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The complete mitochondrial genome of *Parafronurus youi* (Insecta: Ephemeroptera) and phylogenetic position of the Ephemeroptera

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ABSTRACT

The first complete mitochondrial genome of a mayfly, *Parafronurus youi* (Arthropoda: Insecta: Pterygota: Ephemeroptera: Heptageniidae), was sequenced using a long PCR-based approach. The genome is a circular molecule of 15,481 bp in length, and encodes the set of 38 genes. Among them, 37 genes are found in other conservative insect mitochondrial genomes, and the 38th unique gene is *trnM*-like (*trnM2*). The duplication–random loss model can be used to explain one of the translocations at least. The A+T content of the control region is 57%, the lowest proportion detected so far in Hexapoda. Based on the nucleotide dataset and the corresponding amino acid dataset of 12 protein-coding genes, Bayesian inference and maximum likelihood analyses yielded stable support for the relationship of the three basal clades of winged insects as Ephemeroptera+(Odonata+Neoptera).

1. Introduction

The typical arthropod mitochondrial genome is a single circle, ranging in size from approximately 15 to 19 kb and containing 37 genes (13 protein-coding genes, 2 rRNA genes, 22 tRNA genes). Additionally, a control region in insect mtDNA is known as the A+T rich region (Boore, 1999; Wolstenholme, 1992). Although gene content of the insect mitochondrial genome is highly conserved, some exceptions have also been reported. For some species, extra tRNAs present (Cha et al., 2007; Junqueiraa et al., 2004). Due to the nearly constant gene content, maternal inheritance, and lack of recombination, insect mtDNA seemed to be perfectly suited for molecular phylogenetic analyses (Avise, 2000; Carapelli et al., 2007; Podsiadlowski et al., 2007).

Mitochondrial gene order is often used in phylogenetic analysis (Boore et al., 1995; Boore et al., 1998). The most prominent example is the gene translocation of *trnL* to a position between *cox1* and *cox2*, which proved to be a shared derived feature uniting Hexapoda and Crustacea (Boore et al., 1995; Boore et al., 1998). Although the mechanisms underlying gene translocation are not clear, two hypotheses have been suggested. One is the duplication–random loss model (Moritz et al., 1987); the other is the duplication–non-random loss model (Lavrov et al., 2002).

The phylogenetic position of Ephemeroptera within the winged insects (Pterygota) is hotly debated by systematists, and significant disagreement existed in morphological studies. There are three main hypotheses. The first, termed the Paleoptera hypothesis, suggests that Ephemeroptera is sister group to Odonata (dragonflies), forming the group Paleoptera ((Ephemeroptera+Odonata)+Neoptera) (Hennig, 1981). The second hypothesis will be called the basal Ephemeroptera hypothesis and suggests that Ephemeroptera is sister to Odonata+Neoptera (Ephemeroptera+(Odonata+Neoptera)) (Kristensen, 1991; Kukalová-Peck, 1991). The third hypothesis places Odonata as sister to Ephemeroptera+Neoptera and will be referred to as the basal Odonata hypothesis (Odonata+(Ephemeroptera+Neoptera)) (Boudreaux, 1979). The results from different molecular studies (using sequences of H3, 18S rRNA and 28S rRNA genes) were not in consensus either (Giribet and Ribera, 2000; Hovmöller et al., 2002; Kjer, 2004; Misof et al., 2007; Ogden and Whiting, 2003; Ogden et al., 2005; Wheeler et al., 2001).

In this study, we sequenced the complete mitochondrial genome of *Parafronurus youi* Zhou and Braasch, 2003, as a representative of the Ephemeroptera. With the aim of resolving phylogenetic position of the Ephemeroptera among Insecta, we reconstructed phylogenetic

Abbreviations: atp6, ATPase subunit 6; *atp8*, ATPase subunit 8; BI, Bayesian inference analyses; bp, base pairs; *cox1-3*, cytochrome c oxidase subunit I–III; CR, control region; *cob*, cytochrome *b*; *rrnL* and *rrnS*, large and small subunit of ribosomal RMNA genes; ML, maximum likelihood analyses; MP, maximum parsimony analyses; mtDNA, mitochondrial DNA; *nad1-6, 4L*, NADH dehydrogenase subunit 1–6, 4L; PCGs, protein-coding genes; PCR, polymerase chain reaction; *trnX*, genes encoding for transfer RNA molecules with corresponding amino acids *X*.

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Table 1
The primers used in this study

Primer name	Nucleotide sequence (5′–3′)	Annealing temperature (°C)	No. of fragment[a]
CO1F	ACAAATCATAAGGATATTGG	48	1
N-2329	ACTGTAAATATATGATGTGCTCA	48	1
J-3138	AGCGCATCTCCTCTTATAGAAC	55	2
N-3661	CCACAGATTTCTGAACACTGTCCA	55	2
N5-J	AAATCCTTAGAATAAAATCC	46	3
N5-N	ACTCTTGTTACTGCTGG	46	3
J-11338	CATATTCAGCCAGAATGATACTT	50	4
N-11845	ACCACGATATCTTATGATA	50	4
J-12887	CGGTTTGAACTCAGATCATGTAA	52	5
N-13398	CGCCTGTTTATCAAAAACATGTC	52	5
J-2183	CAACATTTATTTTGATTTTTTGG	50	6
N-3014	TCTGATGCACTTTTCTGCCA	50	6
16SC	GCTACCTTTGCACGGTCAAAATACCGCGGCC	60	7
N-2195	ACTTCAGGATGTCCAAAAAATCAA	60	7
CO2	GGGCACCAATGGTATTGAAGTTATGAATACTC	58	8
16SCR	GAACTCTCAAAAAAGATTACGCTGTTATCCCT	58	8
J-760	TTATCTTTAGGCGGATTACCTC	52	9
N-1560	GGTTCCTACTATTCCAGATCT	52	9
J-3571	CGATGTTTTACACTCATGG	48	10
N-5460	TCTACAAAATGTCAATATCA	48	10
J-7260	ACCTGCTACTAACAACAAAAAA	50	11
N-11560	AGAGAAAGTAAACTACGGTCAA	50	11
J-11545	ACATGAATCGGAGCTCGCCCAGT	56	12
N-12945	GCGACCTCGATGTTGGACTAA	56	12
J-13331	TGATTATGCTACCTTTGCACGGT	50	13
N-14588	AAACTAGGATTAGATACCCTATTAT	50	13
J-4500	CCTATGTAGTAACCTTGGGCTT	52	14
N-7030	AAGGATTCACAAGATGTTCGTA	52	14
J-14508	TACGGGACAGGTTCCTCTG	55	15
N-950	TCTGACAGATAGAGTAGGGG	55	15
J-2797	CCTCGACGTTACTCAGATTATC	50	16
N-3785	GGTTTAAGAGACCACTACT	50	16

[a] The orientation is as shown in Fig. 1.

trees based on selected complete mitochondrial genomes from Ephemeroptera, Odonata and seven Neoptera orders, with a silverfish and a bristletail used as outgroup taxa.

2. Materials and methods

2.1. DNA extraction, PCR and sequencing

Specimens of *P. youi* were collected from the type locality, Zijin Mountain, Nanjing, Jiangsu Province, China. DNA extraction from one individual was performed using DNeasy Tissue Kit (Qiagen). All PCRs were performed using a PTC-200 thermal cycler. Initially, five fragments were amplified using the universal primers, CO1F/N-2329, J-3118/N-3661, J-11338/N-11845, J-12887/N-13398 (Simon et al., 1994), as well as N5-J/N5-N (designed for this study) (Table 1 and Fig. 1). Four perfectly matching primers, namely 16SC, N-2195, CO2 and 16SCR (Table 1), were designed on the basis of the sequence information from *cox1*, *cox2* and *rrnS* for two long segments of about 4 kb (16SC/N-2195) and about 12 kb (CO2/16SCR) (Fig. 1). PCR products were gel-purified and used as template for the following PCR reactions. Using universal primers (J-2183/N-3014, J-760/N-1560, J-3571/N-5460, J-13331/N-14588) and those specifically designed primers (J-4500/N-7030, J-2797/N-3785, J-7260/N-11560, J-11545/N-12945, J-14508/N-950) for stretching sequences, nine partially overlapping pieces (No. of fragments 6, 9–16 in Fig. 1 and Table 1) ranging in size from 0.8 to 4 kb were obtained. Normal PCR (or Long PCR) was performed in a 50 μL reaction mixture consisting of 32.5 μl (or 26.5 μl) of sterilized distilled water; 5 μl $MgCl_2$, 25 mM; 5 μl 10 × PCR Buffer (or 5 μl 10 × LA PCR Buffer); 4 μl (or 8 μl) dNTP, 2.5 mM; 1 μl (or 2 μL) of each primer, 5 μM; 1 μl DNA template; 0.5 μl Takara *Taq* (or Takara LA *Taq*) DNA polymerase (Takara Biomedical, Japan). Fragments larger than 1 kb were amplified using Takara LA *Taq* via Long PCR: initial denaturation for 1 min at 94 °C, followed by 35 cycles of 10 s at 98 °C and 10–15 min at 68 °C, and a subsequent 10 min final extension step at 72 °C. Remaining fragments smaller than 1 kb were amplified using Takara *Taq* via normal PCR: initial denaturation for 2 min at 94 °C, followed by 30 cycles of 40 s at 94 °C, 50 s at 46–58 °C, and 50–90 s at 68 °C, and a subsequent 10 min final extension step at 72 °C. PCR products were purified using the Axygen agarose-out kit and sequenced using ABI 3730 system by primer walking.

Sequences were primarily edited and the complete genome sequence was assembled by using Lasergene version 5.0. The locations of 13 protein-coding genes (PCGs) and 2 rRNA (*rrnL* and *rrnS*) genes were determined by comparison with homologous sequences of other insect mtDNA. The tRNA genes were identified by their cloverleaf secondary structure using tRNA-scan SE 1.21 (Lowe and Eddy, 1997). The mitochondrial genome sequence of *P. youi* has been deposited in the GenBank database under the accession no. EU349015.

2.2. Taxa, alignment, and phylogenetic analyses

To elucidate the phylogenetic position of Ephemeroptera within pterygote insects, sequences of the complete mitochondrial genome were obtained from GenBank for 10 species of insects in addition to *P. youi*. The data represent information from 11 orders of Insecta (Table 2). Two nonpterygotes, a silverfish (*Nesomachilis australica*) and a bristletail (*Tricholepidion gertischi*), were used as outgroup taxa.

The nucleotide and putative amino acid regions for each of the 13 mitochondrial protein-coding genes were aligned using Clustal W in Mega 3.0 (Kumar et al., 2004). Each alignment was analyzed with the program Gblocks 0.91b (Castresana, 2000) using default settings, to select conserved regions of the putative amino acids. *Atp8* was not used in the subsequent analyses due to its shortness and strong heterogeneity. We concatenated the alignments of all other 12 mitochondrial protein-coding genes and recovered an alignment consisting of 2943 amino acids residues. An alignment of 8829 nucleotides was obtained using the amino acid alignment as the backbone. A saturation analysis (Xia et al., 2003) was performed for subsets with the first, second and third codon positions using DAMBE

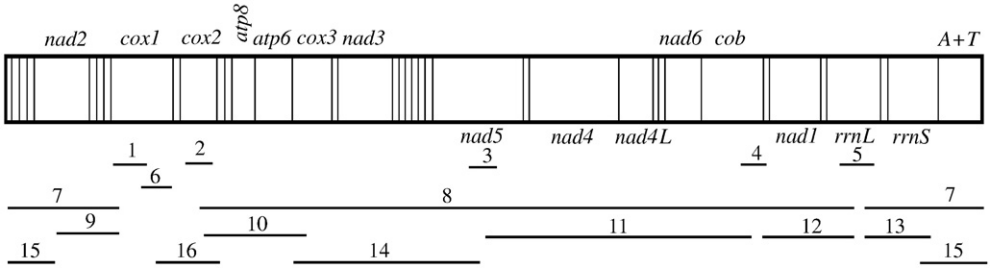

Fig. 1. Schematic representation of amplification strategy employed for the mitochondrial genome of *P. youi*. Lines below the genome map represent the amplification products. Numbers identify the primer pair (listed in Table 1).

Table 2
GenBank accession numbers for taxa used in this study

Order	Species	GenBank accession no.	Reference
Archaeognatha	*Nesomachilis australica*	AY 793551	Cameron et al., 2004
Thysanura	*Tricholepidion gertischi*	NC_005437	Nardi et al., 2003
Ephemeroptera	*Parafronurus youi*	EU 349015	This study
Odonata	*Orthetrum triangulare*	AB 126005	Yamauchi et al., 2004
Blattaria	*Periplaneta fuliginosa*	NC_006076	Yamauchi et al., 2004
Mantophasmatodea	*Sclerophasma paresisensis*	NC_007701	Cameron et al., 2006
Mantodea	*Tamolanica tamolana*	NC_007702	Cameron et al., 2006
Orthoptera	*Locusta migratoria*	NC_001712	Flook et al., 1995
Coleoptera	*Crioceris duodecimpunctata*	NC_003372	Stewart and Beckenbach, 2003
Lepidoptera	*Coreana raphaelis*	NC_007976	Kim et al., 2006
Isoptera	*Reticulitermes hageni*	NC_009501	Cameron and Whiting, 2007

4.2.13 (Xia and Xie, 2001). According to the results, third codon positions were saturated so they were excluded from the final nucleotide alignment and an alignment of 5886 nucleotides was obtained. Model selection for the nucleotide dataset was performed with Modeltest version 3.7 (Posada and Crandall, 1998). The model of GTR+I+G was chosen for the likelihood and Bayesian analyses. Maximum likelihood analyses (ML) of the nucleotide alignment was performed using PAUP* 4.0b10 (Swofford, 2002) with 1000 bootstrap replicates. Model selection for the amino acid dataset was performed with ProtTest ver. 1.4 (Abascal et al., 2005; Carapelli et al., 2007), and under the Akaike information criterion, model MtArt+I+G fit best. For a likelihood analysis, we implemented the MtArt matrix in PHYML (Abascal et al., 2005; Guindon and Gascuel, 2003) and performed an analysis with 100 bootstrap replicates.

Maximum parsimony analyses (MP) with both datasets (nucleotides and amino acids) were performed using PAUP* 4.0b10 (Swofford, 2002). One thousand bootstrap replicates were generated, each with 10 replicates with random taxon order.

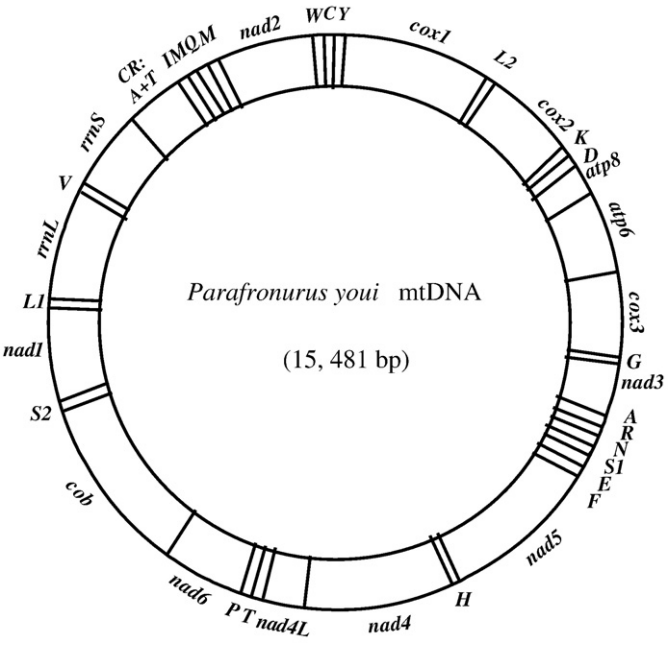

Fig. 2. The complete mitochondrial genome of *P. youi*. Positions and orientations of genes are shown.

Bayesian inference (BI) of nucleotide and amino acid datasets were performed with MrBayes 3.0B4 (Huelsenbeck and Ronquist, 2001), using the GTR+I+G and MtRev+I+G model, respectively. Although the MtArt model (Abascal et al., 2007) and the MtPan model (Carapelli et al., 2007) were reported recently addition to the model selection, but they are not yet included in the MrBayes program. Eight chains ran in parallel for 1,000,000 generations, sampling trees every 1000 generations. Bayesian analyses were repeated two times, always retrieving the same topology. According to the likelihood plots, lnl values stabilized with 20,000 generations so first 20,000 generations were discarded as burn-in (both datasets), while Bayesian posterior probabilities were calculated according to the remaining set of trees.

3. Results

3.1. Genome organization, gene order and non-coding parts

The complete mitochondrial genome of *P. youi* is a circular double helix, 15,481 bp long, containing all 37 genes typically present in Arthropoda (Fig. 2 and Table 3). However, it contains one extra *trnM* (*trnM2*) gene. As in typical arthropod mtDNA, there are only small non-coding regions between genes. In some cases genes overlap, predominantly when the two genes are encoded on different strands (Fig. 2).

The overall AT-content of *P. youi* is 66.4%, which is lower than the values of *Petrobius brevistylis* (67.9%), *Nesomachilis australica* (68.8%), *Thermobia domestica* (68.6%) and *Tricholepidion gertischi* (68.6%). This is the lowest AT-content reported from hexapod mitochondrial genomes. In most animal mitochondrial genomes there is one large

Table 3
Organization of *P. youi* mitochondrial genome

Gene	Strand	Position	Length (nuc.)	Start codon	Stop codon	Intergenic nucleotides[a]
trnI	+	1–67	67			−1
trnM1	+	67–131	65			0
trnQ	−	132–200	69			−1
trnM2	+	200–264	65			−3
nad2	+	262–1296	1035	ATC	TAA	−2
trnW	+	1295–1362	68			−7
trnC	−	1346–1416	71			0
trnY	−	1417–1481	65			−35
cox1	+	1447–3018	1572	ATT	TAA	−7
trnL2	+	3014–3079	66			4
cox2	+	3084–3771	688	ATG	T−	0
trnK	+	3772–3840	69			−1
trnD	+	3840–3905	66			0
atp8	+	3906–4064	159	ATG	TAA	−4
atp6	+	4061–4735	675	ATA	TAA	−1
cox3	+	4735–5523	789	ATG	TAA	−1
trnG	+	5523–5586	64			−3
nad3	+	5584–5940	357	ATA	TAG	−2
trnA	+	5939–6002	64			42
trnR	+	6045–6107	63			0
trnN	+	6108–6172	65			−3
trnS1	+	6170–6235	66			2
trnE	+	6238–6301	64			−2
trnF	−	6300–6363	64			0
nad5	−	6364–8098	1735	ATG	T−	0
trnH	−	8099–8162	64			−1
nad4	−	8162–9508	1347	ATG	TAG	−7
nad4L	−	9502–9798	297	ATG	TAA	2
trnT	+	9801–9864	65			0
trnP	−	9866–9930	65			11
nad6	+	9942–10451	510	ATC	TAA	−1
cob	+	10451–11587	1137	ATG	TAG	−2
trnS2	+	11586–11655	70			16
nad1		11672–12622	951	ATG	TAA	1
trnL1	−	12624–12689	66			0
rrnL	−	12690–13972	1283			0
trnV	−	13973–14043	71			0
rrnS	−	14044–14848	805			0
CR		14849–15481	633			0

[a] Negative numbers indicate that adjacent genes overlap.

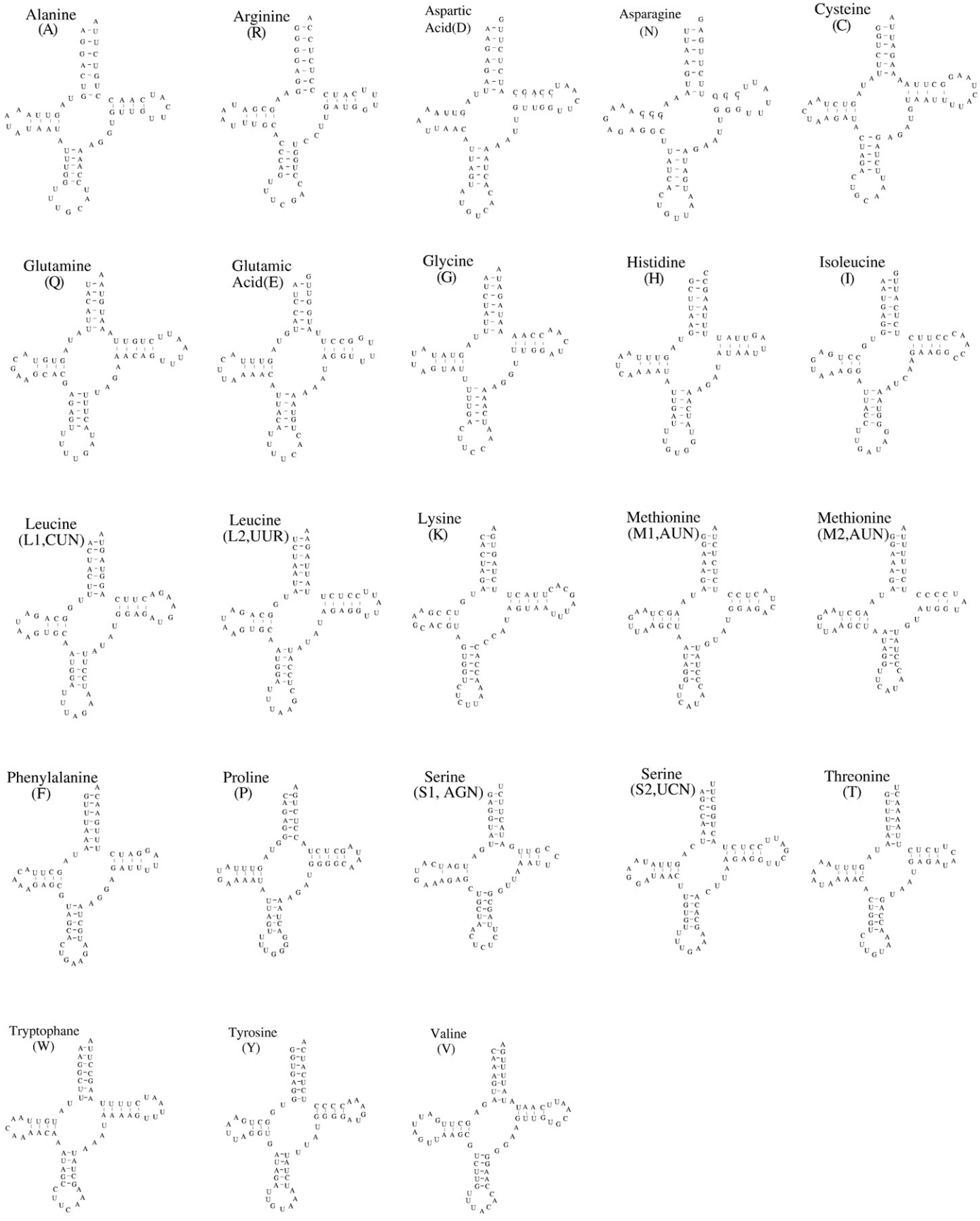

Fig. 3. Putative secondary structures for the tRNA genes of the *P. youi* mtDNA.

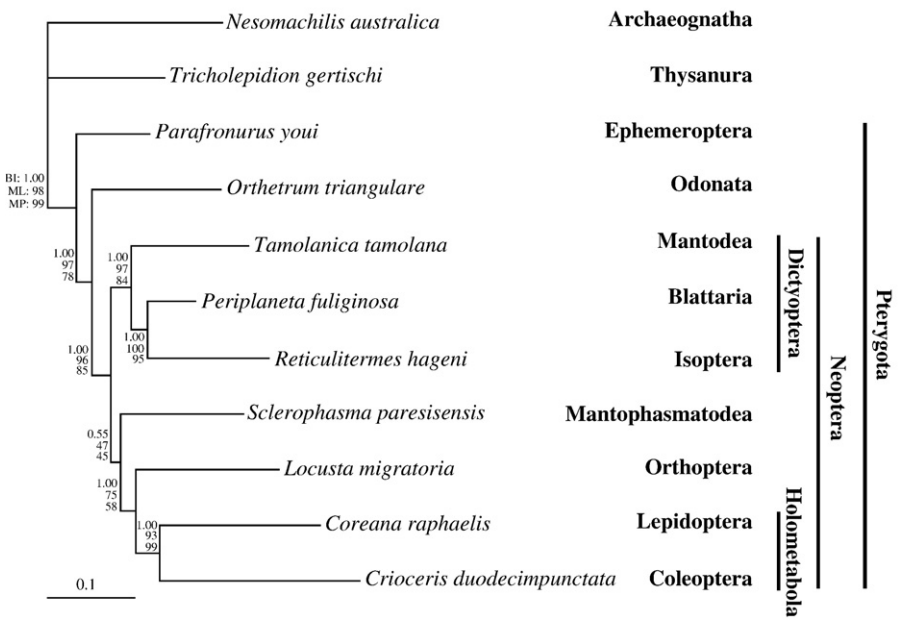

Fig. 4. Phylogenetic tree of the relationships among *P. youi* and 10 other taxa based on the nucleotide dataset of the 12 mitochondrial protein-coding genes. Branch lengths and topology are from the Bayesian analysis. Numbers above branches specify posterior probabilities from Bayesian inference (BI), bootstrap percentages from maximum likelihood (ML, 1000 replicates) and maximum parsimony (MP, 1000 replicates) analyses.

AT-rich non-coding region, referred to as the mitochondrial control region (CR). As in most other arthropods, the mitochondrial control region of *P. youi* is located between *rrnS* and *trnI* (Fig. 2). It consists of 633 bp and has an AT-content of 57%, which is the lowest for Hexapoda to date. In the CR, the repeat region includes 3 tandem repeats of a 94 bp sequence, which has a lower AT-content of 43.6%. The CR has an AT-content of 67% if we exclude these three repeat sequences. Smaller non-coding regions are present between *trnA* and *trnR* (42 bp), between *trnS2* and *nad1* (16 bp) and between *trnP* and *nad6* (11 bp) (Table 3).

The mitochondrial genome of *P. youi* shows no difference in gene order compared to the typical insect pattern. But two copies of the *trnM* gene (with similarity of 72.3%) are located between *trnI* and *trnQ* (*trnM2*), between *trnQ* and *nad2* (*trnM1*) genes, respectively. The *trnM1* is in the typical location of the gene in Arthropoda. The secondary structure predictions of *trnM1* and *trnM2* from tRNA-scan SE 1.21 (Lowe and Eddy, 1997), indicated that these two genes have the same anticodon (AUN, for methionine) and have a similar, cloverleaf-shaped conformation (Fig. 3). Therefore, it is concluded that there is no significant difference between the functionality of *trnM1* and *trnM2*.

3.2. Protein-coding genes, ribosomal RNAs and secondary structure of transfer RNAs

As shown in Table 3, the protein-coding genes show four different start codons, ATG (8 times), ATA (2 times), ATC (2 times) and ATT

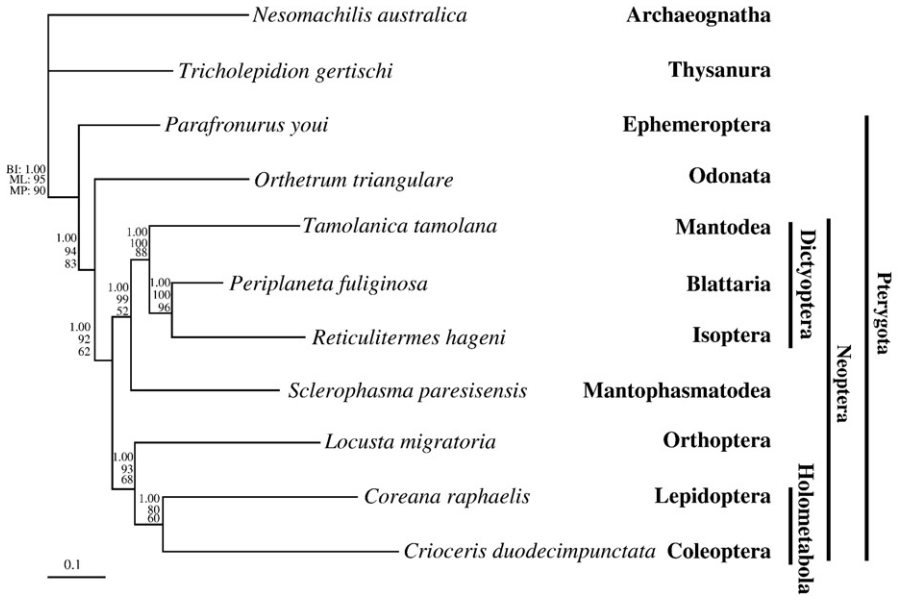

Fig. 5. Phylogenetic tree of the relationships among *P. youi* and other 10 taxa based on the amino acid dataset of 12 mitochondrial protein-coding genes. Branch lengths and topologies from Bayesian analyses. Numbers above branches specify posterior probabilities from Bayesian inference (BI), bootstrap percentages from maximum likelihood (ML, 100 replicates) and maximum parsimony (MP, 1000 replicates) analyses.

(1 time). In the *cox2* and *nad5* genes, the stop codon is truncated (T), whereas the others are TAA (8 times) and TAG (3 times). As in all other mitochondrial genomes sequenced so far, two genes for ribosomal RNAs were present, *rrnL* and *rrnS*, one for the large and one for the small ribosomal subunit, located between *trnV* and *trnL* and between *trnQ* and *trnV*, respectively (Table 2, Fig. 3). All 23 tRNAs can be folded into typical cloverleaf secondary structures (Fig. 3).

3.3. Phylogenetic analyses

Phylogenetic analyses of the 5886 aligned nucleotide positions (1st and 2nd codon positions only) produced identical tree topologies for maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) analyses (Fig. 4). The topology of the tree from 2943 amino acid dataset by BI analysis was similar to that of the nucleotide dataset (Fig. 5), as were those of the ML tree and MP tree. Both datasets yielded a topology of Ephemeroptera+(Odonata+Neoptera). Ephemeroptera as the sister clade of all other Pterygota is well supported by BI analysis with both datasets (nucleotide and amino acid: posterior probabilities 1.0), and by ML analysis with both datasets (bootstrap value 98% in the nucleotide dataset and 95% in the amino acid dataset).

All analyses indicated the Pterygota and Neoptera to be monophyletic. A basal split distinguishes two major clusters of Neoptera: Orthoptera is clustered with (Lepidoptera+Coleoptera), and Mantodea is clustered with (Blattaria+Isoptera), but the position of Mantophasmatodea is not consistently resolved (compare Figs. 4 and 5).

4. Discussion

4.1. Possible explanations for the extra trnM gene

The extra *trnM* gene in *P. youi* is unknown in any other hexapod and may be an autapomorphy of Ephemeroptera. Yet, not much is known about the mechanisms of mitochondrial gene rearrangements. The duplication-random loss model (Moritz et al., 1987) and the duplication-non-random loss model (Lavrov et al., 2002) may account for larger rearrangements. According to the duplication-random loss model, partial duplication of mtDNA caused by errors in replication, such as erroneous initiation or termination (Macey et al., 1997), or strand slippage and mispairing (Madsen et al., 1993), followed by the loss of one copy of each duplicated gene (Moritz et al., 1987; Boore, 2000). It is commonly assumed that the loss of one of the two copies of each duplicated gene happens at random. According to the duplication-non-random loss model, the destiny of each gene copy in the duplicated region is predetermined by its transcriptional polarity and location in the genome (Lavrov et al., 2002). All genes having one polarity would be lost from one genome copy, and all genes having the opposite polarity would be lost from the other. The extra *trnM* gene in *P. youi* can be explained by the duplication-random loss model. The tandem duplication occurred in the region of *trnI–trnQ–trnM1*, followed by deletions of two of the redundant genes (*trnQ* and *trnI*) (Fig. 6).

4.2. Phylogenetic position of Ephemeroptera

Our findings may help resolve the relationships among Ephemeroptera, Odonata and Neoptera. Morphological characters that support the Palaeoptera hypothesis ((Ephemeroptera+Odonata) versus Neoptera) are the inability to fold the wings over the abdomen and the similar wing-base sclerites in Odonata and Ephemeroptera (Hennig, 1981; Kukalová-Peck, 1991). Furthermore, the paired penes, the anal brace, the veinal braces in the wing and aquatic larvae have been called plesiomorphic characters of Ephemeroptera and Odonata (Bechly et al., 2001; Kukalová-Peck, 1991; Kukalová-Peck, 1997; Wootton and Kukalová-Peck, 2000). A combined analysis of nine genes and 170 morphological characters supported the Palaeoptera hypothesis (Kjer et al., 2006). The basal Ephemeroptera hypothesis as (Ephemeroptera versus (Odonata+Neoptera)) is supported by several apomorphic characters shared by dragonflies and neopterans, e.g., the number and position of the articulations of the mandibles, subimago stage, tracheation, annulated caudal filament, paired female genital openings and the loss of several mandibular muscles (Kristensen, 1981; Kristensen, 1991; Staniczek, 2000). The basal Odonata hypothesis (Odonata versus (Ephemeroptera+Neoptera)) is based primarily on the character of sperm transfer mechanisms (Boudreaux, 1979), flight muscles and longitudinal veins in mayflies and neopterans (Willkommen and Hörnschemeyer, 2007; Yoshizawa and Ninomiya, 2007; Zhou, 2007).

The relationships among Ephemeroptera, Odonata and Neoptera have not yet been determined unequivocally by molecular data (Wheeler et al., 2001; Ogden and Whiting, 2003; Kjer et al., 2006). While analyses of the combined 28S rRNA and morphological data supported the basal Ephemeroptera hypothesis, this was not recovered with the 18S rRNA or combined 18S+28S rRNA data (Wheeler et al., 2001). Ogden and Whiting (2003), using 18S rDNA, 28S rDNA and the Histone 3 protein-coding gene (H3), supported the basal Ephemeroptera hypothesis. However, other 18S/28S analysis either supported the basal Ephemeroptera hypothesis (Ogden and Whiting, 2003) or the Palaeoptera hypothesis (Hovmöller et al., 2002). While Bayesian analysis with combined data supported the Palaeoptera hypothesis, nuclear rDNA gene dataset alone supported the basal Odonata hypothesis (Kjer et al., 2006). The basal Odonata hypothesis has been supported by different molecular markers: 18S rRNA (Kjer, 2004; Misof et al., 2007; Yoshizawa and Johnson, 2005) and combined complete 18S rRNA and 28S rRNA (Mallatt and Giribet, 2006). Clearly, nuclear rRNA has difficulty answering this question. Whitfield and Kjer (2008) suggested that the Odonata, Ephemeroptera, and Neoptera present a challenging phylogenetic tree shape, regardless of their true relationships, for the first pterygotes may have emerged up to 400 mya, but the earliest representatives of their extant descendents are Mesozoic. In our analysis, using the complete sequence of mitochondrial genomes including Ephemeroptera, the basal Ephemeroptera hypothesis as (Ephemeroptera versus (Odonata+Neoptera)) is supported. This result received strong support by the nucleotide and amino acid datasets from mitochondrial protein-coding genes with BI and ML analyses. Our results agree with Carapellis et al., (2007) that Pterygota, Neoptera,

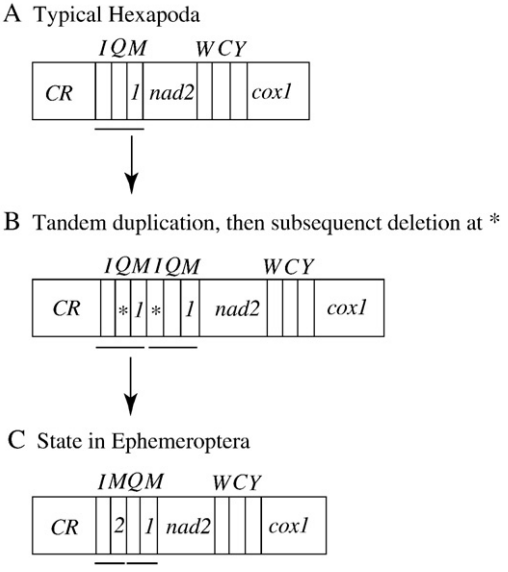

Fig. 6. Proposed mechanism of gene rearrangements in *P. youi* under a model of tandem duplication of gene regions and subsequent gene deletions. (A) Typical Hexapoda gene order in a region of A+T-*cox1*. (B) Tandem duplication in the *trnI-trnQ-trnM* region and subsequent deletions of redundant genes resulting in the derived gene order. (C) State in Ephemeroptera.

Dictyoptera, and Holometabola were recovered as monophyletic (Figs. 4 and 5). Although more genetic data from more taxa of insects are needed, we tentatively conclude that mitochondrial genomes can answer the difficult question of the basic relationships among the winged insects.

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