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Short Communication

Complete sequence and organization of the mitochondrial genome of *Cyclemys atripons* (Testudines, Geoemydidae)

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Abstract

The Black Bridged Leaf Turtle, *Cyclemys atripons* (Testudines; Cryptodira; Geoemydidae), is a poorly known species within the genus *Cyclemys*. We determined the complete nucleotide sequence of the *Cyclemys atripons* mitochondrial genome (mtDNA) and found it to be 16,500 base pairs (bp) in length, with the genome organization, gene order and base composition being identical to that of the typical vertebrate. However, unlike for most turtle mtDNA so far reported, an extra base was not found in the NADH3 gene. The *C. atripons* control region of mtDNA was 981 bp long. Comparisons with three other geoemydids showed that the *C. atripons* control region contained a highly variable region at the 3' end composed of AT enriched tandem repeats containing a fifteen-unit 5'-A $(AT)_3$-3' variable number of tandem repeats (VNTRs).

Key words: control region, *Cyclemys atripons*, mitochondrial genome, tandem repeats.

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Vertebrate mitochondrial (mt) DNA forms a double-stranded circular molecule of about 15-20 kb which generally contain 37 genes encoding 13 proteins, 22 tRNAs, 2 rRNAs and a major noncoding region bearing signals for mitochondrial replication and transcription (Wolstenholme, 1992). Due to its maternal inheritance and relative lack of recombination, the mitochondrial genome has been widely employed as a marker in vertebrate phylogenetic analyses, and have been often used in turtle science. Turtles are easily recognizable by the public, with approximately 270 species in the world (Iverson, 1992) and have been widely studied, with many earlier studies of mtDNA having concentrated on polymorphism analysis using restriction fragment length polymorphism (RFLP) and determining partial sequences (Lamb *et al.*, 1989; 1994). However, the trend in more and more studies is to move to direct sequencing of the complete mtDNA (Peng *et al.*, 2005, 2006; Parham *et al.*, 2006). As of March 2007, complete mitochondrial genomes have been released from GenBank for only 17 turtle species, including 16 cryptodiran turtles and one side-necked turtle, which is far from being sufficient for reliable turtle studies.

The Black Bridged Leaf Turtle, *Cyclemys atripons* (Testudines; Cryptodira; Geoemydidae) is a poorly understood cryptodiran turtle species within the genus *Cyclemys* (Guicking *et al.*, 2002). Previously, only the cytochrome *b* (Cyt *b*) gene of *C. atripons* mtDNA has been published (Spinks *et al.*, 2004), clearly, further studies on this species are necessary. In our study, described in this paper, we sequenced and characterized the complete mitochondrial genome of *C. atripons*, which has laid the foundation for the further comparative analyses between *C. atripons* and other turtles.

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In 2005 a *C. atripons* specimen was obtained from the suburbia of Longzhou city in the Chinese region of Guangxi, after natural death frozen at -80 °C for preservation. Total DNA was extracted from the liver and muscle tissue using the proteinase K method (Sambrook and Russell, 2001) and kept at -20 °C until needed for polymerase chain reaction (PCR) amplification.

Based on partial sequences reported by Spinks *et al.* (2004) and the similarity of mtDNA sequences of the painted turtle (Chrysemys *picta*; GenBank NC_002073) and Reeve's Turtle (Chinemys *reevesii*; GenBank AY676201) we designed 16 pairs of primers for PCR amplification (Table 1). The PCR was carried out in a total volume of 25 μL containing 100 ng of sample genomic DNA, 2.5 μL of 10×Buffer (TaKaRa, Japan), 2 μL of 2.5 mol L^{-1} of $MgCl_2$, 1.5 μL of each dNTP, 0.25 μL of each primer (25 μmol L^{-1}) and 1 unit of Taq DNA polymerase (TaKaRa). The thermal cycles were 95 °C pre-denaturing for 2 min, followed by 35 cycles of 94 °C for 40 s, 51 °C to 58 °C for 45 s and 72 °C for 1 min, plus a final ex-

Table 1 - Polymerase chain reaction primers used in the determination of the complete mitochondrial genome of *Cyclemys atripons*.

Primer	Primers (Y = C/T, R = A /g, W = A /T, M = A/C, H = A/C /T)		Approximate product length (bp)
	Upper light strand (L) 5'→3' sequence	Upper heavy strand (H) 5'→3' sequence	
1	L1 = AAGCATGGCACTGAAGTTGC	H1 = TTTCATCTTTCCTTGCGGTAC	1,116
2	L2 = AAAGCATTCAGCTTACACCTGA	H2 = AAGTTCCACAGGGTCTTCTCG	1,065
3	L3 = TAATGCCTGCCCAGTGACA	H3 = TGATTCCGAGGGTTACTTC	1,104
4	L4 = TCAGGGTGAGCTTCAAACTC	H4 GTAGTTGGGTTTGGTTTARTCC	1,200
5	L5 = ACCTGACAAAAACTAGCCCCA	H5 = ACTATTCCTGCTCAGGCHCCG	1,174
6	L6 = THTTCTCYACTAACCATAAAG	H6 = AAATCYTGCTATGATGGCGAA	1,052
7	L7 = GCTATTCCCACAGGAGTAAAAG	H7 = GCTATCCTGTTTAGCTTCTATAG	1,300
8	L8 = AAGTGGATGCARTCCCAGGACG	H8 = GTTATTAGTAGTGCTGCTGYTGC	1,180
9	L9 = GCCTCTATCTACAAGAAAAC	H9 = GAARAATCGAATTGAGAATGG	960
10	L10 = AGTACAAGTGACTTCCAATCA	H10 = TTTGRTTWCCTCATCGTGTG	1,300
11	L11 = GAACCAACCTCACGAAAACG	H11 = GCTGTTTTTACGGCTGTTTTTG	1,200
12	L12 = AGGATAGAAGTAATCCAGTGG	H12 = TATCTTTCGRATGTCTTGTTC	1,000
13	L13 = CATACACGCMTTCTTYAAAGC	H13 = CTAATAGTGATCCGAAGTTTCAT	1,300
14	L14 = AACCACCGTTGTATTCAACTA	H14 = CAATCTTTGGTTTACAAGACC	1,124
15	L15 = AGCAGCCTCCATTCTWTATTT	H15 = CAGTCTCATTGAGTYGGCAG	800
16	L16 = TTTTACTCTCCCGTGCCCA	H16 = GTCACATTTTACGCCGATT	980

tension at 72 °C for 10 min. The resultant PCR fragments were first resolved on 1% (w/v) agarose gel (Promega, USA). After electrophoreses, gels were stained with ethidium bromide and bands were visualized under ultraviolet. Bands of intended size were excised and recovered with Gel Extract Purification Kit (TaKaRa, Japan). The cleaned PCR products were sequenced in both directions on an ABI3730 automated sequencer (Invitrogen Biotechnology). The sequences obtained from each sequencing reaction averaged 1000 bp in length and each segment overlapped the next contig by roughly 150 bp. The whole mtDNA genome sequence was read at least twice.

Sequence data were analyzed with the EditSeq (DNASTAR) and ClustalX1.8 (Thompson *et al.*, 1997) programs. The locations of protein-coding, rRNA and tRNA genes were identified by the tRNA Scan-SE1.21 and SQUEIN v. 5.35 programs, which were also used for the comparisons with the corresponding sequences from the other turtles cited above. The analysis of the control region sequence was carried out with the DNAsis and BioEdit programs. The resultant complete mitochondrial genome of *C. atripons* (16,500 bp) was deposited in GenBank under accession number EF067858.

The structural organization (Table 2) and gene order (Figure 1) of the complete *C. atripons* mitochondrial genome was identical that of other typical vertebrates, with the genome containing the following: 13 protein-coding genes, all of which except *NADH6* being encoded on the H-strand; 22 tRNA genes, 14 on the H-strand and 8 on the L-strand; 2 rRNA genes, 12S and 16S, both on the H-strand; and one control region. There were few, or small, noncoding intergenic spacer nucleotides, with intervening sequences of 8 bp between cytochrome c oxidase mitochondrial subunit II gene (*COII*) and $tRNA^{Lys}$ plus 13 bp between *NADH4* and $tRNA^{His}$ (Table 2). We found that the base composition of the major coding strand of *C. atripons* mtDNA was A = 34.42%, G = 13.01%, C = 25.36% and T = 27.20%, demonstrating the low G and high A+T bias seen in most other turtles (Pu *et al.*, 2005; Peng *et al.*, 2005, 2006). We also found that in C. atripons mtDNA three protein genes (*COIII*, *NADH6* and Cyt *b*) have an incomplete stop codon such as T, while the cytochrome c oxidase mitochondrial subunit I gene (*COI*) has GTG instead of ATG as a start codon. As in other vertebrate mitochondrial genomes, we found three instances of reading frame overlap, 10 nucleotides for *ATP8* and *ATP6*, 7 for *NADH4L* and *NADH4*, and 5 for *NADH5* and *NADH6* (Table 2).

However, in *C. atripons* our analysis did not find the extra base usually found at a specific position in *NADH3* of most other turtles (Mindell *et al.*, 1998, 1999; Pu *et al.*, 2005; Parham *et al.*, 2006). Such an insertion in the NADH3 gene has been reported in most turtles with the exception of *Pelodiscus sinensis* and *Kinosternon flavescens* (Peng *et al.*, 2005; Pu *et al.*, 2005; Parham *et al.*, 2006). It is generally thought the base could be related to a TAA stop codon frameshift prematurely terminating protein translation if not corrected by RNA editing or other mechanisms (Mindell *et al.*, 1998, 1999). However, since the additional base is apparently absent from *C. atripons* more studies are needed to ascertain whether or not the extra base in *NADH3* is a common characteristic of turtles or is specific to certain species and genera.

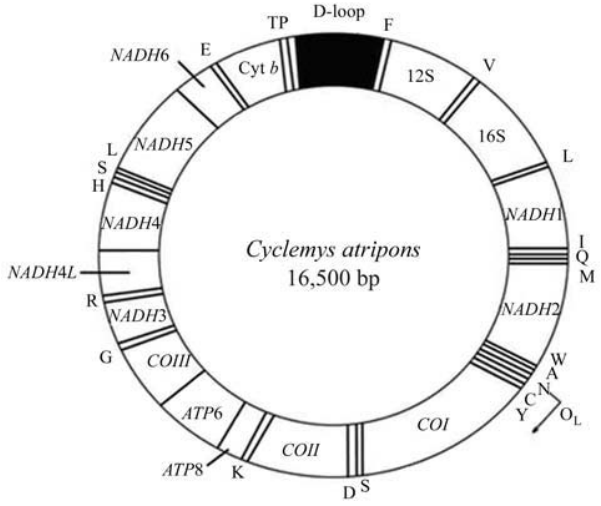

Figure 1 - Circular gene order of the mitochondrial genome of *Cyclemys atripons*. *NADH1-6*, and *NADH4L*: subunits 1-6 and 4L of nicotinamide adenine dinucleotide dehydrogenase; *ATP6* and *8*: subunits 6 and 8 of adenine triphosphatase; *COI-III*: cytochrome c oxidase subunits 1-3; Cyt *b*: cytochrome b; 12S and 16S: 12 and 16S rRNA; D-loop: Control region. Each tRNA gene is identified by the single-letter amino acid code. O_L represent the replication origin of L-strand.

We found that the *C. atripons* mitochondrial genome contained 22 tRNA genes, ranging in size from 66 nucleotides to 76 nucleotides (Table 2), interspersed between the rRNA and protein-coding genes, which is typical of the mtDNAs of other vertebrates. Most of the tRNA genes could be folded into the canonical cloverleaf secondary structure, the exception being the $tRNA^{Ser\ (AGY)}$ gene which lacks the dihydrouridine arm (D arm). The length of the *C. atripons* 12S rRNA gene was 969 nucleotides and the 16S rRNA gene was 1,601 nucleotides long, these genes, as in other vertebrates, being separated by the $tRNA^{Val}$ gene and positioned between the of $tRNA^{Phe}$ and $tRNA^{Leu\ (UUR)}$ genes (Table 2).

In the *C. atripons* mitochondrial DNA, we also found that the light-strand replication origin (31 nucleotides) was located between the $tRNA^{Asn}$ and $tRNA^{Cys}$ genes inside the WANCY tRNA gene cluster (Figure 1). This region has also been discovered in mtDNAs of all other cryptodiran turtles investigated (Pu *et al.*, 2005; Peng *et al.*, 2006), contrasting with its apparent disappearance from *Pelomedusa subrufa* (Zardoya and Meyer, 1998b). This *C. atripons* sequence may potentially fold into a stable stem-loop second-

Table 2 - Organization of the *Cyclemys atripons* mitochondrial genome (16,500 bp)*.

Gene/elements	Position from-to	Size (bp)	Strand (sense)	Codon		5' intergenic space[a]
				Start	Stop	
$tRNA^{Phe}$	1-70	70	H			0
12S rRNA	71-1,035	965	H			0
$tRNA^{Val}$	1,036-1,105	70	H			0
16S rRNA	1,106-2,701	1,596	H			0
$tRNA^{Leu\ (UUR)}$	2,702-2,777	76	H			0
NADH1	2,778-3,749	972	H	ATG	TAG	-1
$tRNA^{Ile}$	3,749-3,818	70	H			-1
$tRNA^{Gln}$	3,818-3,888	71	L			-1
$tRNA^{Met}$	3,888-3,956	69	H			0
NADH2	3,957-4,997	1,041	H	ATG	TAG	-2
$tRNA^{Trp}$	4,996-5,071	76	H			1
$tRNA^{Ala}$	5,073-5,141	69	L			1
$tRNA^{Asn}$	5,143-5,215	73	L			1
O_L	5,217-5,246	30	-			-3
$tRNA^{Cys}$	5,244-5,309	66	L			0
$tRNA^{Tyr}$	5,310-5,380	71	L			1
COI	5,382-6,929	1,548	H	GTG	AGG	-9
$tRNA^{Ser(UCN)}$	6,921-6,991	71	L			2
$tRNA^{Asp}$	6,994-7,063	70	H			0
COII	7,064-7,750	687	H	ATG	TAG	8
$tRNA^{Lys}$	7,759-7,831	73	H			1
ATP8	7,833-8,000	168	H	ATG	TAA	-10
ATP6	7,991-8,674	684	H	ATG	TAA	-1
COIII	8,674-9,457	784	H	ATG	T	0
$tRNA^{Gly}$	9,458-9,526	69	H			0

Table 2 (cont.)

Gene/elements	Position from-to	Size (bp)	Strand (sense)	Codon		5' intergenic space[a]
				Start	Stop	
NADH3	9,527-9,877	351	H	ATG	TAG	-2
$tRNA^{Arg}$	9,876-9,945	70	H			0
NADH4L	9,946-10,245	300	H	ATG	TAA	-7
NADH4	10,239-11,615	1,377	H	ATG	TAA	13
$tRNA^{His}$	11,629-11,698	70	H			0
$tRNA^{Ser(AGY)}$	11,699-11,764	66	H			-1
$tRNA^{Leu(CUN)}$	11,764-11,835	72	H			0
NADH5	11,836-13,641	1,806	H	ATG	TAA	-5
NADH6	13,637-14,161	525	L	ATG	T	0
$tRNA^{Glu}$	14,162-14,229	68	L			4
Cyt *b*	14,234-15,377	1,144	H	ATG	T	0
$tRNA^{Thr}$	15,378-15,449	72	H			1
$tRNA^{Pro}$	15,451-15,519	69	L			0
Control region	15,520-16,500	981	-			

[*] *NADH1-6* and *NADH4L*: NADH dehydrogenase subunits 1-6 and 4L; *COI-III*: cytochrome c oxidase subunits I-III; *ATP6* and *ATP8*: ATPase subunit 6 and 8; Cyt *b*: cytochrome b. T: incomplete stop codon.
[a] Numbers correspond to the nucleotides separating adjacent genes. Negative numbers indicate overlapping nucleotides.

ary structure with a stem comprised of 10 bp and a loop of 10 bp. The secondary structures of the origin of light strand replication (O_L) for 19 cryptodiran turtles (Figure 2) shows that the O_L sequence nucleotides are rather conserved and the secondary structures of these sequences are also similar because 9 bp are identical in the stems, possibly a common characteristic of cryptodiran turtles.

We found that the *C. atripons* D-loop control region was 981 bp long, 69.52% A+T rich and flanked by $tRNA^{Pro}$ and $tRNA^{Phe}$ genes (Table 2). A comparison of the complete control region sequences of four geoemydid turtles is given in the online edition of this paper (Figure S1), Similar to three other geoemydid turtles, three conserved sequence blocks (CSBs) $_{1\text{-}3}$ (Walberg and Clayton, 1981) were identified in the *C. atripons* control region. The whole lengths of four control region sequences ranged from 981 bp in *C. atripons* to 1,379 bp in *Cuora aurocapitata*, mainly resulting from sequences positioned at the 3' end. Interestingly, a large number of AT enriched tandem repeats containing variable number tandem repeats (VNTRs) were revealed at the 3' end (right domain) of the control regions. Furthermore, the composition and number of these tandem units were different for the different species, with the *C. atripons* VNTR being composed of fifteen 5'-A $(AT)_3$-3' units (Figure S1).

The control region is usually considered to be the most variable parts of mtDNAs in terms of nucleotide substitutions, short insertions/deletions and VNTRs dynamics. However, these variations are not distributed randomly across the whole region but occur in particular hypervariable sites and domains at the 5' and 3' ends (Su, 2005). Previous studies of turtles utilizing control region sequences were primarily focused on the 5' end adjacent to $tRNA^{Pro}$ and several regulatory motifs (Lamb *et al.,* 1994; Walker *et al.*, 1997; Walker and Avise, 1998). However, at present most work focuses on the 3' end close to $tRNA^{Phe}$, especially tandemly repeated sequences, including VNTRs (Serb *et al.*, 2001). The length difference between mitochondrial genomes among species is caused mainly by the divergent tandem repeats, which are thought to be generated by strand slippage and mispairing during replication (Fumagalli *et al.*, 1996).

Tandemly repeated control region DNA has been reported from an ever-growing number of taxa. Zardoya and Meyer (1998a) characterized six tandem repeats (containing VNTRs) in the 3' domain of the *P. subrufa* control region and suggested that this sequence might be a potentially informative molecular marker for population studies by its unique localization in the maternally inherited mitochondrial molecule. What is remarkable is that the tandem repeats are present in the four geoemydid turtles discussed in our present paper. The repeat consists of two different repeat cores, "ATTATATC" followed by "AT" in *Pyxidea mouhtii* (DQ659152) than the one "A $(AT)_3$" in *C. atripons*; in *C. reevesii* (AY676201) it is over ten 5'-ATATATC-3' units succeeded by AT-rich repeat; whereas in *C. aurocapitata* (AY874540) an approximately 490 bp AT-rich repeat is in the 3' of the CR , with only a few "G" nucleotides and no "C" nucleotide.

In the mitochondrial genomes of *C. atripons* and other turtles, AT enriched tandem repeats (containing VNTRs) reflect heteroplasmy, suggesting interspecies

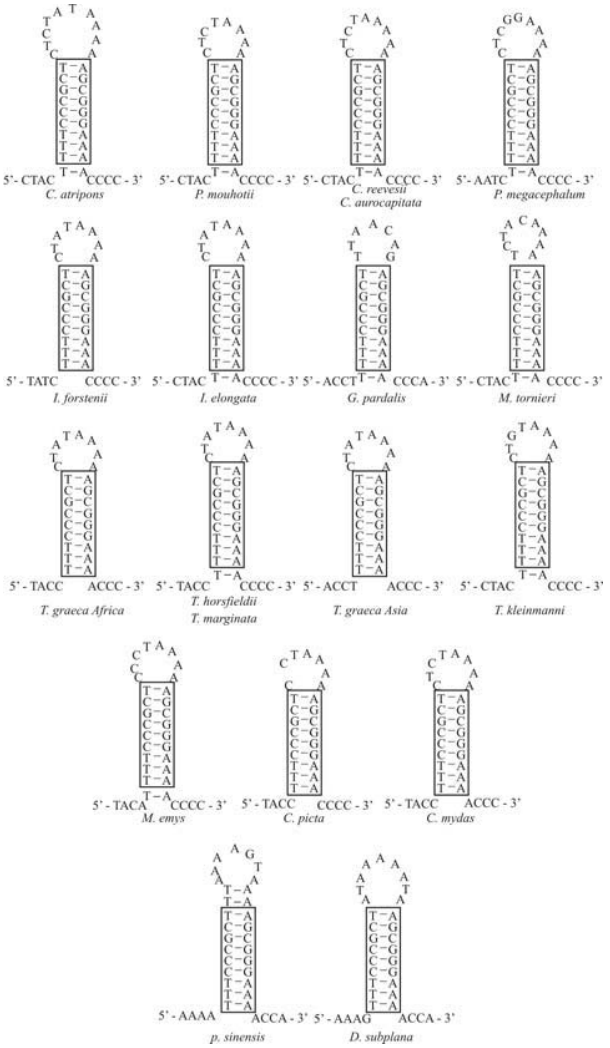

Figure 2 - Proposed secondary structures for the origins of the light strand replication (O_L) of 19 cryptodiran turtles. Frames show nine identical base pairs in the stems. *C. atripons: Cyclemys atripons; P. mouhotii: Pyxidea mouhotii; C. reevesii: Chinemys reevesii; C. aurocapitata: Cuora aurocapitata; P. megacephalum: Platysternon megacephalum; I. forstenii: Indotestudo forstenii; I. elongata: Indotestudo elongata; G. pardalis: Geochelone pardalis; M. tornieri: Malacochersus tornieri; T. graeca: Testudo graeca; T. horsfieldii: Testudo horsfieldii; T. marginata: Testudo marginata; T. kleinmanni: Testudo kleinmanni; M. emys: Manouria emys; C. picta: Chrysemys picta; C. mydas: Chelonia mydas; P. sinensis: Pelodiscus sinensis; D. subplana: Dogania subplana*.

turtle genetic diversity. The occurrence of tandemly repeated mtDNA in the control region could be regarded as a special molecular marker in turtle species researches. However, to confirm this issue and the presence or absence of variation between specimens of the same species (intraspecies polymorphism) more informative characters need to be obtained from more turtle species and specimens. Taken as a whole, our study highlights the need for further work focusing on the 3' domain of the mitochondrial control region.

Acknowledgments

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Internet Resources

Scan-SE1.21 program is available at http://lowelab.ucsc.edu/tRNA.

EditSeq, DNAsis and BioEdit programs are available at http://www.bioon.com/Soft/.

Supplementary Material

The following online material is available for this article:

- Figure S1. The complete control regions of four geoemydids. Dots and dashes represent the same nucleotides as *Pyxidea* and gaps, respectively. The potential regulatory elements (CSBs) $_{1\text{-}3}$ are boxed. Shaded region designates tandem repeat sequences at the 3' end of CRs.

This material is made available as part of the online article from http://www.scielo.br.gmb.

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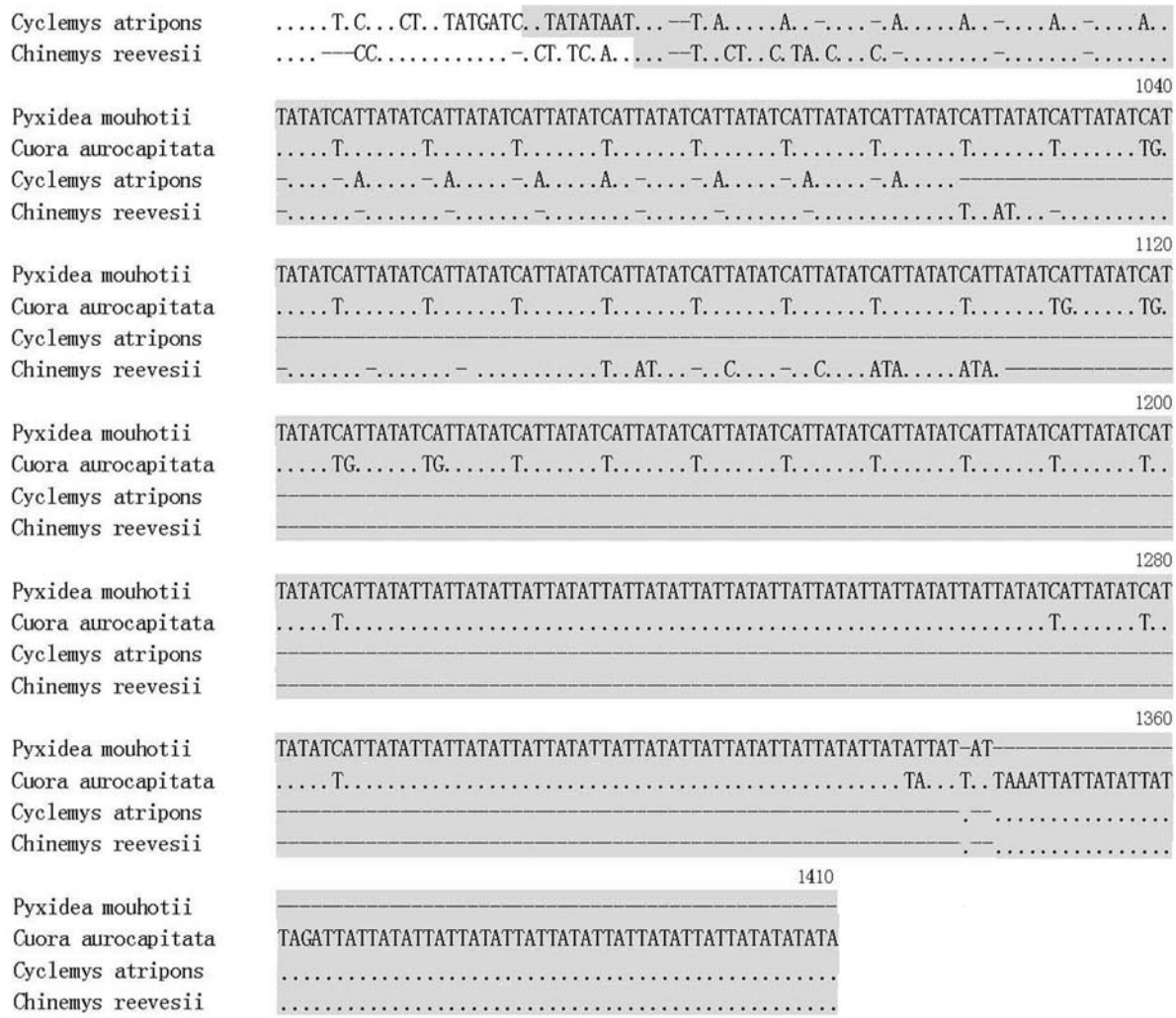
Cyclemys atripons
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1040
Pyxidea mouhotii
Cuora aurocapitata
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