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Organization and variation of mitochondrial DNA control region in pleurodiran turtles

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ABSTRACT. Three complete mitochondrial DNA (mtDNA) control regions (CRs) of *Chelodina rugosa* (Ogilby, 1890), *Chelus fimbriata* (Schneider, 1783), and *Podocnemis unifilis* (Troschel, 1848) were firstly determined using Long-PCR method and the length were 1,016 bp, 1,149 bp, and 985bp, respectively. Together with CRs of *Pelomedusa subrufa* (Bonnaterre, 1789) and nearly complete CR of *Podocnemis expansa* (Schweigger, 1812) obtained from GenBank, the structural and evolutionary characteristics of mtDNA CRs in pleurodiran turtle were analyzed in this study. We identified three functional domains (TAS, CD, and CSB domains) as well as their conservation sequences (TAS, CSB-F, and CSB-1) according to their homology to those of other turtles. Within the TAS domain, an interrupted poly-C stretch was found in *C. rugosa*, *C. fimbriata*, and *P. subrufa*, which also exists in the published mt DNA CRs of *Chrysemys picta* (Schneider, 1783), *Trachemys scripta* (Thunberg in Schoepff, 1792), and *Trionyx triunguis* (Forskål, 1775). The analysis of the origin for the poly-C sequences in TAS domain from six turtles suggested that the poly-C sequences are more related to "goose hairpin" in birds rather than CSB2 in CSB domain. In the CSB domain, CSB2 and CSB3, which were determined in CRs of Cryptodira, were absent in Pleurodira CRs, indicating the regulative mechanisms of transcription may be varied in both two suborders and the lack of CSB2 and CSB3 could be proposed as one of diagnostic characters between Pleurodira and Cryptodira at molecular level. As for CR of other cryptodiran turtles, variable number of tandem repeats (VNTRs) in the 3' end of the CRs was found in the five pleurodiran turtles. Interestingly, the long repeated motifs from each species could form stable stem-loop secondary structures, suggesting that the repeated sequences may play an important role in regulating replication of the mitochondrial genome in Pleurodiran, and the secondary structures of VNTRs may provide some potential information in phylogenetic inference.

KEY WORDS. Control region; CSB; mitochondrial DNA; molecular evolution; Pleurodira.

The mitochondrial genome is highly conserved and compact, usually including genes that code for 13 proteins, 22 tRNAs and two rRNA, as well as an important noncoding sequence (control region, CR) in vertebrates. Particularly, the CR in vertebrate mitochondrial genomes contains two promoters (LSP and HSP) for the transcription, heavy-strand replication origin (O_H), and the displacement loop (D-loop) (Clayton 1982, Chang & Clayton 1986). However, based on the distribution of the variable nucleotide positions and different nucleotide frequencies, the mitochondrial CR is divided into three domains (Brown *et al*. 1986, Saccone *et al*. 1991): termination associated sequence (TAS) domain, central conserved domain (CD), and conserved sequence block (CSB) domain. The TAS domain has been shown to contain sequences associated with termination of newly synthesized H-strands during replication (Doda *et al*. 1981, Brown *et al*. 1986, Sbisà *et al*. 1997). The CD, which contains several areas of highly conserved sequences, is more conserved with respect to the TAS and CSB domains. However, the nature of these conserved sequences varies among vertebrate classes. For example, Anderson *et al*. (1981) indentified several conserved sequence boxes (CSB), B, C, D, E, and F in the CD of the human mitochondrial CR. Of those, only three (CSB B, CSB D, and CSB F) can be found in the avian mitochondrial CD (Rukonen & Kvist 2002). The CSB domain usually contains the origin of the H-strand transcription (Walberg & Clayton 1981, Brown *et al*. 1986, King & Low 1987, Foran *et al*. 1988). In many taxa, the TAS and CSB domains, which are more variable than the CD, have variable numbers of tandem repeats (VNTRs) (Broughton & Dowling 1994, Sbisà *et al*. 1997, Zardoya & Meyer 1998a, Delport *et al*. 2002, Fu *et al*. 2006, Zhang *et al*. 2009, Xiong *et al*. 2010).

Extant turtles have been divided into two monophyletic clades, Pleurodira and Cryptodira. However, virtually all stud-

ies on turtle mitochondrial genomes have focused on Cryptodira. Studies comparing the mitochondrial CRs of pleurodiran turtles have been limited because there is only one published complete CR from one species – *Pelomedusa subrufa* (Bonnaterre, 1789). In the present study, three complete CR of three pleurodiran turtles – *Chelodina rugosa* (Ogilby, 1890), *Chelus fimbriata* (Schneider, 1783), and *Podocnemis unifilis* (Troschel, 1848) – representing two families (Chelidae and Podocnemididae) are characterized. Additionally, together with the complete CR of *P. subrufa* and a nearly complete CR of *Podocnemis expansa* (Schweigger, 1812) obtained from GenBank, we have compared the CR sequences of pleurodiran turtles with the CRs of other vertebrates. Additionally, the features which are shared or not among the pleurodiran turtles and other vertebrates have been identified and are discussed in detail.

MATERIAL AND METHODS

Sample and sequencing

Specimens of *C. rugosa*, *C. fimbriata*, and *P. unifilis* were stored at the Anhui Normal University. Total genomic DNA was extracted from their muscles with the proteinase K method (Sambrook & Russell 2001) and kept at -20°C for PCR amplification.

The mitochondrial CR was amplified by long-PCR. The entire CR and 3 tRNAs ($tRNA^{Thr}$, $tRNA^{Pro}$, and $tRNA^{Phe}$) as well as partial Cyt b and 12S rRNA gene sequences were amplified in one single step using a pair of long-PCR primers:
LCR-F: 5'-CTTCCTATTTGCCTATGCTATC-3'
LCR-R: 5'-TATTTTGGGCTCCTGGTGTA-3'

Long-PCR conditions were: one minute at 95°C, then 30 cycles of 10 seconds at 98°C, five minutes at 55°C, followed by a final extension for 10 minutes at 72°C. PCR products were isolated using a Gel Extract Purification Kit (TaKaRa Co., Ltd, Dalian, China) after 1% agarose gel electrophoresis. The purified Products were sequenced with an ABI3730 automated sequencer.

Sequence analysis

In order to determine the complete CR sequence of the three turtles, the sequences obtained for *C. rugosa*, *C. fimbriata*, and *P. unifilis* were compared with the complete mtDNA of *P. subrufa* (Zardoya & Meyer 1998b), and the tRNAs were identified using tRNAscan-SE 1.21(Lowe & Eddy 1997). The sequence containing almost complete CRs of *P. expansa* was retrieved from GenBank. Subsequently, five pleurodiran turtle sequences were aligned using ClustalX 1.8 software (Thompson *et al.* 1997) and then checked manually in order to define the conserved sequence blocks.

After comparison with published data from other taxa (the sequences used are listed in the Appendix), the conserved box F (CSB F) was delimited in the aligned sequences. The conserved block 1 (CSB1) with the characteristic motif GACATA was also delimited. The boundary of the TAS domains and the Central conserved domain was the starting point of the CSB F. The CSB domain was always set to start with the conserved sequence box 1 (CSB1) (Zhang *et al.* 2009, Xiong *et al.* 2010). The program 'Tandem Repeats Finder' (Benson 1999) was used in order to identify the VNTRs in the CRs. Furthermore, putative secondary structures in the CRs were determined using the software RNA structure (Mathews *et al.* 1999). Subsequently, the computer program RNAdraw (Hofacker *et al.* 1995) was employed to prepare secondary structures for publication.

RESULTS AND DISCUSSION

The length and base composition of pleurodiran turtle CRs

The CRs of *C. rugosa*, *C. fimbriata*, and *P. unifilis* have 1,016 bp, 1,149 bp, and 985bp, respectively. They are rich in adenine and thymine and lack L strand guanines, which is evident in each domain, particularly in the CSB domain (Tab. I). However, the composition of the CR among the three domains is not uniform. The Central conserved domain is poor in adenine and rich in L strand guanines compared to the flanking TAS and CSB domains. Interestingly, among the five turtles compared, the TAS domain was found to be richer in adenine and thymine in three species: *C. rugosa*, *C. fimbriata*, and *P. subrufa*. This TAS domain composition is consistent with that of cryptodiran turtles (Zhang *et al.* 2009, Xiong *et al.* 2010). However, in the other two pleurodiran turtles (*P. unifilis* and *P. expansa*), the TAS domain was rich in cytosine and thymine.

The organization of turtle CRs

Like in most cryptodiran turtles, the CRs in the mitochondrial genomes of pleurodiran turtles are located between the $tRNA^{Pro}$ and $tRNA^{Phe}$ genes. The CSB F and CSB-1 were easy to define in the five turtles (Fig. 1). In the CSB F, 12 of 29 (41.4%) nucleotide positions were fixed among the five turtles. In the CSB1, 9 of 20 (45%) nucleotide positions were fixed. Comparing with the cryptodiran turtles (Zhang *et al.* 2009, Xiong *et al.* 2010), the conserved blocks of pleurodiran turtles varied greatly. This variation may due to the age of the group. The fossil record has shown that most pleurodiran turtles diverged about 100-150 mya (million years ago) (Gaffney 1990). During this long evolutionary history, the conserved blocks sequences of the mtDNA CRs might have changed independently, accumulating more variation than the CRs of cryptodiran turtles, in which most species have diverged after 100 mya (Near *et al.* 2005).

TAS domain

The TAS domain was located between the 5'end of the CR and the beginning of the CSB F. The length of the TAS domain varied from 243 bp to 357 bp, and the sequences were heterogeneous and could not be unambiguously aligned. However, the TAS sequences were easily determined and were identical among the pleurodiran and cryptodiran turtles (Fig. 1).

An interrupted poly-C stretch was found in the TAS domain of *C. rugosa* and *C. fimbriata*. This poly-C stretch also

Species	TAS	CSBF	CSB1
Chelodina rugosa	TACAT	AGC---TATCTTCGAGAAATCACCAATCCTTG	TTA-ATGTTTGTAAGACATAT
Chelus fimbriata	.....	...---..G.C.....T....A....	..C-...A..AGT..G.....
Pelomedusa subrufa	.....	...---..CTCA..T...CATGA..T...C..	...-...C.CAC.G.....A
Podocnemis unifilis	.....	...CCC...TC..AT....C.....TC..CAC	A.CC...CAC.C.G.....A
Podocnemis expansa	.....	...CCC....C..A.....C..G..TC..CCT	...-...C.C.ACG.....A
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Figure 1. The alignment of TAS and conserved blocks from five pleurodiran turtles. Note: Dots indicate same nucleotides as top sequences, and asterisk shows the identical sites.

Table I. The base composition (%) and length of the five pleurodiran turtle CRs.

Species	Complete CR					TAS domain				
	T	C	A	G	Total	T	C	A	G	Total
Chelodina rugosa	32.00	19.9	35.00	13.10	1016.0	32.4	22.50	33.80	11.3	275.0
Chelus fimbriata	31.80	19.8	37.60	10.90	1149.0	28.6	24.90	32.70	13.9	245.0
Pelomedusa subrufa	34.80	18.7	37.90	8.60	1194.0	32.9	25.90	27.20	14.0	243.0
Podocnemis unifilis	32.40	26.0	28.90	12.70	985.0	31.4	32.50	24.60	11.5	357.0
Podocnemis expansa	32.30	25.1	29.80	12.80	1113.0	28.2	33.30	27.10	11.3	354.0
Average	32.66	21.9	33.84	11.62	1091.4	30.7	27.82	29.08	12.4	294.8
Species	Central conserved domain					CSB domain				
	T	C	A	G	Total	T	C	A	G	Total
Chelodina rugosa	37.00	19.20	26.90	16.90	349.0	27.30	18.60	43.10	11.0	392
Chelus fimbriata	37.70	19.00	27.50	15.90	353.0	29.40	18.00	46.30	6.4	551
Pelomedusa subrufa	34.70	24.80	25.30	15.20	363.0	35.70	11.90	50.00	2.4	588
Podocnemis unifilis	32.80	25.30	24.20	17.60	363.0	33.20	18.10	41.10	7.5	265
Podocnemis expansa	32.40	24.80	24.80	18.10	420.0	36.60	16.80	38.90	7.7	339
Average	34.92	22.62	25.74	16.74	369.6	32.44	16.68	43.88	7.0	427

Note: In the CR of *P. expansa*, the TAS and Central conserved domain are complete, and CSB domain is almost complete.

occurs in four other cryptodiran turtles: *Chrysemys picta* (Schneider, 1783), *Trachemys scripta* (Thunberg in Schoepff, 1792), *Trionyx triunguis* (Forskål, 1775), and *P. subrufa* (Zardoya & Meyer 1998a). It is one remarkable feature of the TAS domain sequence in these turtles. The interrupted poly-C stretch is repeated once in *C. picta* and twice in *T. scripta*. The similar poly-C stretch sequence in many birds (Quinn & Wilson 1993, Randi & Lucchini 1998, Ritchie & Lambert 2000) and crocodiles (Ray & Densmore 2002) is referred to as "goose hairpin". In birds, it could potentially form a stable hairpin structure, which is usually characterized by several consecutive cytosine residues separated from the complementary guanine by a putative stop sequence (TCCC). The poly-C stretch in crocodiles (*Crocodylus*) was reported as having a region with high cytosine content that could form a similar stem-loop structure. However, in the present study, the only poly-C stretches that have the capacity to form a similar secondary structure are those of *C. fimbriata* and *P. subrufa* (data not shown). Zardoya & Meyer (1998a) reported that the interrupted poly-C of the TAS domain in *P. subrufa* was remarkably similar to the CSB2. In order to discuss the origin of these similar poly-C sequences, we performed clustering analyses using the Neighbor-joining (NJ) method on the poly-C sequences of the TAS domain of the six turtles, the CSB2s from 31 turtles, and the "goose hairpin" sequence of *Gallus gallus* (Linnaeus, 1758). Interestingly, the sequences grouped into two major clades, A and B (Fig. 2). All CSB2s sequences grouped within Clade A. Though the relationships among these CSB2 may not represent the "species tree" of turtles, the fact that they clustered indicates that the CSB2 of these turtles is conserved. Clade B consists of the poly-C sequences in the TAS domain from the six turtles and the "goose hairpin" sequence from *G. gallus*. Clades A and B indicate that the origin of the poly-C sequences in the TAS domain were closer to "goose hairpin" in the TAS domain rather than the CSB2.

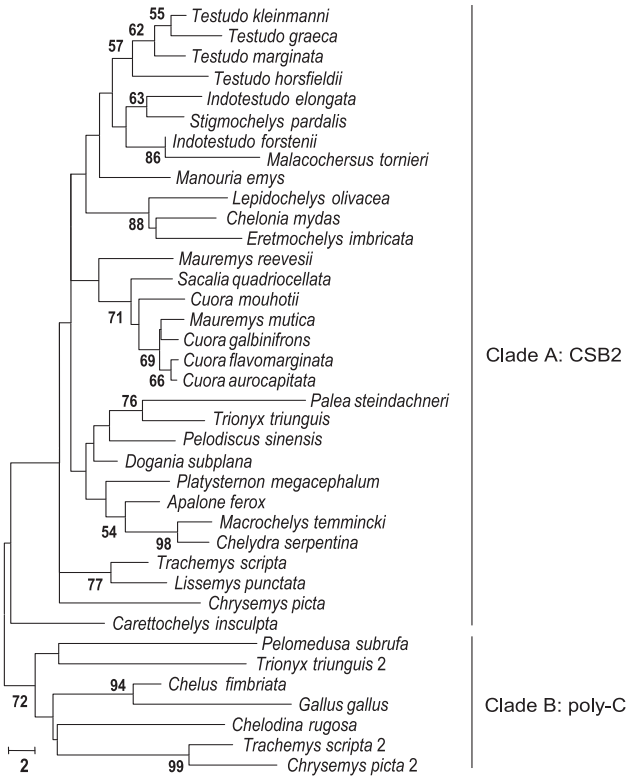


Figure 2. NJ tree based on the CSB2s and poly-C sequences. The tree was reconstructed according to Saitou & Nei (1987) using the absolute number of differences in MEGA (Kumar *et al.* 1993). Sequences used in the analysis are listed in the Appendix. A total of 1000 bootstrap replicates were performed and values greater than 50 are indicated at the respective nodes. Clade A consists of species in which CSB2 is located within the CSB domain, whereas clade B contains the species whose poly-C sequences are within the TAS domain.

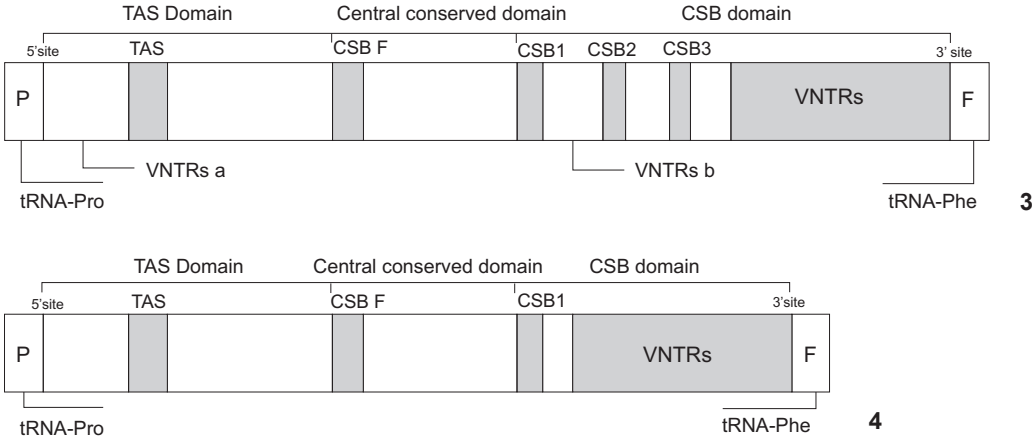
Central conserved domain

The Central conserved domain (CD) is more conserved than the peripheral domains (TAS and CSB domains). It is 353-420bp long in the five turtles. Our resulting alignment had102 conserved sites, 273 variable sites, and 127 parsimony-informative sites. Although the extreme conservation of this region in vertebrates may suggest that it has played an important role in the evolutionary history of the mitochondrial genome, its function remains completely unknown. On other hand, the high degree of similarity makes this domain suitable for phylogenetic inference (Saccone *et al.* 1991). However, we failed to reconstruct the phylogenetic relationships based on it. We believe that the phylogenetic distances in this domain are subject to high statistical fluctuations among family-level taxa of turtles because of the reduced number of sites in this region (353-420bp). Thus, the phylogenetic relationships of the five pleurodiran turtles reconstructed based on this domain were not well resolved.

CSB domain

The CSB domain, ranging from 265bp (*P. unifilis*) to 588bp (*P. subrufa*), has several conserved blocks involved in the regulation of replication and transcription. These short conserved sequence blocks were firstly called CSB1, CSB2, and CSB3 by Walberg & Clayton (1981). Researchers have proposed that they provide regulatory signals for the processing of the RNA primers for replication of the H-strand in human KB and mouse L cells. Since then, these three conserved sequence blocks have been identified in a number of vertebrate mtDNAs, such as fish (Buroker *et al.* 1990, Broughton & Dowling 1994, Liu 2002), amphibians (Dunon-Bluteau *et al.* 1985), lizards(Brehm *et al.* 2003), turtles (Serb *et al.* 2001, Zhang *et al.* 2009, Xiong *et al.* 2010), and mammals (Sbisà *et al.* 1997). However, it is interesting that in turtles only the CSB-1 is found consistently, whereas in marsupials, all three CSBs are consistently present. In placental mammals, CSB-2 and CSB-3 are sometimes missing (Nilsson 2009). Thus, there seems to be a lot of plasticity across vertebrates concerning the CSB1, CSB-2 and CSB-3. Particularly, the CSB1 was found to be a basic element in the CR of the mtDNA of all vertebrates and duplications of the CSB1 have been found in sperm whales, shrews, hedgehogs, opossums (Sbisà *et al.* 1997), and hornbills (Sbisà *et al.* 1997, Delport *et al.* 2002). A sequence such as GACATA, is identical among several vertebrate mtDNA CSB-1 sequences, from fish to mammals, and birds (Buroker *et al.* 1990, Sbisà *et al.* 1997, Ruokonen & Kvist 2002, Nilsson 2009). The conserved sequences and structure of CSB-1 may have important functions. For example, Ghivizziani *et al.* (1994) have reported that the origin of the heavy strand replication (OH) and transition from RNA transcription to DNA replication should occur in the proximity of the CSB-1.

The other two conserved sequence blocks, CSB2 and CSB3, are believed to play an important role in RNA processing during the transcription in mice (Chang & Clayton 1986). In humans, the CSB2 and CSB3 may have another function: Ghivizzani *et al.* (1994) have proposed that together with C residues, they appear to function as domains which avoid interaction with the mitochondrial transcription factor A (mtTFA), thus reinforcing the organization of the mtTFA binding. Although they appear to have an important function in different taxa, both the CSB2 and CSB3 are not present in the CRs of the mtDNA of all vertebrates. In this study, the CSB2 and CSB3 were missing in all five pleurodiran turtles. The same condition has been reported in birds (Ramirez *et al.* 1993, Baker & Marshall 1997, Härlid *et al.* 1997, Randi & Lucchini 1998) and some mammals (Sbisà *et al.* 1997). Interestingly, all mtDNA CRs of cryptodiran turtles published so far have the CSB2 and CSB3. Thus, there are two known types of mitochondrial CR in turtles at present, which correspond to variations in the CSB domain (Figs 3 and 4). In all cryptodiran turtles, the CSB domain contains three conserved blocks (CSB-1, CSB-2, and CSB-3) (Serb *et al.* 2001, Jungt *et al.* 2006, Amer & Kumazawa 2009, Zhang *et al.* 2009, Xiong *et al.* 2010) (Fig. 3) but only one (CSB-1) can be



Figures 3-4. Two typical mtDNA CR structures published in turtles: (3) the structure of mtDNA CR in Cryptodira. VNTRs a and VNTRs b are only identified in CRs of some trionychids; (4) The structure of mtDNA CR in Pleurodira. P: tRNA-Pro gene, F: tRNA-Phe gene.

identified in the CSB region of our five pleurodiran turtles (Fig. 4).This considerable variation shows that the regulative mechanisms of transcription may be different among vertebrates or may involve specific nuclear-mitochondrial co-evolutionary processes (Sbisà *et al*. 1997), and in particular, the mechanisms of regulation may be different between the two suborders (Pleurodira and Cryptodira) in turtles. If the lack of the CSB2 and CSB3 in the CSB region turns out to be a pervading character state in Pleurodira, it could be used as a diagnostic character between Pleurodira and Cryptodira at the molecular level.

VNTRs

VNTRs have been identified in the mtDNA CRs of many animals, including invertebrates and vertebrates (Hoelzel 1993, Fumagalli *et al*. 1996, Goricki & Trontelj 2006, Ji *et al*. 2008). In cryptodiran turtles, VNTRs are inserted in the two peripheral domains, i.e. TAS and CSB domains (Fig. 3). VNTRs are only found within the TAS domain in some trionychids, such as *Apalone ferox* (Schneider, 1783), *Dogania subplana* (Geoffroy Saint-Hilaire, 1809), *Pelodiscus sinensis* (Wiegmann, 1835), and *Palea steindachneri* (Siebenrock, 1906) (Xiong *et al*. 2010). VNTRs located between the CSB1 and the CSB2, identified in many mammalian species (Gemmell *et al*. 1996, Sbisà *et al*. 1997, Larizza 2002, Peng *et al*. 2007, Sun *et al*. 2009), are also presented in *P. sinensis* (Xiong *et al*. 2010) and *Indotestudo forstenii* (Schlegel and Müller, 1845). As for most turtles, VNTRs were commonly found at the 3'end of the CSB domain (Zardoya & Meyer 1998a, Serb *et al*. 2001, Jungt *et al*. 2006, Amer & Kumazawa 2009, Zhang *et al*. 2009, Xiong *et al*. 2010). Generally, VNTRs located between the CSB1 and CSB2, or within the TAS domain, or at the 3'end of the CRs identified in most turtles (Zardoya & Meyer 1998a, Zhang *et al*. 2009, Xiong *et al*. 2010) support the idea that the CR can tolerate deletion/insertion events and accumulate repeats without loss of function (Sbisà *et al*. 1997).

The VNTRs of the new determined mtDNA CRs from the three pleurodiran turtles, and two additional pleurodiran turtles (*P. expansa* and *P. subrufa*), which were only located at the 3'end of the mtDNA, were further analyzed in this study (Tab. II). As in pleurodiran turtles, the repeated motifs of each species were not homogenous at the end of the CR. The motifs of VNTRs in five pleurodiran turtles were similar in length. The lengths of the VNTRs are closer in closely related species.

Table II. Comparison of the repeat units in CR of the five pleurodiran turtles analyzed in the present study.

Species	Repeated motif	Sequences
C. rugosa	(74 bp)×3	5'-ATTATACACCAAGTGCAGTGTCGAAAGATAATTATTTTTACACGCACCAAACACTGTGCCGAAATTATATTAAC-3'
C. fimbriata	(76 bp)×4	5'-GAAAAAACAGACTACTTTTATATAAAAAAATTAATTGTTTTTTATTCAAAAATTTAAACAACTAACTATCTTTGTC-3'
	(36 bp)×5	5'-GAAAATTAACCCTAAAACACTACCAAGTCAAGTACC-3'
P. unifilis	(2 bp)×19	5'-AT-3'
	(66 bp)×2	5'-CTATAAATTCTCCTAGACTTACTAAGAAATTTACCCTAATCGCTAATATATATACATATTAATAGC-3'
P. expansa	(69 bp)×4	5'-CAATAATTTATTAAATTCTCCTAGACTTACTAAGATATTTACCTCAATTGCTAATTGTATATATATATA-3'
P. subrufa	(65 bp)×6	5'-CAAACATATATACAAACAAAGTACTAATTATACTAATCATATAAATATATATATATATATATATA-3'

Potential secondary structures of the repeated sequences in the five pleurodiran turtles' CR were constructed in this study, and the long repeated motif from each species formed a stable stem-loop structure (Figs 5-9). In hornbills, these VNTRs' stem-loop structures are believed to function as a termination sequence in mitochondrial genome replication (Delport *et al*. 2002). In turtles, whether these VNTRs have some function or not needs further study, but it is noteworthy that the potential secondary structures of the repeated sequences were very similar among *P. unifilis*, *P. expansa*, and *P. subrufa* (Figs 7-9).

Although the mechanism for generating the VNTRs in the mitochondrial genome is not well understood at present, it is believed that the VNTRs can be used as molecular markers and provide sufficient phylogenetic information in molecular phylogenetics, population genetics, species identification, as well as genetic diversity and conservation, because they can be used to differentiate among genera, species, populations, and even individuals (Zardoya & Meyer 1998a, Zhang *et al*. 2009). Pelomedusids and podocnemidids were placed into Pelomedusidae (sensu lato) based on morphological characteristic (Gaffney 1975, Gaffney & Meylan 1988). Although de Broin (1988) suggested that the extant Pelomedusinae and Podocneminae (Gaffney & Meylan 1988) should be recognized as two families, i.e. Pelomedusidae (consisting of the extant African *Pelusios*

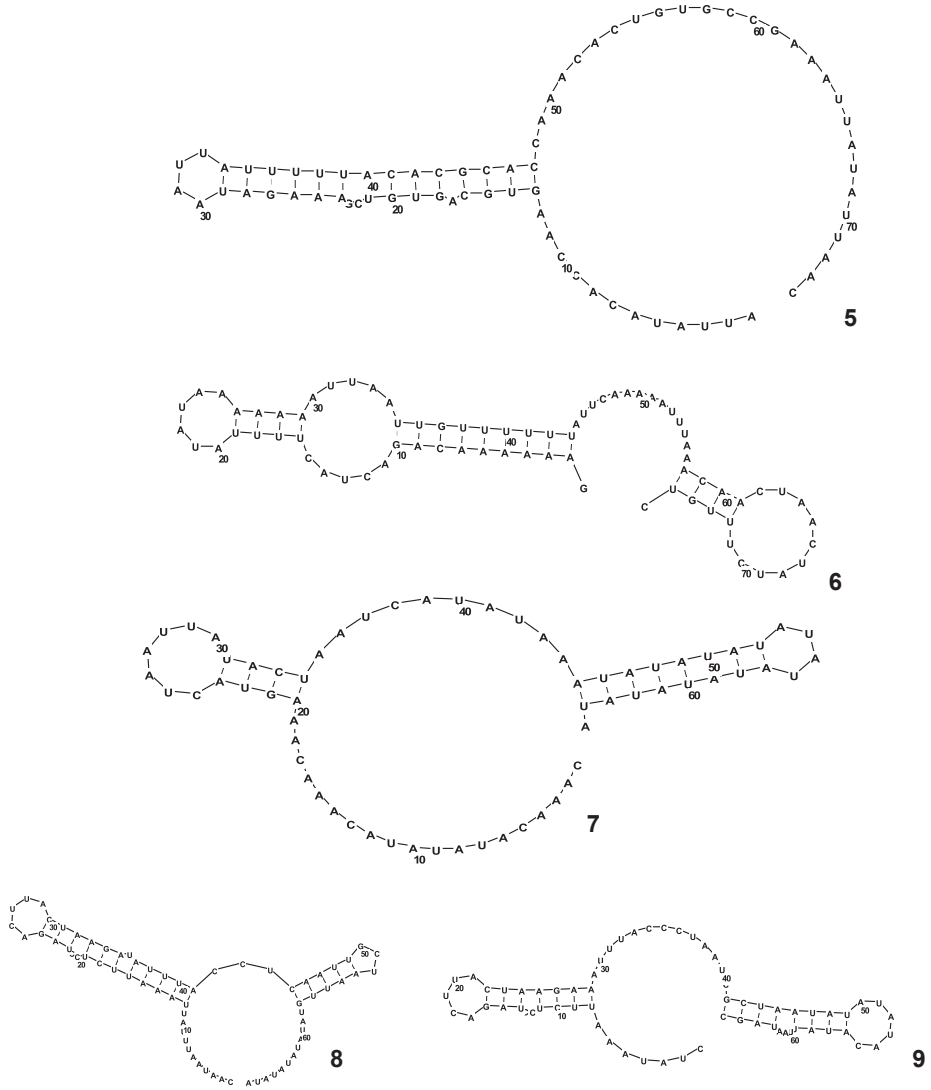


Figure 5-9. Secondary structure of repeat units constructed from VNTRs of the five pleurodiran turtles: (5) the 74 bp repeat unit of *C. rugosa*; (6) the 76 bp repeat unit of *C. fimbriata*; (7) the 65 bp repeat unit of *P. subrufa*; (8) the 69 bp repeat unit of *P. expansa*; (9) the 66 bp repeat unit of *P. unifilis*.

and *Pelomedusa*) and Podocnemidae (consisting of extant Madagascan *Erymnochelys*, and the extant South American *Podocnemis* and *Peltocephalus*), morphological studies still support the monophyly of Pelomedusidae (sensu lato). Recently, molecular evidence has also suggested that the Pelomedusidae (sensu lato) may be monophyletic (Georoges *et al*. 1998, Noonan 2000, Fujita *et al*. 2004, Noonan & Chippindale 2006, Thomson & Shaffer 2010). In the present study, these secondary structures of VNTRs for *P. subrufa* (Pelomedusinae), *P. expansa*, and *P. unifilis* (Podocneminae) also supports the monophyly of Pelomedusidae (sensu lato). We suggest that comparative analyses of secondary structures of VNTRs may provide some potential information for phylogenetic inferences.

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Appendix. Species and associated GenBank accession numbers used in the present study.

Family name	Species name	GenBank accession number
Trionychidae	*Trionyx triunguis*	NC_012833
	Apalone ferox	FJ890514
	Palea steindachneri	FJ541030
	Dogania subplana	AF366350
	Lissemys punctata	NC_012414
	Pelodiscus sinensis	AY687385
Carettochelyidae	*Carettochelys insculpta*	FJ862792
Pelomedousidae	*Pelomedusa subrufa*	AF039066
Podocnemididae	*Podocnemis unifilis*	this study
	Podocnemis expansa	AY572983
Chelidae	*Chelus fimbriata*	this study
	Chelodina rugosa	this study
Geoemydidae	*Mauremys reevesi*	AY676201
	Cuora aurocapitata	AY874540
	Cuora flavomarginata	EU708434
	Mauremys mutica	DQ453753
	Cuora mouhotii	DQ659152
	Sacalia quadriocellata	EF088646
	Cuora galbinifrons	NC_014102

Continue

Appendix. Continued.

Family name	Species name	GenBank accession number
Chelydridae	*Chelydra serpentine*	NC_011198
	Macrochelys temmincki	EF071948
Emydidae	*Chrysemys picta*	AF069423
	Trachemys scripta	FJ392294
Cheloniidae	*Chelonia mydas*	AB012104
	Eretmochelys imbricata	NC_012398
	Lepidochelys olivacea	AM258984
Testudinidae	*Testudo kleinmanni*	DQ080048
	Stigmochelys pardalis	DQ080041
	Malacochersus tornieri	DQ080042
	Manouria emys	DQ080040
	Indotestudo elongata	DQ080043
	Testudo horsfieldii	DQ080045
	Testudo graeca	DQ080049
	Indotestudo forstenii	DQ080044
	Testudo marginata	DQ080047
Platysternidae	*Platysternon megacephalum*	DQ016387
Phasianidae	*Gallus gallus*	AY235571

Note: The taxononmical classification provided by Genbank entries was used for the species and the classification of Rhodin *et al.* (2010) was used for the following four species: *Macroclemys temminckii* (Troost in Harlan, 1835) was replaced by *Macrochelys temminckii* (Troost, 1835); *Chinemys reevesii* (Gray, 1831) should be *Mauremys reevesii* (Gray, 1831); *Geochelone pardalis* (Bell, 1828) should be *Stigmochelys pardalis*; and *Pyxidea mouhotii* (Gray, 1864) should be *Cuora mouhotii* (Gray, 1862).