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Two complete mitogenomes of Ocypodoidea (Decapoda: Brachyura), *Cleistostoma dilatatum* (Camptandriidae) and *Euplax* sp. (Macrophthalmidae) and its phylogenetic implications

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Abstract

Complete mitochondrial genomes (mitogenomes) can provide useful information for phylogenetic relationships, gene rearrangement, and molecular evolution. In the present study, two newly sequenced mitogenomes of Ocypodoidea (*Cleistostoma dilatatum* and *Euplax* sp.) were reported for the first time, which are 15 444 bp and 16 129 bp in length, respectively. *Cleistostoma dilatatum* is the first species in the family Camptandriidae whose complete mitogenome was sequenced. Each mitogenome contains an entire set of 37 genes and a putative control region, but their gene arrangements are largely different. Tandem duplication and random loss model is proposed to account for their gene arrangements. Comparative genomic analyses of 19 mitogenomes clustering in one branch reveal that 18 of them shared the same gene rearrangement, while that of *C. dilatatum* mitogenome was consistent with the ancestral gene arrangement of Brachyura. The dN/dS ratio analysis shows that all PCGs are evolving under purifying selection. Phylogenetic analyses show that all Macrophalmidae species cluster together as a group, and then form a sister clade with Camptandriidae. Moreover, the polyphyly of three superfamilies (Ocypodoidea, Eriphioidea, and Grapsoidea) is reconfirmed. These findings help to confirm the phylogenetic position of Camptandriidae, as well as provide new insights into the phylogeny of Brachyura.

Key words: Camptandriidae, Macrophthalmidae, mitogenome, gene rearrangement, phylogenetic analysis

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1 Introduction

With the rapid development of next-generation sequencing (NGS) technologies that can effectively analyze huge pools of molecular data, an increasing number of mitogenomes provide important insights into species evolution and phylogenetic relationships (Tan et al., 2018; Ruan et al., 2020, Yang et al., 2021). Generally, gene order in most vertebrate mitogenomes is considered conserved, e.g., less than 4% rearrangement ratio in fish mitogenomes (Li et al., 2013). However, extensive gene rearrangements have been observed in invertebrate mitogenomes (Wu et al., 2012; Liu et al., 2017; Jiang et al., 2018). Recent studies have shown that some of these rearrangements contain useful information for phylogeny, and many scholars have applied gene rearrangement as a new molecular marker in phylogenetic studies. For example, Akasaki et al. (2006) compared the gene rearrangement of subclass Coleoidea and proposed that the arrangements of mitochondrial genes in Oegopsida and Sepiida were derived from those of Octopoda. This conclusion is consistent with the results of their phylogenetic analysis based on mitochondrial genes. Through a comparative study of gene rearrangement and phylogenetic relationships of five species from the superfamily Tellinoidea, Yuan et al. (2012) suggested that the genus *Sinonovacula* should be placed in the superfamily Solenoidea instead of the superfamily Tellinoidea. Besides, Tan et al. (2018) compared the published mitogenome sequences of two infraorders (Anomura and Brachyura) and affirmed the potential value of using rearrangement information to investigate the phylogeny of Anomura.

In contrast, there are also some scholars consider that gene order is not suitable for phylogenetic reconstruction. For example, Xie et al. (2019) demonstrated that approach based on gene order alone is clearly inferior to sequence-based approaches to resolve major phylogenetic relationships. In their research, none of the relationships among major stylommatophoran groups were resolved in the gene order tree. Recently, Zhang et al. (2021b) reconstructed the phylogeny of Paguroidea based on both sequence data and gene order. The results indicated that gene order data did not seem to work well for phylogenetic analysis within families. From their gene order tree, the relationships within families are suspicious because two close relatives belonging to the same genus (*Dardanus arrosor* and *D. aspersus*) owned two different gene rearrangements. Of course, increasing the availability of mitogenomic data from different taxa will help to validate the applicability of gene order data in inferring phylogenetic relationships.

The infraorder Brachyura contains approximately 7 250 known species inhabiting marine, freshwater, and terrestrial habitats (Chen et al., 2018; Ma et al., 2019). Brachyura, as the old-

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est crab, originated in the Jurassic period (Schweitzer and Feldmann, 2010; Davie et al., 2015a), and a group of its members with extremely diverse morphology and ecology was finally formed after massive radiative evolution. However, the diversity has also caused remarkably challenges for species identification, and their real phylogenetic relationships remain controversial (Camargo et al., 2020; Tan et al., 2018). Grapsoidea and Ocypodoidea, two of the most abundant and economically important groups in Brachyura, are of commercial value to fisheries and aquaculture. However, the classification of Grapsoidea and Ocypodoidea has been controversial for a long time. Previous studies based on morphological features considered them to be monophyletic clades (Ng et al., 2008; Davie et al., 2015b). Recently, an increasing number of molecular studies have challenged the monophyly of these taxa (Chen et al., 2018, 2019; Lu et al., 2020). For example, molecular study of Wang et al. (2020) revealed that Ocypodoidea and Grapsoidea are divided into three clades, and similar findings were presented in Tan et al. (2018). Larger taxon samples are required to fully understand the phylogenetic relationships between Ocypodoidea and Grapsoidea in future studies.

Members of the family Camptandriidae Stimpson, 1858 are commonly found in the estuarine, mangrove mudflat, and open mudflat habitats in the Indo-West Pacific regions (Jones and Clayton, 1983). Species of this family share a distinct condition in the male first gonopod, in which the distal part is bent or twisted over the proximal base by about 180°, producing a strongly recurved structure (Naruse et al., 2015). Initially, this family was regarded as a subfamily of Ocypodidae. Subsequently, Camptandriinae was raised to the family level and a complete diagnosis of this family was carried out (Cheryl, 1997). According to WoRMS (http://www.marinespecies.org/), Camptandriidae consists of 42 species belonging to 24 genera. Most studies of this family focused on morphological features (Naderloo, 2017a; Trivedi et al., 2017). Although there are few researches on molecular level, most of them were based on partial mitochondrial gene sequences (Kitaura et al., 1998; Miura et al., 2007). To date, no complete mitogenome from Camptandriidae has ever been reported. The phylogenetic relationships among Camptandriidae and even the evolutionary status of this family have not been well resolved due to limited mitogenomic data.

Members of the family Macrophthalmidae Dana, 1851 occur throughout the Indo-West Pacific, with most of the known species living in intertidal habitat (Mendoza and Ng, 2007). The macrophthalmids are distinguished primarily by having antennules that fold transversely or obliquely, a narrow inter-antennulary septum, external maxillipeds that do not completely close the buccal cavern, and eyestalks that are usually elongate (Davie, 2002). Although this family had long been regarded as a subfamily of Ocypodidae, Kitaura et al. (2002) provided clear molecular evidence that it should be regarded as a distinct family. At present, it includes 86 species belonging to 13 genera. Similarly, the genus *Euplax* H. Milne Edwards, 1852, it was initially regarded as a subgenus of *Macrophthalmus* Desmarest, 1823. Afterward, McLay et al. (2010) updated it to a valid genus. According to WoRMS, the genus *Euplax* only contains two species, *Euplax dagohoyi* (Mendoza and Ng, 2007) and *Euplax leptophthalmus* (H. Milne Edwards, 1852). To date, only three complete mitogenomes of this family are available from the National Center for Biotechnology Information (NCBI) dataset, and all of them belong to the genus *Macrophthalmus*. The phylogenetic relationships among Macrophthalmidae have been poorly resolved.

Accordingly, in the present study, two newly sequenced mitogenomes of Ocypodoidea (*C. dilatatum* and *Euplax* sp.) were reported for the first time, one of which (*C. dilatatum*) is the first species in the family Camptandriidae whose complete mitogenome was sequenced. The characteristics of these two mitogenomes and the other 17 mitogenomes clustering in one branch of the phylogenetic tree were compared. Genome collinearity analysis of 19 mitogenomes showed that 18 of them shared the same gene rearrangement, while that of *C. dilatatum* mitogenome was consistent with the ancestral gene arrangement of Brachyura. Possible models were proposed to explain the current mitogenomic rearrangements. The phylogeny of Brachyura was reconstructed and the evolutionary status of Camptandriidae was revealed for the first time from the mitogenomic level. These results will not only enrich the mitogenomes of Ocypodoidea and mitogenomic rearrangements, but also lay a foundation for further evolutionary studies of Brachyura.

2 Materials and methods

2.1 *Sampling, DNA extraction, mitogenome sequencing, and assembly*

Specimens of *C. dilatatum* and *Euplax* sp. were collected from Jiangsu Province, China (34°47′48.80″N, 119°13′42.38″E) and Hainan Province, China (18°24′39.48″N, 109°58′20.60″E), respectively. Specimens were immediately preserved in 95% ethanol until DNA extraction. According to the key morphological features of crabs, these two specimens were identified with a stereo dissecting microscope (Naderloo, 2017a, 2017b). The SQ Tissue DNA Kit (OMEGA) was used to extract the total genomic DNA from muscle tissue following the manufacturer's instructions. The genomic DNA was sent to Shanghai Origingene Biopharm Technology Co., Ltd. for library preparation and high-throughput sequencing. The libraries were constructed by using the VAHTS Universal Plus DNA Library Prep Kit, with an insert size of 150 bp. Paired-end sequencing with a read length of 150 bp was performed on an Illumina Hiseq 6000 platform. Adapters and low-quality bases were removed using cutadapt v1.16 (Martin, 2011) with the following parameters: q, 20; m, 20. Trimmed reads shorter than 50 bp were discarded. Quality control of raw and trimmed reads was performed using FastQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The filtered clean data were assembled and mapped to complete mitogenome sequence using NOVOPlasty v2.7.2 (Dierckxsens et al., 2017).

2.2 *Mitogenome annotation and sequence analysis*

The newly assembled mitogenomes of *C. dilatatum* and *Euplax* sp. were annotated using the software of Sequin (version 15.10, http://www.ncbi.nlm.nih.gov/Sequin/). The boundaries of protein-coding and ribosomal RNA genes were performed using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov). Transfer RNA genes were manually plotted, according to the secondary structure predicted by the MITOS Web Server (Bernt et al., 2013) and tRNAscan-SE 1.21 (Lowe and Chan, 2016). The control region was determined by the locations of adjacent genes. Finally, circular mitogenome maps of *C. dilatatum* and *Euplax* sp. were drawn with the BLAST Ring Image Generator v0.95 (Alikhan et al., 2011).

The base composition and relative synonymous codon usage (RSCU) were obtained using MEGA X (Kumar et al., 2018). The strand asymmetry was calculated using the following formulas: AT-skew = (A – T)/(A + T); GC-skew = (G – C)/(G + C) (Perna and Kocher, 1995). Synteny analysis between the genomes was performed using Mauve v2.4.0 (Darling et al., 2004). To estimate the

evolutionary-selection constraints on 13 PCGs, the nonsynonymous (dN) and synonymous (dS) substitution rates were calculated using Mega X. The genetic distances of 13 PCGs were also estimated using Mega X based on the Kimura 2-parameter (K2P) substitution model.

2.3 *Phylogenetic analysis*

The phylogeny of Brachyura was inferred based on 107 available complete mitogenomes and two newly determined ones (Table S1). The species *Pagurus nigrofascia* and *P. gracilipes* from Anomura were used as outgroups. PhyloSuite (Zhang et al., 2020a) was used to extract the nucleotide sequences of 13 PCGs for each of the above species from the GenBank files. The MAFFT program (Katoh et al., 2002) integrated into PhyloSuite was executed to align multiple sequences in normal-alignment mode, and ambiguously aligned regions were identified and moved by Gblocks (Talavera and Castresana, 2007). The alignments of individual genes were then concatenated and used to generate input files (Phylip and Nexus formats) for phylogenetic analysis. The best-fit models were selected by ModelFinder (Kalyaanamoorthy et al., 2017) based on the Bayesian Information Criterion (BIC). Phylogenetic trees were built under maximum likelihood (ML) and Bayesian inference (BI) methods. The ML analysis was carried out in IQ-TREE (Nguyen et al., 2015) using an ML+rapid bootstrap (BS) algorithm with 1 000 replicates. The BI analysis was performed in MrBayes 3.2.6 (Ronquist et al., 2012) with default parameters and 3×106 Markov Chain Monte Carlo generations. The trees were sampled every 1 000 generations with a burn-in of 25%. The average standard deviation of split frequencies below 0.01 was considered to reach convergence.

3 Results and discussion

3.1 *General features of C. dilatatum and Euplax sp. mitogenomes*

The complete mitogenomes of *C. dilatatum and Euplax* sp. are 15 444 bp and 16 129 bp in length, respectively (GenBank accessions MW191756 and MT176431; the order of the following data is the same as these) (Fig. 1; Tables 1 and 2). These two mitogenomes both contain a typical set of 37 genes (13 PCGs, 22 tRNAs, and two rRNAs) and a putative control region (CR). Nine PCGs and 14 tRNAs are encoded by the heavy (H-) strand, while the remaining genes are encoded by the light (L-) strand. There are 140 intergenic nucleotides dispersed in 13 locations in *C. dilatatum* mitogenome, and 537 intergenic nucleotides in 17 locations in *Euplax* sp. mitogenome; respectively. The longest one is 53 bp (between *ND5* and *ND4*) and 169 bp (between *ND4L* and *ND6*) in these two mitogenomes (Tables 1 and 2). The base composition of *C. dilatatum* mitogenome is 34.4% A, 34.7% T, 11.4% C, 19.5% G, and that of *Euplax* sp. is 34.9% A, 34.0% T, 10.4% C, 20.7% G; the AT contents are 69.1% and 68.9%, suggesting a strong AT bias (Tables S2 and S3).

All the 13 PCGs initiate with typical ATN codons in the two mitogenomes. The majority of PCGs terminate with TAA or TAG, while four PCGs in *C. dilatatum* mitogenome (*COI*, *COII*, *COIII*, and *Cyt b*) and three PCGs in *Euplax* sp. mitogenome (*COII*, *COIII*, and *Cyt b*) use a single T as a stop codon (Tables 1 and 2). Incomplete stop codons are common in metazoan mitogenomes and may be recovered via post-transcriptional polyadenylation (Ojala et al., 1981). The GC-skew values of nine PCGs (*COI*, *COII*, *ATP8*, *ATP6*, *COIII*, *ND3*, *ND6*, *Cyt b*, and *ND2*) are negative, indicating they are encoded by the H-strand, whereas the remaining four exhibit positive values, indicating they are encoded by the L-strand (Tables S2 and S3). The most frequently used amino acids are Leu and Ser. In comparison, the least common amino acids are Cys and Arg (Figs 2a, b). The RSCU values of each codon in these two mitogenomes are roughly identical (Figs 2c, d; Table S4). It is worth noting that the RSCU values for the codons NNU and NNA are usually greater than one, suggesting a strong AT bias in the third codon position.

Twenty-two tRNAs are scattered throughout the entire mitogenome (Tables 1 and 2). All of them can be folded into typical cloverleaf secondary structures except for S_1 in both two mitogenomes (Figs S1 and S2). The lack of DHU arm in S_1 is thought to be a common phenomenon in metazoan mitogenomes (Gong et al., 2020; Lu et al., 2020; Ruan et al., 2020). The *16S rRNA* and *12S rRNA* genes of *C. dilatatum* mitogenome are located between L_1 and *V*, *V* and CR, respectively. While *Euplax* sp. mitogenome shares different rRNA arrangements (L_1- *16S- 12S- H*).

3.2 *Comparative mitogenomic analysis clustering in one branch*

To estimate the evolutionary-selection constraints on 13 PCGs in 19 mitogenomes, we perform dN/dS analysis for each

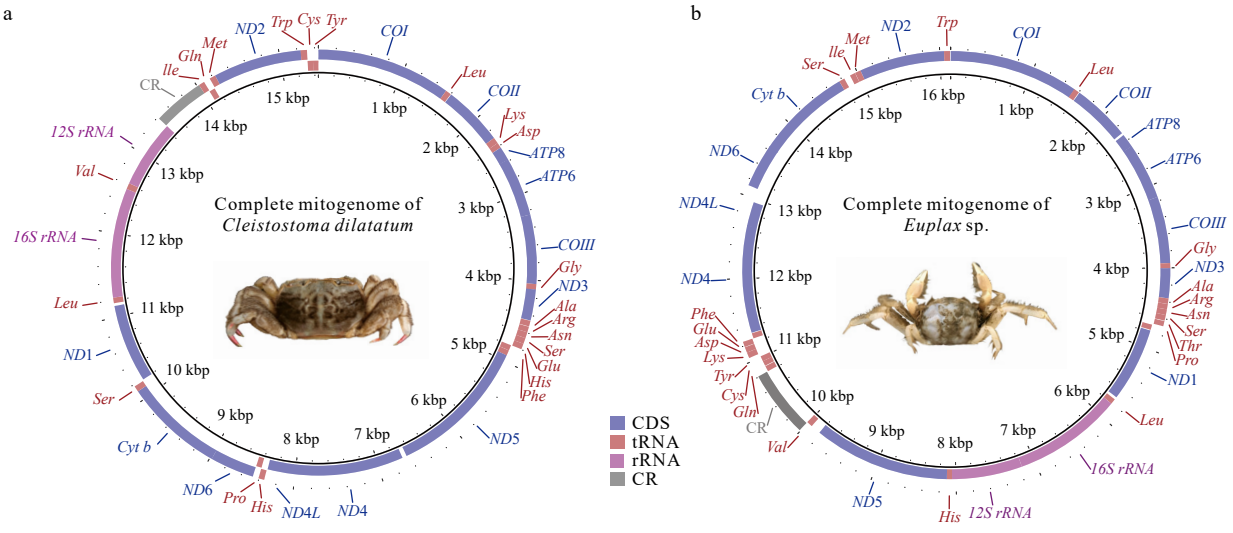

Fig. 1. Gene maps of *Cleistostoma dilatatum* (a) and *Euplax* sp. (b) mitogenomes. Genes encoded on the heavy or light strands are shown outside or inside the circular gene map, respectively.

Table 1. Features of the mitochondrial genome of *Cleistostoma dilatatum*

Gene	Position		Length/bp	Amino acid	Start/Stop codon	Anticodon	Intergenic region	Strand
	From	To						
COI	1 bp	1 534 bp	1 534	511	ATG/T	–	0	H
Leu (L_2)	1 535 bp	1 599 bp	65	–	–	TAA	6	H
COII	1 606 bp	2 293 bp	688	229	ATG/T		0	H
Lys (*K*)	2 294 bp	2 363 bp	70	–	–	TTT	0	H
Asp (*D*)	2 364 bp	2 424 bp	61	–	–	GTC	1	H
ATP8	2 426 bp	2 584 bp	159	52	ATG/TAA	–	–4	H
ATP6	2 581 bp	3 252 bp	672	223	ATA/TAA	–	–1	H
COIII	3 252 bp	4 041 bp	790	263	ATG/T	–	0	H
Gly (*G*)	4 042 bp	4 105 bp	64	–	–	TCC	–3	H
ND3	4 103 bp	4 456 bp	354	117	ATT/TAA	–	4	H
Ala (*A*)	4 461 bp	4 525 bp	65	–	–	TGC	4	H
Arg (*R*)	4 530 bp	4 593 bp	64	–	–	TCG	0	H
Asn (*N*)	4 594 bp	4 662 bp	69	–	–	GTT	0	H
Ser (S_1)	4 663 bp	4 729 bp	67	–	–	TCT	0	H
Glu (*E*)	4 730 bp	4 795 bp	66	–	–	TTC	2	H
His (*H*)	4 798 bp	4 862 bp	65	–	–	GTG	1	L
Phe (*F*)	4 864 bp	4 928 bp	65	–	–	GAA	–1	L
ND5	4 928 bp	6 643 bp	1 716	571	ATT/TAA	–	53	L
ND4	6 697 bp	8 034 bp	1 338	445	ATG/TAA	–	–7	L
ND4L	8 028 bp	8 330 bp	303	100	ATG/TAA	–	9	L
Thr (*T*)	8 340 bp	8 405 bp	66	–	–	TGT	0	H
Pro (*P*)	8 406 bp	8 470 bp	65	–	–	TGG	2	L
ND6	8 473 bp	8 976 bp	504	167	ATT/TAA	–	–1	H
Cyt b	8 976 bp	10 110 bp	1 135	378	ATG/T	–	0	H
Ser (S_2)	10 111 bp	10 177 bp	67	–	–	TGA	15	H
ND1	10 193 bp	11 131 bp	939	312	ATA/TAA	–	34	L
Leu (L_1)	11 166 bp	11 232 bp	67	–	–	TAG	0	L
16S	11 233 bp	12 546 bp	1 314	–	–	–	0	L
Val (*V*)	12 547 bp	12 619 bp	73	–	–	TAC	0	L
12S	12 620 bp	13 435 bp	816	–	–	–	0	L
CR	13 436 bp	14 024 bp	589	–	–	–	0	H
Ile (*I*)	14 025 bp	14 090 bp	66	–	–	GAT	–3	H
Gln (*Q*)	14 088 bp	14 156 bp	69	–	–	TTG	8	L
Met (*M*)	14 165 bp	14 234 bp	70	–	–	CAT	0	H
ND2	14 235 bp	15 245 bp	1 011	336	ATT/TAG	–	–2	H
Trp (*W*)	15 244 bp	15 315 bp	72	–	–	TCA	1	H
Cys (*C*)	15 317 bp	15 380 bp	64	–	–	GCA	0	L
Tyr (*Y*)	15 381 bp	15 444 bp	64	–	–	GTA	–1	L

Note: – represents no data.

PCG. It is commonly accepted that dN/dS>1, dN/dS=1, and dN/dS<1 generally indicate positive selection, neutral mutation, and purifying selection, respectively (Yang, 2006). All of the dN/dS ratios are lower than one (<1), indicating that all 13 PCGs are evolving under purifying selection. *ATP8* gene exhibits a highly relaxed purifying selection with the highest dN/dS value (0.619), whereas *COI* gene exhibits the strongest purifying selection with the lowest dN/dS value (0.077) (Fig. 3). The lowest dN/dS value of *COI* gene indicates that this gene is bound by the protein-coding function and bears strong natural selection pressure, thus ensuring the normal function of its encoded protein, which means that *COI* gene has an important role in the survival and evolution of the above species. Besides, we conduct genetic distance analysis for 13 PCGs. *COI* gene possesses the least genetic distance (average 0.214), and *ATP* gene captures the largest value (average 0.409), representing the most conserved and variable genes, respectively (Fig. 3).

Genomic synteny analysis reveals that four large genomic homologous regions are prevalent in all 19 mitogenomes (marked A–D in Fig. 4). It is evident that the homologous regions B and C are rearranged in *C. dilatatum* mitogenome when choosing *Eriocheir sinensis* (Brachyura: Varunidae) mitogenome as the reference sequence (Fig. 4). The two homologous regions show a C-B order in *C. dilatatum* mitogenome, while that the remaining crabs display a B-C order (Fig. 4). Further analysis indicated that *C. dilatatum* mitogenome was consistent with the ancestral gene arrangement of Brachyura, while that of the remaining crabs shared exactly the same gene rearrangements.

3.3 *Gene rearrangement*

Gene arrangements in *C. dilatatum* and *Euplax* sp. mitogenomes are shown in Fig. 5. For *C. dilatatum* mitogenome, only a single *H* moves from the downstream of *ND5* to downstream of *E* (Fig. 5A①) when compared with the gene order in ancestral crus-

Table 2. Features of the mitochondrial genome of *Euplax* sp.

Gene	Position		Length/bp	Amino acid	Start/Stop codon	Anticodon	Intergenic region	Strand
	From	To						
COI	1 bp	1 539 bp	1 539	512	ATG/TAA	–	–5	H
Leu (L_2)	1 535 bp	1 600 bp	66	–	–	TAA	8	H
COII	1 609 bp	2 296 bp	688	229	ATG/T	–	28	H
ATP8	2 325 bp	2 486 bp	162	53	ATT/TAA	–	–4	H
ATP6	2 483 bp	3 154 bp	672	223	ATA/TAA	–	–1	H
COIII	3 154 bp	3 943 bp	790	263	ATG/T	–	0	H
Gly (*G*)	3 944 bp	4 006 bp	63	–	–	TCC	–3	H
ND3	4 004 bp	4 357 bp	354	117	ATA/TAA	–	1	H
Ala (*A*)	4 359 bp	4 422 bp	64	–	–	TGC	1	H
Arg (*R*)	4 424 bp	4 487 bp	64	–	–	TCG	0	H
Asn (*N*)	4 488 bp	4 554 bp	67	–	–	GTT	0	H
Ser (S_1)	4 555 bp	4 621 bp	67	–	–	TCT	6	H
Thr (*T*)	4 628 bp	4 689 bp	62	–	–	TGT	16	H
Pro (*P*)	4 706 bp	4 770 bp	65	–	–	TGG	10	L
ND1	4 781 bp	5 707 bp	927	308	ATA/TAG	–	33	L
Leu (L_1)	5 741 bp	5 807 bp	67	–	–	TAG	0	L
16S	5 808 bp	7 170 bp	1 363	–	–	–	0	L
12S	7 171 bp	8 048 bp	878	–	–	–	0	L
His (*H*)	8 049 bp	8 113 bp	65	–	–	GTG	–1	L
ND5	8 113 bp	9 813 bp	1 701	566	ATG/TAA	–	125	L
Val (*V*)	9 939 bp	10 011 bp	73	–	–	TAG	0	L
CR	10 012 bp	10 806 bp	795	–	–	–	0	H
Gln (*Q*)	10 807 bp	10 875 bp	69	–	–	TTG	7	L
Cys (*C*)	10 883 bp	10 944 bp	62	–	–	GCA	0	L
Tyr (*Y*)	10 945 bp	11 010 bp	66	–	–	GTA	37	L
Lys (*K*)	11 048 bp	11 116 bp	69	–	–	TTT	0	H
Asp (*D*)	11 117 bp	11 182 bp	66	–	–	GTC	4	H
Glu (*E*)	11 187 bp	11 249 bp	63	–	–	TTC	–1	H
Phe (*F*)	11 249 bp	11 314 bp	66	–	–	GAA	7	L
ND4	11 322 bp	12 659 bp	1 338	445	ATG/TAA	–	–7	L
ND4L	12 653 bp	12 955 bp	303	100	ATG/TAA	–	169	L
ND6	13 125 bp	13 649 bp	525	174	ATT/TAA	–	–20	H
Cyt b	13 630 bp	14 764 bp	1 135	378	ATG/T	–	0	H
Ser (S_2)	14 765 bp	14 830 bp	66	–	–	TGA	76	H
Ile (*I*)	14 907 bp	14 971 bp	65	–	–	GAT	2	H
Met (*M*)	14 974 bp	15 042 bp	69	–	–	CAT	0	H
ND2	15 043 bp	16 053 bp	1 011	336	ATG/TAG	–	–2	H
Trp (*W*)	16 052 bp	16 121 bp	70	–	–	TCA	7	H

Note: – represents no data.

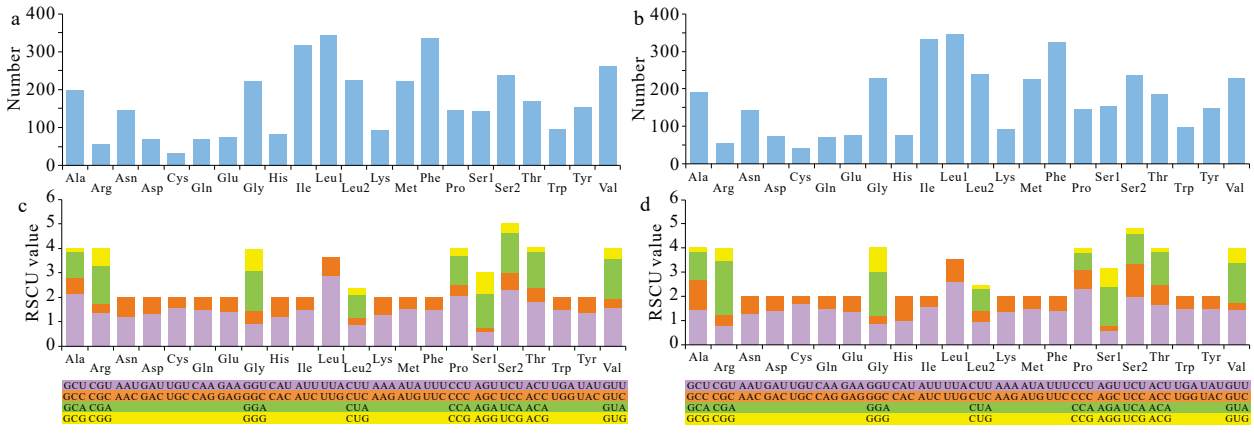

Fig. 2. Amino acid composition in the mitogenome of *Cleistostoma dilatatum* (a) and *Euplax* sp. (b); relative synonymous codon usage in the mitogenome of *C. dilatatum* (c) and *Euplax* sp. (d). RSCU: relative synonymous codon usage.

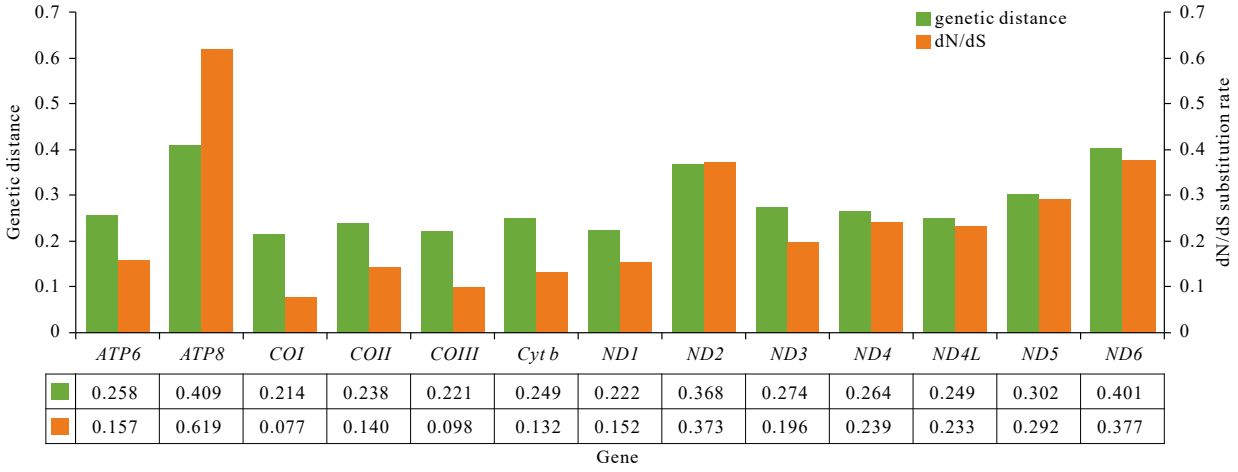

Fig. 3. Genetic distance (on average) and dN/dS substitution rates of 13 PCGs among 19 mitogenomes.

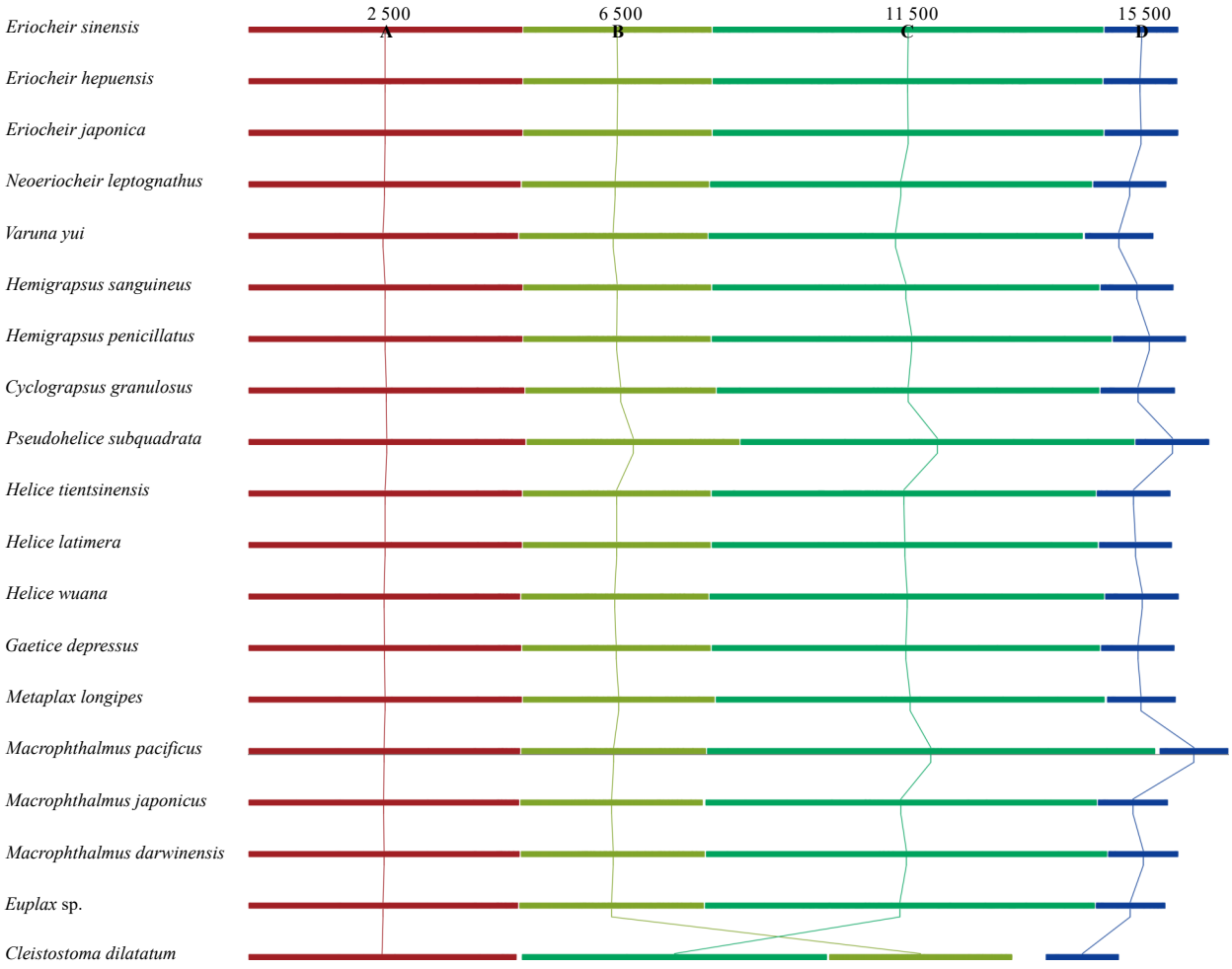

Fig. 4. Multiple genome alignments of 19 mitogenomes. The mitogenome of *Eriocheir sinensis* at the top as the reference genome. All genomes are started from the *COI* gene. The number at the top of each genome shows nucleotide positions. Within each of the alignments, local collinear blocks are represented by blocks of the same color connected by lines.

taceans (the pancrutacean ground pattern) mitogenomes (Boore, 1999). In contrast, gene order in *Euplax* sp. mitogenome underwent large-scale gene rearrangements. At least nine gene clusters (or genes) significantly differ from the typical order, involving 12 tRNA genes (*K*, *D*, *E*, *F*, *H*, *T*, *P*, L_1, *V*, *Q*, *C*, and *Y*), two rRNAs (*16S rRNA* and *12S rRNA*), one PCG (*ND1*), and a putative CR (Fig. 5B). Of these gene rearrangements, three tRNA gene pairs (*K-D*, *E-F*, and *C-Y*) and two single tRNA genes (*V* and *Q*) are moved into the *ND5* and *ND4* junction (Fig. 5B①②⑥⑧⑨), forming an eight-tRNA cluster (*V-Q-C-Y-K-D-E-F*) if CR is not considered. The CR is shifted from the typical area between *12S rRNA* and *I* to the *V* and *Q* junction (Fig. 5B⑦). A single *H* gene, one tRNA gene

pair (*T-P*), and the *ND1- L_1-16S-12S* gene cluster are moved to the position between S_1 and *ND5* (Fig. 5B③④⑤).

Currently, four widely-accepted mechanisms have been used to account for mitogenomic rearrangements, including tandem duplication and random loss (TDRL) model (Moritz and Brown, 1987), intramitochondrial recombination model (Poulton et al., 1993), tandem duplication and non-random loss model (Lavrov et al., 2002), and double replications and random loss model (Shi et al., 2014). How did the gene orders in these two newly sequenced mitogenomes emerge? Here, we proposed that the TDRL mechanism resulted in the generation of these two mitogenomes. The hypothesized intermediate steps are as follows. Firstly, the *F-ND5-H* genes underwent a complete copy, forming a dimeric block, (*F-ND5-H*)-(*F-ND5-H*). Consecutive copies were then followed by a random loss of the duplicated genes, forming a novel *H-F-ND5* gene order (Fig. 6B). The *H-F-ND5* gene cluster is a common phenomenon in the mitogenome of ancestral and most living species of Brachyura (Lu et al., 2020; Zhang et al., 2020b), including Portunidae, Grapsidae, Ocypodidae, Leucosiidae, Eriphiidae, and the *C. dilatatum* mitogenome in this study. In the second rearrangement event, the gene block from *K* to *Y* underwent a complete copy, forming a dimeric block (*K-D-ATP8-ATP6-COIII-G-ND3-A-R-N-S_1-E-H-F-ND5-ND4-ND4L-T-P-ND6-Cyt b-S_2-ND1-L_1-16S-V-12S-CR-I-Q-M-ND2-W-C-Y*)-(*K-D-ATP8-ATP6-COIII-G-ND3-A-R-N-S_1-E-H-F-ND5-ND4-ND4L-T-P-ND6-Cyt b-S_2-ND1-L_1-16S-V-12S-CR-I-Q-M-ND2-W-C-Y*). Consecutive copies were then followed by a random loss of supernumerary genes, forming a new gene block, (*K-D-ATP8-ATP6-COIII-G-ND3-A-R-N-S_1-E-F-ND4-ND4L-T-P-ND6-Cyt b-S_2-ND1-L_1-16S-12S-I-M-ND2-W-H-ND5-V-CR-Q-C-Y*). In the following step, the newly formed gene block from *K* to *Y* underwent a second copy and likewise experienced a random loss of redundant genes. Finally, the ultimate gene arrangement in *Euplax* sp. mitogenome was generated (Fig. 6C), which is consistent with the ancestral gene arrangement of Varunidae and Macrophthalmidae (Wang et al., 2020). Summarily, all the rearrangement events mentioned above can be explained by TDRL model, which supposes that the rearranged gene order occurs via tandem duplications followed by random deletion of certain duplications (Moritz et al., 1987).

3.4 *Phylogenetic analysis*

The phylogenetic trees obtained using BI and ML methods resulted in identical topological structures except for supporting values. Here, only one topology (BI) with both support values was presented (Fig. 7). The results show that all Macrophalmidae species cluster together as a group, wherein *Euplax* sp. shows the closest relationship with *Macrophthalmus darwinensi*. Our phylogenetic trees firstly show the evolutionary status of Camptandriidae that it has the most closely related relationship with Macrophalmidae. These two families (Camptandriidae and Macrophalmidae) as a group then form a sister clade with Varunidae. Macrophalmidae and Varunidae sharing exactly the same mitogenomic rearrangements gather together in the phylogenetic tree, which is in consistence with most molecular results (Chen et al., 2018; Wang et al., 2020; Zhang et al., 2021a). Camptandriidae mitogenome, however, capturing the conserved gene arrangement (ancestral gene arrangement of Brachyura) forms a clade with the taxa that share the identically large-scale gene rearrangements. Similar phenomena have been reported in increasing number of crab mitogenomes (Tan et al., 2018; Li et al., 2020; Zhang et al., 2020c, 2021b). For instance, our recent work found that two closely related species belonging to the same genus (*D. arrosor* and *D. aspersus*) possessed two different gene rearrangements (Zhang et al., 2021b). More complex situations exist in Potamidae mitogenomes (Zhang et al., 2020c). Thus it echoes the viewpoint that the mitogenomic gene rearrangement is likely a continuous and dynamic process and may occur very recently even after speciation events (Zhang et al., 2021b). Of course, since here *C. dilatatum* is the only species of the family Camptandriidae, the phylogenetic status of Camptandriidae and the aforesaid thought-provoking hypothesis should be confirmed with more species.

Of the 30 families in our phylogenetic tree, except for Xanthidae, Gecarcinidae, and Homolidae, each family forms a monophyletic clade (Fig. 7). Regarding the non-monophyly of Xanthidae, four Xanthidae species are divided into two clades.

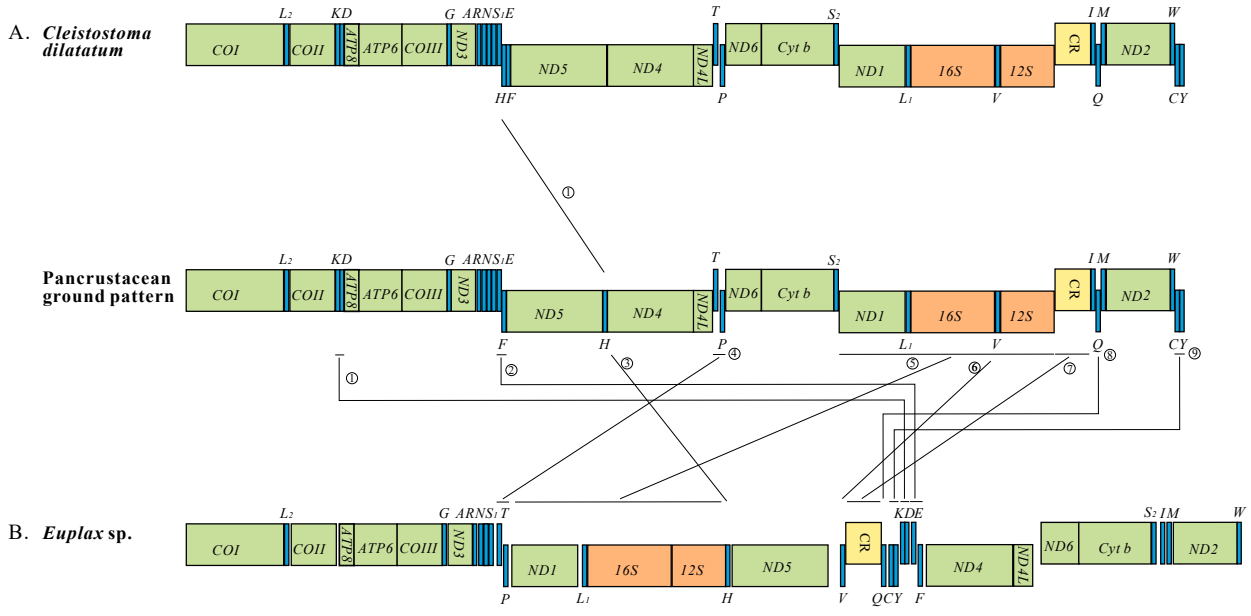

Fig. 5. Gene arrangements in *Cleistostoma dilatatum* (A) and *Euplax* sp. (B) mitogenome.

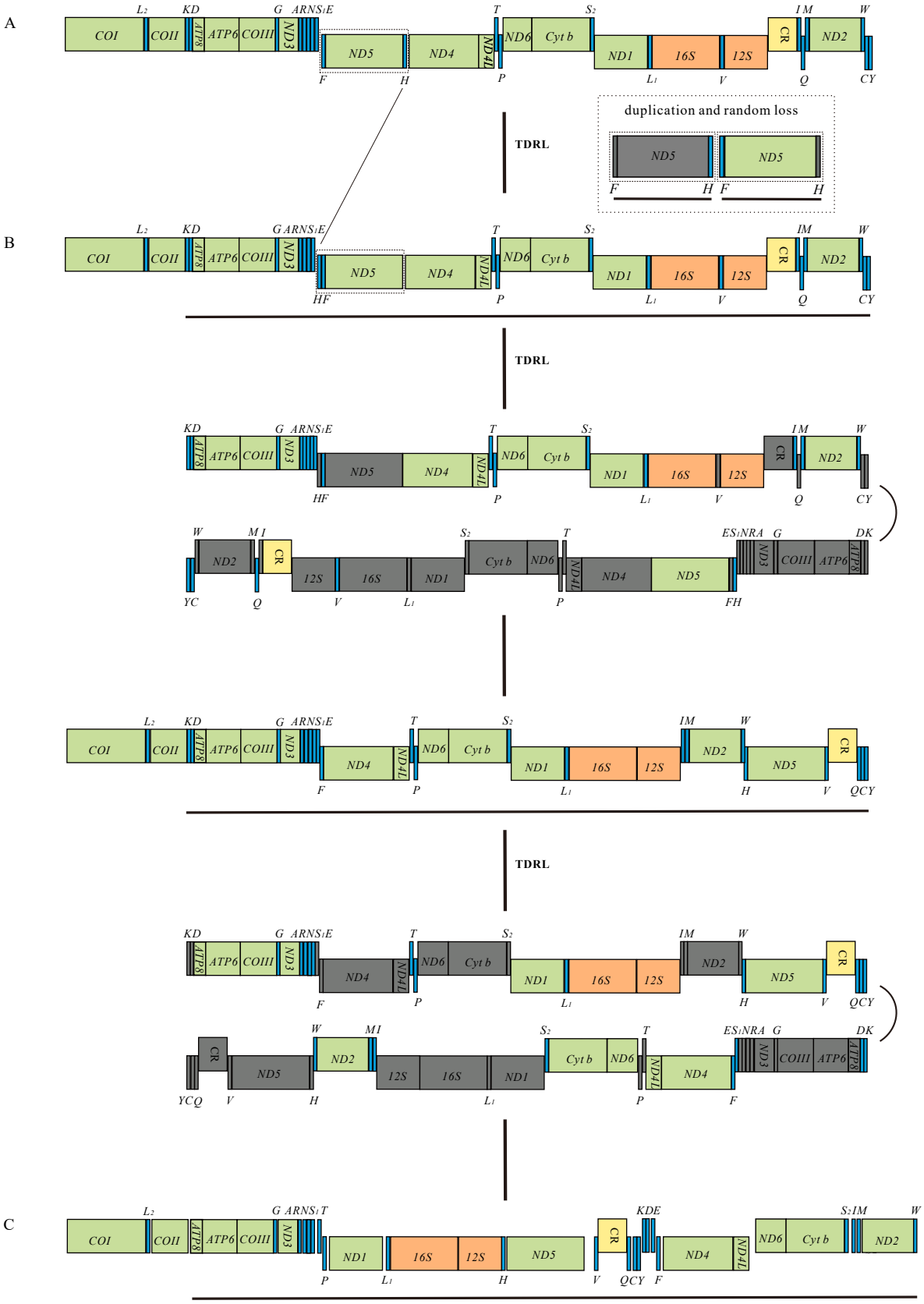

Fig. 6. Inferred intermediate steps between the ancestral gene arrangement of crustaceans and two newly sequenced mitogenomes. The ancestral gene arrangement of crustaceans (A); the results of one tandem duplication and random loss (TDRL) event, the ancestral gene arrangement in Brachyuran mitogenome, and the final gene arrangement in *Cleistostoma dilatatum* mitogenome (B); the results of two TDRL events, the ancestral gene arrangement in Varunidae and Macrophthalmidae mitogenomes, and the final gene arrangement in *Euplax* sp. mitogenome (C). The duplicated gene block is underlined and the lost genes are labeled with gray.

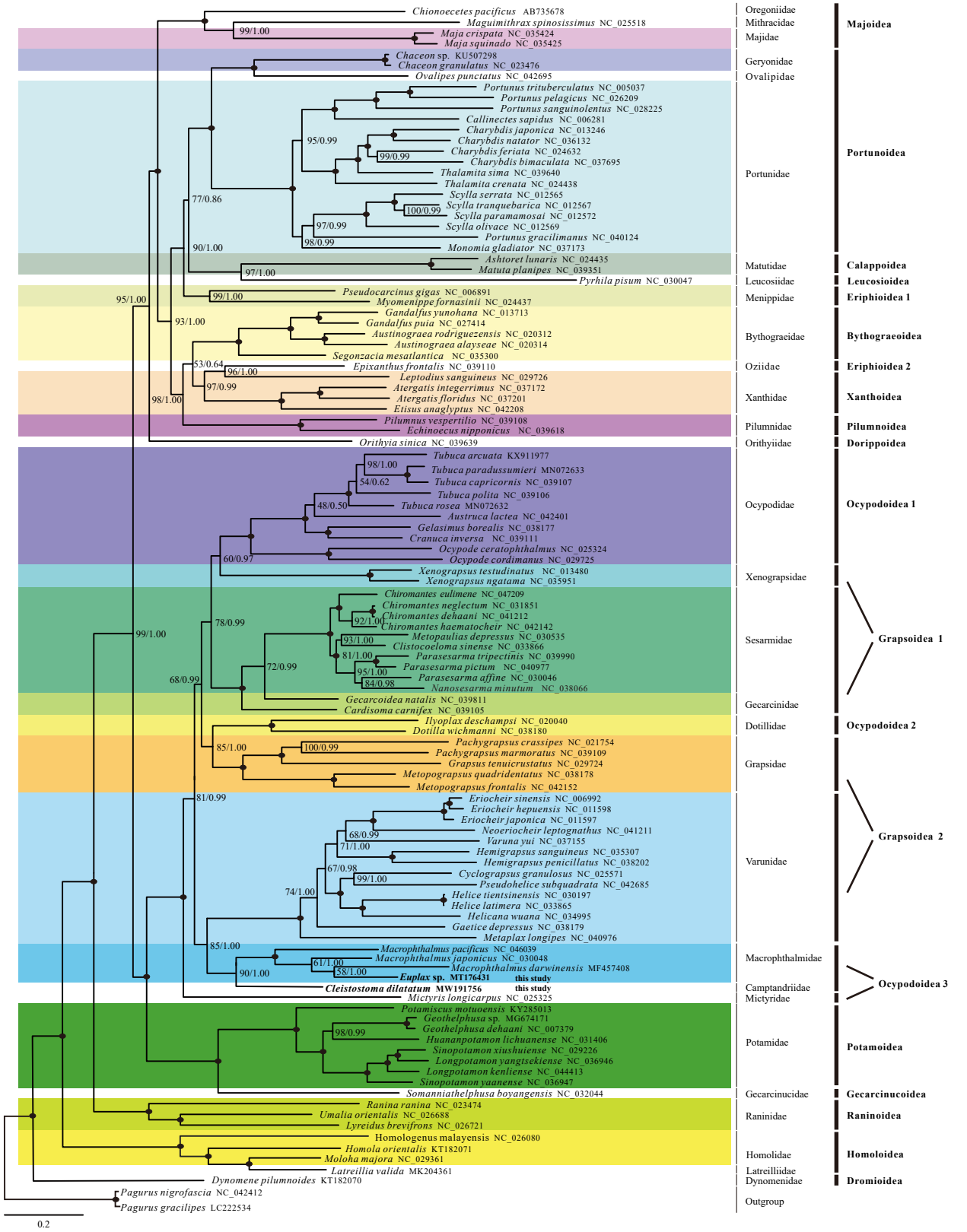

Fig. 7. Phylogenetic tree of brachyuran species inferred from the nucleotide sequences of 13 PCGs based on maximum likelihood (ML) and Bayesian inference (BI) analyses. The node marked with a solid circle indicates 100 ML bootstrap support and 100% BI posterior probability. The numbers after the species name are the GenBank accession number.

Three of them cluster together as a clade, and the remaining one (*Leptodius sanguineus*) forms a sister clade with the single representative of the family Oziidae (*Epixanthus frontalis*), which calls attention to authoritative identification of these two species (*L. sanguineus* and *E. frontalis*). Of course, the increasing samples of Oziidae will also help to clarify the suspicious classi-

fication and relationships. For two Gecarcinidae species, one of them (*Gecarcoidea natalis*) forms a sister clade with Sesarmidae species, and then clusters with the remaining one (*Cardisoma carnifex*). As far as the non-monophyly of Homolidae, the single representative of Latreilliidae (*Latreillia valida*) forms a sister clade with a member of the family Homolidae (*Moloha majora*), which calls attention to authoritative identification of *L. valida*. Furthermore, it is worth noting that almost one-third of the families (11/30) include only one representative, so the non-monophyly of relevant families should be treated with caution.

Viewed from a higher taxonomic level, most superfamilies of Brachyura are found to be monophyletic, with the exception of Eriphioidea, Ocypodoidea, and Grapsoidea (Fig. 7). Although the polyphyly of the above three superfamilies is well supported in our phylogenetic tree, the interrelationships of these groups remain largely disputable. Regarding the interrelationships among Ocypodoidea and Grapsoidea, no consensus has been reached in current studies. For example, Sesarmidae (Grapsoidea) have a close relationship with Gecarcinidae (Grapsoidea), and Dotillidae (Ocypodoidea) form a sister clade with Grapsidae (Grapsoidea) in our phylogenetic tree. However, in Tan et al. (2018) , Sesarmidae (Grapsoidea) first clustered with Dotillidae (Ocypodoidea), and then formed a sister clade with Gecarcinidae (Grapsoidea). While in Wang et al. (2020) , Dotillidae (Ocypodoidea) and Xenograpsidae (Grapsoidea) formed a sister clade, and then clustered with Sesarmidae (Grapsoidea). These three families as a group then formed a clade with Gecarcinidae (Grapsoidea). Therefore, more sampling across a breadth of taxonomic groups and integration of additional molecular data need to be mined in order to substantially resolve the interrelationships of these groups.

4 Conclusions

In this study, two newly sequenced mitogenomes of Ocypodoidea, *C. dilatatum* and *Euplax* sp., were reported for the first time. TDRL model is proposed to be involved in the evolution of these two mitochondrial gene rearrangements. Comparative mitogenomic analyses of the species clustering in one branch in the tree display two types of gene arrangements. The dN/dS ratio analysis of all PCGs indicates that purifying selection plays a leading role in the evolution of mitochondrial PCGs. Phylogenetic analyses show that Camptandriidae and Macrophalmidae are the most closely related species, and the polyphyly of three superfamilies (Ocypodoidea, Eriphioidea, and Grapsoidea) is well supported. Nevertheless, large-scale taxonomic samplings are still needed to confirm the phylogenetic status of Camptandriidae and the non-monophyly of relative families due to limited representatives. Also, the authentic relationships within Brachyura will be better understood with the help of increasing samplings and data.

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Supplementary information:

Fig. S1. Potential secondary structures of 22 inferred tRNAs in *Cleistostoma dilatatum* mitogenome.

Fig. S2. Potential secondary structures of 22 inferred tRNAs in *Euplax* sp. mitogenome.

Table S1. List of 109 Brachyuran species and two outgroups used in this paper.

Table S2. Composition and skewness of *Cleistostoma dilatatum* mitogenome.

Table S3. Composition and skewness of *Euplax* sp. mitogenome.

Table S4. Relative synonymous codon usage in the mitogenomes of *Cleistostoma dilatatum* and *Euplax* sp.

The supplementary information is available online at https://doi.org/10.1007/s13131-022-2054-9 and http://www.aosocean.com/. The supplementary information is published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.