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Complete mitochondrial genome of the *Cyclemys dentata* and phylogenetic analysis of the major family Geoemydidae

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ABSTRACT. In the present study, the complete mitochondrial (mt) genome of *Cyclemys dentata* was determined using PCR reactions. The structural organization and gene order of *C. dentata* were equivalent to those of most other vertebrates. The mt genome was 16,489 bp in length, has rich A+T content, consisting of 13 protein-coding genes, 2 ribosomal RNA genes, 22 transfer RNA genes, and a control region (D-loop). All protein-coding genes started with ATG, many genes have complete stop codons, except *ND2*, *COX3*, *ND3*, and *cyt-b* genes had incomplete stop codons of T. The light-strand replication origin (O_L) of *C. dentata* might fold into a stable stem-loop secondary structure, and its loop had 2 nt less than that of the *Cyclemys atripons* O_L sequence. The D-Loop of *C. dentata* contained a central domain (CD), 2 extended termination associated sequences (ETAS1, ETAS2) and 3 conserved sequence blocks (CSB1, CSB2, CSB3). The average length of 20 turtles' mt genomes was 16,692.5 bp, including 34.1% A, 27.0% T, 26.0% C and 12.9% G. The *C. dentata* mitochondrial genome

could provide useful data for further studies on phylogenetics and conservation genetics of this species. The phylogenetic relationships of the family Geoemydidae were analyzed by maximum-likelihood (ML) and neighbor-joining (NJ) based on concatenated sequences of 13 protein-coding genes from 20 turtle species. The ML and NJ trees had homologous topologies. The results support the existing classification of the genera of Geoemydidae, that *C. dentata* was a sister species of *C. atripons*, *Pyxidea* nested in *Cuora*, and *Chinemys* was synonymous with *Mauremys*.

Keywords: *Cyclemys dentata*; Mitochondrial genome; Phylogenetic relationships

INTRODUCTION

In vertebrates, the mitochondrial (mt) genome is a small double-stranded circular DNA, ranging in size from 15 to 20 kb. The typical mt genome contains 38 components, including 13 protein-coding genes, 2 ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes, and one control region (D-loop) (Boore, 1999). Because of its maternal inheritance, relative lack of recombination, and a higher rate of base substitution than most nuclear genes, the mt genome has been widely used to study taxonomy, phylogeny, genetic structure, and biological identification (Jiang et al., 2011). It has been employed as a marker in turtle science (Avise et al., 1992; Bowen et al., 1994; Shaffer et al., 1997; Kumazawa and Nishida 1999; Cao et al., 2000; van der Kuyl et al., 2002; Spinks et al., 2004; Krenz et al., 2005; Parham et al., 2006; Fritz et al., 2008; Stuart and Fritz, 2008; Zhang et al., 2008b; Wiens et al., 2010).

Turtles are one of the most instantly recognizable life forms on Earth; their characterization is dated back more than 200 million years ago (Krenz et al., 2005). With regard to the more recent diversity of turtles and tortoises throughout the world, there are 452 taxa comprising modern chelonians. Of these, 11 taxa have been extinct since 1500 AD; thus, currently, there are 441 taxa comprising living chelonians, including 322 species and 119 additional subspecies. The living chelonians are divided into 2 reciprocally monophyletic clades, including Pleurodira and Cryptodira (van Dijk et al., 2012). Geoemydidae, a Cryptodira family, is the world's largest turtle family and is comprised of 23 genera and approximately 73 species (Iverson, 1992; van Dijk et al., 2012).

The Asian Leaf Turtle, *Cyclemys dentata* (Gray 1831), belongs to the family Geoemydidae and is widely distributed from northern India to the Philippines; it can also be found in southern China (Stuart and Fritz, 2008). Although *C. dentata* is currently categorized as "Near Threatened" in the Red List by the International Union for the Conservation of Nature (IUCN, 2012), a large number of Asian leaf turtles are often caught and sold for the pet trade, food trade, or use in traditional Chinese medicine; thus, the number of wild turtles is declining. *C. dentata* is captive bred in China, which minimizes the impact on wild populations. However, the phylogeny of *C. dentata* is still poorly understood. Only the cytochrome b (*cyt-b*) gene of *C. dentata* mitochondrial DNA (mtDNA) and 3 nuclear DNA sequences (i.e., *C-mos* and *Rag2* genes, and *R35* intron) have been used to study the diversity of *Cyclemys* species (Fritz et al., 2008; Stuart and Fritz, 2008). Clearly, further study on *C. dentata* is necessary

for species identification and conservation. In this paper, we sequenced and characterized the complete mt genome of *C. dentata*, and compared it to the mtDNA of other turtle species to acquire available molecular genetic information. We constructed molecular phylogenetic trees based on the mitochondrial genes to confirm relationships among the genera of the family Geoemydidae.

MATERIAL AND METHODS

DNA sample

The muscle sample from a 5-year-old, female *C. dentata* was obtained from the Zhong-Yi Turtle Farm of Qin-Zhou city in Guangxi Province, China. Total DNA was extracted from the muscle sample according to the method described by Sambrook and Russell (2001) and was used to sequence the complete mt genome.

Polymerase chain reaction (PCR) and sequencing

Sixteen pairs of primers were designed based on the complete mt genome of *Cyclemys atripons* (GenBank accession No. EF067858) and were used to amplify the mtDNA fragments of *C. dentata* via PCR, with each fragment sequence overlapping the next by >50 base pairs (bp). These primers are listed in Table 1. The PCR cycles were as follows: 5 min at 94°C; followed by 35 cycles of 30 s at 94°C, 45 s at 55-56°C, and 2 min at 72°C; with a final extension at 72°C for 5 min. PCR was carried out in a 50-µL reaction mixture containing 100 ng total DNA, 1X PCR buffer, 0.5 mM dNTPmix, 1.5 mM $MgCl_2$, 1.0 µM of each primer, and 2.0 U *Taq*DNA polymerase (TaKaRa, Dalian, China). The 16 PCR products were purified from 1% agarose gel with the DNA Agarose Gel Extraction Kit (TaKaRa) and then sequenced directly by the primer walking method in an ABI3730 (Invitrogen Biotechnology Co. Ltd., USA). More than 3 copies of each fragment were sequenced to verify the reliability of the data.

Table 1. Sixteen PCR primers used in amplification of the complete mitochondrial genome of *Cyclemys dentata*.

No.	Primers (5'→3')		Size	Tm
	forward	reverse	(bp)	(°C)
1	CATGGCACTGAAGTTGCCAAG	TTTCGTCTTTCCCTTGCGGTA	1209	56
2	CCCACCATAAACCAATTAAAAC	GTTTTATTTTACTCCGAGGTCG	1121	55
3	TAAGACGAGAAGACCCTGTGGAAC	AGGAAGAATAAGGCGAATGGG	1275	55
4	GCACCATTTGATTTAACCGAA	ATTATTCATCCTAGATGGGCGA	1129	55
5	GGGGACTAAATCAAACCCAAC	TATACTGTTCATCCTGTGCCCG	1287	55
6	GTACCCTTAATAATTGGAGCGC	TTCTTGGGTTTGTACATGGGC	1278	56
7	TAGAATGATTACATGGCTGTCCACC	TTGCTTAGGGTTGGGAAGATGAT	1219	56
8	CCAATATAAACCCATGAACCTG	TATACACCGTCAGCGATTGTG	1265	55
9	CCAACCGAAACCAAACTATTC	CTATGGGTTAATGCGGTAGGT	1176	55
10	GCTGGCATAGGCTTATCATT	TGGCTGAGAACCATCATAGG	1150	55
11	CTAACACGAACAGAATGAGCA	TTCTGTTCGTCCTCGTCATC	1160	55
12	GCCAACAACATACTTCAACTT	TTTCATACCAGGATAGGTCG	1256	55
13	CCAACTCATTAGCCTACTTCA	TTGGCCTCATGGTAAGACAT	1228	55
14	CATCGGTCGAGGACTTTACTAC	GGGTTGCTTATCTCTCGTGAT	1278	55
15	TATGTATTATCGTGCATTCAT	GTGTCAGTTTAGTTGCTCTC	720	55
16	GAGAGCAACTAAACTGACACA	AGTAAGGCTAGGACCAAATCT	269	56

Tm = temperature.

Data analysis

Sequence data were analyzed with the DNASTAR Lasergene 7.0 software (DNASTAR Inc., USA). Overlapping contigs were compiled using SeqMan to assemble a continuous sequence. The locations of protein-coding genes, ribosomal RNA(rRNA), tRNA, and the control region were identified by tRNAscan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/), MegAlign program (DNASTAR), and a Blastn (http://www.ncbi.nim.nih.gov/BLAST/) comparison to other known turtle mt genomes. The complete mtDNA sequence of *C. dentata* was submitted to GenBank. Structure analysis of the control region was conducted in accordance with previously published methods (Sbisà et al., 1997; Zhang et al., 2008b).

Phylogenetic analyses of turtles were performed using MEGA version 5.2 (Tamura et al., 2011). The maximum composite likelihood (MCL) method was used to estimate evolutionary distances between DNA sequences. Nineteen mtDNA sequences of Geoemydidae were used to conduct the phylogenetic analyses, and *Manouria impressa* (Cryptodira; Testudinidae) mtDNA was used as the outgroup (Table 2). Thirteen protein-coding genes of these mtDNAs were aligned, and phylogenetic trees were constructed by maximum-likelihood (ML) and neighbor-joining (NJ) using MEGA 5.2. Each node of the tree was assessed with bootstrap percentages computed based on 1000 replications.

Table 2. Twenty turtles were used to conduct a phylogenetic analysis and nucleotide composition contrast of mitochondrial genomes.

Family	Genus	Species	Accession No.	Nucleotide proportion (%)				mtDNA size (bp)
				T	C	A	G	
Geoemydidae	*Cuora*	*Cuora amboinensis*	FJ763736	26.7	26.4	33.8	13.1	16708
		Cuora aurocapitata	AY874540	27.4	26.0	33.6	13.0	16890
		Cuora bourreti	JN020145	26.8	26.2	33.9	13.1	16649
		Cuora galbinifrons	EU809939	27.6	25.8	34.1	12.5	17244
		Pyxidea mouhotii	DQ659152	27.3	25.8	34.0	12.8	16837
		Cuora pani	GQ889364	27.4	25.9	33.7	13.0	16922
		Cuora picturata	JF712890	26.9	26.1	34.0	13.0	16623
	Cyclemys	*Cyclemys atripons*	EF067858	27.2	25.4	34.4	13.0	16500
		Cyclemys dentata	JX455823	27.2	25.4	34.3	13.1	16489
	Heosemys	*Heosemys annandalii*	JF742646	26.7	25.9	35.1	12.3	16604
	Mauremys	*Mauremys japonica*	GU938833	26.5	26.5	34.0	13.0	16443
		Mauremys sinensis	FJ871126	26.2	26.8	33.8	13.2	16461
		Mauremys mutica	DQ453753	26.5	26.5	33.8	13.2	16609
		Chinemys reevesii	AY676201	26.6	26.4	34.0	12.9	16576
		Mauremys reevesii	FJ469674	27.0	26.1	34.1	12.8	16781
		Mauremys megalocephala	HM132059	27.2	25.9	34.1	12.8	16783
	Notochelys	*Notochelys platynota*	HQ853256	28.1	25.3	34.4	12.2	16981
	Sacalia	*Sacalia bealei*	GU183364	26.9	25.9	34.2	13.1	16561
		Sacalia quadriocellata	EF088646	26.9	25.9	34.3	12.9	16551
Testudinidae	*Manouria*	*Manouria impressa*	EF661586	26.3	26.4	34.2	13.1	16642
			Average:	27.0	26.0	34.1	12.9	16692.5

RESULTS

Mitochondrial genome features

The mitochondrial genome of *C. dentata* was initiallysequenced. It was 16,489 bp

in length and has been deposited into GenBank (accession No. JX455823). The structural organization (Table 3) and gene order (Figure 1) of the complete *C. dentata* mtDNA was identical to that of other typical vertebrates, with the mt genome containing the following: 13 protein-coding genes, 2 rRNAs, 22 tRNAs, and a control region (D-Loop). The overall base compositions of the *C. dentata* mtDNA were as follows: A = 34.3, T = 27.2, C = 25.4, and G = 13.1%. A+T was observed more frequently than G + C, demonstrating the low G and high A + T bias that is seen in other turtle species. The average base frequencies for the mtDNA of 20 turtle species were as follows: A = 34.1, T = 27.0, C = 26.0, and G = 12.9% (Table 2). This A+T rich pattern reflects the typical sequence characteristic of the vertebrate mt genome. All protein-coding genes of the *C. dentata* mtDNA started with ATG. The *ND2*, *COX3*, *ND3*, and *cyt-b* genes had incomplete stop codons of T; the *COX1* and *ND6* genes ended with AGG, the remaining protein-coding genes terminated with TAA (Table 3). The incomplete termination codon (i.e., T--) was common in animal mtDNAs and can be converted into a termination codon (i.e., TAA) after transcription (Boore, 2001).

Table 3. Structural organization of the *Cyclemys dentata* mitochondrial genome.

Gene/elements	Position		Size	Strand	Codon	
	Origin	Stop	(bp)	(sense)	start	stop
$tRNA^{Phe}$	1	70	70	H		
12S rRNA	71	1036	966	H		
$tRNA^{Val}$	1037	1106	70	H		
16S rRNA	1107	2705	1599	H		
$tRNA^{Leu(UUR)}$	2706	2781	76	H		
ND1	2782	3750	969	H	ATG	TAA
$tRNA^{Ile}$	3753	3822	70	H		
$tRNA^{Gln}$	3822	3892	71	L		
$tRNA^{Met}$	3892	3960	69	H		
ND2	3961	5002	1042	H	ATG	T - -
$tRNA^{Trp}$	5003	5077	75	H		
$tRNA^{Ala}$	5079	5147	69	L		
$tRNA^{Asn}$	5149	5221	73	L		
O_L	5223	5250	28	L		
$tRNA^{Cys}$	5248	5313	66	L		
$tRNA^{Tyr}$	5314	5384	71	L		
COX1	5386	6933	1548	H	ATG	AGG
$tRNA^{Ser(UCN)}$	6925	6995	71	L		
$tRNA^{Asp}$	6998	7067	70	H		
COX2	7068	7754	687	H	ATG	TAA
$tRNA^{Lys}$	7756	7828	73	H		
ATP8	7830	7997	168	H	ATG	TAA
ATP6	7988	8671	684	H	ATG	TAA
COX3	8671	9454	784	H	ATG	T - -
$tRNA^{Gly}$	9455	9523	69	H		
ND3	9524	9872	351	H	ATG	T - -
$tRNA^{Arg}$	9873	9942	70	H		
ND4L	9943	10242	300	H	ATG	TAA
ND4	10236	11612	1377	H	ATG	TAA
$tRNA^{His}$	11626	11695	70	H		
$tRNA^{Ser(AGY)}$	11696	11761	66	H		
$tRNA^{Leu(CUN)}$	11761	11832	72	H		
ND5	11833	13638	1806	H	ATG	TAA
ND6	13634	14158	525	L	ATG	AGG
$tRNA^{Glu}$	14159	14226	68	L		
Cyt *b*	14231	15370	1150	H	ATG	T - -
$tRNA^{Thr}$	15375	15446	72	H		
$tRNA^{Pro}$	15448	15516	69	L		
Control region	15517	16489	973	H		

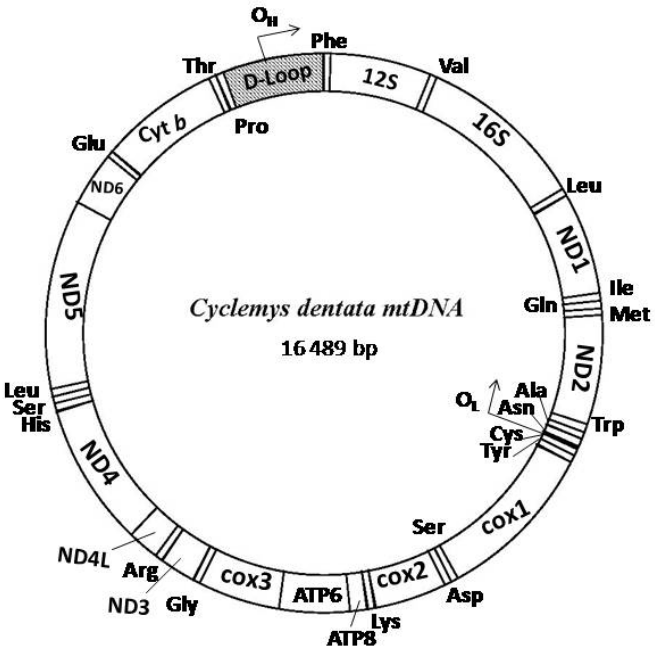

Figure 1. Order of the circular genes of the mitochondrial genome of *Cyclemys dentata*. 12S and 16S: 12S rRNA and 16S rRNA; ND1-6, ND4L: NADH dehydrogenase subunits 1-6, 4L; COX1-3: cytochrome oxidase subunits 1-3; Cyt b: cytochrome b; ATP6, 8: ATP synthase subunits 6, 8; D-loop: control region; each tRNA gene was identified by the 3-letter amino acid code; O_H, O_L: the replication origin of the H-strand and light strand replication.

Noncoding sequences

The lengths of the *C. dentata 12S* and *16S* rRNA genes were 966 and 1599 bp, respectively, which were similar to other vertebrate species. Two genes were separated by the tRNA-^{Val} and positioned between the tRNA^{Phe} and tRNA$^{Leu\ (UUR)}$ (Table 3). There were 22 tRNA genes in the *C. dentata* mt genome, ranging in size from 66 to 76 bp (Table 3), that were interspersed between the protein-coding genes and rRNA, which are typical animal mtDNA genes that can be folded into the analogous cloverleaf secondary structure.

The light-strand replication origin (O_L) of *C. dentata* was 28 bp in length, which was positioned in the WANCY tRNA genes cluster and located between tRNA^{Asn} and tRNA^{Cys} (Table 3). The O_L sequence of *C. dentata* might potentially fold into a stable stem-loop secondary structure, with a stem comprised of 10 bp and a loop of 8 nucleotides (nt); this loop had 2 nt less than that of the *Cyclemys atripons* (*C. atripons*) O_L sequence (Figure 2).

The length of the *C. dentata* mtDNA control region was 973 bp, 68.35% A+T rich, and flanked by the tRNA^{Pro} and tRNA^{Phe} genes (Table 3). Similar to other turtle species, 2 extended termination associated sequences, one central domain, and 3 conserved sequence blocks were identified in the *C. dentata* control region (Figure 3). There were AT enriched tandem repeats at the 3' end of this control region. The composition and number of these tandem units were variable for the different species, with 27 5'-TA-3' repeats in the *C. dentata* control region.

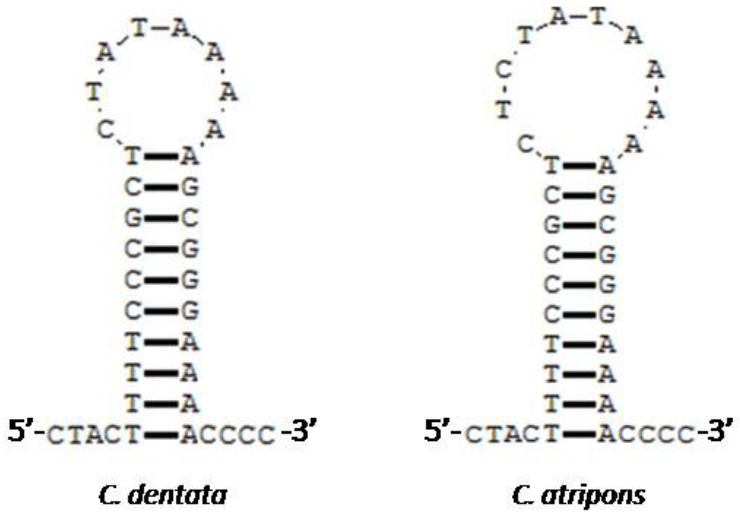

Figure 2. Secondary structures of the origins of the O_L of 2 turtles.

Figure 3. Structural elements of the control region (D-loop) of *Cydemys dentata*. ETAS1, 2: extended termination associated sequences 1, 2; CD: central domain; CSB1-3: conserved sequence blocks 1-3.

Phylogenetic analysis

The NJ and ML phylogenetic trees, which were constructed based on the mtDNA of the protein-coding genes from 20 turtle species, had similar topologies (Figure 4). According to clusters of the phylogenetic trees, *C. dentata* was a sister species to *C. atripons* (BP=100%), which initially clustered with the genus *Sacalia*, followed by clustering with the families *Heosemys* and *Notochelys*. Seven turtles of the family *Cuora* clustered with the monophyly of 6 turtles of the family *Mauremys*. Nineteen representative turtles of Geoemydidae were divided into 6 families.

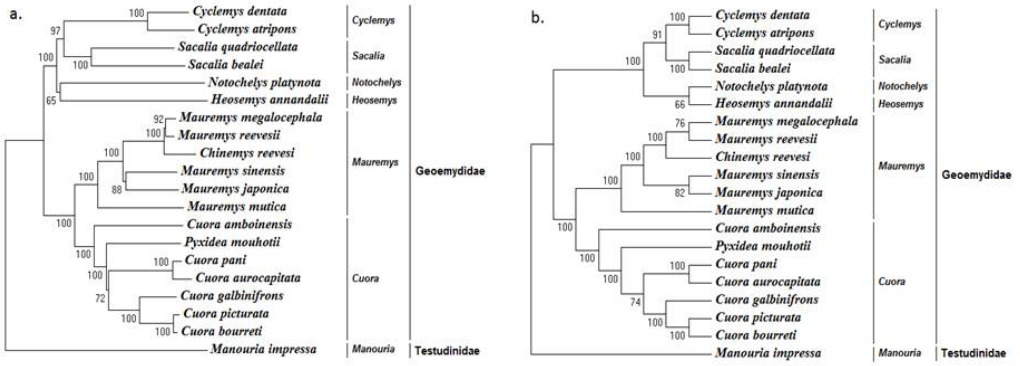

Figure 4. Phylogenetic trees constructed based on protein-coding genes from 20 turtles. Numbers above the branches indicate bootstrap proportions (BPs). *Manouria impressa* were used as outgroups.

DISCUSSION

The mitochondrial genome of vertebrates universally contained 37 genes and one control region, and the organization of the genes was almost analogous (Boore, 1999). The mt genome of *C. dentata* was 16,489 bp in length, and contained 37 genes and a control region. The organization and gene order of *C. dentata* mtDNA were similar to those of most other turtle species (Zhang et al., 2008a,b; Kumazawa and Nishida, 1999), except those of *Platysternon megacephalum*, whose complete mtDNA contained 2 control regions (Parham et al., 2006) and other distinctive features (Peng et al., 2006). We found that the size and nucleotides occupying the *C. dentata* mtDNA were the same as those of *C. atripons*. In addition, we did not find insertions in the *ND3* gene; these extra nucleotides can be found in some turtle species (Mindell et al., 1998). The origin of the L-strand replication (O_L) sequence nucleotides and secondary structure were conserved and may fold into a stable stem-loop structure. However, the base numbers of the stem or loop were among the different species. The stem and loop primarily contained 9 to 11 and 7 to 10 nt, respectively (Zhang et al., 2008b). The O_L of *C. dentata* contained a 10-bp stem and 8-nt loop, which was different from that of *C. atripons* (i.e., 10-bp stem and 10-nt loop). The *C. dentata* control region was rich in A + T, and its 3' end had 27 5'-TA-3' tandem repeats. The base composition of the repeat was different from that of other turtle species; there were 5'-A (AT) 3-3' repeats in *C. atripons* (EF067858) and 5'-ATATATC-3' units in *C. reevesii* (AY676201). The difference in the AT enriched tandem repeats may reflect species genetic diversity in turtles, and these repeats could be regarded as special genetic markers for use in genomic studies of turtles.

The Geoemydidae is the largest turtle family, contains rich species-level diversity, and is widely distributed throughout most of the planet's continents (Iverson et al., 1992; Spinks et al., 2004). However, the family Geoemydidae is known to be poor and in flux. In the present study, *Cuora*, *Cyclemys*, and *Pyxidea* were classified into the *Cyclemys* group, which was derived from a *Heosemys*-like ancestor, and the 4 genera were united into a *Heosemys* complex (Bramble, 1974). Afterwards, the *Cuora* were divided into *Cuora* and *Cistoclemmys* based on turtle morphology and 4 chromosomal characteristics (Hirayama, 1984; Yasukawa et al., 2001). However, Honda (2002) recommended synonymizing *Cistoclemmys* and *Pyxidea* with *Cuora* based on a phylogenetic analysis of rRNA sequences from mtDNA (Honda

et al., 2002). A phylogenetic analysis of *Cyclemys* with the *cyt-b* gene of mtDNA and nuclear DNA data strongly support that *Cyclemys* contains 3 known species (i.e., *C. pulchristriata*, *C. atripons*, and *C. oldhamii*) and 2 undescribed species (Guicking et al., 2002). Spinks (2004) used mt and nuclear DNA data to study the phylogenetic relationships among 65 species and subspecies representing all 23 genera of Geoemydidae. The results suggest that the genus *Mauremys* is paraphyletic with *Ocadia* and *Chinemys* (Spinks et al., 2004). In the present study, we compiled the complete 13 protein-coding genes sequences of mtDNA to reconstruct the phylogenetic relationships among 19 the genera of Geoemydidae. The topologies were consistent between the NJ and ML trees. Our results supported the nesting of *Pyxidea* within *Cuora* and that *Chinemys* was synonymous with *Mauremys*.

Leaf turtles (genus *Cyclemys*, Geoemydidae) (Bell, 1834) were previously thought to belong to a genus that included only one or 2 species (Iverson, 1992); later, additional species were identified (Guicking et al., 2002; Fritz et al., 1997, 2008; Stuart and Fritz, 2008). According to morphology and preliminary nuclear DNA data, the *Cyclemys* could have 6 species, including 2 morphologically distinct groups, a yellow-bellied species group (*C. dentata*, *C. atripons*, and *C. pulchristriat*a) and a dark-bellied species group (*C. enigmatica*, *C. fusca*, and *C. gemeli*) (Fritz et al., 2008). In our study, *C. dentata* and *C. atripons* were clustered together, indicating that they are sister species. Fritz et al. (2008) found that the mtDNA sequences of *C. enigmatica* did not differ from those of *C. dentata* but were highly distinct with regard to nuclear genomic markers, suggesting that the original mt genome of *C. enigmatica* was lost due to introgressive hybridization (Fritz et al., 2008). Hybridization among species might lead to a loss in biodiversity within the genus *Cyclemys*. On the other hand, the pet trade, food trade, and use in traditional Chinese medicine threaten wild populations of *Cyclemys* species. Thus, it is critical to conduct a comprehensive investigation on the genetic diversities of *Cyclemys* species and draft effective measures for the protection of wild turtles.

CONCLUSION

The structural organization and gene order of the *C. dentata* mitochondrial genome were similar to those of most other turtles; the complete mtDNA contained 37 genes and one control region. Based on the complete 13 protein-coding gene sequences, we reconstructed the phylogenetic relationships for 20 turtle species. The results support the existing classification of genera of Geoemydidae. We plan to conduct further studies on the genetic diversity of *Cyclemys* species in the near future.

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