indotestudo forstenii* as being

distinct from *Indotestudo elongata* appears intuitive. Although the wild living *Indotestudo* species are difficult to tell apart by morphological characteristics, the existing taxonomical characters supported the collected specimens as *Indotestudo elongata* (Figures 1B,B1) and *Indotestudo forstenii* (Figures 1C,C1), indicating that they inhabit the boundaries of Assam and Mizoram (23.36 N 93.00 E). However, morphological variations among the congeners of *Indotestudo*, predominantly in the length of interhumoral suture relative to interpectoral suture as observed in this study (Figures 1B1,C1), may relate to ecotypic variation among distant populations of the same species [40-43]. This study has given the morphological key evidences of *Indotestudo forstenii*, with existence in NE India eliminating the prevailing hypothesis of endemisms in Indonesia. In the study, it is further noted that the nuchal scute, an identifying character in *Indotestudo elongata*, does not remain always narrow but varies in width (posterior end wider than the anterior end) among different individuals at different life stages.

The perplexing species *Indotestudo elongata*, *Indotestudo forstenii*, and *Indotestudo travancorica* exhibited morphological differences, but displayed less distinctive genetic divergences at the species level in barcode sequences. The NJ phylogeny reflects nonsynonymous clustering by all the congeners of *Indotestudo*, which had the least interspecific divergences from the other species, and which violated the sequence divergence criteria for distinct species, as reported for several other turtles and tortoises [28]. It is evident in a few cases, however, that low rates of both molecular evolution and chromosomal rearrangement in turtles [42,45] may make hybridization possible by delaying the evolution of genetic barriers to reproduction. Further, because mitochondrial genes tend to accumulate differences at a rate several-fold slower in turtles than in other vertebrates, species considered 'recent radiations' will probably be nearly alike [28]. It is evident in this study that the congeners of *Indotestudo* are genetically very much identical at the COI partial gene segment. If, the replica sequences of *Indotestudo* congeners are enriched in the database from wide geographical ranges the synapomorphic character among the congeners would be substantiated and rectify the ambiguous species taxonomy.

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