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Comparative mitochondrial genome analysis of Varunidae and its phylogenetic implications

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Abstract

Complete mitochondrial genomes (mitogenomes) can indicate phylogenetic relationships, as well as useful information for gene rearrangement mechanisms and molecular evolution. Currently, the phylogenetic location of the genus *Varuna* (Brachyura: Varunidae) has not been well resolved mainly because of limited representatives (only two extant species). Here, we determined a new mitogenome of this genus (*Varuna litterata*) and added the published mitogenomes to reconstruct the phylogeny of Varunidae. The 16 368-bp mitogenome contains the entire set of 37 genes and a putative control region. The characteristics of this newly sequenced mitogenome were described and compared with the other 15 Varunidae mitogenomes. All 16 analyzed mitogenomes have identical gene order and similar molecular features. The sliding window and genetic distance analyses demonstrate highly variable nucleotide diversity, with comparatively low variability of *COI* and *COII*, and high variability of *ND6*. The nonsynonymous/synonymous substitution rates (dN/dS ratio) analysis shows that all 13 PCGs are under purifying selection and *ATP8* gene evolves under the least selective pressure. Twelve tRNA genes, two rRNAs, one PCG, and the putative control region are found to be rearranged with respect to the pancrustacean ground pattern gene order. Tandem duplication/random loss model is adopted to explain the large-scale gene rearrangement events occurring in Varunidae mitogenomes. Phylogenetic analyses show that all Varunidae species are placed into one group, and form a sister clade with Macrophthalmidae. Nevertheless, the phylogenetic relationships within Varunidae are not completely consistent based on the two different datasets used in this study. These findings will contribute to a better understanding of gene rearrangement and molecular evolution in Varunidae mitogenomes, as well as provide insights into the phylogenetic studies of Brachyura.

Key words: varunid crab, *Varuna litterata*, mitogenome, gene rearrangement, tandem duplication/random loss, phylogeny

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1 Introduction

The typical metazoan mitochondrial genome (mitogenome) is a closed-circular molecule of 14–20 kb. It generally contains 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), two ribosomal RNA genes (*12S* and *16S*), and an AT-rich region (also called control region, CR) (Boore, 1999). The mitogenome has been widely used as an ideal tool for population genetics, comparative genomics, and phylogenetic studies. The reasons for its popularity are as follows: (1) the small genome size and simple gene structure are convenient for PCR amplification and sequencing; (2) the strict maternal inheritance can avoid the complexity of parental inheritance; (3) the relatively high evolutionary rate and low level of recombination are suitable for conducting evolutionary and phylogenetic analysis (Gyllensten et al., 1991; Sato and Sato, 2013; Ma et al., 2015; Sanchez et al., 2016; Tan et al., 2018). Besides, comparative analyses of the complete mitogenomes of closely related taxa can provide key information for gene rearrangements and phylogenetic studies (Liu and Cui, 2010; Zhuang and Cheng, 2010; Xin et al., 2017). With the advent and maturation of next-generation sequencing (NGS) technologies, comparative mitogenomics has become an important method for adaptive evolution and phylogenetic analysis (Li et al., 2020; Wang et al., 2020; Zhang et al., 2020b). For instance, Wang et al. (2020) demonstrated the common origin of Macrophthalmidae and Varunidae, and supported the previous hypotheses of paraphyly of Ocypodoidea and Grapsoidea. In Zhang et al. (2020b), comparative analysis of mitogenome of a deep-sea crab *Chaceon granulates* revealed positive selection and novel genetic features, which should be useful for studies on crab evolution and adaptive mechanisms.

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The true crabs (infraorder Brachyura), with over 7 250 known species inhabiting marine, freshwater, and terrestrial habitats, are one of the most successful aquatic invertebrate taxa worldwide (Basso et al., 2017; Chen et al., 2018; Ma et al., 2019). However, the extreme morphological and ecological diversity have made them difficult to be identified, and thus darken the real phylogenetic relationships (Tan et al., 2018; Camargo et al., 2020). The Grapsoidea is one of the largest superfamilies within Brachyura, and its classification and phylogeny have long been disputed. It is traditionally recognized as a monophyletic group based on morphological characteristics (Martin and Davis, 2001; Ng, 2008; Davie et al., 2015); however, an increasing number of molecular studies have challenged the monophyly of this taxon (Chen et al., 2018, 2019; Tan et al., 2018; Lu et al., 2020). Moreover, members of the current family Varunidae (Brachyura; Grapsoidea) were previously grouped as a subfamily (Varuninae) of Grapsoidae (Alcock, 1900). Afterward, it was elevated to family level based on larval morphology and molecular data (Schubart et al., 2000, 2002). Among them, *Varuna* is a commercially important genus that is widely distributed in the Indo-West Pacific (Jamieson et al., 1996; Ng, 2006). According to World Register of Marine Species (http://www.marinespecies.org/), there are only two extant species in this genus (*V. yui* and *V. litterata*). Currently, most studies of this genus have focused on the morphology and growth (Tu, 1992; Ng, 2006), and there have been few researches on molecular phylogeny (Lin et al., 2018). To date, the phylogenetic location of the genus *Varuna* has not been well resolved mainly because of limited representatives.

Accordingly, in the present study, we determined a new mitogenome of this genus (*V. litterata*) and added the published mitogenomes to reconstruct the phylogeny of Varunidae. A detailed comparison of 15 published Varunidae mitogenomes was also performed to reveal the gene rearrangements and genomic evolution. Additionally, the most comprehensive molecular phylogenetic analysis of 109 Brachyuran species was conducted based on the nucleotide and amino acid sequences of 13 PCGs. These results will help to better understand the rearrangement processes and possible mechanisms of Varunidae mitogenomes and lay a foundation for further evolutionary relationships within Brachyura.

2 Materials and methods

2.1 *Ethics statement*

The species was not involved in the endangered list of International Union for Conservation of Natural Resource (https://www.iucnredlist.org/). Specimen collection and maintenance were performed in strict accordance with the recommendations of Animal Care Quality Assurance in China. All experimental protocols were approved by the Institutional Ethics Committee of Zhejiang Ocean University.

2.2 *Sample collection and identification*

An individual specimen of *V. litterata* was collected from Hainan Province, China (18°20′18″N, 109°30′50″E). The specimen was immediately preserved in absolute ethanol after collection and then stored at –20°C. Identification of this specimen was performed with a stereo dissecting microscope based on the key morphological features of crabs (Aiyun and Siliang, 1991; Ng, 2006). After identification, we measured the width and length of its carapace, which were about 3.2 cm and 2.9 cm, respectively. Also, the total body weight of this specimen was about 7.2 g.

2.3 *DNA extraction, mitogenome sequencing and assembly*

The SQ Tissue DNA Kit (OMEGA) was used to extract the total genomic DNA following the manufacturer's instructions. The mitogenome was obtained using a next-generation sequencing method with Illumina Hiseq 4000 at Shanghai Origingene Biopharm Technology Co. Ltd. Raw sequence data were deposited in Short Read Archive database (https://www.ncbi.nlm.nih.gov/sra/) with the accession No. SRX7809615. Clean data without sequencing adapters were de novo assembled by the NOVOPlasty 2.7.2 software (Dierckxsens et al., 2017). To assess the single-base accuracy of the assembled genome, we compared it with three confirmed sequences by PCR and Sanger sequencing methods.

2.4 *Mitogenome annotation and sequence analyses*

The complete mitogenome was manually annotated using the software of Sequin (version 15.10, http://www.ncbi.nlm.nih.gov/Sequin/). The PCGs were determined by the Open Reading Frame following the invertebrate mtDNA translation table. The boundaries of rRNA and tRNA genes were performed using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov) and tRNAscan-SE 1.21 (Lowe and Chan, 2016), respectively, comparing with the related species. Transfer RNA genes were manually plotted, according to the secondary structure predicted by tRNAscan-SE 1.21 (Lowe and Chan, 2016) and MITOS Web Server (Bernt et al., 2013) with invertebrate mitochondrial genetic codes. The control region was determined by the locations of adjacent genes. The mitogenome map was drawn by CGView Server version 1.0 (Stothard and Wishart, 2005). The tandem repeats in the control region were detected using Tandem Repeats Finder 4.09 (Benson, 1999). The base composition was obtained using MEGA X (Kumar et al., 2018). The following formulae were used to calculate strand asymmetries: AT-skew = (A–T) / (A+T), GC-skew = (G–C) / (G+C) (Perna and Kocher, 1995).

Sixteen complete mitogenomes including the newly sequenced one were selected to better understand the mitogenomic evolution of Varunidae species. The concatenated sequences of the aligned 13 PCGs and two rRNAs from these Varunidae mitogenomes were used to perform the DNA polymorphism sliding window analysis. The nucleotide diversity (Pi) of each PCG and rRNA gene and a sliding window of 200 bp at a step size of 20 bp were conducted using DnaSP version 6.12.03 (Rozas et al., 2017). The nonsynonymous (dN) and synonymous (dS) substitution rates were deduced from the data of PCGs sequences alignment of 16 varunid species using Mega X (Kumar et al., 2018). The genetic distances of 13 PCGs were also calculated using Mega X with Kimura-2-parameter. Besides, pairwise comparisons of gene order among Decapoda, Brachyura, and Varunidae were executed by CREx program (Bernt et al., 2007). The rearrangement mechanism for Varunidae was deduced using the common intervals parameter, which considers events of transpositions, inverse transpositions, inversions, and tandem duplication random loss.

2.5 *Phylogenetic analysis*

One hundred and ten complete mitogenome sequences downloaded from Genbank database (https://www.ncbi.nlm.nih.gov/genbank), and one newly determined sequence (*V. lit-*

terata) was used to reconstruct the phylogenetic relationships among Brachyura (Table S1). The nucleotide and amino acid sequences of 13 PCGs for each species were extracted from the GenBank files using PhyloSuite (Zhang et al., 2020a). All genes were aligned in batches with MAFFT (Katoh et al., 2002) integrated into PhyloSuite, using normal-alignment mode. Gblocks (Talavera and Castresana, 2007) was used to identify and remove the ambiguously aligned sequences using default settings. The sequences were then concatenated and used to generate input files (phylip and nexus format) for phylogenetic analyses. The best-fit model was selected by ModelFinder (Kalyaanamoorthy et al., 2017) based on the Bayesian Information Criterion (BIC). GTR+F+I+G4 and mtMet+F+R6 were selected as the best-fit models for nucleotide and amino acid sequences, respectively. Both maximum likelihood (ML) and Bayesian inference (BI) were employed for phylogenetic analyses. The ML analysis was conducted using IQ-TREE (Nguyen et al., 2015), under an ML+rapid bootstrap (BS) algorithm with 1 000 replicates. The BI analysis was carried out in MrBayes 3.2.6 (Ronquist et al., 2012) with default parameters. Two independent runs of four Markov chain Monte Carlo (MCMC) chains (one cold chain and three hot chains) were simultaneously run for 3×10^6 generations, with sampling conducted every 1 000 generations. The final average standard deviation of split frequencies fell to 0.001 8, which was considered to reach convergence (<0.01). The first 25% samples were discarded as burn-in, and the remaining trees were used to calculate Bayesian posterior probabilities (BPP) in a 50% majority-rule consensus tree.

3 Results

3.1 *Sequence features of V. litterata mitogenome*

The complete mitogenome of *V. litterata* is a circular double-stranded DNA molecule of 16 368 bp in length (Fig. S1). The sequence has been deposited in GenBank under accession number MT193719. It contains 13 PCGs, 2 rRNAs, 22 tRNAs, as well as a putative CR (Fig. S1, Table 1). The majority of 37 genes are encoded by the heavy (H-) strand, except 4 PCGs and 2 rRNAs (Table 1). The overall nucleotide composition is 35.2% A, 36.2% T, 10.8% G, and 17.8% C, which reveals a strong AT bias (71.4%) (Table 2). The nucleotide skew statistics show negative AT-skew (−0.014) and negative GC-skew (−0.243) (Table 3). Overall, the genes in *V. litterata* mitogenome are closely arranged with overlapping (eight overlappings totaling 22 bp) and interval (19 intergenic spacers totaling 382 bp) phenomena (Table 1).

All the 13 PCGs are initiated by the canonical start codon ATN. Most PCGs terminate with TAA or TAG, whereas *COI* and *Cyt b* use an incomplete stop codon T (Table 1). Incomplete stop codons are common in metazoan mitogenomes and may be recovered via post-transcriptional polyadenylation (Ojala et al., 1981). Both the AT-skew and GC-skew of the 13 PCGs are negative, −0.177 and −0.011; respectively (Table 2), showing an obvious bias toward the use of Ts and a slight bias toward Cs in the entire protein-coding gene sequence. Besides, the GC-skew values of four PCGs (*ND5*, *ND4*, *ND4L,* and *ND1*) are positive, indicating they are encoded by the L-strand, whereas the remaining nine exhibit negative values, indicating they are encoded by the H-strand (Table 2).

The 22 tRNA genes are interspersed throughout the entire genome. The total length is 1 454 bp, with each tRNA gene ranging from 63 bp to 73 bp. Most tRNAs can fold into the typical cloverleaf structure except of *tRNA-Ser* (TCT) that lacking the dihydrouridine arm (Fig. S2), which seems to be a common feature in metazoan mitogenomes (Wang et al., 2015; Gong et al., 2019, 2020). Except for the Watson-Crick base pairs (A-T and G-C) and G-U matches, a total of three mismatched base pairs are found, including two U-U base pairs in *tRNA-Asn* and *tRNA-His*, and one C-A base pair in *tRNA-Met*. Such mismatches are probably corrected through posttranscriptional RNA editing (Lavrov et al., 2000; Masta and Boore, 2004). The *16S* and *12S rRNA* genes are located between *tRNA-Leu* (L_1) and *tRNA-His (H)*, with a total length of 2 262 bp (Fig. S1, Table 1). Both of them exhibit positive GC-skew (Table 2), indicating they are encoded by the L-strand.

3.2 *Comparative analysis of Varunidae mitogenomes*

The sequence features of the new mitogenome in this study (*V. litterata*) and the other 15 Varunidae mitogenomes are compared. The mitogenomes possess variations in size that range from 15 915 bp (*V. yui*) to 16 898 bp (*Pseudohelice subquadrata*). The concatenated PCGs, tRNAs, and rRNAs are quite conserved in length except the unusually larger size of rRNAs in *P. subquadrata* (Fig. S3, Table S1). The maximum length diversification is detected in the rapidly evolving CR. It ranges from 643 bp to 1 096 bp, which is the primary contributor to variations in Varunidae mitogenome sizes. Besides, the size of mitogenome is also related to the overlapping regions and intergenic spacers dispersing in the mitogenomes.

The nucleotide composition of 16 Varunidae mitogenomes shows that all of them are rich in As and Ts, with the A+T content ranging from 67.7% (*P. subquadrata*) to 74.6% (*Neoeriocheir leptognathus*) (Table 3). Skew metric shows that all mitogenomes are highly consistent with negative base skewness, except *Metaplax longipes* and *P. subquadrata* exhibit positive AT-skew values (Table 3). Further analysis reveals that GC-skew values of PCGs in the L-strand are much higher than those in the H-strand (Fig. 1a). In contrast, there is no significant difference in AT-skew values between the two strands. Most AT-skew values are negative, except that of *COII* and *ATP8* genes in *M. longipes* mitogenome (Fig. 1b).

In the ancestral crustacean mitogenomes without gene rearrangements, CR is typically located between *12S rRNA* and *tRNA-Ile* genes. However, it moves to the location between *tRNA-Val* and *tRNA-Gln* genes in all 16 Varunidae mitogenomes. Tandem repeat elements are one of the most common structures in CR (Li and Liang, 2018; Li et al., 2019). Here, each mitogenome of Varunidae has tandem repeat units except *Cyclograpsus intermedius*. The motif (consensus nucleotide) length varies from 2 bp to 113 bp and the copy number ranges from 2 to 139.5 (Fig. 2). The tandem repeat units differ in nucleotide composition, length, and copy number across these varunid species, thus leading to the length heteroplasmy of CR (Fig. 2).

Nucleotide diversity of 16 Varunidae mitogenomes is conducted using the DNA polymorphism sliding window analysis. Due to the instability of tRNA and non-coding CR, we only calculate the nucleotide diversity of the 13 PCGs and 2 rRNAs. The result indicates a highly variable nucleotide polymorphism of these genes. Among them, *ND6* gene presents the highest polymorphism (Pi=0.267), followed by *ATP8* (Pi=0.240) and *ND2* (Pi=0.237). Inversely, *COII* (Pi=0.161) and *COI* (Pi=0.163) show the lowest values, suggesting they are the conserved genes. Two rRNA genes

Table 1. Features of the mitochondrial genome of *Varuna litterata*

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	1 600 bp	66	–	–	TAA	5	H
COII	1 606 bp	2 310 bp	705	234	ATG/TAA	–	12	H
ATP8	2 323 bp	2 484 bp	162	53	ATG/TAA	–	–7	H
ATP6	2 478 bp	3 152 bp	675	224	ATT/TAA	–	–1	H
COIII	3 152 bp	3 943 bp	792	263	ATG/TAA	–	–1	H
Gly (*G*)	3 943 bp	4 007 bp	65	–	–	TCC	0	H
ND3	4 008 bp	4 358 bp	351	116	ATT/TAA	–	1	H
Ala (*A*)	4 360 bp	4 422 bp	63	–	–	TGC	2	H
Arg (*R*)	4 425 bp	4 487 bp	63	–	–	TCG	–1	H
Asn (*N*)	4 487 bp	4 551 bp	65	–	–	GTT	0	H
Ser (S_1)	4 552 bp	4 618 bp	67	–	–	TCT	22	H
Thr (*T*)	4 641 bp	4 705 bp	65	–	–	TGT	2	H
Pro (*P*)	4 708 bp	4 771 bp	64	–	–	TGG	20	L
ND1	4 792 bp	5 727 bp	936	311	ATG/TAA	–	31	L
Leu (L_1)	5 759 bp	5 825 bp	67	–	–	TAG	0	L
16S	5 826 bp	7 198 bp	1 373	–	–	–	0	L
12S	7 199 bp	8 087 bp	889	–	–	–	0	L
His (*H*)	8 088 bp	8 150 bp	63	–	–	GTG	6	L
ND5	8 157 bp	9 890 bp	1 734	577	ATA/TAA	–	126	L
Val (*V*)	10 017 bp	10 089 bp	73	–	–	TAC	0	L
CR	10 090 bp	11 185 bp	1 096	–	–	–	0	H
Gln (*Q*)	11 186 bp	11 254 bp	69	–	–	TTG	11	L
Cys (*C*)	11 266 bp	11 328 bp	63	–	–	GCA	0	L
Tyr (*Y*)	11 329 bp	11 393 bp	65	–	–	GTA	2	L
Lys (*K*)	11 396 bp	11 465 bp	70	–	–	TTT	–2	H
Asp (*D*)	11 464 bp	11 531 bp	68	–	–	GTC	6	H
Glu (*E*)	11 538 bp	11 602 bp	65	–	–	TTC	2	H
Phe (*F*)	11 605 bp	11 669 bp	65	–	–	GAA	14	L
ND4	11 684 bp	13 021 bp	1 338	445	ATG/TAG	–	–7	L
ND4L	13 015 bp	13 326 bp	312	103	ATA/TAA	–	87	L
ND6	13 414 bp	13 923 bp	510	169	ATT/TAA	–	–1	H
Cyt b	13 923 bp	15 057 bp	1 135	378	ATG/T	–	0	H
Ser (S_2)	15 058 bp	15 124 bp	67	–	–	TGA	20	H
Ile (*I*)	15 145 bp	15 209 bp	65	–	–	GAT	4	H
Met (*M*)	15 214 bp	15 281 bp	68	–	–	CAT	0	H
ND2	15 282 bp	16 292 bp	1 011	336	ATC/TAG	–	–2	H
Trp (*W*)	16 291 bp	16 358 bp	68	–	–	TCA	9	H

Note: – represents no data. CR is abbreviation of control region.

are moderately polymorphic, with nucleotide diversity values of 0.204 in *16S rRNA* and 0.178 in *12S rRNA*, respectively (Fig. 3a). Congruent results are observed based on the K2P genetic distance analysis. Both *COI* and *COII* genes possess the least genetic distance (average 0.187), and *ND6* gene captures the largest value (average 0.348), representing the most conserved and variable genes, respectively (Fig. 3b).

To investigate the selective pressure imposed on the PCGs in these varunid species, we perform dN/dS analyses for each PCG. All of the dN/dS ratios are lower than one (<1), indicating that all 13 PCGs are evolving under purifying selection. The dN/dS ratios of individual genes are different, reflecting the different levels of functional constraints (Muse, 2000). *COI* gene exhibits the strongest purifying selection with the lowest dN/dS value (0.024), whereas *ATP8* gene exhibits a highly relaxed purifying selection with the highest dN/dS value (0.469) (Fig. 3b). The dN/dS ratio analysis indicates that the evolution of Varunidae mitogenomes has been dominated by purifying selection.

3.3 *Gene rearrangement*

Compared with the gene arrangements in the mitogenomes of ancestral crustaceans (the pancrustacean ground pattern) (Boore et al., 1998), the gene order in *V. litterata* and 15 other Varunidae mitogenomes underwent a large-scale rearrangement. At least nine gene rearrangements dramatically altered the gene order, involving 12 tRNA genes (*K*, *D*, *E*, *F*, *H*, *T*, *P*, L_1, *V*, *Q*, *C*, and *Y*), 2 rRNAs (*16S* and *12S rRNA*), 1 PCG (*ND1*), and a putative CR (Fig. S4). Of these gene rearrangements, 3 tRNA gene pairs (*K-D*, *E-F*, and *C-Y*) and 2 single tRNA genes (*V* and *Q*) are moved into the *ND5* and *ND4* junction, forming an 8-tRNA cluster (*V-Q-C-Y-K-D-E-F*) if CR is not considered. The CR is shif-

Table 2. The percentage content of composition and skewness of *Varuna litterata* mitogenome

	A/%	T/%	G/%	C/%	(A+T)/%	AT-skew	GC-skew	Length/bp
Mitogenome	35.2	36.2	10.8	17.8	71.4	−0.014	−0.243	16 368
PCGs	28.2	40.3	15.6	16.0	68.4	−0.177	−0.011	11 195
COI	27.8	35.2	16.9	20.1	63.0	−0.118	−0.088	1 539
COII	32.5	34.8	12.8	20.0	67.2	−0.034	−0.221	705
ATP8	35.8	48.8	3.7	11.7	84.6	−0.153	−0.520	162
ATP6	28.9	39.0	11.7	20.4	67.9	−0.148	−0.272	675
COIII	26.8	37.0	15.2	21.1	63.8	−0.160	−0.164	792
ND3	30.8	41.6	10.3	17.4	72.4	−0.150	−0.258	351
ND1	25.4	43.5	20.7	10.4	68.9	−0.262	0.333	936
ND5	28.1	40.7	20.9	10.3	68.8	−0.184	0.342	1 734
ND4	28.1	42.6	19.7	9.6	70.7	−0.205	0.342	1 338
ND4L	27.9	47.4	18.6	6.1	75.3	−0.260	0.506	282
ND6	26.7	47.5	6.9	19.0	74.1	−0.280	−0.470	549
Cyt b	26.9	37.5	14.4	21.2	64.4	−0.166	−0.193	1 135
ND2	29.5	43.7	8.1	18.7	73.2	−0.195	−0.395	1 011
16S rRNA	40.4	39.0	13.6	7.0	79.4	0.018	0.322	1 373
12S rRNA	41.4	38.7	12.7	7.2	80.1	0.034	0.277	889
tRNAs	38.0	35.8	14.9	11.3	73.8	0.031	0.134	1 454
CR	38.0	42.2	11.1	8.7	80.2	−0.053	0.124	1 096

Table 3. The percentage content of composition and skewness of mitogenome in 16 Varunidae species

Species	A/%	T/%	G/%	C/%	(A + T)/%	AT-skew	GC-skew	Length/bp
Cyclograpsus granulosus	33.1	36.1	11.2	19.5	69.3	−0.043	−0.272	16 300
Pseudohelice subquadrata	34.2	33.5	10.5	21.7	67.7	0.010	−0.347	16 898
Helicana wuana	33.0	35.5	11.5	20.0	68.4	−0.037	−0.269	16 359
Helice latimera	34.0	35.1	11.0	19.9	69.1	−0.017	−0.290	16 246
Helice tientsinensis	33.9	35.1	11.0	19.9	69.1	−0.017	−0.289	16 212
Cyclograpsus intermedius	34.7	35.9	10.7	18.7	70.6	−0.017	−0.270	16 184
Eriocheir hepuensis	35.1	36.4	10.8	17.7	71.5	−0.018	−0.245	16 335
Eriocheir sinensis	35.3	36.4	10.7	17.7	71.6	−0.015	−0.248	16 354
Eriocheir japonica	35.2	36.5	10.7	17.7	71.6	−0.018	−0.245	16 352
Neoeriocheir leptognathus	35.6	39.0	10.1	15.3	74.6	−0.046	−0.206	16 143
Hemigrapsus penicillatus	34.1	36.4	11.4	18.1	70.5	−0.033	−0.229	16 486
Hemigrapsus sanguineus	34.3	35.5	11.2	19.1	69.8	−0.018	−0.260	16 275
Metaplax longipes	37.6	33.8	10.6	17.9	71.4	0.053	−0.257	16 305
Varuna litterata	35.2	36.2	10.8	17.8	71.4	−0.014	−0.243	16 368
Varuna yui	35.7	36.5	10.2	17.6	72.2	−0.011	−0.265	15 915
Gaetice depressus	35.4	37.6	10.5	16.5	73.0	−0.030	−0.223	16 288

ted from the typical area between *12S rRNA* and *I* to the *V* and *Q* junction. In addition, the *ND1*-L_1-*16S*-*12S* gene cluster, 1 tRNA gene pair (*T*-*P*), and a single *H* gene are moved to the position between *ND3* and *ND5*. Correspondingly, the *A*-*R*-*N*-S_1-*E*-*F* cluster order is changed into *A*-*R*-*N*-S_1-*T*-*P* order.

3.4 *Phylogenetic analysis*

To investigate the phylogenetic status of the genus *Varuna* within Varunidae and the phylogenetic relationships of Brachyura, the phylogenetic analysis of 109 Brachyuran species was conducted based on the concatenated nucleotide and amino acid sequences of 13 PCGs using ML and BI methods (Figs 4 and 5, Figs S5 and S6). All trees show that *V. litterata* is most closely related to the species of the same genus, *V. yui*. All 16 Varunidae species cluster into a clade and form a sister group with Macrophthalmidae, supporting the recent molecular researches (Wang et al., 2019, 2020; Lu et al., 2020). Nevertheless, the phylogenetic relationships within Varunidae are not completely consistent with each other and the support values are relatively low (Fig. 4). The nucleotide sequences place *Gaetice depressus* at the most basal position in both ML and BI trees, whereas it is replaced by *Metaplax longipes* and nests within the Varunidae clade in the amino acid trees. Furthermore, *Cyclograpsus granulosus* and *C. intermedius* belonging to the same genus cluster together as the closest relatives in the amino acid trees as a matter of course, however, *P. subquadrata* replaces *C. intermedius*'s position and *C. intermedius* scatters in the clade of the nucleotide trees.

In the family-level relationship within Brachyura, the phylogenetic trees (ML tree and BI tree) based on nucleotide sequences show an identical topology; thus, only one topology (BI) with both support values is displayed (Fig. 5). However, the phylogenetic trees based on amino acid sequences are not consistent, and both trees are slightly different from the nucleotide

trees (Figs S5 and S6). Of the 29 families included in this phylogeny, the monophyly of each family is well supported except Xanthidae and Homolidae (Fig. 5, Figs S5 and S6). However, it is worth noting that the monophyly of Gecarcinidae is presented in the amino acid trees, whereas it consists of two clades in the nucleotide trees, one of which forms a sister clade with Sesarmidae (Fig. 5, Figs S5 and S6). Viewed from a higher taxonomic level, most superfamilies of Brachyura are found to be monophyletic, with the exception of Eriphioidea, Ocypodoidea, and Grapsoidea. Although the polyphyly of the above three superfamilies is well supported in both the amino acid and nucleotide trees, the interrelationships of the three groups are inconsistent (Fig. 5, Figs S5 and S6). An in-depth analysis of a larger number of Brachyuran samples is required to further elucidate the phylogenetic relationships of Eriphioidea, Ocypodoidea, and Grapsoidea.

4 Discussion

4.1 *Possible gene rearrangement mechanism*

How did the mitogenome structure of Varunidae species emerge? Four dominating mechanisms have been proposed to account for mitogenomic rearrangements, including tandem duplication/random loss (TDRL) model (Moritz and Brown, 1987), tandem duplication/non-random loss (TDNL) model (Lavrov et al., 2002), recombination model (Lunt and Hyman, 1997), and tRNA mis-priming model (Cantatore et al., 1987; Jacobs et al., 1989). With the help of CREx, three TDRL events are found to have occurred in the mitogenomes of 16 varunid crabs. The hypothesized intermediate steps are as follows. Firstly, one single tRNA gene *H* underwent a transposition from the *F-ND5-H* to *H-F-ND5* order, which is a common phenomenon in the ancestral mitogenomes of Brachyura and is widely explained by the TDRL model (Xin et al., 2017; Chen et al., 2019; Wang et al., 2020). The gene block (*F-ND5-H*) tandemly duplicated and generated two sets of the same gene cluster (*F-ND5-H*)-(*F-ND5-H*). Subsequently, a novel *H-F-ND5* gene order was formed after a random loss of the duplicated genes (Fig. 6b). In the second rearrangement event, the gene block from *K* to *Y* underwent a complete copy, forming a dimeric block. 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*-, and *Y*) (Fig. 6b). 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 (Fig. 6b). Finally, the ultimate gene arrangements in Varunidae mitogenomes was generated, namely *COI*-, L_2-, *COII*-, *ATP8*-, *ATP6*-, *COIII*-, *G*-, *ND3*-, *A*-, *R*-, *N*-, S_1-, *T*-, *P*-, *ND1*-, L_1-, *16S*-, *12S*-, *H*-, *ND5*-, *V*-, *CR*-, *Q*-, *C*-, *Y*-, *K*-, *D*-, *E*-, *F*-, *ND4*-, *ND4L*-, *ND6*-, *Cyt b*-, S_2-, *I*-, *M*-, *ND2*- and *W* (Fig. 4c).

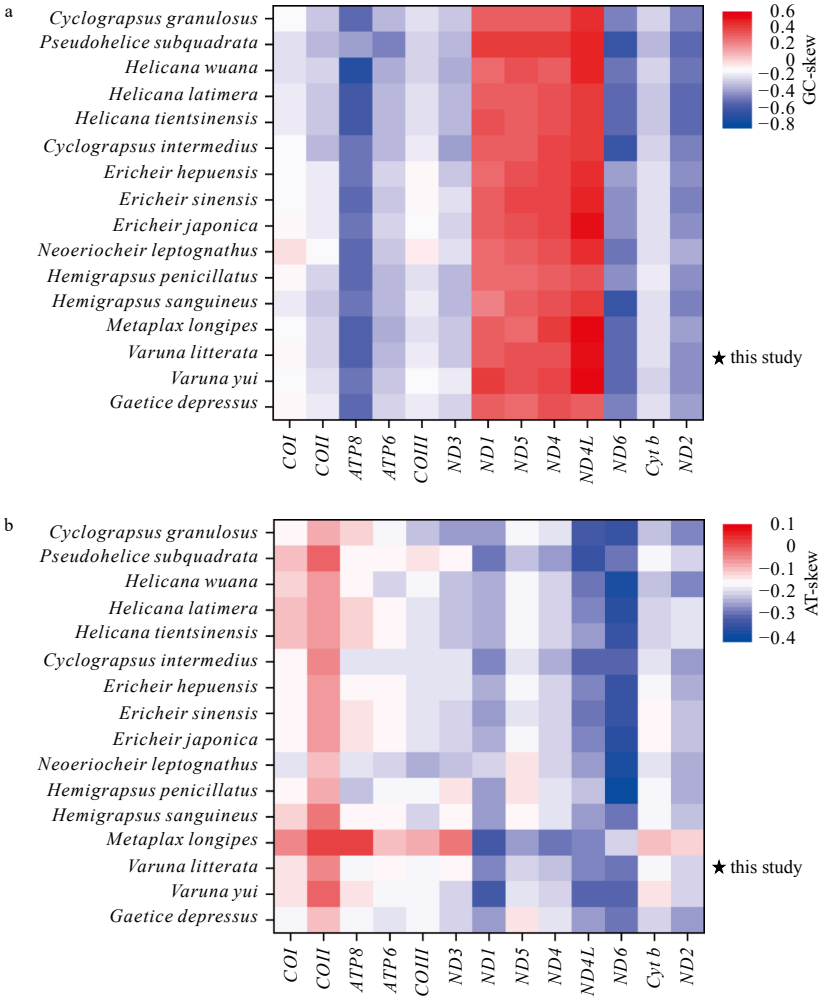

Fig. 1. The base skews of 13 PCGs of 16 Varunidae mitogenomes. a. GC-skews, b. AT-skews.

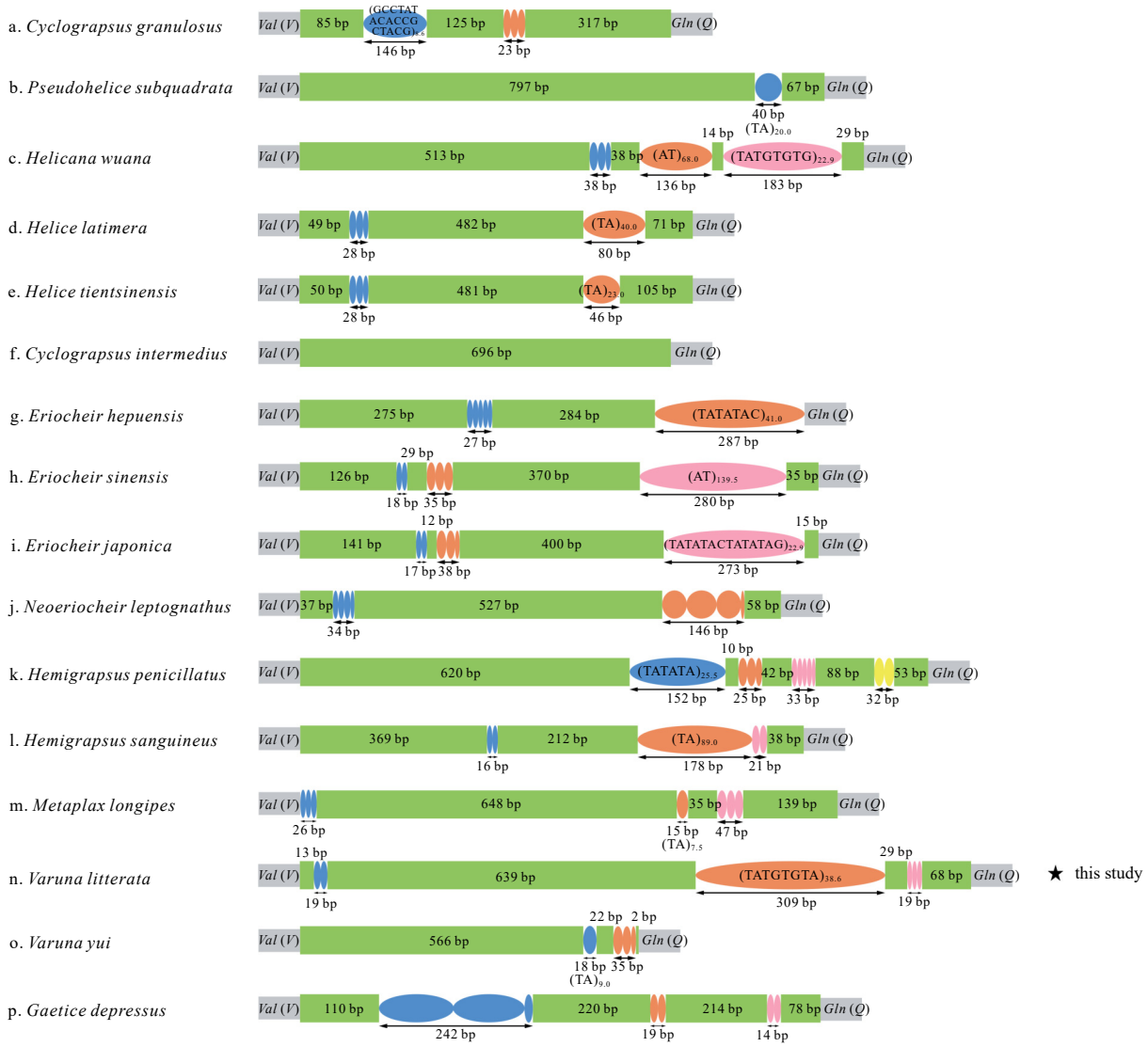

Fig. 2. Structure of control region in 16 Varunidae mitogenomes. The colored ovals indicate the tandem repeats; the remaining regions are shown with green boxes. The tandem repeat with copy number exceeding 7 is displayed in the format of $(motif)_n$.

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; Arndt and Smith, 1998). The hypothesis of TDRL model leading to rearrangements in Varunidae mitogenomes accords with Chen et al. (2018) and Wang et al. (2020).

4.2 *Phylogenetic relationship within Varunidae and Brachyura*

At present, it seems to be a common phenomenon that the intergeneric relationship within Varunidae is unstable. In Tang et al. (2018), the genus *Eriochei* form a sister clade with *Cyclograpsus*. In contrast, *Eriochei* are closely relative to *Hemigrapsus*, while *Cyclograpsus* are placed at the most basal branch in Chen et al. (2018). Besides, the genus *Varuna* have a relatively distant relationship with *Eriocheir* in our phylogenetic trees, while in Wang et al. (2020), *Varuna yui* and the genus *Eriocheir* cluster together as a sister group. Limited representative genus and scarce samples of each genus might be responsible for the inconsistent relationships of Varunidae species. Accordingly, large-scale taxonomic samplings are needed to resolve the interrelationships within Varunidae in future studies.

The main topological structures in our phylogenetic trees follow the previous results, but some differences are also observed. Here, all trees show that Varunidae and Macrophthalmidae form a sister group and are quite distantly related to Mictyridae, which is congruent with most previous studies (Chen et al., 2019; Tan et al., 2019; Wang et al., 2020). While the phylogenetic relationships of these three families were exhibited as ((Macrophthalmidae + Mictyridae) + Varunidae) in recent study (Tan et al., 2018). Furthermore, the amino acid trees support the viewpoint of Chen et al. (2019) that Sesarmidae and Gecarcinidae are more closely related, and Dotillidae is the sister clade to Grapsidae. While the sister-group relationship between Sesarmidae and Dotillidae is revealed in the nucleotide trees, supporting the findings of Basso et al. (2017) and Tan et al. (2018). The unstable topology structures in phylogenetic trees might be caused by the limited representatives of the main taxa. In future studies, extensive samples, more accurate molecular markers, and integrated molecular and

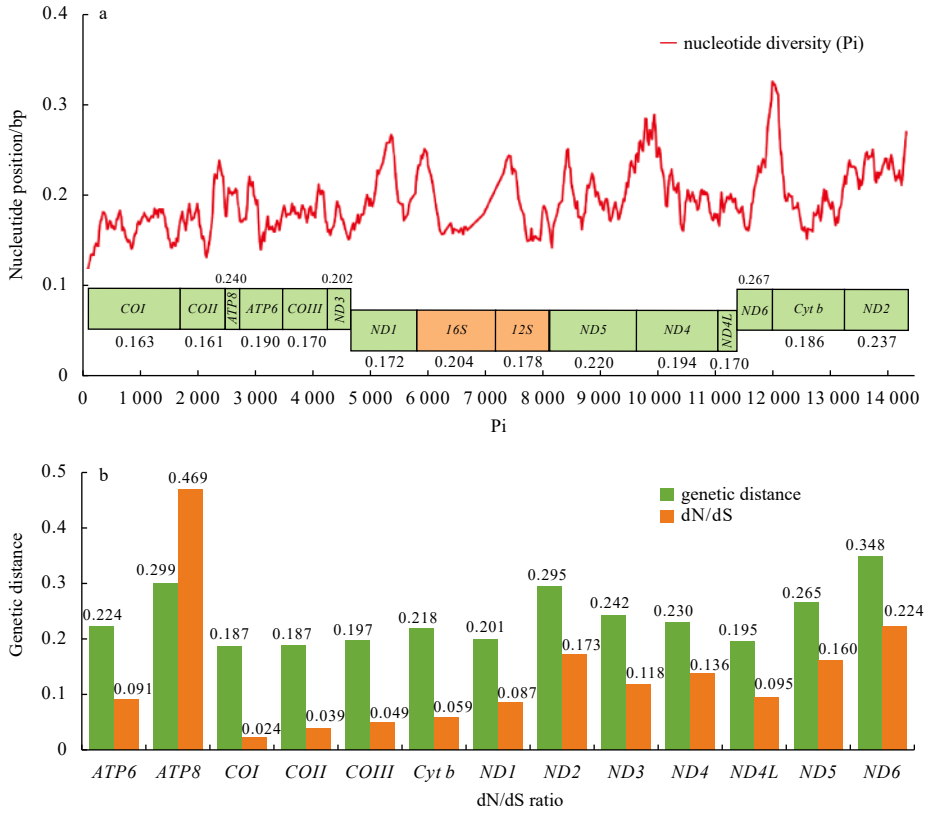

Fig. 3. Sliding window analyses of 13 PCGs (green box) and 2 rRNAs (orange box) among 16 Varunidae mitogenomes (a). The red line shows the value of nucleotide diversity (Pi) in a sliding window analysis (a sliding window of 200 bp with a step size of 20 bp). Gene names and the Pi value of each gene are indicated above or below the graph. Genetic distance (on average) and nonsynonymous/synonymous substitution rates (dN/dS) of 13 PCGs among 16 Varunidae species (b).

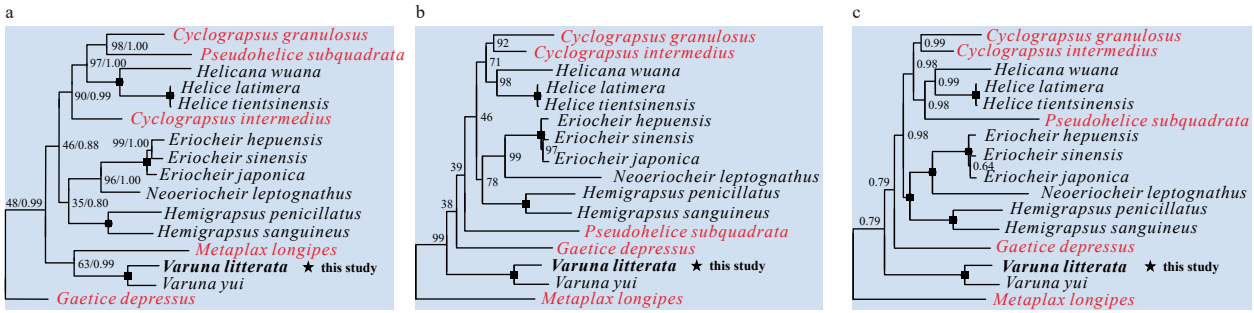

Fig. 4. Phylogenetic trees of Varunidae species inferred from 13 PCGs based on different methods. a. Nucleotide sequences based on maximum likelihood (ML) and Bayesian inference (BI) analysis; b. amino acid sequences based on ML analysis; c. amino acid sequences based on BI analysis. Node marked with a solid circle indicates 100 maximum likelihood bootstrap value and 100% supporting value.

morphological data are needed to deduce the authentic phylogenetic relationships within Brachyura.

5 Conclusions

In this article, we determined and described the complete mitogenome of *V. litterata*. The 16 368-bp mitogenome contains 37 genes and 1 AT-rich region, as is typical of metazoan mitogenomes. This newly sequenced mitogenome shares similar genomic features with 15 other previously reported Varunidae mitogenomes. Evolutionary analysis reveals that all 13 PCGs evolve under purifying selection, while the *ATP8* gene evolves under a highly relaxed selection. All these 16 Varunidae mitogenomes capture the same gene rearrangements, which can be well explained by the TDRL model. Phylogenetic analyses indicate that all Varunidae species cluster into a clade, and form a sister clade with Macrophthalmidae. The polyphyly of three superfamilies (Eriphioidea, Ocypodoidea, and Grapsoidea) is reconfirmed. In future researches, large-scale taxonomic samplings and more classification markers are still required to further investigate the mitogenomic evolution in Varunidae and to better resolve the taxonomy and phylogenetic relationships within Brachyura.

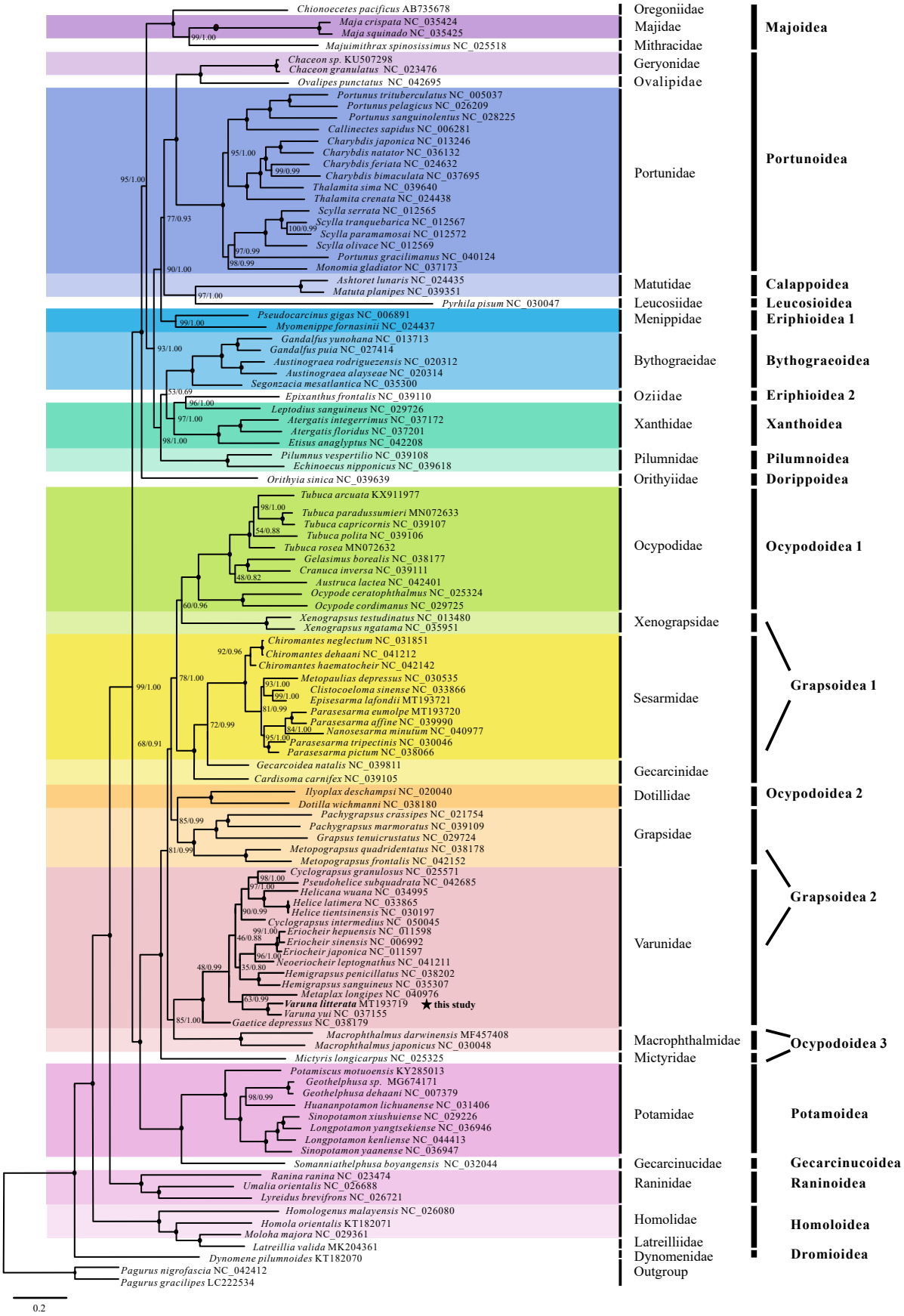

Fig. 5. Phylogenetic tree of Brachyuran species inferred from the nucleotide sequences of 13 PCGs based on maximum likelihood (ML) and Bayesian inference (BI) analysis. Node marked with a solid circle indicates 100 maximum likelihood bootstrap value and 100% supporting value.

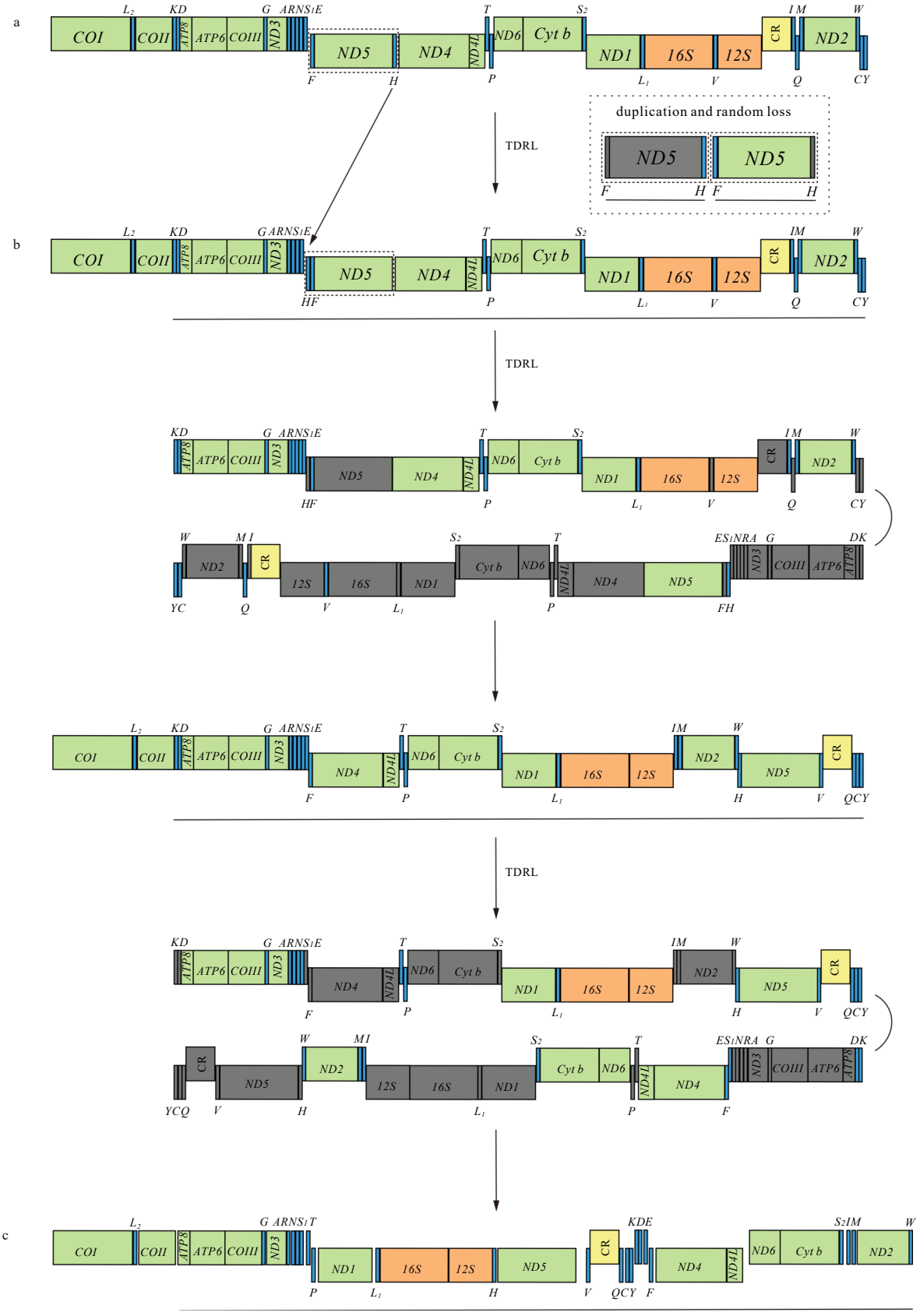

Fig. 6. Inferred intermediate steps between the ancestral gene arrangement of crustaceans and Varunidae mitogenomes. a. The ancestral gene arrangement of crustaceans; b. the results of one tandem duplication/random loss (TDRL) event and the ancestral gene arrangement of Brachyura; c. the results of two TDRL events and the final gene arrangement in *Varuna litterata* and 15 other varunid species. The duplicated gene block is underlined and the lost genes are labeled with gray.

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Supplementary information:

Fig. S1. Gene map of the *Varuna latterata* mitogenome.

Fig. S2. Potential secondary structures of 22 inferred tRNAs in *Varuna latterata* mitogenome.

Fig. S3. The lengths of protein-coding genes (PCGs), rRNAs, tRNAs, and control regions among 16 Varunidae mitogenomes. The number marked with an asterisk indicates the total length of the mitogenome, including the intergenic spaces.

Fig. S4. Gene rearrangements in *Varuna latterata* mitogenome.

Fig. S5. Phylogenetic tree of Brachyuran species inferred from the amino acid sequences of 13 PCGs based on maximum

likelihood (ML) analysis.

Fig. S6. Phylogenetic tree of Brachyuran species inferred from the amino acid sequences of 13 PCGs based on Bayesian inference (BI) analysis.

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

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