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Complete mitochondrial genome sequence of the humphead wrasse, *Cheilinus undulatus*

X.Z. Qi[1,2], S.W. Yin[3], J. Luo[2] and R. Huo[2]

[1]Key Laboratory of Tropic Biological Resources, Hainan University, Haikou, China
[2]Central Lab of Marine Biology of the Ocean College, Hainan University, Haikou, China
[3]College of Life Sciences, Nanjing Normal University, Nanjing, China

Corresponding author: S.W. Yin
E-mail: yinshaowu@163.com

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ABSTRACT. The humphead wrasse (*Cheilinus undulatus*) is a large coral fish that has become threatened due to habitat loss and fishing pressure. We sequenced the mitochondrial genome of *C. undulatus*, using a normal PCR method. The complete mtDNA sequence encoded 13 protein genes, 22 tRNA genes and 2 rRNA genes. It was found to be 16,613 bp in length and had an overall H-strand base compositions of 27.3 for A, 30.9 for C, 16.8 for G, and 25.0% for T. Compared with the sequences of 8 other members of the family Labridae, gene content, genome organization, and nucleotide compositions were similar. All tRNAs formed a typical clover-leaf structure, except $tRNA^{Ser}$ (AGY), and most of the size variations among tRNAs stemmed from variations of length in the arm and loop of TΨC, and loop of DHU.

Key words: Mitochondrial genome; *Cheilinus undulatus*; Normal PCR method

INTRODUCTION

The humphead wrasse (*Cheilinus undulatus*) is a large coral reef fish that is widely distributed in the tropical Indo-Pacific region, and it is the largest living member of the family Labridae, with a maximum size exceeding 2 m and 190 kg (Sadovy et al., 2003). The humphead wrasse is the most valuable fish in the live reef food fish trade in Asia. It is particularly susceptible to overexploitation due to its life history, which involves slow growth, late sexual maturity, longevity, sex reversal, and low replenishment rates (Sadovy, 2002).

In 1996, the humphead wrasse was listed as a vulnerable species in the International Union for Conservation of Nature Red List, the first to focus on the status of marine fishes specifically. It was listed due to the concerns of rapidly declining populations in many areas during the last decade (Sadovy and Vincent, 2002). Therefore, it is important to evaluate the genetic diversity of the humphead wrasse for the conservation of its germplasm resource.

Mitochondrial DNA (mtDNA), with the characteristics of maternal inheritance, compact size, multiple copies per cell, rapid evolutionary rate, and rare recombination, has been widely used as a source of molecular markers to analyze populations and the evolution of fishes (Craig et al., 2001; Maggio et al., 2005; Ding et al., 2006). Most of these markers such as cytochrome b, 12S ribosomal RNA, cytochrome c oxidase subunit I, and NADH dehydrogenase subunit 2 are identified in a few mitochondrial regions, and adequate resolution of evolutionary relationships requires longer DNA sequence (Miya and Nishida, 2000). Thus, entire mitochondrial genome sequences containing more information are required to construct phylogenetic trees, rather than the simple collection of individual gene sequence. The examination of mitochondrial genomes may reveal important genome-level characteristics, such as length variation, base compositional bias, codon usage, gene rearrangement, RNA secondary structures, and control modes of replication and transcription (Sheffield et al., 2008).

In the present study, we amplified the mitochondrial genome of *C. undulatus* using a normal polymerase chain reaction (PCR) method and described its gene organization and control region. Complete mtDNA sequence data would provide important information not only for population studies of *C. undulatus*, but also for phylogenetic studies of the genus *Cheilinus*.

MATERIAL AND METHODS

Fish samples and DNA extraction

The *C. undulatus* specimen was obtained from Qing Li Fisheries Company of Sanya, and the harvested tissues for DNA extraction were immediately frozen at -80°C. Total genomic DNA was extracted from dorsal muscle tissues by the following procedure. Approximately 0.1 g tissue was homogenized in 1 mL digestion buffer (10 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 mM NaCl, 1% sodium dodecyl sulfate, 10 mg/mL dithiothreitol, 0.5 mg/mL proteinase K) and incubated at 37°C for 8-16 h, followed by standard phenol-chloroform extraction. The DNA samples were stored in 50 µL TE buffer.

Amplification, cloning and sequencing of PCR products

A series of primers based on the complete mitochondrial genome sequence of *Hali-*

choeres melanurus (GenBank accession No. NC009066) were designed (Table 1). PCR was carried out using a Mastercycler gradient (Eppendorf) with a 25-μL reaction mixture containing 50 ng total genomic DNA, 0.2 mM dNTPs, 0.2 μM of each primer, 2.5 μL 10X ExTaq reaction buffer, and 2 U ExTaq polymerase (TaKaRa). The PCR parameters were set up as initial denaturation at 94°C for 4 min, followed by 30 cycles composed of DNA denaturation at 94°C for 30 s, annealing at 47-53°C for 30 s and extension at 72°C for 2 min, and a final extension at 72°C for 7 min. The amplified PCR products were purified and ligated into pMD18-T vector (TaKaRa). The sequences of resultant plasmids were verified at Shanghai Bio-Engineering Technology Co., Ltd.

Data analysis

The sequence of each identified gene was compared with the mitochondrial genome sequence of *H. melanurus*. To comparatively analyze the control region of mitochondrial genomes, 6 complete mitochondrial genome sequences of Labridae were collected from GenBank. Homologous sequences for each gene are initially aligned using ClustalX (Thompson et al., 1997), and further analysis was carried out by MEGA version 4.0 (Tamura et al., 2007).

RESULTS

Primer design strategy

The normal PCR method included template selection, PCR primer design, PCR amplification, and sequence splicing. *Parajulis poecilepterus, H. melanurus* and *Pseudolabrus sieboldi* are important members of the family Labridae. The complete mitochondrial genome sequences of three species and four mitochondrial gene sequences of *C. undulatus* from GenBank [EF609322 (COI), EU601399 (Cyt b), EU601220 (tRNA-Phe and 12S), and AY279685 (16S)] were downloaded. After sequences of three complete mitochondrial genomes and four mitochondrial genes were subjected to alignment analysis by the NCBI BLAST software, the complete mitochondrial genome sequence of *H. melanurus* was selected as the template for the design of PCR primers.

The design strategies and criteria of PCR primers are shown in Figure 1. 1) The length of PCR products was controlled in the range of 1300-1500 bp for the following reasons: a) the reaction can only analyze the sequence in the range of 800-1000 bp using current DNA sequencing technology; b) adjacent amplified fragments must have some overlap for facilitating splicing; c) research cost should be low. 2) We designed primers for two rounds of PCR. The primer pairs in each round had half the total number of primer pairs and the distance between fragments amplified by adjacent primer pairs was approximately 1000-1200 bp, as shown in Figure 1A. 3) Sequence alignment analysis was conducted by the BLAST software for the sequencing results of the first-round PCR products and the PCR primer template. The first-round PCR products were used to replace the corresponding parts of the template sequence. 4) The new sequence generated from the first round PCR products was used as the template for the design of the second-round PCR primers. Moreover, during the primer design for the second-round PCR, each primer pair must be included in the new sequence to ensure some overlap between the second-round PCR products and the new sequence in the template (Figure 1B). 5) For the primers with low efficiency, we also developed a new primer pair outside the original primer that did not work for nested PCR amplification (Figure

1C). Totally, 12 primer pairs were designed for the first-round PCR, as shown in Table 1. Agarose gel electrophoresis of the first-round PCR products showed that 3 primer pairs (Sumei-A4sen and Sumei-A4ant; Sumei-A6sen and Sumei-A6ant; Sumei-A7sen and Sumei-A7ant) were of low efficiency and could not complete PCR. Therefore, we designed 3 new primer pairs (Sumei-A4-2sen and Sumei-A4-2ant; Sumei-A6-2sen and Sumei-A6-2ant; Sumei-A7-2sen and Sumei-A7-2ant) outside the original primers for nested PCR amplification, as shown in Table 2. The sequence alignments between the sequencing results of the first-round PCR products and the template of PCR primers were conducted to replace the corresponding parts of the template sequence using the first-round PCR products. Based on the newly generated sequences as the template, 10 primer pairs were designed for the second-round PCR, as shown in Table 1.

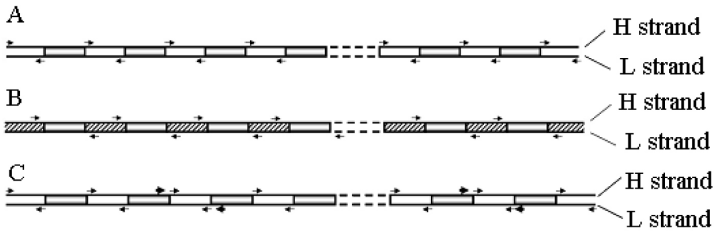

Figure 1. Design strategies of PCR primers. Two rounds of PCR primers were designed. **A.** The mtDNA genome sequence of *Halichoeres melanurus* was selected as the template for the first-round PCR primers, and the distance between fragments amplified by adjacent primer pairs was approximately 1000-1200 bp. **B.** The first-round PCR products were used to replace the corresponding parts of the mtDNA genome sequence of *H. melanurus*, and the new sequence was used as the template for the design of the second-round PCR primers. **C.** For the primers with low efficiency, a new primer pair outside the original primer was developed for nested PCR amplification.

Table 1. Primer pairs of the complete mitochondrial genome sequences of humphead wrasse.

First-round PCR primers		Second-round PCR primers	
Primer name	Primer sequence (5'-3')	Primer name	Primer sequence (5'-3')
Sumei-A1sen	ACCCTAGAAAGTTCCGAGAGC	Sumei-B1sen	ACCCTGAAGTCTACATCCTC
Sumei-A1ant	CTTCATTTCGGTTTTCAGCC	Sumei-B1ant	GGCTAAGTCTTGGGTAGG
Sumei-A2sen	AGAGCACAAAGGCTTGGTCC	Sumei-B2sen	CTTCTCCCTCTTTCTCGTCCT
Sumei-A2ant	CCTTCCCTTGCGGTACTTAAC	Sumei-B2ant	GCTCGGCTAGGTGGGTAAT
Sumei-A3sen	CCCCCAACCCTCCTTTACAA	Sumei-B3sen	TTCATTGACGCTCGCATAAG
Sumei-A3ant	GACCAATGGCCATCATAGCTCA	Sumei-B3ant	TGTGCCTGATACCAGCTCCT
Sumei-A4sen	ATGGTGGCAGAGCACGGCAA	Sumei-B4sen	CTCCGAAAGTCTCAACCCAC
Sumei-A4ant	TCATGCAAGTAGCCAGTGGGAGC	Sumei-B4ant	TTATTCCGTAACGAAGGCCT
Sumei-A5sen	CCCTCACCTAACCATTAAAGCC	Sumei-B5sen	CTATCGCATTCGCAGGTCTC
Sumei-A5ant	TGGAATCAGACAGCAAGGCC	Sumei-B5ant	GGCAGATGCTCGCTGGTTA
Sumei-A6sen	CTAGCCCTCACAGGAATG	Sumei-B6sen	ATCAACGGACCAAGTTACCC
Sumei-A6ant	GACGAAGGTGGTTATTGC	Sumei-B6ant	GAGGCGTGCCTTTAGCAG
Sumei-A7sen	TAGCCATGCTTGCCTTAA	Sumei-B7sen	GGTAAGTGTACCGGAAGGTG
Sumei-A7ant	CGTTGGCGTGTATGTTTC	Sumei-B7ant	GAGGAGGTTAGGACAGATTGAG
Sumei-A8sen	ACTATGGTCGTAGCAGGGAT	Sumei-B8sen	GTACATCCCCTCATTACCCACCT
Sumei-A8ant	ATGGTGAGCGGAGATGTT	Sumei-B8ant	GGGAGGGCATTCTCACGG
Sumei-A9sen	CTCGCTCCGATTCTTCCT	Sumei-B9sen	CTTCCCGAACGAACCGACT
Sumei-A9ant	GCCCGTTATGATTACACCTC	Sumei-B9ant	GAGGAGTACAGTGCCGAGGG
Sumei-A10sen	GGGAGGCAAGTCGTAACAT	Sumei-B10sen	CAGGTCTCCCACCACAACTT
Sumei-A10ant	TCCCCTTTAGATACACCAGTG	Sumei-B10ant	CCTGTCTAGGGAGGGCTTTA
Sumei-A11sen	GAAACTTAACGGACCCAACA		
Sumei-A11ant	TGCTAAGGGATTCAGGATGT		
Sumei-A12sen	ACACCCACAGTTAGGTCAGC		
Sumei-A12ant	GGGAGGGCTTTAGCTTAATT		

Table 2. Second-round PCR primer pairs for the complete mitochondrial genome sequences of humphead wrasse and the new primer pairs outside the low-efficiency primers.

Primer name	Primer sequence (5'-3')	Remarks
Sumei-A4-2sen	GCACGGCAATTGCGAAAGG	The new primer pair outside the
Sumei-A4-2ant	GCCAAGGCCAATTAGAAGTGTGG	4th primer that did not work for PCR
Sumei-A6-2sen	CATAACTCAGCGAGGACAAC	The new primer pair outside the
Sumei-A6-2ant	GACTGGTGTAGGGCCTTCT	7th primer that did not work for PCR
Sumei-A7-2sen	AGACGCAATCATCGAATCAC	The new primer pair outside the
Sumei-A7-2ant	GTGGCAATGTCAGAGGTGTAG	3rd primer that did not work for PCR

Besides the reduction of the experimental cost and technical difficulties, the normal PCR method can avoid the inherent complexities of long PCR techniques and result in amplified PCR products with the convenience of sequencing. Compared with conventional long PCR techniques, the normal PCR method can identify a series of technical parameters and form a new method for amplifying mtDNA. For example, two adjacent sections of PCR products should have approximately 100 bp overlap. Because some regions are not conserved and since the primers located in those regions are of low efficiency, we designed the inlaid PCR primers for nested PCR amplification to ensure the success of the experiments.

General features of mitochondrial genome in *C. undulatus*

The total length of the mitochondrial genome in *C. undulatus* was 16,613 bp, and similar to that of other animal mitochondrial genomes (Boore, 1999). The organization and location of the mitochondrial genome of *C. undulatus* was in accordance with the pattern observed in other teleostean mtDNAs (Figure 2). This mitochondrial genome included 13 protein genes, 22 tRNA genes, and 2 rRNA genes. It also had an overall H-strand base composition of 27.3 A, 30.9 C, 16.8 G, and 25.0% T. Meanwhile, the sequence of the control region (D-loop) located between tRNA-Pro and tRNA-Phe was 903 bp in size, comprising 5.44% of the whole genome. The complete mitochondrial genome sequence of *C. undulatus* was deposited in GenBank with the accession No. GU296101.

DISCUSSION

Protein-coding genes

The mtDNA of *C. undulatus* encoded 13 protein genes. Among these 13 protein genes, ND1, ATP8, ND5, and ND6 use ATG as the start codon and TAA as the stop codon; ND2, ND3, ND4, ND4L, ATP6, COX2, COX3, and Cyt-b use ATG as the start codon, but do not possess proper stop codons although a terminal T or TA where the TAA appears is created via post-transcriptional polyadenylation (Ojala et al., 1981). The exception is only COX1 with GTG as the start codon and AGG as the stop codon. The gene pairs of ATP8-ATP6, COX1-$tRNA^{Ser}$ and ND5-ND6 exhibited overlap lengths of 9, 8 and 3 bp, respectively. Eight tRNA genes ($tRNA^{Gln}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{Ser}$, $tRNA^{Glu}$, and $tRNA^{Pro}$) and one protein gene (ND6) were located on the light strand. The direction, location, and encoding-strand selection of the genes from *C. undulatus* were identical to those of the typical bony fishes (Figure 2 and Table 3).

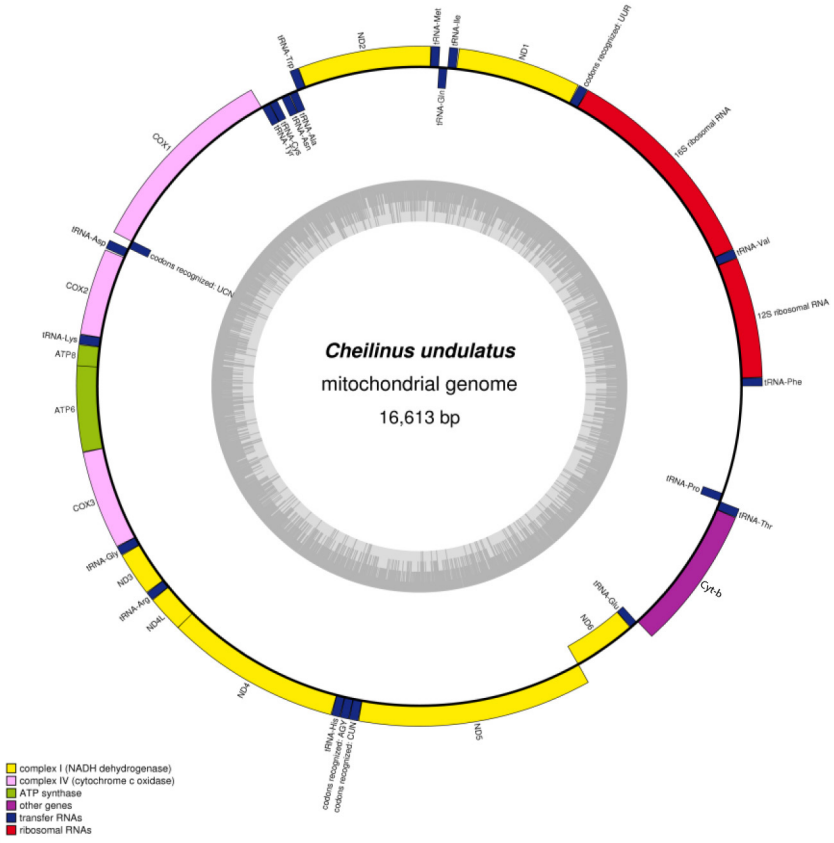

Figure 2. Structure of the mitochondrial genome and annotation of protein-coding genes from *Cheilinus undulatus*. ND1 = NADH dehydrogenase subunit 1 gene; ND2 = NADH dehydrogenase subunit 2 gene; ND3 = NADH dehydrogenase subunit 3 gene; ND4L = NADH dehydrogenase subunit 4L gene; ND4 = NADH dehydrogenase subunit 4 gene; ND5 = NADH dehydrogenase subunit 5 gene; ND6 = NADH dehydrogenase subunit 6 gene; COX1 = cytochrome C oxidase subunit 1 gene; COX2 = cytochrome C oxidase subunit 2 gene; COX3 = cytochrome C oxidase subunit 3 gene; ATP8 = ATP synthase F0 subunit 8 gene; ATP6 = ATP synthase F0 subunit 6 gene; Cyt-b = cytochrome B gene; small spacer regions = D-loop region.

RNA genes

The mitochondrial genome of *C. undulatus* contained 22 tRNA genes interspersed between the rRNA and protein-coding genes. The lengths of the 22 tRNA genes ranged from 68 to 77 bp. The predicted secondary structures of tRNAs are shown in Figure 3. Most of the size variation between the tRNAs stemmed from length variation in the arm and loop of TΨC and loop of DHU.

All tRNAs could form a typical clover-leaf structure except for $tRNA^{Ser}$ (AGY) (Figure 3), and all putative secondary clover-leaf structures contained 7 bp in the amino acid acceptor (AA) stem, while the majority had 5 or 4 bp in the TψC stem, 5 or 4 bp in the anticodon stem, and 4 or 3 bp in the DHU stem.

Table 3. Gene regions in mitochondrial genome of *Cheilinus undulatus*.

Gene/control region	Position	Size (bp)	AA	Strand	Gene/control region	Position	Size (bp)	AA	Strand
tRNA-Phe	1-68	68		H	*tRNA^{Lys}*	7903-7979	77		H
rRNA12sr	69-1024	956		H	*ATP8* gene	7981-8148	168	55	H
tRNA-Val	1025-1096	72		H	*ATP6* gene	8139-8821	683	227	H
rRNA16sr	1097-2798	1702		H	*COX3* gene	8822-9606	785	261	H
tRNA-Leu	2799-2871	73		H	*tRNA^{Gly}*	9607-9678	72		H
ND1 gene	2874-3848	975	324	H	ND3 gene	9680-10031	352	117	H
tRNA-Ile	3853-3922	70		H	*tRNA^{Arg}*	10032-10100	69		H
tRNA-Gln	3924-3994	71		L	*ND4L* gene	10101-10396	296	98	H
tRNA-Met	3994-4063	70		H	*ND4* gene	10391-11772	1382	460	H
ND2 gene	4064-5108	1045	348	H	*tRNA^{His}*	11773-11841	69		H
tRNA-Trp	5109-5179	71		H	*tRNA^{Ser}*	11842-11912	71		H
tRNA-Ala	5182-5250	69		L	*tRNA^{Leu}*	11913-11985	73		H
tRNA-Asn	5253-5325	73		L	*ND5* gene	11986-13824	1839	612	H
tRNA-Cys	5366-5433	68		L	*ND6* gene	13821-14351	531	176	L
tRNA-Tyr	5434-5504	71		L	*tRNA^{Glu}*	14350-14420	71		L
COX1 gene	5506-7065	1560	519	H	*Cyt-b* gene	14426-15566	1141	380	H
tRNA-Ser	7057-7127	71		L	*tRNA^{Thr}*	15567-15639	73		H
tRNA-Asp	7131-7203	73		H	*tRNA^{Pro}*	15639-15710	72		L
COX2 gene	7212-7902	691	230	H	D-loop	15711-16613	903		-

H = heavy strand; L = light strand.

The 12S rRNA and 16S rRNA genes were 956 and 1702 bp in length, respectively. As in other vertebrates, both genes are located between genes of *tRNA^{Phe}* and *tRNA$^{Leu(UUR)}$*, and separated by the *tRNA^{Val}* gene (Hou et al., 2007). The overall content of the bases was similar to that observed for other Labridae (data not shown).

Control region

In vertebrates, each mtDNA strand (heavy strand and light strand) has its own control region, which forms a stable stem-loop structure (Pereira, 2000). The major portion of mtDNA involved in transcription and replication of the heavy strand in vertebrates is called the D-loop region and has been well characterized (Shadel and Clayton, 1997; Sbisa et al., 1997). The vertebrate D-loop region typically located between *trnP* and *trnF* is divided into three major domains: extended termination-associated sequences (ETAS), central domain (CD), and conserved sequence block (CSB). Each of these domains carries particular conserved functional motifs (Bourlat et al., 2009).

However, in most bony fishes, the origin of light-strand replication (O_L) in *C. undulatus* was between *tRNA^{Asn}* and *tRNA^{Cys}* and was 40 nucleotides in length. The region had the potential to fold into a typical stem-loop secondary structure with 11 bp in the stem and 13 bp in the loop (Figure 4A). As shown in Figure 4B, the O_L for 7 species from the family Labridae was submitted to alignment analysis by ClustalX, and the results showed that the secondary structure of O_L was composed of a conserved stem (CCCCCGCCT**/GGGGGCGGA**) and two-variable base (asterisks) loop structure.

Compared with the structure of the control region (D-loop) located between tRNA-Pro and tRNA-Phe in other fishes (Cao et al., 2007; Tang et al., 2009; Tan et al., 2010), the ETAS, CD and CSB were identified, as shown in Figure 5. The TAS domain was 300-320 bp in length and the ETAS (TACATTGCTTGCAGTATTACAT) was observed in this domain. Two conserved blocks (CSB-F and CSB-D) in CD and three CSB (CSB-1, CSB-2 and CSB-3) were also identified in the CSB domain (Figure 5).

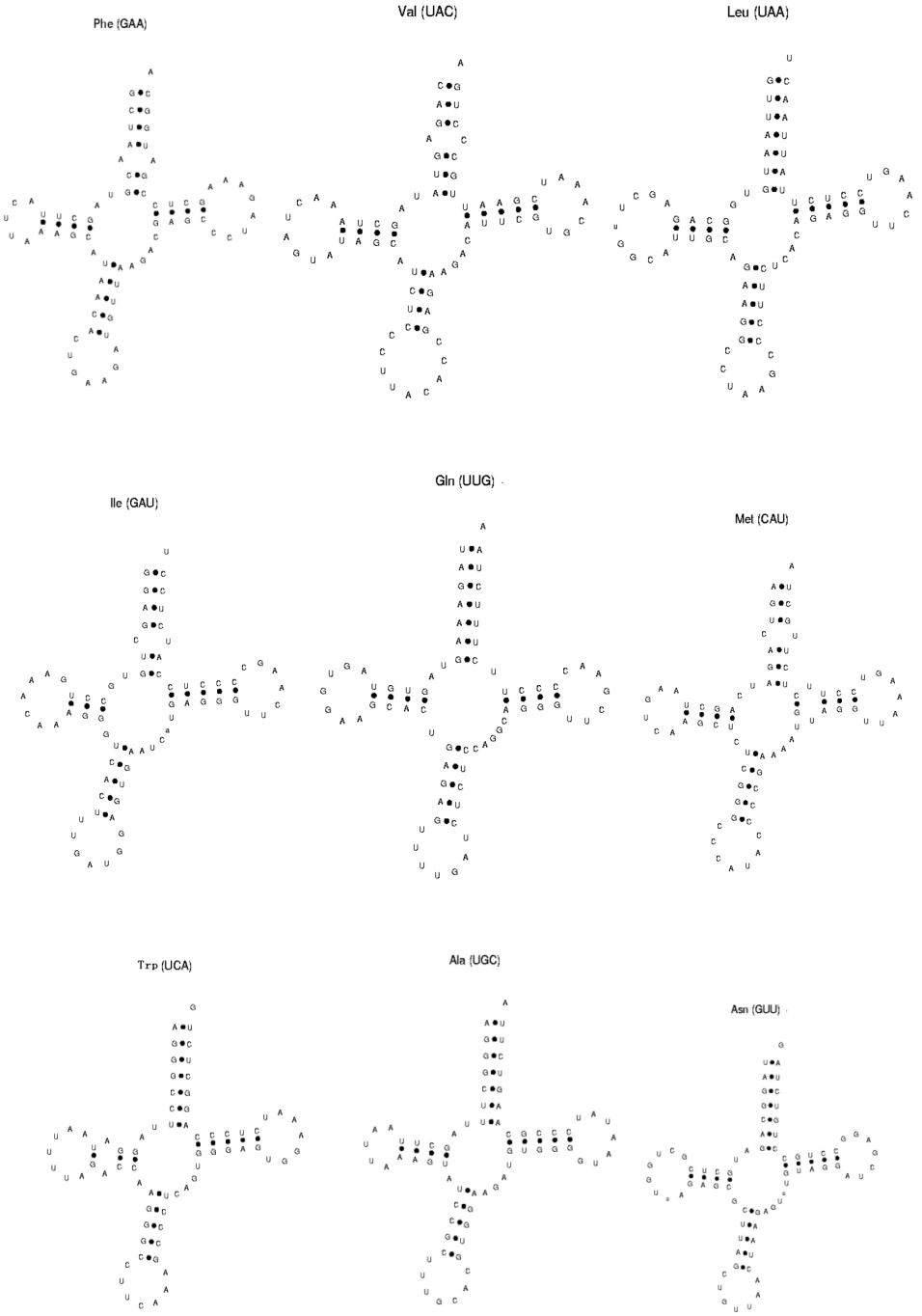

Figure 3. Inferred secondary structures of 21 tRNAs from mitochondrial genome of *Cheilinus undulatus*. The tRNAs are labeled with abbreviations of their corresponding amino acids.

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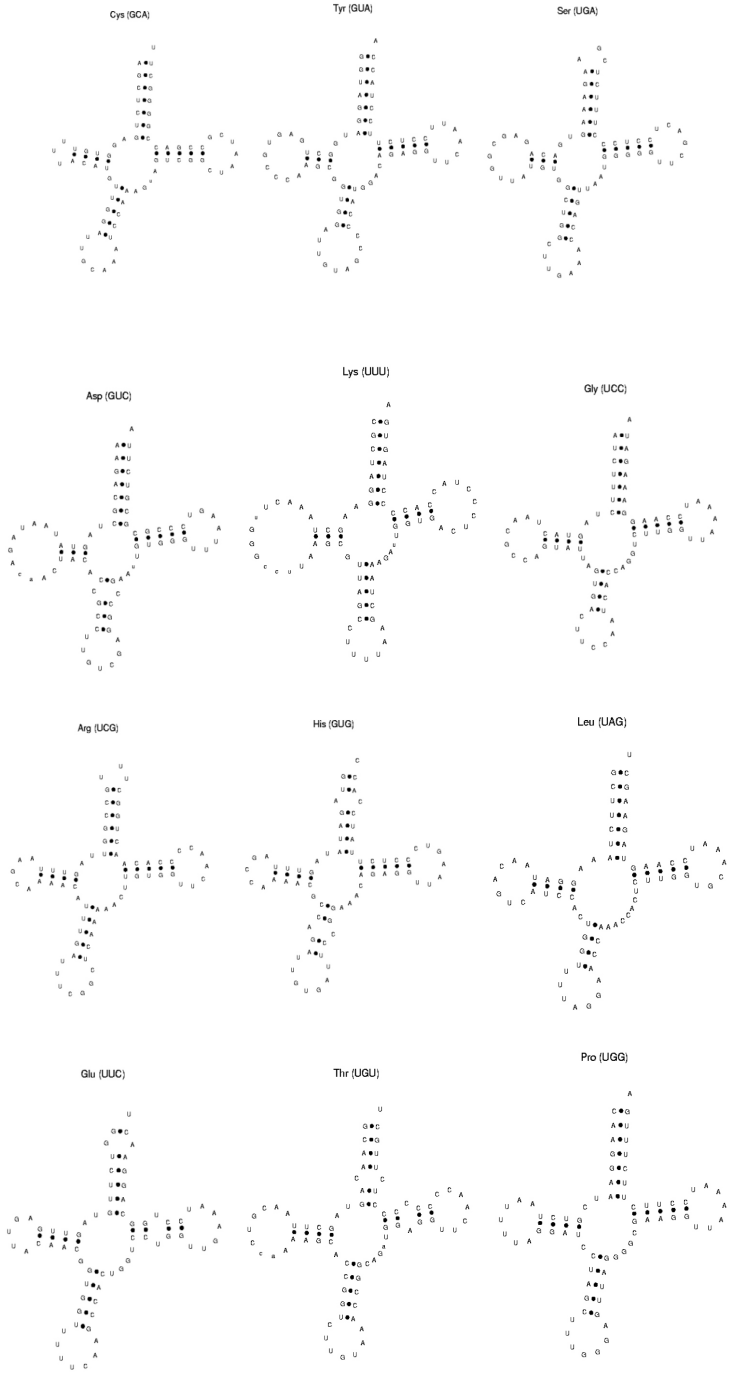
Figure 3. Continued.



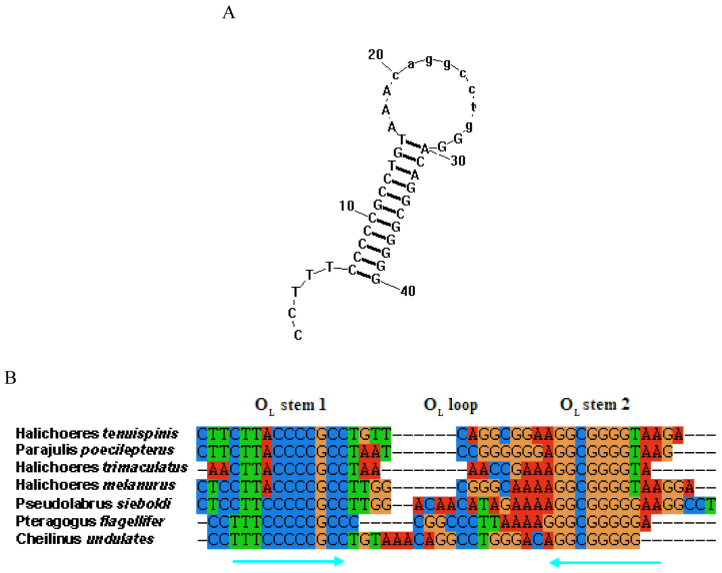


Figure 4. A. Secondary structure at the origin of light-strand replications (O_L) of *Cheilinus undulatus* mitochondrial DNA. **B.** Alignment of the O_L stem-loop sequences from 7 species (*C. undulatus*, *Halichoeres melanurus*, *H. tenuispinis*, *H. trimaculatus*, *Parajulis poecilepterus*, *Pseudolabrus sieboldi*, and *Pteragogus flagellifer*) of the family Labridae.

ATCACACAAACGCCCAGCATAATTATGATATCACACGCCTGCCTAATCCACACATTATGC
GCTCATGTCTGTGCCCGCAACCCATTATACTAAGCCCACCCAATAAAATGGGGCCACAC
ETAS1
ATACATTGCTTGCAGTATTACATATAGCTGACTGCGTGCATACGCCATGGTAATCACCCA
GGTATGTGCTTCGACCATTGAACTGCATTGCCCCAGTACCATGACTTAGTACTACCCAC
AGCTTAGCAGTACCCGGATGTTAGCGGCGTACCCTTGCGTCCAAGGCGTACAACCAGT
CSB-F
GTGTCAAACATTCCACTACACCATGCTAGCCCCCAAGGGCTGGCCGGCGAGAACCTAC
CATTACGGTCTATAGACTTGCTAGGTCTTCCTGATATTCAAGGACAGGCTAAGGGAAGC
CSB-D
GTCAGAGATACTGATCTATTTCTTGCCTCCGGTTGTTACTTCAGGCTCATTACGGCCGA
ACACACCCCTGTCTTTCATCGACGCTTGCATGAATTGATGCTTCGAACACATAATCTTC
GTCACCCCGCATGCCAAGCGTTCTCTCCACGGGTGTAAGGGGTTTTTTAGTTTTTTGTA
AGTTTGATCCATACTTCAACAGCTCGTGATTCCCAAGTAGTGCCCAAATCTAGGAGAAC
TGGCTCGTATCAACGGAAGCAATGAAGAAGACGGGGGTCCGGCAGACCATAAAGGAC
CSB-1
TGTCGCTCGCTAGCATTCCACATAAGGATATCGAGTACATACTATAACCTCCGACAGCC
CSB-2
CCCCACACCCCCCACTGCTGACAATCGCTTAAGGCCCTAGCAACCAAGAACATAAAGA
AAGCTCCCACTAGCTTATCTGCTTGCAGCTATCACGTCATCTATATTACAGTATCTTAATA
CSB-3
TTGCAATACTGTAAAAAAC

Figure 5. Complete sequences of the *Cheilinus undulatus* mtDNA control region. The conserved sequences including extended termination-associated sequences (ETAS), conserved sequence block (CSB), and central domain (CSB-F and CSB-D), are given in boxes.

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