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Unexpected lack of genetic and morphological divergence in a widespread tortoise – Phylogeography of *Indotestudo elongata*

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Abstract. The diverse climatic and geological history of Southeast Asia has been a driving force behind significant Pleistocene and Holocene range dynamics and the consequent biological diversification in the region. Among the species exhibiting wide distributions, extending across several well-known zoogeographic barriers, the Elongated Tortoise (*Indotestudo elongata*) is noted for its extensive variability in size, shape, and colouration. To examine phylogeographic differentiation within *I. elongata*, we analysed three mitochondrial gene fragments (ND4, cyt *b*, and COI) for 52 individuals including fourteen historic specimens and 25 sequences obtained from GenBank. Phylogenetic analyses of the concatenated alignment revealed genetic homogeneity across the range, supported by a shallow mean uncorrected p distance of 1.2% in cyt *b*. This corresponds to a surprising lack of biogeographic structuring. Concordantly, multivariate statistical analyses of morphometric and colouration-related characters of 166 adult tortoises (79 males and 87 females) from Myanmar, Thailand, Cambodia, and Vietnam showed no statistically significant differences between biogeographic units. The lack of any phylogeographic signal and morphological differentiation despite the presence of significant biogeographic barriers is unusual for a terrestrial vertebrate from this region. Archaeological evidence suggests that Elongated Tortoises have been used as a food item for millennia. It could be that their current wide distribution and the absence of genetic differentiation are the result of a significant range expansion caused by extensive human-mediated dispersal over the prehistoric and historic tortoise trade. The observed lack of phylogeographic structure considerably simplifies conservation measures such as conservation breeding, reintroduction, and population reinforcement.

Key words. Testudines, Testudinidae, biogeography, geographic variation, historical DNA, human-mediated transport, mitochondrial DNA, molecular genetics, morphology, museum specimens, Southeast Asia.

Introduction

The Indo-Burma biodiversity hotspot is among the most diverse regions on earth (Myers et al. 2000). The area's rich biodiversity is the result of its intricate tectonic, environmental, and geological past which facilitated significant Pleistocene and Holocene range dynamics and biological diversification (Woodruff 2010, Hall 2012). Repeated glacial cycles and associated climate and sea level fluctuations led repeatedly to the expansion and contraction of habitat types and the formation of land bridges (Voris 2000) that served as corridors for migrations. Tectonic processes created prominent mountain ranges and freshwater river systems that act as barriers to dispersal for reptiles and amphibians (e.g., Bain & Hurley 2011, Poyarkov et al. 2023). Phylogeographic research provided significant insights on how geographic barriers contributed to genetic divergence and speciation processes within many widespread amphibian (e.g., Stuart et al. 2006, Geissler et al. 2015, Wogan et al. 2016, Chen et al. 2018, Gorin et al. 2020), chelonian and squamate taxa in this region (e.g., Fritz et al. 2008, Wood et al. 2012, Ihlow et al. 2016b, Klabacka et al. 2020, Wagner et al. 2021, Grismer et al. 2022, Gong et al. 2023).

In this study, we examine the phylogeography of the widely distributed Elongated Tortoise, *Indotestudo elongata* (Blyth, 1854). The genus *Indotestudo* Lindholm, 1929 currently comprises three species: the Travancore Tortoise, *I. travancorica* (Boulenger, 1907), endemic to the Western Ghats in India; the Sulawesi Tortoise, *I. forstenii* (Schlegel & Müller, 1845), which is found in Indonesia on the island of Sulawesi and possibly on Halmahera; and the Elongated Tortoise, *I. elongata* (see TTWG 2021). Habitat loss and unsustainable exploitation for consumption and trade have led to a decline of at least 80% over the last 90 years, with slow recovery rates further exacerbating the decline. The Elongated Tortoise is listed as Critically Endangered and in need of effective conservation measures (Rahman et al. 2019).

The distribution of *I. elongata* includes parts of eastern India (states of Odisha, West Bengal, Jharkhand, and Bihar) and northern India (Uttar Pradesh and Uttarakhand) and ranges eastwards through Nepal, Bhutan, Bangladesh, and Indochina to the Guangxi Province of China, and southwards to peninsular Malaysia (Ihlow et al. 2016a, Khan et al. 2020, TTWG 2021). Across its range, *I. elongata* inhabits a variety of low- to mid-elevation habitats (Thirakhupt & van Dijk 1995, van Dijk 1998, Zug et al. 1998, Grismer et al. 2007, Das 2010, Platt et al. 2012), but is absent from elevations exceeding 795 m above sea level. The species' extensive range covers several geographic features that have been demonstrated to impede the dispersal of amphibians and squamates (e.g., Bain & Hurley 2011, Geissler et al. 2015, Poyarkov et al. 2023) including mountain ranges, like the Northwest Uplands, the Arakan-Joma Mountains, and the Annamite Range, but also significant river systems such as the Brahmaputra, the Ayeyarwady (Irrawaddy), the Thanlwin (Salween), the Mekong, the Chao Phraya, and the Red River that undoubtedly constitute dispersal barriers for terrestrial tortoises. Additionally, the distribution of *I. elongata* spans across biogeographic and climatic transition zones, such as the Isthmus of Kra and the Kangar–Pattani Line, which delineate Indochinese from Sundaic biota, representing significant divides between distinct climatic regions (Inger 1999, Hughes et al. 2003, 2011, Poyarkov et al. 2023). *Indotestudo elongata* exhibits a crepuscular activity pattern, aestivates during the dry season, and maintains relatively small home ranges, ranging between 0.7 and 70 ha in size (Ihlow et al. 2014, 2016a), suggesting limited activity and movement. Given these ecological traits, along with the species' vast but fragmented habitat, prominent zoogeographic barriers are assumed to restrict dispersal and gene flow, suggesting the presence of clear phylogeographic structuring. Thus, to examine whether geographic isolation has resulted in the formation of geographically distinct lineages in *I. elongata*, we compare phylogeography (mtDNA sequence variation) and external morphology against five predefined biogeographic units that account for biogeographic barriers. If differentiation exists, we expect that phylogeographic clades and morphological clusters match our biogeographic units.

Methods

Sampling

Legislative restrictions, such as the Convention on Biological Diversity (CBD) and, since 2014, the Nagoya Protocol on Access and Benefit-sharing, have made the acquisition of fresh genetic samples for research increasingly challenging, acting counterproductive both to research and conservation (Neumann et al. 2018, Prathapan et al. 2018, Fritz et al. 2022). Therefore, this study benefitted much from sequencing historic DNA (hDNA) from museum specimens. The challenges of DNA degradation, fragmentation, and contamination in preserved specimens necessitated the application of strict protocols specifically designed for ancient DNA (aDNA) analysis, and a clean room working environment (see detailed descriptions in Kehlmaier et al. 2023; however, for the present study Sanger sequencing approaches were used).

Blood and tissue samples of *I. elongata* were collected from thirteen living tortoises and fourteen museum specimens that were preserved for various times (Table S1). Sample sites cover a major portion of the species' distribution range (Fig. 1). In addition, a single sample of a living specimen of the closely related Indonesian *I. forstenii* was studied. Samples were preserved dry or in pure ethanol and kept at -80 °C until processing. All samples were exported to Germany prior to October 2014 when the 'Nagoya Protocol on Access and Benefit-sharing' took effect. Our sole sequence from Odisha, located on the Chota Nagpur Plateau in northeastern India, was processed in India.

Laboratory procedure

DNA was extracted from fresh samples using the Analytik Jena InnuPrep DNA Mini Kit or the InnuPrep Blood Kit (Analytik Jena GmbH) according to the manufacturer's instructions, but with overnight incubation in lysis buffer instead of 15 min. The three mitochondrial gene fragments cytochrome *b* (cyt *b*), the partial NADH dehydrogenase subunit 4 gene with adjacent genes coding for tRNA His, tRNA Ser, and tRNA Leu (ND4), and the barcoding gene cytochrome c oxidase subunit 1 (COI) were amplified and sequenced using standard primers, except for COI, where a newly designed reverse primer was used (Table S2). PCRs for fragments from fresh samples were performed in a 25 µl volume containing 2.5 µl Bioron PCR complete buffer (50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, 0.5% Triton X-100, pH 8.5), 1 unit of Taq DNA polymerase (Bioron), 0.2 mM of each dNTP (Fermentas), 0.4 µM of each primer, and 20 ng DNA. After the initial denaturation for 5 min at 94 °C, 35 cycles were performed with denaturing 45 s at 94 °C, annealing 30 s at 56 °C, and primer extension for 60 s at 72 °C, followed by a final elongation of 10 min at 72 °C. Extraction of DNA from historic specimens was performed in a clean room facility for aDNA. Lysis of tissue and DNA extraction were performed on separate workbenches. For historic samples, the sbeadex forensic kit (LGC Genomics) was used for DNA extraction according to the manufacturer's instructions, but with overnight incubation of tissue with proteinase K (instead of 10 min) and a reduced elution volume of with 50 µl instead of 100 µl. The PCR setup for these samples was also conducted in a cleanroom. The PCR mix for hDNA from museum specimens included Bioron PCR incomplete buffer (50 mM KCl and 10 mM Tris-HCl, 0.5% Triton X-100, pH 8.5), additional 4 mM $MgCl_2$, and

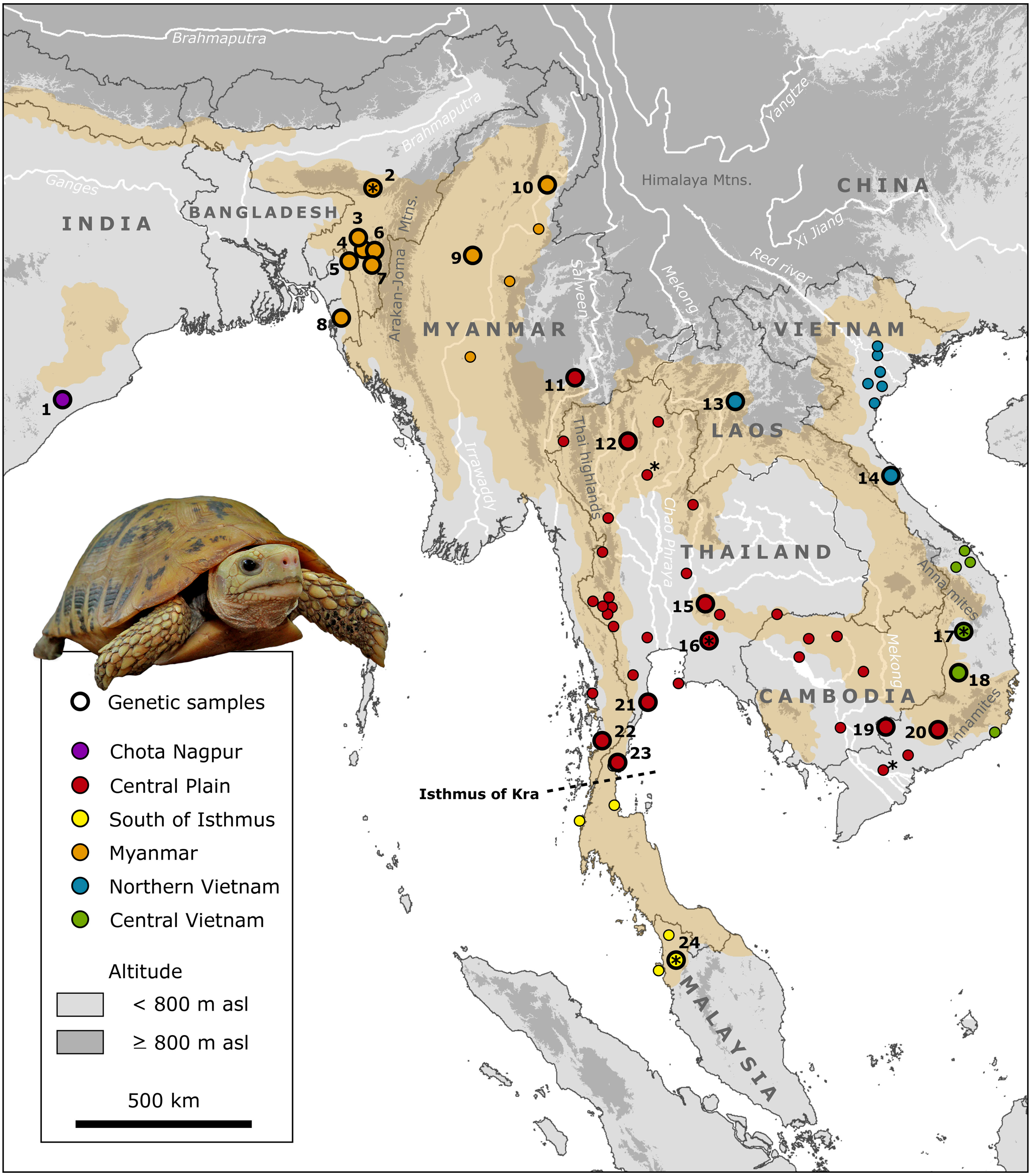

Figure 1. Distribution range of *Indotestudo elongata* (orange-shaded; according to TTWG 2021) and sampling sites (small coloured circles: morphological data, large coloured circles: genetic samples). Symbols with asterisks indicate imprecise locality information. Major rivers are labelled and shown as white lines.

10 µg of bovine serum albumin (Thermo Scientific). Otherwise, protocols and thermocycling conditions were identical as for fresh samples.

The alignment containing fresh samples was used to identify variable sites for each gene. Depending on their age, the historic samples were amplified in a series of short overlapping fragments of 160–270 base pairs (bp) each (representing the previously identified variable sites), using a set of taxon-specific internal primers designed for each gene based on sequences from fresh samples using OligoAnalyser 1.0.3 (Table S3). All PCR products were purified using ExoSap-IT enzymatic clean-up (GE Healthcare; adding 0.2 µl ExoSap-IT soluted in 0.8 µl H_2O to each sample; 37 °C for 30 min, 94 °C for 15 min) and sequenced on an ABI 3730 Genetic Analyzer (Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). For purification, 400 µl Sephadex (GE Healthcare; 1:20 dilution) per well in a Performa DTR V3 96-Well Short Plate was used (Edge Biosystems).

For 23 specimens, complete sequences for cyt *b* (1105 bp), ND4 (718 bp), and COI (1491 bp) were successfully obtained, whereas four samples yielded only partial sequences. European Nucleotide Archive (ENA) accession numbers and sampling sites are summarized in Table S1.

Genetic data analysis

Newly generated sequences were aligned in BIOEDIT version 7.2.5 (Hall 1999). The data set was supplemented with 31 analogous GenBank sequences, including 25 sequences uploaded as *I. elongata*, two as *I. travancorica*, and six as *I. forstenii* (Table S1). These mtDNA alignments were concatenated for phylogenetic calculations. Homologous sequences of *Testudo graeca* were extracted from a complete mitochondrial genome (GenBank accession number DQ080049) and incorporated as outgroup. The final concatenated dataset consisted of 3313 bp for 59 *Indotestudo* sequences (1105 bp for cyt *b*, 718 bp for ND4, and 1491 bp for COI) and was partitioned by gene and codon position. The optimal partition scheme and best-fitting nucleotide substitution models for Bayesian analyses were inferred using PartitionFinder 2 (Lanfear et al. 2016) and the Bayesian Information Criterion (BIC) (Table S4). Bayesian trees were calculated with MrBayes 3.2.7 (Ronquist et al. 2012) and the implemented Markov chain Monte Carlo (MCMC) algorithm. Two independent runs (each with four chains) were performed with 10 million generations each, sampling every 500th generation, until the average standard deviation of split frequencies fell below 0.01. Results of the MCMC runs were summarized and the initial 25% of each run were discarded as burn-in; parameters were examined using Tracer 1.7.1 (Rambaut et al. 2018). Maximum Likelihood trees were calculated using RAxML 8.2.10 (Stamatakis 2014) using the default GTR + G model for all partitions. Five independent fast bootstrap searches were conducted to compute ML trees, starting from distinct randomized Maximum Parsimony trees. Subsequently, thorough bootstrap replicates were plotted on the best tree. Trees were rooted with *T. graeca* (GenBank accession number DQ080049) as outgroup. Uncorrected p distances were calculated in MEGA X using the pairwise deletion option (Kumar et al. 2018).

Morphology

One hundred and sixty-six adult tortoises, corresponding to 79 males and 87 females, from Vietnam, Cambodia, Myanmar, and Thailand were examined for 42 morphometric and two colour-related characters. Metric characters of carapace and plastron were measured to the nearest mm using digital callipers for straight-line characters and measuring tape for curved measurements. The area of dark plastron and carapace pigmentation was determined as percentages of the total area using a colour threshold method in ImageJ (Abràmoff et al. 2004). As the carapace is highly domed in *I. elongata*, pigmentation of the third vertebral scute was used as a representative measure for the carapace. Using binary images, a threshold that fully captured the dark pigmentation was selected and subsequently used to process all images. For a complete list of examined characters and their respective contribution to the first four PCs, see Tables 1a, 1b, S5, and S6.

According to their provenance, specimens were assigned to biogeographic units (Fig. 1) following the biogeographic subregion concept by Bain & Hurley (2011). These groups are: 'Myanmar' (MY) with specimens from west of the Salween River, 'Central Plain' (CP) with tortoises from central Thailand and the Cambodian lowlands, 'south of Isthmus' (SI) with individuals collected south of the Isthmus of Kra on the Malayan Peninsula, 'Northern Vietnam' (NV) with specimens from the north-eastern and north-western uplands of northern Vietnam, and 'Central Vietnam' (CV) with specimens from the lowlands of central and southern Vietnam.

As *I. elongata* is known to be sexually dimorphic (Boulenger 1889, Taylor 1970, van Dijk 1998, Ihlow et al. 2016a), morphometric analyses were separately performed for males and females. Sexes are not distinguishable with certainty in specimens with a straight carapace length (SCL) below ~15 cm (McCormick 1992). Therefore, juveniles and individuals with irregular shell patterns were excluded from the analyses. Carapace and plastron were analysed separately. To avoid size-dependent intercorrelation effects in the morphometric data, regression residuals of the metric variables were calculated using SCL as a co-variable. To investigate whether geographic units are distinguishable in morphological space, we conducted a Principal Component Analysis (PCA) using mixed variables as implemented in the ADE4 package (Dray & Dufour 2007) for R 3.0.1 (R Core Team 2013). This method can handle quantitative as well as categorical variables (Kiers 1994), allowing for the inclusion of colouration data. A Multivariate Analysis of Variance (MANOVA) was performed on the colouration characters to check whether variation in pigmentation is linked to the geographic units.

Table 1a. Summary statistics for morphological characters (carapace) for all examined specimens of *Indotestudo elongata*. * Metric characteristics given in mm; mean values and standard deviation SD given below. SCL: straight carapace length, SCW: straight carapace width, CCL: curved carapace length, CCW: curved carapace width, HT: shell height, NL: length of nuchal scute, NW: width of nuchal scute, V1L, V2L, V3L, V4L, V5L: length of vertebral scutes 1–5, V1W1, V1W2, V2W, V3W, V4W, V5W: width of vertebral scutes 1–5, CauL: length of caudal scute, CauW1, CauW2: width of caudal scute, C1L1, C2L2, C2L, C3L, C4L: length of costal scutes, C1W, C2W, C3W, C4W: width of costal scutes, Ccol%: pigmentation of carapace.

Character *	*I. elongata* ♀ (n=87)	*I. elongata* ♂ (n=79)
SCL	174–305 241.62±24.24	178–308 240.09±25.27
SCW	107–192 147.48±13.91	113–186 146.19±14.87
CCL	211–365 292.86±28.02	219–382 301.7±32.69
CCW	184–318 247.02±13.91	181–300 239.57±24.32
HT	73–119 98.33±9.12	62–118 92.49±9.46
NL	9.1–29.39 19.81±3.4	9.6–28.96 21.53±4.12
NW	1.8–13.77 7.31±2.49	2.48–23.62 7.25±2.88
V1L	32.39–56.84 43.21±4.79	33.15–55.35 44.27±4.73
V1W1	28.7–53.51 38.64±4.89	28.6–48.77 39.27±4.69
V1W2	25.98–59.09 42.21±6.69	32.63–65.69 41.62±6.02
V2L	29.68–51.3 41.73±4.74	30.03–57.74 39.72±4.92
V2W	39.34–70.3 54.81±6.5	36.83–70.08 53.37±6.2
V3L	28.07–58.76 41.93±5.88	30.39–49.92 39.5±4.69
V3W	40.87–80.64 62.29±8.0	44.77–77.03 59.57±7.16
V4L	33.48–64.45 47.9±6.49	30.63–65.72 48.63±6.94
V4W	32.47–83.14 56.24±7.88	39.32–70.81 55.87±7.04
V5l	35.76–67.65 52.35± 7.01	38.5–78.28 59.11±8.93
V5W	49.99–87.67 65.92±7.96	47.45–90.27 68.24±8.32
CauL	26.11–53.8 39.67±6.2	26.69–53.09 41.97±5.44
CauW1	40.24–85.44 67.05±8.82	41.23–92.04 70.31±9.59
CauW2	27.94–87.27 44.39±8.00	34.11–88.32 44.15±8.1
C1L1	33.27–59.51 46.89±5.07	32.35–62.81 46.64±5.33
C1L2	38.93–65.56 53.37±5.44	39.54–69.94 53.16±5.98
C1W	38.55–64.46 51.94±5.78	36.33–67.75 51.16±5.66
C2L	28.09–54.87 43.51±4.97	30.5–62.23 41.11±5.34
C2W	42.32–77.4 58.96±6.79	40.09–72.3 56.21±6.21
C3L	28.24–56.99 41.89±5.31	29.13–56.04 40.31±5.36
C3W	42.47–76.62 57.97±6.37	42.79–75.12 56.82±6.59
C4L	21.2–47.36 29.99±4.41	20.45–46.86 30.59±5.78
C4W	34.4–62.73 46.13±5.2	32.31–67.39 45.87±6.84
Ccol%	0–81 33.02±20.57	1–74 33.32±18.28

Table 1b. Summary statistics for morphological characters (plastron) for all examined specimens of *Indotestudo elongata*. * Metric characteristics given in mm; mean values and standard deviation SD given below. SPL: straight plastron length, CPL: curved plastron length, SPW: straight plastron width, CPW: curved plastron width, GulL, HumL, PecL, AbdL, FemL, AnL: medial seam length of plastral scutes, Af1L, Af2L: length of anal fork, Pcol%: pigmentation of plastron.

Character *	♀ (n=87)	♂ (n=79)
SPL	138–225 183.05±16.27	142–222 179.72±17.76
CPL	143–232 189.91±16.96	146–227 186.43±18.84
SPW	97–390.9 135.17±32.45	96–162 128.08±14.52
CPW	108–185 141.84±13.09	105–180 141.31±16.48
GulL	16.46–35.22 25.01±3.92	13.04–37.02 26.15±4.51
HumL	12.49–36.39 21.52±4.74	9.02–34.97 21.81±5.06
PecL	18.44–38.08 28.72±4.38	18.99–39.74 28.4±4.18
AbdL	51.73–93.93 71.74±8.91	45.26–87.84 64.49±8.4
FemL	24.16–46.93 35.88±4.55	27.11–50.36 37.61±5.47
AnL	1.43–11.88 5.51±1.6	0–44.7 7.52±5.14
Af1L	20.6–42.97 31.16±3.54	22.87–52.14 35.69±5.14
Af2L	23.66–44.59 34.17±3.45	25.51–53.5 38.69±5.25
Pcol%	0–54 14.29±12.55	1–49 12.82±9.78

Results

Mitochondrial phylogeny and phylogeography

The branching patterns of the Bayesian and ML calculations (Fig. 2) found, in agreement with previous studies (Iverson et al. 2001, Le et al. 2006, Fritz & Bininda-Emonds 2007), *I. elongata* as the sister taxon of the allopatric *I. travancorica*, while *I. forstenii* was the successive sister taxon. However, four GenBank sequences identified as *I. forstenii*, albeit with a putative provenance in India (i.e., far beyond the distribution range of the species), represented misidentified *I. elongata* (accession numbers KC354731, KC354732, KF894793, JX049140). Within *I. elongata*, sequences were moderately variable, with a mean uncorrected p distance

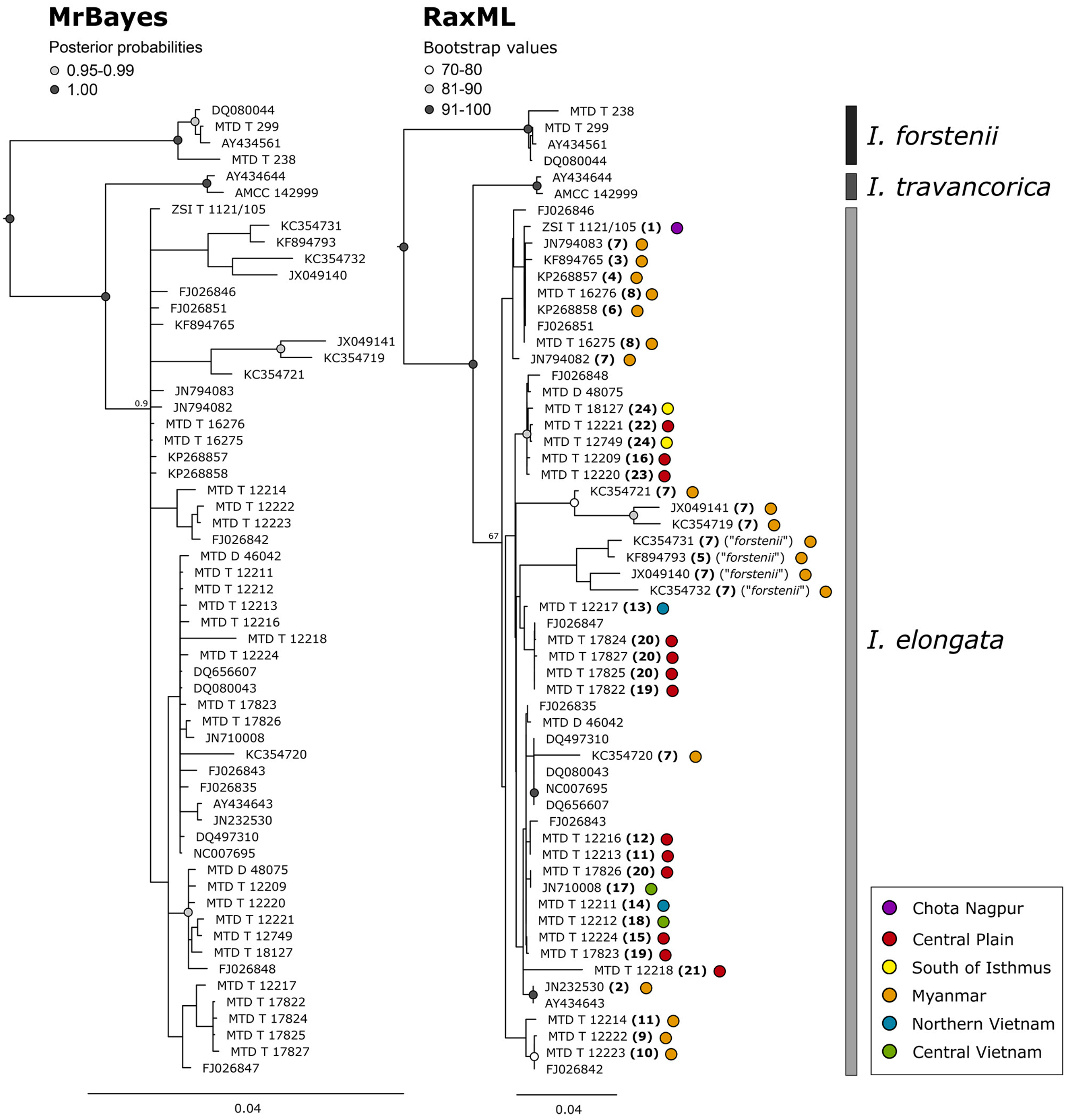

Figure 2. Mitochondrial phylogeny of *Indotestudo elongata* based on 3508 bp of mitochondrial DNA obtained from Bayesian and Maximum Likelihood analyses. Outgroup (*Testudo graeca*) removed for clarity; posterior probabilities < 0.95 and bootstrap values < 70 are not shown. The monophyly of *I. elongata* sequences is supported by a posterior probability of 0.90 and a bootstrap value of 67, respectively. The bold numbers in brackets correspond to the sites shown in Figure 1.

of 1.2% in cyt *b* (Fig. 2). Yet, the intraspecific relationships were not well resolved and all branching patterns received low statistical support. There was a basal polytomy, which branches into several terminal clades with shallow divergences. There is no clear geographic pattern. Compared to the other sequences, the four GenBank sequences misidentified as *I. forstenii* and two further GenBank sequences (JX049141, KC354719) have long branches, indicating many singletons suggestive of base-calling errors.

Morphology

Morphometric traits and shell colouration of the carapace could be used for 79 female and 70 male tortoises; data for the plastron were analysed for 73 females and 68 males. There was significant variation in body shape and colouration. Nonetheless, no correlation between these traits and the predefined geographic units was found (Fig. 3). The MANOVA performed on the colouration characters did not reveal any significant differences in plastron or carapace colouration between the geographic groups ($p = 0.812$, Wilks' $\lambda = 0.960$).

Discussion

Based on sequence divergences of the mitochondrial cyt *b* gene, Iverson et al. (2001) inferred that *I. forstenii* branched off from its congeners before *I. elongata* and

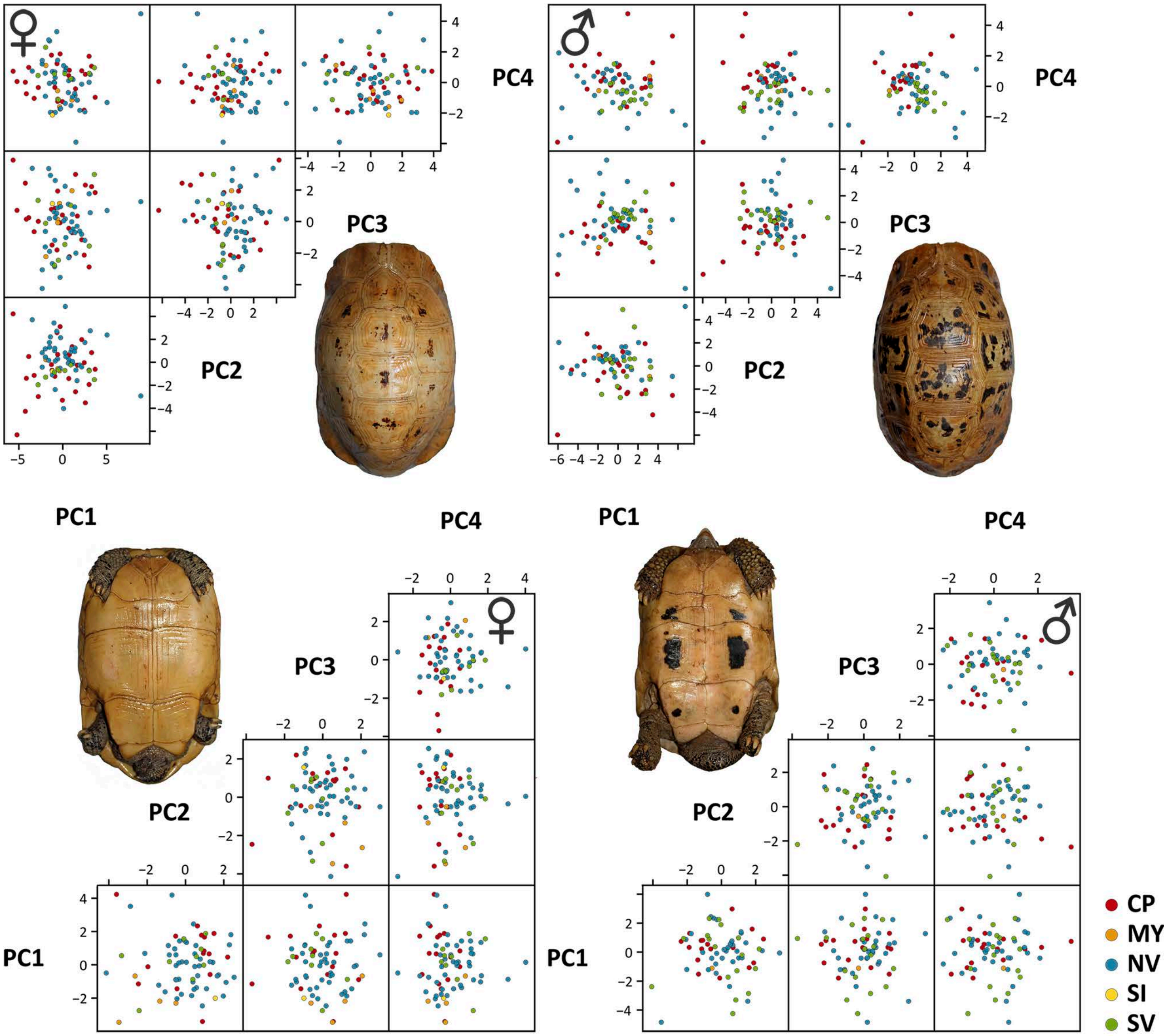

Figure 3. Morphological variation in shell measurements of *Indotestudo elongata*. Principal Component Analyses were performed separately for each sex. Only the first four PCs (eigenvalue > 3) are shown. Colours correspond to geographic groups as defined in Figure 1.

I. travancorica separated. This scenario was later confirmed by phylogenetic analyses of 15 nuclear loci (Thomson et al. 2021). Considering the complex paleogeographic history of Southeast Asia, the ancestor of *I. forstenii* was hypothesized to have dispersed to present-day Sulawesi via the exposed Sunda Shelf during a period of low sea levels and crossed the deep-water channel corresponding to the Wallace Line (Lovich 1994, Voris 2000, Meijaard et al. 2004, Sathiamurthy & Voris 2006). Using a dated phylogeny for mitogenomes, Kehlmaier et al. (2023) estimated that *I. forstenii* diverged in the late Miocene, ca. 10.2 million years ago. This is slightly younger than the estimate of Thomson et al. (2021) using 15 nuclear genes. Thomson et al. (2021) estimated that *I. elongata* and *I. travancorica* separated ca. 5 million years ago.

It has been speculated that the disjunct population of *I. elongata* in the Chota Nagpur Plateau in northeastern India represents a relic of a historically wider distribution and was isolated from the Himalayan foothills through the formation of the Indo-Gangetic Plain (Hora 1948, Smith 1931, Frazier 1992). If so, this population should diverge genetically. It is separated by wide distribution gaps from the nearest populations in the east and north. The delta formed by the Ganges, Brahmaputra and Meghna Rivers and the Ganges River and its tributaries are further barriers to the east and north, respectively. We included one sample from this disjunct population in our present investigation and it was not differentiated (Figs 1 and 2; sample from Barbara Reserve Forest, Odisha). This is remarkable because the Brahmaputra River and the Khasi-Garo-Jaintia Mountain Range south of the river act as a major biogeographic barrier for vertebrates between northeastern India and the rest of the country (Mani 1974, Ripley & Beehler 1990, Pawar et al. 2007, Deepak & Karanth 2018), even though some forest-dwelling species extend into Odisha, indicating some level of migration across this barrier (Köhler et al. 2023).

Uncorrected p distances of the cyt *b* gene have been often used as a yardstick to quantify phylogeographic and taxonomic differentiation in chelonians. We observed among our data for *I. elongata* average sequence divergences of 1.2% for cyt *b*, resembling those in other wide-ranging tortoise species such as *Testudo hermanni* (1.5%), *T. horsfieldii* (1.0%), *Chelonoidis carbonarius* (2.0%), and *Kinixys belliana* (1.9%) (Fritz et al. 2006, 2009, Vargas-Ramírez et al. 2010, Kindler et al. 2012). However, in contrast to *I. elongata*, these species are phylogeographically structured. The lacking differentiation in *I. elongata* is also mirrored by our morphological results.

Thus, contrary to our expectations and despite many significant biogeographic barriers, *I. elongata* is phylogeographically unstructured. In the face of the species' preference for low- and mid-elevation habitats and the presence of high mountain chains, this is unexpected. Several wide-ranging and co-distributed chelonians inhabiting lowlands across mainland Indochina show clear phylogeographic, morphological and taxonomic structuring that reflect zoogeographic barriers. These include Asian leaf turtles (*Cyclemys* spp.), Asian softshell turtles (*Amyda* spp.), and snail-eating turtles (*Malayemys* spp.; Fritz et al. 2008, 2014, Ihlow et al. 2016b). However, the Indochinese Box Turtle *Cuora couro kamaroma* (previously *C. amboinensis kamaroma*) exhibits a pattern more akin to that of *I. elongata*, with little genetic structuring across mainland Indochina, north of the Isthmus of Kra and east of the Arakan-Joma Mountains (Blanck et al. 2023).

While population connectivity and gene flow in aquatic and semi-aquatic species, like *C. c. kamaroma*, is enhanced by seasonal floods which inundate and connect large portions of the Southeast Asian lowlands, this is not necessarily true for terrestrial species as *I. elongata*. Numerous squamate reptile groups contain terrestrial lowland species or species complexes that inhabit similar habitats and exhibit pronounced genetic structuring linked to zoogeographic barriers, e.g., agamids (Hartmann et al. 2013, Grismer et al. 2014a, Wagner et al. 2021), lacertids (Arnold 1997, Lin et al. 2002), scincids (Geissler et al. 2011), geckos (Grismer & Van Tri 2007, Wood et al. 2012, Grismer et al. 2014b), and snakes (Lukoschek et al. 2011, Ratnarathorn et al. 2023). However, none of the zoogeographic barriers relevant to these taxa has significantly influenced *I. elongata*.

Thus, the central questions are: How has *I. elongata* maintained genetic connectivity across its range, or why has the species not evolved in phylogeographically structured populations? One possibility could be that human-mediated dispersal has contributed to the observed absence of any phylogeographic structure. Humans have long utilized *I. elongata* for subsistence consumption as evidenced by tortoise remains recovered from deposits in different rock shelters across Thailand and Cambodia that date back to between the Late Pleistocene and the first half of the Holocene (Mudar & Anderson 2007, Bochaton et al. 2023, Paleobiology database: https://paleobiodb.org). When fossil chelonian remains are identified, *I. elongata* often stands out as the most abundant species, indicating that Hoabinhian hunter-gatherer groups have preferred to exploit *I. elongata* over other species (Cheung & Dudgeon 2006, Rahman et al. 2019, Bochaton et al. 2023). There are other cases known where the prehistoric and historic use of chelonians has impacted their genetic diversity. A prominent example is the genus *Pelodiscus*, for which millennium-old farm breeding and the translocation of turtles obscured the underlying natural pattern (Gong et al. 2018). It is also known that prehistoric humans transported tortoises on the Bahamas between different islands, obscuring the original phylogeographic pattern (Kehlmaier et al. 2021). Similar observations were made for other giant tortoises both on Galapagos (e.g., Caccone 2002) and several islands in the Western Indian Ocean (Kehlmaier et al. 2023). Also, for the Indian Star Tortoise *Geochelone elegans* (Vamberger et al. 2020, Kundu et al. 2022, but see Sahoo et al. 2024) and the Central American River Turtle *Dermatemys mawii* (González-Porter et al. 2011) massive translocations seem to have homogenized genetic structure.

In the case of *I. elongata*, however, different genetic lineages do not occur together, as observed for instance in *Pelodiscus* (Gong et al. 2018) or the extinct Bahamian giant tortoises (*Chelonoidis alburyorum*, Kehlmaier et al. 2021). Instead, we observed a complete absence of genetic structuring. One hypothesis explaining our findings is that *I. elongata* originally had a much smaller distribution range and that prehistoric and historic humans distributed it across the relevant biogeographic barriers, leading to the establishment of the species in formerly tortoise-free regions. Turtles and tortoises are ideal provision during journeys, and frequently have been dispersed overseas by humans as "living cans" (see the examples and discussion in Fritz et al. 2013). A similar scenario seems plausible on the mainland, in particular when tortoises were an esteemed food item in the trade between local communities.

Collection pressure for local, regional, and international trade of *I. elongata* have greatly increased in modern times (Holloway 2003, Emmett 2009, Ihlow et al. 2014, Horne et al. 2022). A major conservation concern has been genetic pollution from the release of individuals confiscated from trade without regard to their origin (e.g., Ihlow et al. 2014). However, the observed lack of phylogeographic structure considerably simplifies urgently needed conservation measures such as conservation breeding, reintroduction, and population reinforcement of this critically endangered species.

Further interdisciplinary research is needed to test our hypothesis of a human-mediated prehistoric or historic range expansion of *I. elongata*. Both genomic tools, reliably allowing for the detection of changes in population size and genetic diversity, and further archaeological investigations could contribute additional valuable insights.

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Supplementary data

The following data are available online:

Supplementary document 1. Studied samples of *Indotestudo* and mtDNA sequences obtained from GenBank.