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A Comprehensive Description and Evolutionary Analysis of Testudines Mitochondrial Genomes

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ABSTRACT

Background: There are not many species of turtles and some species have become rare or even endangered due to the changes in the ecological environment, the destruction of human pet market trade, the use of food and medicinal materials and other factors. The phylogenetic study of *Geoemyda spengleri* and their related species will help to protect turtle germplasm resources.

Methods: The sample was collected from nature reserves in Guangxi, China and processed for DNA isolation and confirmed with Polymerase chain reaction (PCR). Maximum-likelihood (ML) were conducted based on concatenated sequences of 13 protein-coding genes from mitochondrial genomes of 25 taxa.

Result: The complete mitochondrial genome (17,448 bp) from the Black-breasted leaf turtle (*Geoemyda spengleri*) was determined. The genome content, gene order and base composition conformed to the consensus vertebrate type mtDNA. However, a remarkable feature was found in this molecule: a small number of (ATATTATTATATTATTATATATC)n direct tandem repeats followed by a AT-enriched microsatellite sequence at the 3' end of the control region (D-loop), which might be useful as molecular markers for studying population genetics and helpful for species identification and conservation. The results strongly supported that 1) *Geoemyda spengleri* and the most recent common ancestor of *Batagur trivittata* and *Pangshura sylhetensis* formed a monophyletic clade, whereas most other species of Geoemydidae formed another branch, suggesting that Geoemyda and *Batagur trivittata* may have more closely relationships than other genera; 2) the Geoemydidae with Testudinidae was a sister group rather than with the Emydidae. Furthermore, In order to analyze the relationship between habitat distribution and the phylogenetic evolution of turtles, the habitat distribution map was plotted based on the habitat distribution of species of Geoemydidae. The results also supported that *Geoemyda spengleri* and *Batagur trivittata* may relatively have intimate relationships.

Key words: Control region, *Geoemyda spengleri*, Mitochondrial genome, Phylogenetic relationships.

INTRODUCTION

With a long and successful evolutionary history, turtles are one of the most specialized and ancient groups of reptiles. It appears in the carboniferous, at the same time as the mighty dinosaurs, which disappeared at the end of the cretaceous period. However, tortoises and turtles have survived and multiplied so far. Therefore, tortoises and turtles have played an important role in the systematic evolution of vertebrates (Zhang *et al*., 2007). There are not many species of turtles and some species have become rare or even endangered due to the changes in the ecological environment, the destruction of human pet market trade, the use of food and medicinal materials and other factors. Therefore, it is of great theoretical and practical significance to study turtle species in terms of the protection of biological diversity and the sustainable utilization of turtle resources. According to statistics, there are about 13 families, 89 genera and more than 270 species of tortoises in the world (Zhou *et al*., 2004), among which Batagurinae are the largest family. There are 23 genera in Batagurinae, which include Batagur, Callagur, Chinemys, Hieremys, Kachuga, Ocadia, Malayemys, Hardella, Morenia, Geoclemys, Orlitia, Siebenrockiella, Annamemys, Geoemyda, Mauremys, Melanochelys, Notochelys, Sacalia, Rhinoclemmys, Cuora, Pyxidea, Cyclemys and Hoesemys; more than 60 species are included; they are mainly distributed in Europe, North Africa and Asia, with another genus distributed in central and South America.

Previous work has been done on morphology (Gauthier *et al*., 1988; DeBraga and Rieppel, 2010) and chromosomes (Hedges and Poling, 1999) have been studied, but it is difficult to reach a consistent conclusion.

The complete mitochondrial genome sequence, critical information (Guangxin, 2015) for the study of genome evolution and species phylogeny, remains unavailable for most of the Geoemydidae. We determined one complete mitochondrial genome of *Geoemyda spengleri* and examined the relationships among 23 Testudines species

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based on phylogenetic analyses of mitochondrial genome. We generated complete nucleotide sequence for the mitochondrial genomes and determined the mitochondrial genomic structure. Finally, we conducted phylogenetic analyses based on the mitochondrial sequence data with the main aim of investigating the phylogenetic relationships within *Geoemydidae*.

MATERIALS AND METHODS

The experiment was conducted in the engineering center of Zhejiang Ocean University from 2018-6 to 2019-5.

Sample collection and DNA extraction

Tissue sample of *G. spengleri* was obtained from a mountain forest in Guangxi, China (21.7°N,108.0°E). We took the muscle tissue from the severed tail and disinfected the turtle's wound with alcohol. After the turtle has recovered, we released it into its habitat.The part of the tail muscle was used to determine the complete mitochondrial sequence. Both morphological and PCR-based (COI gene) were used for species identification; the tail muscle was sampled and preserved in 95% ethanol. Total DNA was extracted from the muscle tissue using the proteinase K method (Sambrook and Russell, 2001) and kept at -20°C for PCR amplification. The rest of the tail sample was stored in the Marine Biological Museum of Zhejiang Ocean University, under the registration codes: 1508504.

PCR amplifications and sequencing

Sixteen pairs of degenerate primers were designed (Supplementary Table 1), based upon the reported complete mitochondrial genome sequences for *Mauremys mutica* and *Sacalia quadriocellata*, using the program: 95°C pre-denaturing for 2 min, followed by 35 cycles of 94°C for 40 s, 51°C -58°C for 45 s and 72°C for 1 min and a final extension at 72°C for 10 min. Each reaction contained 100 ng template DNA, 2.5 µL 10× Buffer (TaKaRa, Dalian, China), 2 µL MgCl 2 (2.5 mol/L), 1.5 µL of each dNTP, 0.25 µL of each primer (25 mol/L) and 1 U Taq DNA polymerase (5 U/µL, TaKaRa). PCR products were electrophoresed on a 1.0% agarose gel and DNA fragments of intended sizes were recovered using a Gel Extract Purification Kit (TaKaRa, Dalian, China). Then the cleaned PCR products were sequenced in both directions with an ABI3730 automated sequencer (Invitrogen Biotechnology Co., Ltd, USA). To ensure the reliability of the data, we amplified and confirmed multiplicity until almost all the sequencing chromatograms displayed single peak and the resultant sequences were as the expected length.

Sequence analysis

The Sequencher TM (GeneCode, AnnArbor, MI, USA) program was used for editing and assembling the contiguous, overlapping sequences. The most DNA sequence data were characterized using BLAST searches at NCBI. Sequence data of *G. spengleri* was analyzed with EditSeq (DNASTAR). The locations of protein-coding, rRNA and tRNA genes were identified by tRNA Scan-SE1.21 (http://lowelab.ucsc.edu/tRNA Scan-SE) and SQUEIN (ver.5.35) program as well as comparisons with the corresponding sequences of other known turtles. Besides, analyses of the control region were performed with DNAsis program. The complete mtDNA sequence of *G. spengleri* was deposited in GenBank under accession number KU641028.

For phylogenetic analyses of major turtle lineages, a total of 23 turtle mtDNA sequences available in the database were chosen (Supplementary Table 2). In addition, Crocodylia and Sauria two clades of diapsid reptile, were used as the outgroup, based on the alliance between turtles, crocodilians and saurians. One typical and uncontroversial crocodilians, *Mecistops cataphractus* (# MT554033) and one typical and uncontroversial saurians *Basiliscus vittatus* (#NC 012829), were used here. We aligned the nucleotide data for the 13 protein-coding genes resulting in 11,244 nucleotide sites. Phylogenetic trees were constructed by maximum-likelihood (ML) using MEGA7.0.26.

RESULTS AND DISCUSSION

Genome features

The mt genome of *G. spengleri* was 17,448 bp long and conformed to other consensus vertebrate mitochondrial form (Fig 2). It consisted of 13 protein-coding, 2 rRNA, 22 tRNA genes and one control region (D-loop), all of which were similar in length to their counterparts in other turtles (Supplementary Table 1). There were few or small noncoding intergenic spacers, whereas a 26 bp intervening sequence existed between tRNAAsn and tRNACys genes, a 16 bp intervening sequence existed between ND4 and tRNAHis genes,and three partially overlapping, *i.e.*, ATP8-ATP6, ND4L-ND4 and ND5-ND6 sharing 22, 7 and 5 nucleotides, respectively, were found (Table 1).

Totally, it encodes 3,775 amino acids. The most frequently used amino acids was Leu (16.2%), Thr (9.2%), Ile (8.1%) and Ser (7.5%), while the least common amino acids were Cys (0.9%), Asp (1.8%), Arg (1.8%) and Lys (2.3%) (Fig 1A). Relative synonymous codon usage (RSCU) values for the third positions of the 13 PCGs was shown in Fig 1B. The usage of both two-fold and four-fold degenerate codons was biased toward the use of codons abundant in T or A, in accord with other turtles.

The nucleotide compositions of the 18 mitochondrial genomes were shown in Table 2. The base composition of the major coding strand of *G. spengleri* mtDNA was A, 33.7%; G, 13.1%; C, 25.6% and T, 27.6%, showing high average A%+T% and low G% contents as in most other vertebrates. Strand asymmetry of nucleotide composition is usually described by AT and GC skews. The mitochondrial genome AT skews of these chelonian species ranged from 0.098 to 0.136, while the GC skews were all negative, arranging from -0.375 to -0.320. Most of the *G. spengleri* mt genome protein-coding genes had a start codon of ATG, ND2 start with an ATA initiation codon, COI start with a GTG initiation codon,

while ND6 uses CCT as the initiation codon. Protein-coding genes of six terminated with TAA, three ended with TAG, two ended with CAT, one ended with AGG and the remainder of the reading frames had an incomplete termination of a single nucleotide T (Table 1), where the post-transcriptional polyadenylation could produce a TAA stop codon. Besides, we discovered an extra base "G" at a specific position in the ND3 gene of *G. spengleri*.

Ribosomal and transfer RNA genes

16S rRNA is located between the $tRNA^{Ile}$ and $tRNA^{Val}$ genes and 12S rRNA is located between the $tRNA^{Val}$ and $tRNA^{Trp}$

Supplementary Table 1: PCR and sequencing primers used in this study.

PCR	Sequence (5′→3′) L primers	Sequence (5′→3′) H primers
1	TTATACAAGAAACTCAAATTAACAGAAAATCGGC	TCCTTTTTCAATTGGGCTGTACCCC
2	CTCAATAAAAGAATATAAGTTCAACC	GTAAGGTAAGGCTTGTTTTTAGTAT
3	GGAGTTTACGACCTCGATGTTG	TCAAAAGCATAGTGCTAAGGTGATT
4	TCTAAATCCCGCCCACATC	ATGATGTTATTATTACGGAGATTTTGT
5	CCCACCTAGGATGAATAATCACAAT	TTCCTGAGGAGGCTAGGAGTAGAA
6	AGCCCATGCCTTTATTATAATCTTC	GCAGCCATGTAATCATTCTACATTAG
7	AGATGCATACACTCTATGGAATTCTATC	AGATTTTTGGTTGTAAGATTACGGTATA
8	CAATCCGAATATTAATCTCAGCCG	TGGATGGTTTGGTTTCGGTTAGT
9	GCTTACGTGTTTGTTTTACTTCTGTG	GGGCTATAAATAGGGATAGTATTATTCC
10	ATTTACACTTTTACCTGAGCCTTC	GAGTAGATGTTCTCGTGTATGGGT
11	AATACCCCTATACGGGTTACACC	CAGGATATGCTAAGAATTAGTCCAAT
12	CTTATACGTCACATGATCCATCCTG	CGATTAATGTTAGGAGCAGGGC
13	CGCTCTCACCCAAAATGACATC	TGTTGTAGTTTTTGTACAAATGGGTTG
14	GCAATCCCACTAAAATCTAAACGA	GAGTAAATGTACAGCTGCTAAACCG
15	TGAGGGGGATTCTCAGTAGACAAT	TATGCATTTAAGAACACCGTATGTCA
16	TTAAGTTAATGGTTTAAGGACATAAAC	GTTTTGTAATAGTACCTATGGCTAGC

Supplementary Table 2: 23 representative turtles used for phylogenetic analysis.

Order	Suborder	Family	Species	Accession no.
Crocodylia		Crocodylidae	*Mecistops cataphractus*	MT554033
Sauria	Iguania	Corytophanidae	*Basiliscus vittatus*	NC_012829
Testudines	Cryptodira	Emydidae	*Chrysemys picta bellii*	NC_023890
			Trachemys scripta	KM216749
			Malaclemys terrapin	NC_031300
		Geoemydidae	*Sacalia quadriocellata*	EF088646
			Cyclemys dentata	NC 018793
			Notochelys platynota	HQ853256
			Cuora mouhotii	DQ659152
			Mauremys annamensis	HM131942
			Heosemys annandalii	JF742646
			Geoemyda spengleri	KU641028
			Batagur trivittata	NC 032300
			Pangshura sylhetensis	MK580979
		Testudinidae	*Manouria emys*	DQ080040
		Carettochelyidae	*Carettochelys insculpta*	FJ862792
		Trionychidae	*Pelochelys cantorii*	KT962834
		Dermochelyidae	*Dermochelys coriacea*	MF460363
		Cheloniidae	*Natator depressus*	NC 018550
		Platysternidae	*Platysternon megacephalum*	DQ256377
		Chelydridae	*Macrochelys temminckii*	NC 009260
		Kinosternidae	*Kinosternon leucostomum*	FJ915117
	Pleurodira	Chelidae	*Chelodina longicollis*	NC 024667
		Pelomedusidae	*Pelusios castaneus*	NC 026049
		Podocnemididae	*Podocnemis unifilis*	JF802204

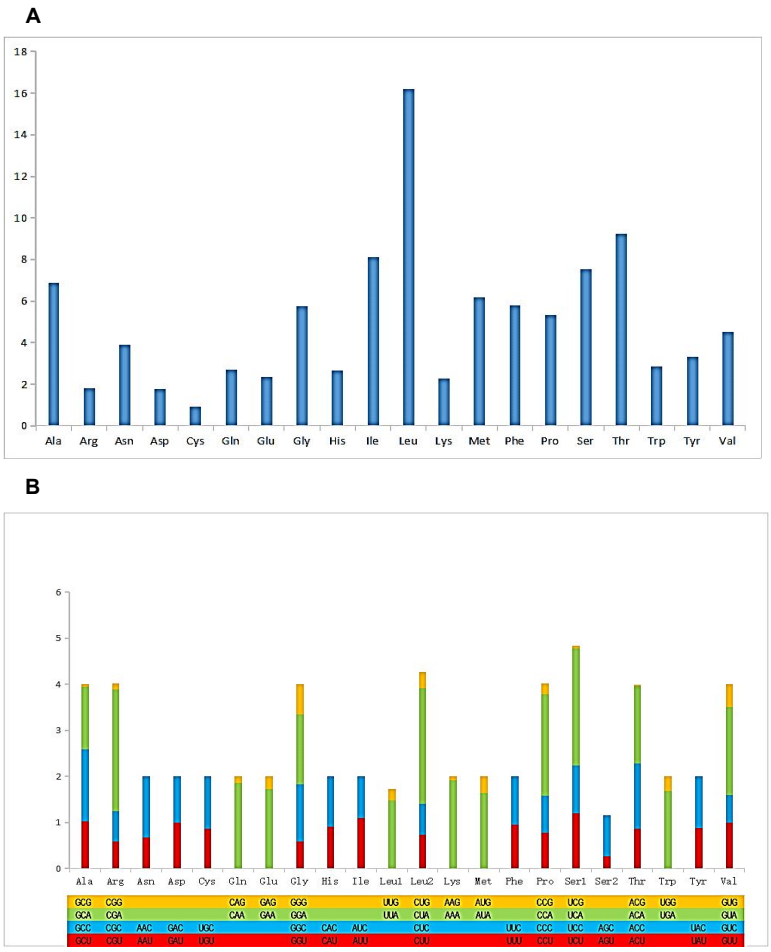


Fig 1: Amino acid composition in *Geoemyda spengleri*. mitogenome (A); Relative synonymous codon usage in *Geoemyda spengleri* mitogenome (B).

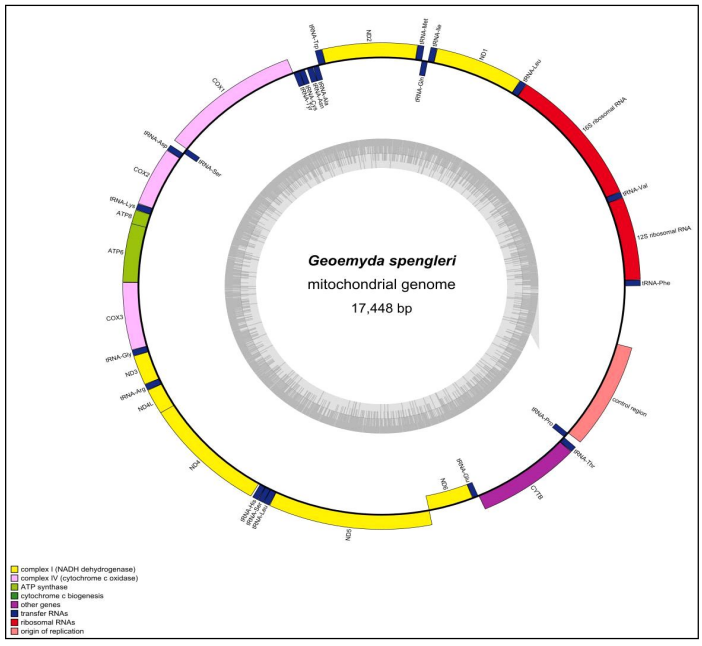


Fig 2: Gene map for mitochondrial genomes of *Geoemyda spengleri.*

genes. The lengths of 12S rRNA and 16S rRNA genes of *G. spengleri* are 964bp and 1681bp, respectively. All the mitochondrial genomes of the 18 chelonian species contain 22 tRNAs, 14 of which are encoded by the H-strand and 8 encoded by the L-strand. Among the 22 tRNAs, two forms of $tRNA^{Leu}$ (UUR and CUN) and two forms of $tRNA^{Ser}$ (UUR and CUN) were observed in all chelonian species, ranging in size from 62 to 76 bp and in few of their end sequences

Table 1: Mitochondrial genome characteristics of the Geoemyda spengleri*.

Gene	Position		Size(bp)		Codon		Intergenic nucleotidesa	Strand
	From	to	Nucleotide	Amino acid	Initiation	Stop		
$tRNA^{Phe}$	1	70	70				0	H
12S rRNA	71	1034	964				0	H
$tRNA^{Val}$	1035	1104	70				0	H
16S rRNA	1105	2715	1681				0	H
$tRNA^{Leu}$	2716	2790	75				0	H
ND1	2791	3762	972		ATG	TAG	-1	H
$tRNA^{Ile}$	3762	3831	70				-1	H
$tRNA^{Gln}$	3831	3901	71				0	L
$tRNA^{Met}$	3902	3971	70				0	H
ND2	3972	5012	1041		ATA	TAG	-2	H
$tRNA^{Trp}$	5011	5086	76				1	H
$tRNA^{Ala}$	5088	5156	69				1	L
$tRNA^{Asn}$	5158	5230	73				26	L
$tRNA^{Cys}$	5257	5322	66				0	L
$tRNA^{Tyr}$	5323	5393	71				1	L
COI	5395	6942	1548		GTG	AGG	0	H
$tRNA^{Ser}$	6943	7004	62				2	L
$tRNA^{Asp}$	7007	7076	70				0	H
COII	7077	7763	687		ATG	TAA	1	H
$tRNA^{Lys}$	7765	7837	73				1	H
ATP8	7839	8006	168		ATG	TAA	-22	H
ATP6	7985	8680	696		ATG	TAA	-1	H
COIII	8680	9463	784		ATG	CAT	0	H
$tRNA^{Gly}$	9464	9531	68				0	H
ND3	9532	9883	352		ATG	TAG	-3	H
$tRNA^{Arg}$	9881	9951	70				0	H
ND4L	9952	10248	297		ATG	TAA	-7	H
ND4	10242	11618	1377		ATG	TAA	16	H
$tRNA^{His}$	11635	11703	69				0	H
$tRNA^{Ser}$	11704	11767	64				1	H
$tRNA^{Leu}$	11769	11840	72				0	H
ND5	11841	13634	1794		ATG	TAA	-5	H
ND6	13630	14154	525		CCT	CAT	4	L
$tRNA^{Glu}$	14155	14222	68				4	L
CYTB	14227	15370	1144		ATG	T	0	H
$tRNA^{Thr}$	15371	15441	71				1	H
$tRNA^{Pro}$	15443	15511	69				0	L
Control region	15512	17448	1937					H
CSB-1	16139	16158	20					
CSB-2	16224	16245	22					
CSB-3	16277	16305	29					
Tandem repeat	17321	17448	128					

*ND1-6 and ND4L: NADH dehydrogenase subunits 1-6 and 4L; COI-III: Cytochrome c oxidase subunits I-III; ATP 6 and 8: ATPase subunit 6 and 8; CYTB: Cytochrome b; T: Incomplete stop codon.

a Numbers correspond to the nucleotides separating adjacent genes. Negative numbers indicate overlapping nucleotides.

Table 2: Genome sizes and nucleotide compositions for the complete mitochondrial genomes of 25 species analyzed in this study.

	T (%)	C (%)	A (%)	G (%)	Genome size (bp)	AT skew	GC skew
Cuora mouhotii	27.3	25.8	34	12.8	16837	0.109	-0.337
Mauremys annamensis	26.9	26.4	33.7	13	16844	0.113	-0.338
Sacalia quadriocellata	26.8	25.9	34.1	13.2	16816	0.121	-0.327
Heosemys annandalii	26.7	25.9	35.1	12.3	16604	0.136	-0.356
Cyclemys dentata	27.2	25.4	34.3	13.1	16489	0.115	-0.32
Platysternon megacephalum	27.4	25.6	34	13	19043	0.107	-0.329
Geoemyda spengleri	27.6	25.6	33.7	13.1	17448	0.098	-0.321
Manouria emys	25.9	26.8	34.1	13.2	16455	0.137	-0.341
Pangshura sylhetensis	25.9	27.2	33.3	13.6	16568	0.125	-0.334
Chrysemys picta bellii	26.6	26	34.4	13	16875	0.127	-0.335
Malaclemys terrapin	27	25.9	34	13.1	16717	0.113	-0.328
Trachemys scripta	27	25.9	34.3	12.9	16810	0.119	-0.336
Batagur trivittata	24.5	28.6	33.6	13.3	16463	0.156	-0.367
Macrochelys temminckii	27.4	25.2	34.7	12.7	16569	0.117	-0.332
Pelochelys cantorii	24.1	28.6	35.2	12.1	17424	0.188	-0.405
Carettochelys insculpta	23.8	28.1	36.3	11.7	16439	0.209	-0.412
Natator depressus	25	27.6	35.3	12	16281	0.171	-0.392
Dermochelys coriacea	26.3	26.1	35.5	12.1	16501	0.148	-0.368
Kinosternon leucostomum	28.2	23.8	36	12	16559	0.121	-0.328
Chelodina longicollis	26.5	25	35.7	12.8	16647	0.148	-0.322
Pelusios castaneus	27.2	28.2	32.4	12.2	16761	0.087	-0.396
Podocnemis unifilis	28	26.4	33.5	12.1	16493	0.089	-0.369
Notochelys platynota	28.1	25.3	34.4	12.2	16981	0.101	-0.347
Mecistops cataphractus	25.8	27.4	32.6	14.3	16924	0.116	-0.314
Basiliscus vittatus	25.9	27.3	33.4	13.5	16948	0.127	-0.339

AT(Skew)=(A-T)/(A+T) GC(Skew)=(G-C)/(G+C).

overlapping with neighboring tRNAs or rRNAs. All tRNAs except $tRNA^{Ser}$ (AGY) could be folded into the typical clover-leaf secondary structure.

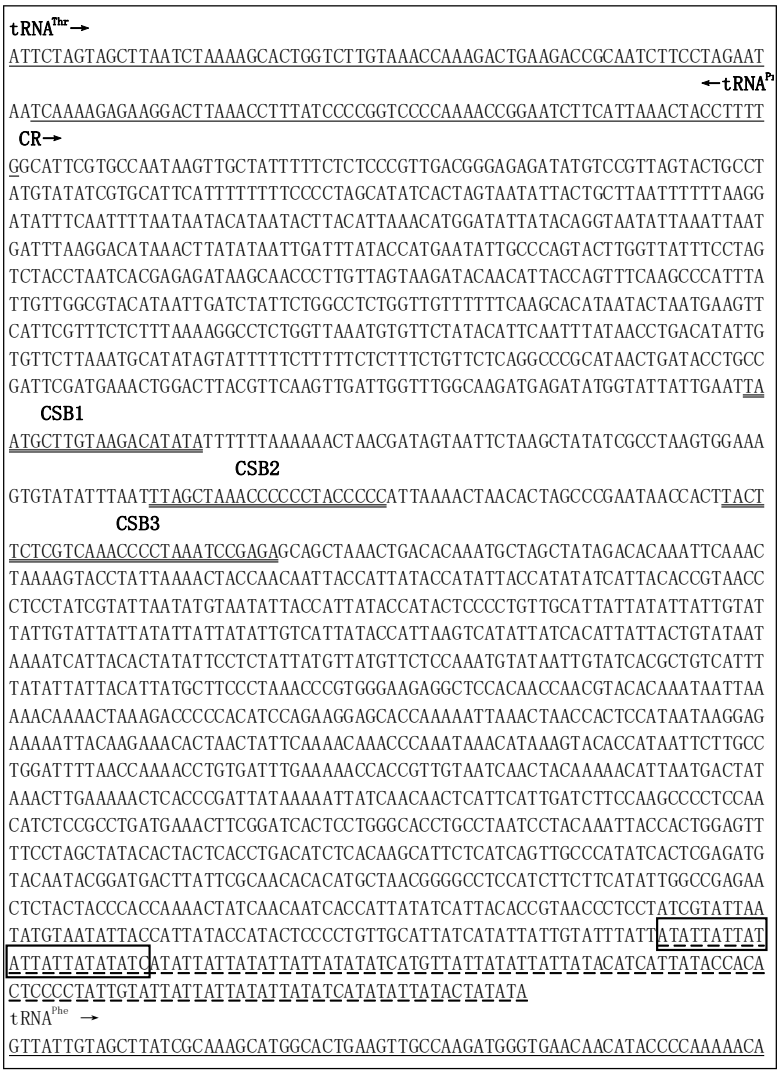
Control region (CR)

The major noncoding CR of G. spengleri was 1,937 bp long, flanked by $tRNA^{Pro}$ and $tRNA^{Phe}$ genes (Table 1). In this 67.99% A+T rich region, several conserved motifs including three conserved sequence blocks (CSB1, CSB2 and CSB3) were identified. The most striking feature of this control region was the presence of longer tandem repeats of (ATATTATTATATTATTATATATC) n (n=3) at the 3' end, downstream to the CSB3. Furthermore, it was followed by a AT-enriched microsatellite sequence flanking the $tRNA^{Phe}$ gene (Supplementary Fig 1).

Phylogenetic analysis

The ML trees are illustrated in Fig 3. From the resultant ML tree, the ingroup species were divided into two major clades: the Pleurodira (*Chelodina longicollis, Pelusios castaneus, Podocnemis unifilis*) and an assemblage of 20 cryptodires. Within the Cryptodira, 9 geoemydids formed a clade with a bootstrap value of 100%. The phylogenetic tree shows that Geoemydidae was monophyletic in origin and the genera and species in the family were gradually clustered. There were four clades in the family: 1. The *C. dentata-S. quadriocellata* assemblage and *N. platynota* formed a clade. 2. *C. mouhotii* and *M. annamensis* formed a clade. 3. *Heosemys annandalii* was a separate clade. 4. *G. spengleri* and the *B. trivittata-P. sylhetensis* assemblage forms a clade. In general, the Geoemydidae was the sister group to the monophyly of turtle (*M. emys*) of Testudinidae and was then clustered with the *D. coriacea-N. depressus* assemblage fllowed by a clade comprising of *P. megacephalum* and three representative turtles of Emydidae. The assemblage of the above species was in order clustered with *M. temminckii* followed by *K. leucostomum* and *P. cantorii*. Finally, the Carettochelyidae comprising of *C. insculpta* was sister group to all remaining Cryptodira used in this study.

Nine controversial geoemydid genera (nine species) were studied, based on morphological characters, Bramble (Bramble, 1974) hypothesized that Cyclemys, Pyxidea and Cuora formed a closely related assemblage (Cyclemys group) and originated from a Heosemys-like ancestor. In our resultant trees, Cyclemys, Sacalia and Notochelys formed a monophyletic group, Cuora and Mauremys formed another monophyletic group. These two monophyletic groups and their common ancestor formed a new monophyletic group and the intimate relationships between the common ancestor of all the descendants of this new monophyletic group and Heosemys were well supported (100% BP under ML), which seems to be consistent with their morphological characters.



Suplementary Fig 1: Nucleotide sequences of the control region and flanking tRNA genes from *Geoemyda spengleri* mitochondrial genome. The potential regulatory elements (CSB1, CSB2 and CSB3) are double-underlined, flanking tRNA genes and tandem repeats (containing AT microsatellites) are underlined with solid and dashed lines, respectively. A tandem repeated motif is boxed.

As far the relationships of the Geoemydidae, Testudinidae and Emydidae, some authors suggested a closer affinity of the geoemydid turtles with the emydids (Ernst and Barbour, 1989; Iverson, 1992) and the others suggested the Geoemydidae and Testudinidae originated from a common ancestor and sister-relationship of the two families inferred from morphological and molecular data (*e.g.,* Shaffer *et al*., 1997; Hirayama, 1984; Honda *et al*., 2002; Lee, 2019).Our ML analyses supported the latter view that the geoemydid turtles have closer affinities to the testudids than to emydids with high statistical supports (Fig 3).

Based on morphological and partial mtDNA data, the Chelydridae and Platysternidae were considered as sister taxa (Gaffney and Meylan, 1988; Gaffney *et al*., 1991; Shaffer *et al*., 1997). In contrast, most other workers elucidated that Platysternon was more similar to emydids and testudinoids based on morphological evidence (Whetstone,1978), karyotype characteristics (Haiduk and Bickham,1982) and molecular data (Parham *et al*., 2006). The present ML analysis implied that Platysternon is a sister taxon to the Emydidae (*C. picta*, *M. terrapin* and *T. scripta*) with a strong support of 98%. Obviously, Platysternon had more intimate relationship with emydidae than Chelydridae according to the phylogram. But some workers agreed with placing Platysternon within Testudinoidea (Wu, 1999). Therefore, further verifications are definitely needed to determine their exact phylogenetic relationships.

In this study, the distribution maps of 9 species of the Geoemydidae were drawn to illustrate the species distribution of 9 genera of the Geoemydidae. Looking at the habitat distribution of *G. spengleri*, the *B. trivittata* and the *Pangshura sylhetensis*, we found something interesting (Fig 4). All three species are found in countries or regions with abundant water resources, but there is no overlap in

their habitats. In addition, these three species are only distributed in the southern hemisphere and are mostly located in the area between 8 degrees and 28 degrees North latitude.

Several other species of the family have a wider range of habitats in terms of latitude than these three species. The latitudinal zonality is mainly manifested by the regular change of climate, soil, organisms and their environment

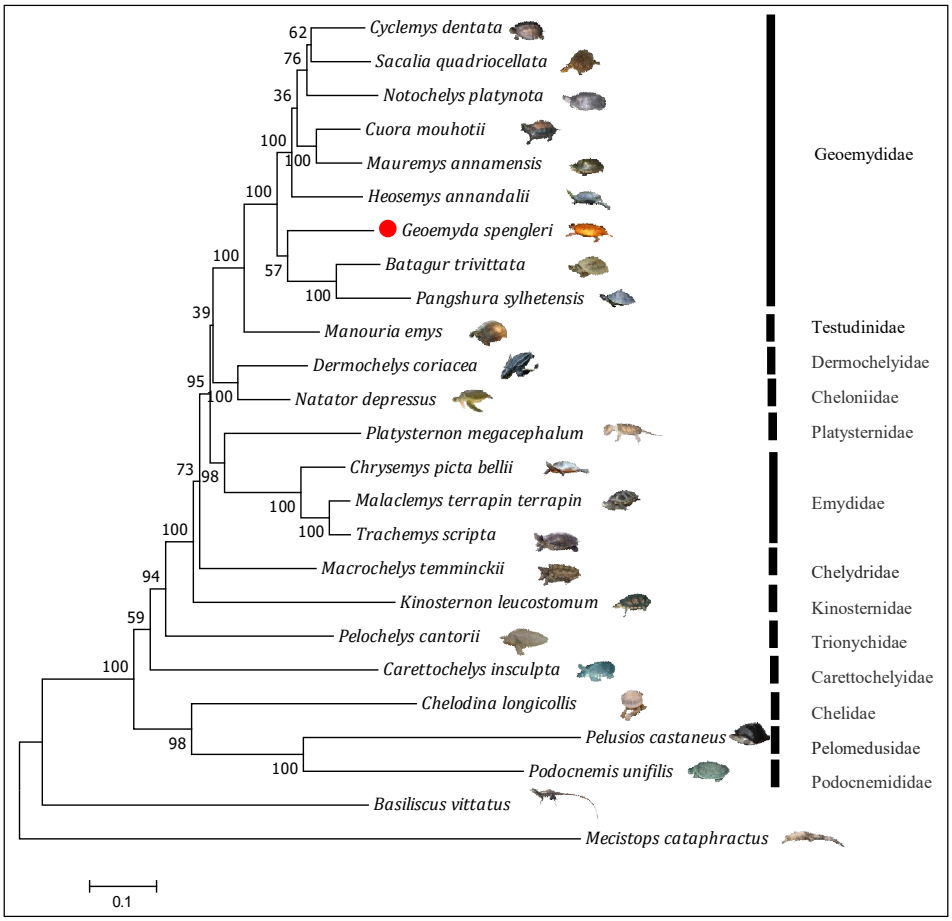


Fig 3: Maximum-likelihood (ML) phylogram derived from 13 protein combined sequence data of 25 taxa.The percentage of trees in which the associated taxa clustered together is shown next to the branches. *Basiliscus vittatus* and *Mecistops cataphractus* are used as outgroups.

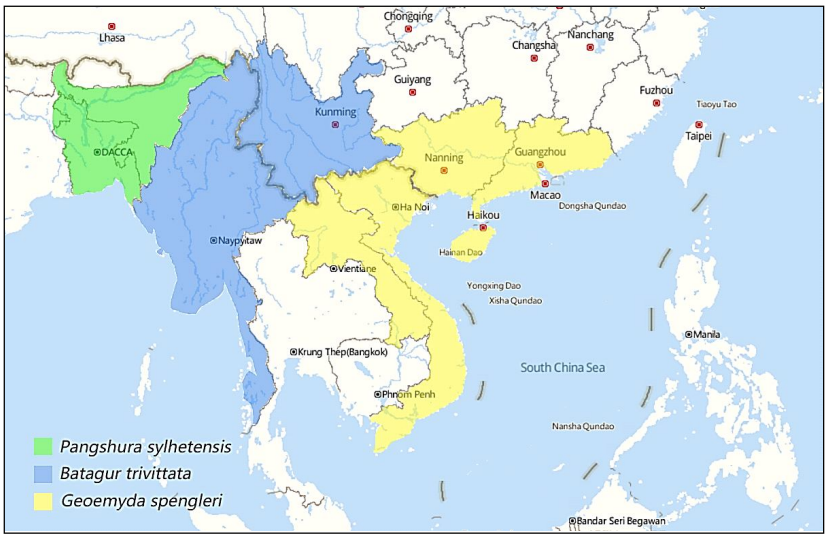


Fig 4: Habitat distribution of *P. sylhetensis*, *B. trivittata* and *G. spengleri.*

from the equator to the poles (Das, 2018). Because the latitude distribution of these three species is more similar than that of the other turtles and the longitude distribution of the habitat of *B. trivittata* and *G. spengleri* is more similar than that of *P. sylhetensis*, we guess that *G. spengleri* and *B. trivittata* are more closely related. This is also consistent with the results shown in the phylogenetic tree.

CONCLUSION

In summary, our mtDNA genome sequence data provided a new stock of useful information to review the relationships among major turtle lineages and verify preliminary hypotheses to turtle evolution further. Obviously, larger number of sample groups and more gene information are needed towards getting a definitive understanding of the grand evolution problem.

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