

Short Note

Already too late? Massive trade in Indian star tortoises (*Geochelone elegans*) might have wiped out its phylogeographic differentiation

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Abstract. *Geochelone elegans* is one of the most heavily traded tortoise species of the world, and confiscated tortoises are frequently released into the wild, without knowledge about their origin. Using for the first time samples from Pakistan and Sri Lanka, we examined phylogeographic differentiation of *G. elegans* using 2289 bp of mitochondrial DNA. We found weak intraspecific differentiation without a clear geographic pattern. We suggest that natural phylogeographic differentiation may have been already destroyed by massive releases of confiscated non-native tortoises. The presence of two distinct clades on Sri Lanka, however, could also be the result of a natural range expansion of a mainland lineage into the distribution range of a lineage endemic to Sri Lanka during Pleistocene low sea level stands. We propose that a systematic screening of the genetic differentiation of wild *G. elegans* should be conducted across its entire distribution range to provide a sound basis for the relocation of confiscated tortoises.

Keywords: confiscation, conservation, India, Pakistan, release of confiscated tortoises, South Asia, Sri Lanka.

The Indian star tortoise (*Geochelone elegans*) is one of the most heavily traded tortoise species of the world. It is estimated that annually nearly 100,000 star tortoises are illegally collected and exported from India alone (de Silva et al., 2019). This species is thought to represent approximately 11% of all global seizures of chelonians, and between 2000 and 2015 more than 34,000 live *G. elegans* were reported to be confiscated, most of them (more than 21,300 individuals) within India. Many of these confiscated tortoises are subsequently released (D’Cruze et al., 2018). Until today, little is known about a

possible phylogeographic differentiation of Indian star tortoises. The species has a disjunct distribution range. A northwestern distribution patch, corresponding to southeastern Pakistan and adjacent India (Gujarat, Madhya Pradesh, Rajasthan), is widely separated by a gap of 600-700 km from a patch in southeastern India (Andhra Pradesh, Karnataka, Tamil Nadu) and Sri Lanka (TTWG, 2017). This gap coincides with two well-known biogeographic divides (Goa Gap, Godavari River). Moreover, Sri Lanka constitutes also a distinct biogeographic region that is separated by the Palk Strait from India (Ramachandran et al., 2017), so that phylogeographic structuring is expected in *G. elegans*.

Until today, there is no comprehensive study about the genetic variation of Indian star tortoises. A pioneering investigation by Gaur et al. (2006) using six microsatellite loci and two mitochondrial markers (cytochrome *b* = *cyt b* gene, control region) suggested indeed that tortoises from the disjunct distribution patches are

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distinct. However, Gaur et al. (2006) used only tortoises originating in India, and the majority of their samples came from confiscated individuals. In wild Indian tortoises, Gaur et al. (2006) found two *cyt b* haplotypes differing by two sites (GenBank accession numbers AY776255 and AY776256; both 823 bp). One of these haplotypes occurred in tortoises from northwestern India and the other in tortoises from southeastern India. In the present study, we compare for the first time samples of *G. elegans* from India, Pakistan, and Sri Lanka. We include for comparative purposes also data of *G. platynota*, the Burmese star tortoise. This species is closely related to *G. elegans* and currently regarded as the only other species of the genus *Geochelone* (TTWG, 2017).

Forty-six samples of *G. elegans* from Pakistan (7), India (10), and Sri Lanka (22) were studied, besides seven samples from pet trade tortoises and six samples of *G. platynota*. All samples from India and nine samples from Sri Lanka came from tortoises in the live collection of Turtle Island, Graz (Austria). These tortoises are long-term captives with reliable provenance, obtained by Reiner Praschag (Graz) during the early 1970s in India and Sri Lanka. The samples of *G. platynota* were taken from animals confiscated in Germany; the remaining samples were collected from wild tortoises (supplementary table S1).

Three mitochondrial genes were sequenced (12S, *cyt b*, ND4 with adjacent DNA coding for tRNAs). Details of DNA isolation, PCR and sequencing are described in Kindler et al. (2012). The obtained 12S fragments were up to 370 bp long; *cyt b* fragments, up to 1067 bp; and mtDNA fragments comprising the partial ND4 gene plus adjacent DNA coding for tRNAs, up to 852 bp. Sequences were aligned and inspected using BioEdit 7.0.5.2 (Hall, 1999). All sequences aligned perfectly; gaps occurred only in non-protein-coding DNA sections.

For phylogenetic analyses, the individual mtDNA fragments were concatenated, resulting in an alignment of 2289 bp length. *Centrocorys sulcata*, the sister taxon of *Geochelone* (Le et al., 2006; Fritz and Bininda-Emonds, 2007), was used for tree rooting. European Nucleotide Archive (ENA) accession numbers and collection sites are given in supplementary table S1. The best partitioning scheme was assessed using PartitionFinder (Lanfear et al., 2012), the Akaike Information Criterion (AIC), and the 'greedy' option. Phylogenetic relationships were inferred with MrBayes 3.2.3 (Ronquist et al., 2012) using the best partition schemes and evolutionary models of supplementary table S2 and default parameters. Two parallel runs, each with four chains, were conducted. The chains ran for 10 million generations with every 500th generation sampled. The calculation parameters were analyzed using a burn-in of 2.5 million generations to assure that both runs converged. Subsequently, only the plateau of the remaining trees was sampled, and a 50% majority rule consensus tree was generated. In addition, phylogenetic relationships were estimated using RAxML 7.2.8 (Stamatakis, 2006) and the GTR+G substitution model across all partitions; exploratory RAxML calculations for the *cyt b* gene only included also the two haplotypes of wild Indian star tortoises from Gaur et al. (2006). For our final trees, five independent Maximum Likelihood searches were performed using different starting conditions and the fast bootstrap algorithm. Then, 1000 non-parametric thorough bootstrap replicates were calculated and the values were plotted against the best tree.

Using PopART (<http://popart.otago.ac.nz>), a parsimony network was drawn to display mutational differences of haplotypes. Since the underlying TCS algorithm is sensitive to missing data, concatenated data sets with lacking genes were excluded. For the same reason, all individual missing sites were removed, resulting in an alignment of 2062 bp length comprising sequences of 43 tortoises.

For the *cyt b* gene, uncorrected *p* distances were calculated in MEGA 7.0.21 (Kumar, Stecher and Tamura, 2016) using the pairwise deletion option.

Phylogenetic analyses revealed only negligible variation within *G. elegans*, with three moderately supported mtDNA clades (fig. 1). One clade corresponded to our five samples from Gujarat, India (clade A in fig. 1), and another clade to four samples from Sri Lanka (clade B). In the third clade (clade C) occurred 37 sequences of Indian star tortoises from all sampled regions except Gujarat, along with sequences from trade tortoises. Weak sequence divergence was also reflected by the parsimony network that showed a maximum of 17 mutation steps among haplotypes that grouped in three weakly differentiated clusters (fig. 1). With respect to the *cyt b* gene, often used for barcoding in turtles (e.g. Kindler et al., 2012; Petzold et al., 2014), the two *Geochelone* species differed on average by an uncorrected *p* distance of 4.49%. The divergences among the three clades within *G. elegans* ranged from 0.45% to 0.71% (table 1). While the divergence between the two *Geochelone* species falls into a range observed also for other congeneric testudinid species (Fritz et al., 2012; Kindler et al., 2012), the lower values within *G. elegans* support the conspecificity of all studied samples.

Our results are unexpected in that our samples do not match completely with the differentiation pattern described in a previous investigation (Gaur et al., 2006). According to this study, *G. elegans* from northwestern India differ from their conspecifics from southeastern India. Our samples from Gujarat (clade A), from the northwestern Indian distribution patch, were indeed different from the samples from Tamil Nadu (clade C), representing the disjunct southeastern distribution range. Our exploratory RAxML analyses confirmed that the haplotype from northwestern India described by Gaur et al. (2006) represents our clade A and their haplotype from southeastern India, our clade C. However, our Pakistani samples did not

cluster with the samples from the nearby Indian state of Gujarat (clade A), as expected, but with our samples from Tamil Nadu (clade C). In the same weakly supported clade C, which was also revealed as a distinct cluster in haplotype network analysis (fig. 1), occurred also samples from Sri Lanka. Yet, four other samples from Sri Lanka were distinct and represented another clade (clade B). These four samples constituted in the network a cluster that was clearly distinct from the remaining samples. From other Sri Lankan samples, this cluster was separated by a minimum of 10 mutation steps, and the variation between the two clusters including Sri Lankan samples corresponded to the observed maximum sequence divergence (17 steps). The four wild-caught tortoises belonging to clade B originated from sites in northwestern and southeastern Sri Lanka. In one site, another tortoise representing clade C was found (supplementary table S1).

It is obvious that further research is needed to elucidate this intricate situation. In the face of the massive trade in Indian star tortoises, one possible interpretation is that the genetic signal of released confiscated tortoises obscures a natural phylogeographic pattern. This pattern could soon be lost entirely, if genetically uncontrolled releases continue. Especially the difference between tortoises from Pakistan and Gujarat is surprising and difficult to explain. However, with respect to Sri Lanka, we cannot exclude that the presence of two distinct genetic clusters reflects a natural range expansion of a mainland lineage into the distribution range of another lineage endemic to Sri Lanka during Pleistocene low sea level stands. On the other hand, Sri Lanka is known to be the central hub for the illegal trade in *G. elegans*, and many star tortoises are smuggled from India to Sri Lanka (Malsinghe et al., 2017; de Silva et al., 2019). This could also have contributed to the presence of the mainland lineage in Sri Lanka.

We suggest that a systematic screening of the genetic differentiation of wild *G. elegans* is conducted across its entire distribution range

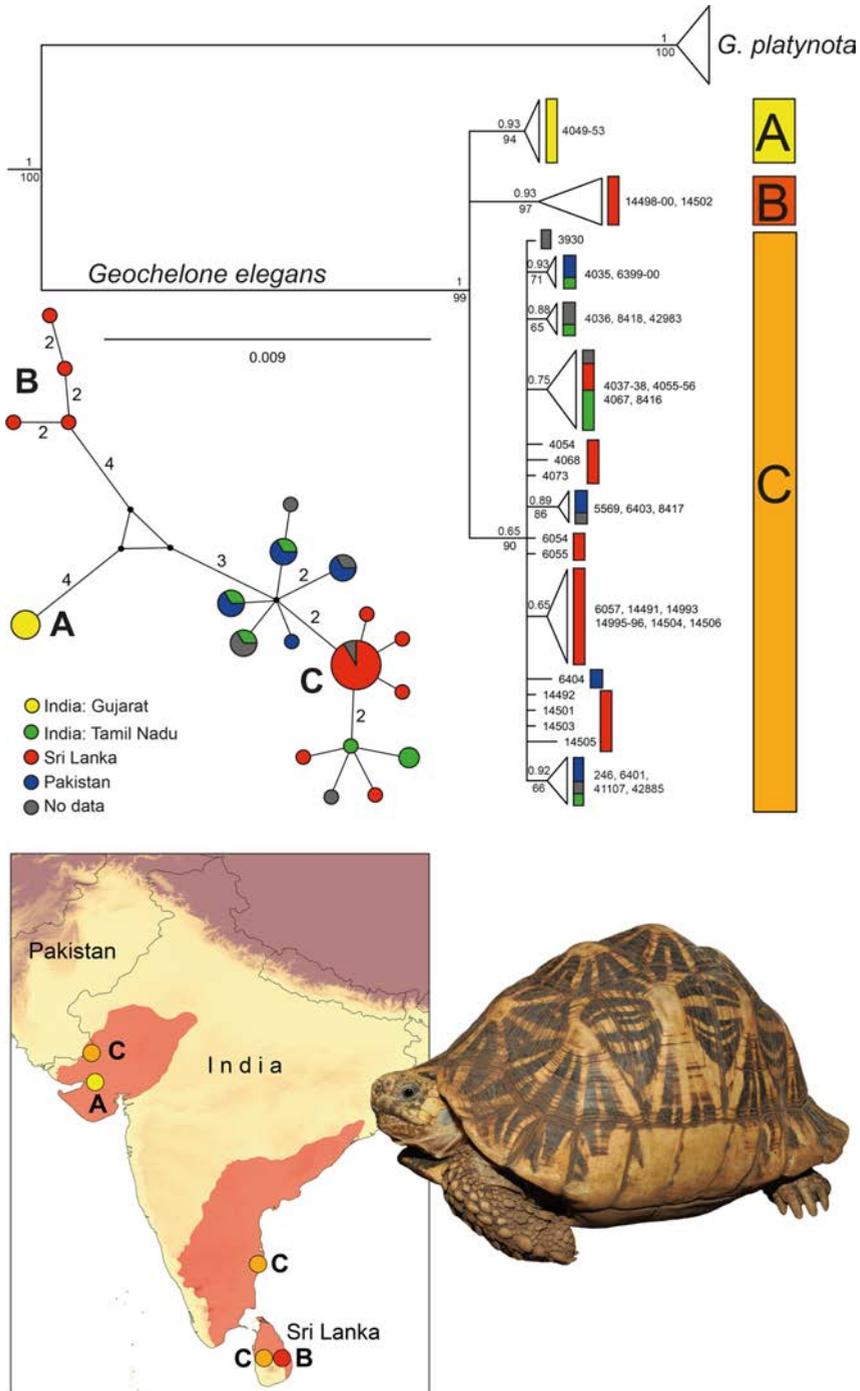


Figure 1. Top: Bayesian tree for mitochondrial DNA sequences (2289 bp) of *Geochelone elegans* and *G. platynota*. Outgroup (*Centrochelys sulcata*) removed for clarity. Values above nodes are posterior probabilities; below nodes, thorough ML bootstrap values greater than 50. Centre: Parsimony network of mtDNA sequences (2062 bp) of 43 *G. elegans*. Haplotype size corresponds to number of individuals. Missing node haplotypes are shown as small black circles; lines connecting haplotypes represent one mutation step except when otherwise indicated by numbers. Bottom: Distribution range of *G. elegans* (from TTWG, 2017) and approximate sampling sites. The photo by Peter Praschag shows a star tortoise from Sri Lanka.

Table 1. Uncorrected *p* distances (means, expressed as percentages) between and within *Geochelone elegans* and *G. platynota* using the cytochrome *b* gene (1067 bp). Within-clade divergences on the diagonal in bold.

	<i>n</i>	<i>G. elegans</i> (all)	Clade (A)	Clade (B)	Clade (C)	<i>G. platynota</i>
<i>G. elegans</i> (all)	46	0.30				
Clade (A)	4	–	0			
Clade (B)	4	–	0.66	0.20		
Clade (C)	38	–	0.45	0.71	0.20	
<i>G. platynota</i>	6	4.49	4.18	4.60	4.25	0.10

to serve in future as a sound basis for the relocation of confiscated tortoises. This will be a major challenge because legislative restrictions massively impede biodiversity research (Neumann et al., 2018; Prathapan et al., 2018), contributing therefore to the erosion of biodiversity and thwarting the original spirit of any national and international conservation measures and conventions.

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Supplementary material. Supplementary material is available online at:

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