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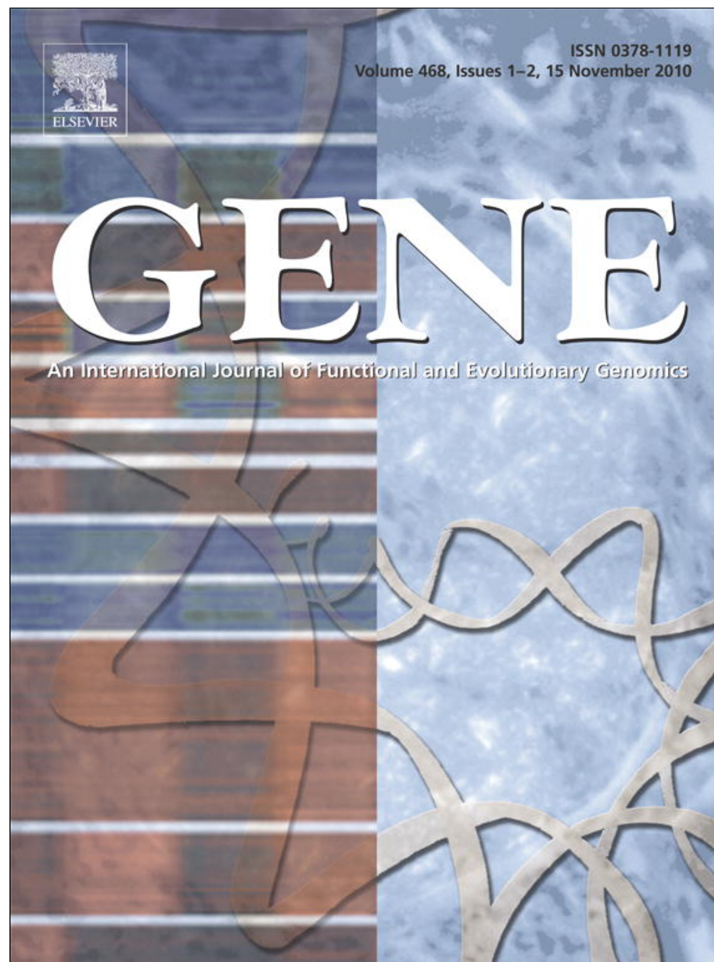

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The complete mitochondrial genome and phylogenomics of a damselfly, *Euphaea formosa* support a basal Odonata within the Pterygota

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ABSTRACT

This study determined the first complete mitochondrial genome of a damselfly, *Euphaea formosa* (Insecta: Odonata: Zygoptera), and reconstructed a phylogeny based on thirteen protein-coding genes of mitochondrial genomes in twenty-five representative hexapods to examine the relationships among the basal Pterygota. The damselfly's mitochondrial genome is a circular molecule of 15,700 bp long, and contains the entire set of thirty-seven genes typically found in insects. The gene arrangement, nucleotide composition, and codon usage pattern of the mitochondrial genome are similar across the three odonate species, suggesting a conserved genome evolution within the Odonata. The presence of the intergenic spacer s5 likely represents a synapomorphy for the dragonflies (Anisoptera). Maximum parsimony, maximum likelihood, and Bayesian analyses of both nucleotide and amino acid sequences cannot support the three existing phylogenetic hypotheses of the basal Pterygota (Palaeoptera, Metapterygota, and Chiastomyaria). In contrast, the phylogenetic results indicate an alternative hypothesis of a strongly supported basal Odonata and a sister relationship of the Ephemeroptera and Plecoptera. The unexpected sister Ephemeroptera + Plecoptera clade, which contradicts with the widely accepted hypothesis of a monophyletic Neoptera, requires further analyses with additional mitochondrial genome sampling at the base of the Neoptera.

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1. Introduction

Insects were the first group of organisms to take to the skies and the only invertebrates to have acquired functional wings, starting approximately 400 million years ago in the early Devonian period (Engel and Grimaldi, 2004). Researchers have regarded the origin of wings as the most important morphological innovation for the success of insects, which allows them to colonize every terrestrial and freshwater ecosystem through increased locomotion and dispersal ability (Hennig, 1981; Brodsky, 1994; Grimaldi and Engel, 2005). The successive structural and functional wing modifications in derived insect lineages, such as the harden elytra of beetles, further facilitate the radiation and domination of insects in diverse habitats. The winged insects (Pterygota) are the most diverse organisms and the ecologically predominant lineages over all life forms. However, the origin and evolution of these tremendous radiations are far from clear, largely due to unresolved phylogenetic relationships among the basal Pterygota, including the Palaeoptera and the remaining primitive winged insects. The Palaeoptera contains merely two extant lineages, dragonflies (Odonata) and mayflies (Ephemeroptra). Nevertheless, the relationships of palaeopteran groups and their phylogenetic positions within the winged or secondarily wingless insect orders (Neoptera) remain controversial. Earlier studies propose three different phylogenetic hypotheses for the relationships among the basal Pterygota: 1) the Palaeoptera (Hennig, 1981), 2) the Metapterygota (Börner, 1904; Kristensen, 1991), and 3) the Chiastomyaria (Boudreaux, 1979) (Fig. 1).

The Palaeoptera hypothesis suggests that the Odonata is the sister to the Ephemeroptera, which together forms a monophyletic group (Palaeoptera) sister to the remaining winged insects of the Neoptera ((Odonata + Ephemeroptera) + Neoptera) (Hennig, 1981; Brodsky, 1994) (Fig. 1A). The Palaeoptera is mainly supported by the "palaeopterous condition", the incapability of flexing the wings over the abdomen during rest, and other morphological traits such as the anal brace, the bristle-like antennae, the formation of

Abbreviations: *atp6* and *atp8*, ATPase subunits 6 and 8; BIC, Bayes information criteria; BI, Bayesian inference; BPP, Bayesian posterior probability; CDspT, codons per thousands codons; *cob*, cytochrome b; *cox1–3*, cytochrome c oxidase subunits 1–3; EST, expressed sequence tag; LB, likelihood bootstrap; *l-rRNA*, large subunit of ribosomal gene; MCMC, Markov chain Monte Carlo; ML, maximum likelihood; MP, maximum parsimony; mtDNA, mitochondrial DNA; *nad1–6, 4L*, NADH dehydrogenase subunits 1–6, 4L; nt1, nt2, and nt3, the first, second, and third nucleotide positions; PB, parsimony bootstrap; PCR, polymerase chain reaction; PCGs, protein-coding genes; RSCU, Relative Synonymous Codon Usage; *trnX*, gene encoding for transfer RNA corresponding to amino acids X.

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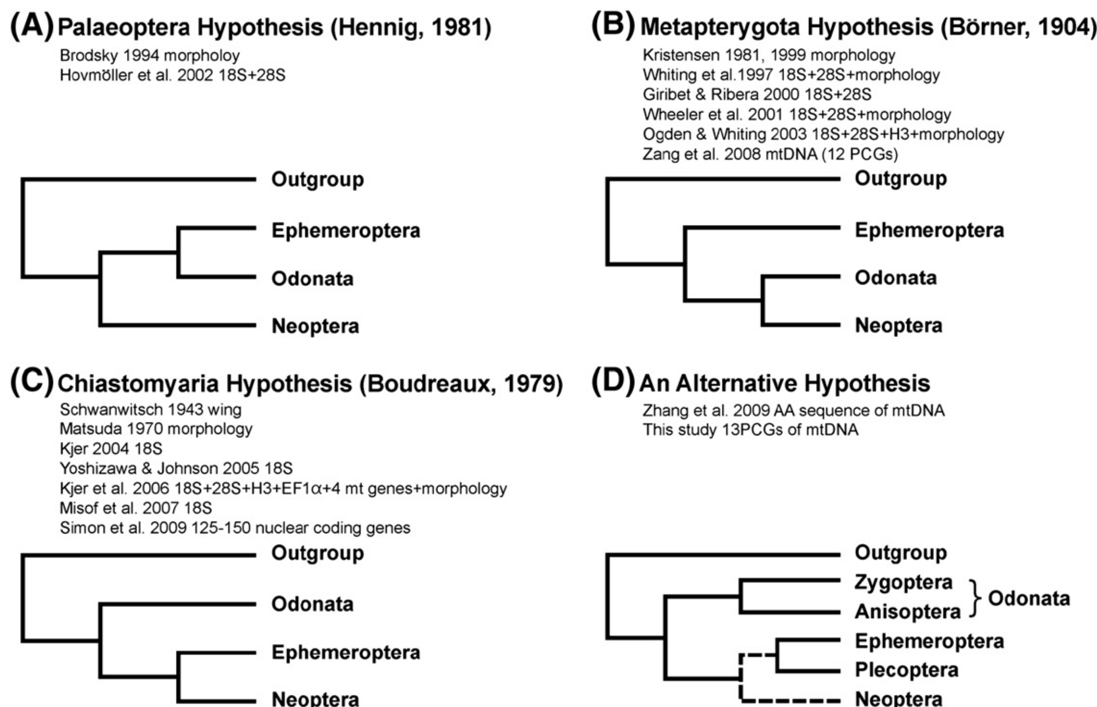

Fig. 1. Phylogenetic hypotheses for the basal pterygotes. A. Palaeoptera hypothesis, B. Metapterygota hypothesis, C. Chiastomyaria hypothesis, and D. this study. Below the trees is the list of morphological and molecular studies supporting the particular topology. Dashed lines indicate tree branches without strong support in the present data set.

intercalary veins, and the aquatic larval lifestyle (Martynov, 1925; Hennig, 1981; Kukalová-Peck, 1991; Staniczek, 2000; Bechly et al., 2001). Studies from 18S and 28S rDNA also support the Palaeoptera hypothesis (Hovmöller et al., 2002). On the contrary, other authors have considered the Odonata + Neoptera as a natural group, the Metapterygota, based largely on the absence of a subimago (winged adult but not sexually mature), the lack of caudal filament, and the fixation of the anterior mandibular articulation (Ephemeroptera + (Odonata + Neoptera)) (Börner, 1904; Kristensen, 1981, 1991) (Fig. 1B). Various combinations of morphological and molecular data sets have supported the Metapterygota (Whiting et al., 1997; Giribet and Ribera, 2000; Wheeler et al., 2001; Ogden and Whiting, 2003; Zhang et al., 2008). Alternatively, the Chiastomyaria hypothesis proposed that the Odonata, with its indirect sperm transfer, is sister to the Ephemeroptera + Neoptera (Chiastomyaria), which have the direct sperm transfer (Odonata (Ephemeroptera + Neoptera)) (Schwanwitsch, 1943; Matsuda, 1970; Boudreaux, 1979). The Chiastomyaria has gained increased support from recent molecular data (Kjer, 2004; Kjer et al., 2006; Misof et al., 2007; Simon et al., 2009). The latest phylogenomic analysis of a large EST dataset supported the Odonata as the most basal winged insect lineage within the Pterygota (Simon et al., 2009).

A robust phylogeny for ancient rapid radiation, such as those among the basal Pterygota, requires a combination of suitable phylogenetic methodology, large numbers of informative sequences from multiple genes, and adequate taxon sampling (Caterino et al., 2000; Simon et al., 2006; Whitfield and Kjer, 2008). Compared to the whole genome sequencing approach (e.g., Ellegren, 2008), the lower cost of obtaining an entire mtDNA offers a molecular marker, in which substantially large amounts of informative data from diverse insect taxa are quickly accumulated and readily available (Feijao et al., 2006; Lee et al., 2009). Animal mtDNA has continued to provide useful genetic markers for studies of molecular evolution, population genetics, and phylogenetic reconstruction in many metazoan lineages because of its high sequence variability, shorter coalescent time, and easy amplification and comparison across different organisms (Harrison, 1989; Simon et al., 1994; Gray et al., 1999; Avise, 2004; Ballard and Rand, 2005; Simon et al., 2006; Rubinoff, 2006; Galtier et al., 2009). Nevertheless, animal mtDNA can also suffer potential pitfalls of substitutional biases, among-site rate heterogeneity, and substitutional saturation, especially in reconstructing deep phylogenetic splits such as insect ordinal relationships (e.g., Lin and Danforth, 2004; Cameron et al., 2006; Fenn et al., 2008). Among insects, the mitochondrial genome is a circular molecule of sizes ranging from approximately 15 to 20 kbp, and mostly consisting of two rRNA genes, twenty-two tRNA genes, thirteen PCGs, and an A + T-rich control region showing substantial length variation among taxa (Simon et al., 1994, 2006). Phylogenetic analyses of insect mitochondrial genomes to date, have indicated that genome rearrangements are not useful for interordinal and higher relationships, due to gene order conservation among major insect lineages (Cameron et al., 2006; Carapelli et al., 2006), or extensive gene order variations within a particular derived lineage (Shao et al., 2001, 2003; Dowton et al., 2003; Cameron et al., 2007). Nevertheless, the nucleotide sequences of PCGs in insect mitochondria are informative and useful sources of interordinal or lower level relationships after accommodating the effects of base compositional biases, unequal rates of nucleotide substitution, and asymmetric mutation with more realistic models of DNA evolution (Lin and Danforth, 2004; Hassanin et al., 2005; Cameron et al., 2006; Carapelli et al., 2007; Simon et al., 2006; Fenn et al., 2008; Hua et al., 2008; Zhang et al., 2008).

The extant Odonata has been traditionally recognized as three major groups, Anisoptera (dragonflies), Anisozygoptera (one genus with two species), and Zygoptera (damselflies), that comprises a morphologically diverse suborder with twenty families (Corbet, 1999; Schorr et al., 2008). To date, a completely sequenced mitochondrial genome of the Odonata is only available for two dragonfly species, *Davidius lunatus* (Gomphidae) (Lee et al., 2010) and *Orthetrum triangulare melania* (Libellulidae) (Yamauchi et al., 2004). This study sequences the first representative of the complete mitochondrial genome of a damselfly, *Euphaea formosa* Hagen, 1869 (Euphaeidae), and examines the relationships among palaeopteran lineages using the mitochondrial phylogenomic approach. We compared the

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genome organization, structure, and composition of mtDNA among the three available odonates and reconstructed phylogenies based on thirteen PCGs of mitochondrial genomes from exemplars of nine wingless insects and thirteen major basal pterygote lineages. This investigation used phylogenetic analyses and statistical methods to test the validity of the three hypotheses proposed for the basal Pterygota.

2. Materials and methods

2.1. Sequencing mitochondrial genome of E. formosa

The *E. formosa* specimen (code EfRa010) used for this study was collected from Fongkang River (22°13′50″N–120°47′10″E) of southern Taiwan in 2006. The insect was preserved in 95% EtOH and kept in a −80 °C freezer. We extracted total genomic DNA from the thorax muscle of the specimen using standard CTAB protocol outlined in Lin and Wood (2002). Vouchers consisting of the remaining damselflies were deposited in the insect collection of Tunghai University. We amplified the whole mitochondrial genome as two DNA fragments using long PCR primer sets, L2020-CO1/H12230-16S and L12167-16S/H5244-CO3, following the recommended thermal cycle profile (Yamauchi et al., 2004). PCRs were performed on a thermal Mastercycler (EPPENDORF, USA) with a total volume of 25 µl containing 1 µl (approximately 100 ng) of genomic DNA, 1 µl of each primer (10 mM), 4 µl of dNTP (1 mM), 2.5 µl of 10× buffer, and 1.25 U of TaKaRa LA *Taq*™ polymerase (Takara Bio Inc., Japan). The amplified long PCR fragments were isolated using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan) and cloned into pCR® 2.l-TOPO vector (Invitrogen). DNA sequencing was performed using primer walking approach with a combination of 5 published and 17 newly designed primers (Online supplementary data), cycle sequencing, and dye terminator methodologies on an ABI PRISM™ 377 automatic sequencer (Perkin Elmer, USA) by the Mission Biotech, Taiwan.

2.2. Sequence annotation and tRNA folding

The mtDNA sequences were edited and assembled using SegMan program in the Lasergene (v. 7.1, DNAStar, Madison, WI). Chromatograms of contig sequences were checked manually for ambiguous base calls. PCGs, rRNA genes, and intergenic spacers were identified through BLAST searches in GenBank and by comparison with homologous sequences of other insect mtDNA. Nomenclature of genes and strands followed Simon et al. (1994). The abundance of codon families and the pattern of CDspT and RSCU in PCGs for the three available mtDNAs of the Odonata were analyzed in DAMBE (v. 4.2.13, Xia and Xie, 2001). The start and incomplete stop codons were excluded from the analyses. The transfer RNA identification and analysis was conducted using DOGMA (Dual Organellar Genome Annotator, Wyman et al., 2004), with the COVE threshold set to a lower value of 7 to allow all putative tRNAs. We chose the tRNA based on its COVE score and the quality of predicted secondary folding with reference to published insect's mitochondrial tRNAs. The complete mitochondrial genome of *E. formosa* was deposited in the GenBank (accession no. HM126547).

2.3. Taxon sampling, sequence alignment, and phylogenetic analyses

To reconstruct the basal relationships of pterygote insects, the DNA sequences of the complete mitochondrial genome of representative taxa of 21 basal insect orders and three Collembolan outgroups were obtained from GenBank (Table 1). Taxon sampling was focused on available mitochondrial genomes in the basal pterygote lineages. The mitochondrial genomes of the more derived taxa, such as the paraneopterous or holometabolous insects were available but not included in the present data set because some of these highly divergent mitochondrial genomes can violate the assumptions found in nucleotide substitution models and result in incorrect tree topologies. The nucleotide sequences of all 13 PCGs were first translated into amino acid sequences in MacClade (v. 4.06, Maddison and Maddison, 2000) with the invertebrate mitochondrial genetic code (translation Table 5) and aligned using Clustal W method with a gap penalty of 15 and a gap length penalty of 6.6 in MegAlign of Lasergene package. The alignment of nucleotide sequences was obtained by converting aligned amino acid sequences back to nucleotide sequences. The level of substitution saturation of the first (nt1), second (nt2), and third (nt3) codon positions of nucleotide sequences was evaluated using a saturation test with F84 model implemented in DAMBE. We excluded all nt3 positions from the phylogenetic analyses because of substitution saturation in these sites (see Section 3.2).

Maximum parsimony analyses of nucleotide and protein sequences were performed using PAUP* (v. 4.0b10, Swofford, 1998), with 400 replicates of parsimony ratchet procedure implemented in Pauprat (Sikes and Lewis, 2000). Parsimony bootstrap (PB) values were calculated using 1000 replicates and 100 random taxon additions in PAUP*. For Bayesian inference (BI) and maximum likelihood (ML) analyses of nucleotide sequences, we divided the nucleotide sequences into 26 character partitions corresponding to genes and codon positions (nt1 and nt2). The best-fit model of nucleotide substitution for each partition was selected on the basis of the Bayes Information Criteria (BIC) in Modeltest (v. 3.7, Posada and Crandall, 1998). The chosen models for each partitions were as follows: GTR + G model for nt2 of *nd2* and nt1 of *nd4L*; GTR + I + G model for nt1 of *atp6*, *cox2*, *cob* and *nd2*, nt2 of *atp8*, nt1 and nt2 of *cox1*, *cox3*, *nd1*, *nd4* and *nd5*; HKY + I + G model for nt1 of *nd6*; K81uf + I + G model for nt1 of *atp8*; TIM + I + G model for nt1 of *nd3*; and TVM + I + G model for nt2 of *atp6*, *cox2*, *cob*, *nd3* and *nd6*. These substitution models were used in the site-specific BI analyses in MrBayes (v. 3.1.2, Huelsenbeck and Ronquist, 2001), with all model parameters unlinked and rate multipliers variable across sites. Two independent Markov chain Monte Carlo (MCMC) processes each containing four Markov chains were performed simultaneously for 4×10^6 generations, with Markov chains being sampled for every 100 iteration. We terminated MCMC searches after the average split frequencies of two processes falling below the value of 0.01 and the convergence diagnostic potential scale reduction factor reaching 1, which indicated the convergence of separate MCMC processes. The initial 20,000 MCMC samples were discarded as burnin. The remaining 20,000 trees were used to compute a 50% majority rule tree, with the percentage of trees recovering the node representing the node's Bayesian posterior probability (BPP).

For the ML analyses of nucleotide sequences, we conducted the tree searches and parameter optimization using a rapid approximation algorithm implemented in RAxML (Stamatakis, 2006) to reduce the computational time and memory requirement. Because RAxML does not allow less complex models, such as HKY + I + G or TVM + I + G, to be applied separately to individual partitions, we reconstructed the ML tree using the GTRCAT GAMMAI model to approximate the site-specific substitution rates for individual partitions, and to accommodate the rate heterogeneity within each partition using four discrete rate categories of a gamma distribution (GAMMA) and an estimation of the proportion of invariable sites (I). We conducted 100 iterations in each ML analyses and identified the optimal ML tree by comparing the likelihood values among them. To assess the support for internal nodes of the ML tree, we calculated 1000 likelihood bootstrap (LB) replications with the GTRGAMMAI model.

For phylogenetic analyses of protein sequences, we conducted BI in MrBayes using the MtRev model, with the rate heterogeneity accommodations (G + F: *atp8*, *nad2*, and *nad4L*; I + G: *cox1*, *cox3*, and *cob*; and I + G + F: *atp6*, *cox2*, *nad1*, *nad3*, *nad4*, *nad5*, and *nad6*)

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Table 1
List of taxa and their mtDNAs analyzed in this study.

Order	Family	Species	Acc. number	References
Collembola	Entomobryidae	*Orchesella villosa*	NC_010534	Carapelli et al. (2007)
	Neanuridae	*Bilobella aurantiaca*	NC_011195	Carapelli et al. unpublished
	Sminthuridae	*Sminthurus viridis*	NC_010536	Carapelli et al. (2007)
Diplura	Campodeidae	*Campodea fragilis*	NC_008233	Podsiadlowski et al. (2006)
	Japygidae	*Japyx solifugus*	NC_007214	Carapelli et al. (2005)
Archaeognatha	Machilidae	*Pedetontus silvestrii*	NC_011717	Zhang et al. (2008)
	Machilidae	*Petrobius brevistylis*	NC_007688	Podsiadlowski (2006)
	Machilidae	*Trigoniophthalmus alternatus*	NC_010532	Carapelli et al. (2007)
	Meinertellidae	*Nesomachilis australica*	NC_006895	Cameron et al. (2004)
Zygentoma	Lepismatidae	*Thermobia domestica*	NC_006080	Cook et al. (2005)
	Lepidotrichidae	*Tricholepidion gertschi*	NC_005437	Nardi et al. (2003)
	Nicoletiidae	*Atelura formicaria*	NC_011197	Comandi et al. (2009)
Ephemeroptera	Ephemeridae	*Ephemera orientalis*	NC_012645	Lee et al. (2010)
	Heptageniidae	*Parafronurus youi*	EU349015	Zhang et al. (2008)
Odonata	Euphaeidae	*Euphaea formosa*	HM126547	This study
	Gomphidae	*Davidius lunatus*	NC_012644	Lee et al. (2010)
	Libellulidae	*Orthetrum triangulare melania*	AB126005	Yamauchi et al. (2004)
Plecoptera	Pteronarcyidae	*Pteronarcys princeps*	NC_006133	Stewart and Beckenbach (2006)
Isoptera	Rhinotermitidae	*Reticulitermes hageni*	NC_009501	Cameron and Whiting (2007)
Blattaria	Blattidae	*Periplaneta fuliginosa*	NC_006076	Yamauchi et al. (2004)
Mantodea	Mantidae	*Tamolanica tamolana*	NC_007702	Cameron et al. (2006)
Mantophasmatodea	Mantophasmatidae	*Sclerophasma paresisense*	NC_007701	Cameron et al. (2006)
Phasmatodea	Phasmatidae	*Ramulus hainanense*	NC_013185	Hua et al. unpublished
	Timematidae	*Timema californicum*	DQ241799	Cameron et al. (2006)
Orthoptera	Acrididae	*Locusta migratoria*	NC_001712	Flook et al. (1995)

selected separately for each gene partitions in ProtTest (v. 2.4, Abascal et al., 2005). Two independent MCMC processes were performed simultaneously for 1×10^5 generations and terminated after the average split frequencies of two processes falling below the value of 0.01. Trees and parameters were sampled for every 100 iterations, with a burnin of 1×10^4 generations. We conducted ML tree searches using the MtArt + I + G model of amino acid changes (Abascal et al., 2007), and performed bootstrap analyses of 100 replicates in PHYML (v. 3.0, Guindon and Gascuel, 2003).

2.4. Testing alternative phylogenetic hypotheses

We tested the three alternative phylogenetic hypotheses of basal Pterygota (Fig. 1) by calculating the probability values of the tree topologies (Table 2) using the Kishino–Hasegawa (KH), Shimodaira–Hasegawa (SH), weighted Kishino–Hasegawa (WKH), weighted Shimodaira–Hasegawa (WSH), and approximately unbiased (AU) test calculated from the multi-scale bootstrap in CONSEL (Shimodaira and Hasegawa, 2001). The likelihood values of alternative tree topologies were obtained from ML heuristic searches of 5×10^6 generations using the constrained topologies and GTR + I + G model in GARLI (Zwickl, 2006). A p-value of less than 0.05 was interpreted as strong evidence for rejecting the phylogenetic hypothesis.

3. Results

3.1. Genome organization, structure, and composition

The complete mtDNA of the *E. formosa* is a circular molecule of 15,700 bp long. This length is within the range of the other two odonate mitochondrial genomes (incomplete 14,033 bp in *O. triangulare melania* and 15,913 bp in *D. lunatus*). The mtDNA of *E. formosa* contains the entire set of thirty-seven genes (13 PCGs, 22 tRNA genes, two rRNA genes and an A + T-rich control region), with the gene arrangement typically found in an insect's mitochondria (Boore, 1999; Simon et al., 1994), and identical to the other two odonate genomes (Fig. 2). This genome contains four non-coding intergenic spacers (s1–s4), ranging from 13 to 35 bp. The result also found three of these four spacers (s2–s4) in another two odonate mtDNA, while *E. formosa* has a unique s1 and is missing an intergenic spacer (s5, between *nd1* and *trnL2*), shared by *O. triangulare melania* and *D. lunatus*. Six gene junctions in the mtDNA of *E. formosa* have short overlaps, with the largest one being eight nucleotides long and found at the junction of *trnC–trnY*. The base frequency of the entire *E. formosa* mtDNA is as follows: A = 42.2%; T = 28.3%; C = 17.3%; G = 12.0%, with a typically high A + T content of 70.3% within the range of values found in *O. triangulare melania* (73.9%) and *D. lunatus* (70.1%).

Eleven of the thirteen PCGs in *E. formosa* employ the standard start codons for invertebrate mtDNA: eight use ATA and ATG (*nad2*, *cox1*, *cox2*, *atp6*, *cox3*, *nad4*, *nad4L*, and *cob*), which encode for methionine (M); three use ATC and ATT (*atp8*, *nad5*, and *nad6*), encoding for isoleucine (I). Findings showed an exception in *nad1* and *nad3* genes, which initiate with the non-canonical putative start codon TTG encoding for leucine (L). The current work recognized a standard stop codon in ten PCGs (TAA for *cox1*, *atp8*, *atp6*, *nad3*, *nad4*, *nad4L*, *nad6* and *cob*; TAG for *nad1* and *nad2*). The *cox2*, *cox3* and *nad5* have an incomplete stop codon of a single T. The *atp8–atp6* and *nad4–nad4L* are the PCG pairs having a nucleotide overlap. The A + T content of all PCGs in *E. formosa* is highly biased (70.5%), with *atp8* having the highest (84.3%) and *cox1* having the lowest values (64%). *Cox1* of *O. triangulare melania* and *D. lunatus* also has the lowest A + T content, but the PCG with the highest A + T content for *D. lunatus* is *nad4L*.

Table 2
Maximum likelihood values and statistics for alternative phylogenetic hypotheses of basal Pterygota calculated in GARLI and CONSEL program.

Hypothesis	Topological constraints	LnL	AU	KH	SH	WKH	WSH
Palaeoptera	((Odonata, Ephemeroptera), Neoptera)	−103,878.5042	0.006*	0.032*	0.161	0.014*	0.034*
Metapterygota	(Ephemeroptera, (Odonata, Neoptera))	−103,866.8527	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Chiastomyaria	(Odonata, (Ephemeroptera, Neoptera))	−103,860.7880	0.150	0.139	0.397	0.139	0.359
This study		−103,842.0322	0.875	0.861	0.921	0.861	0.958

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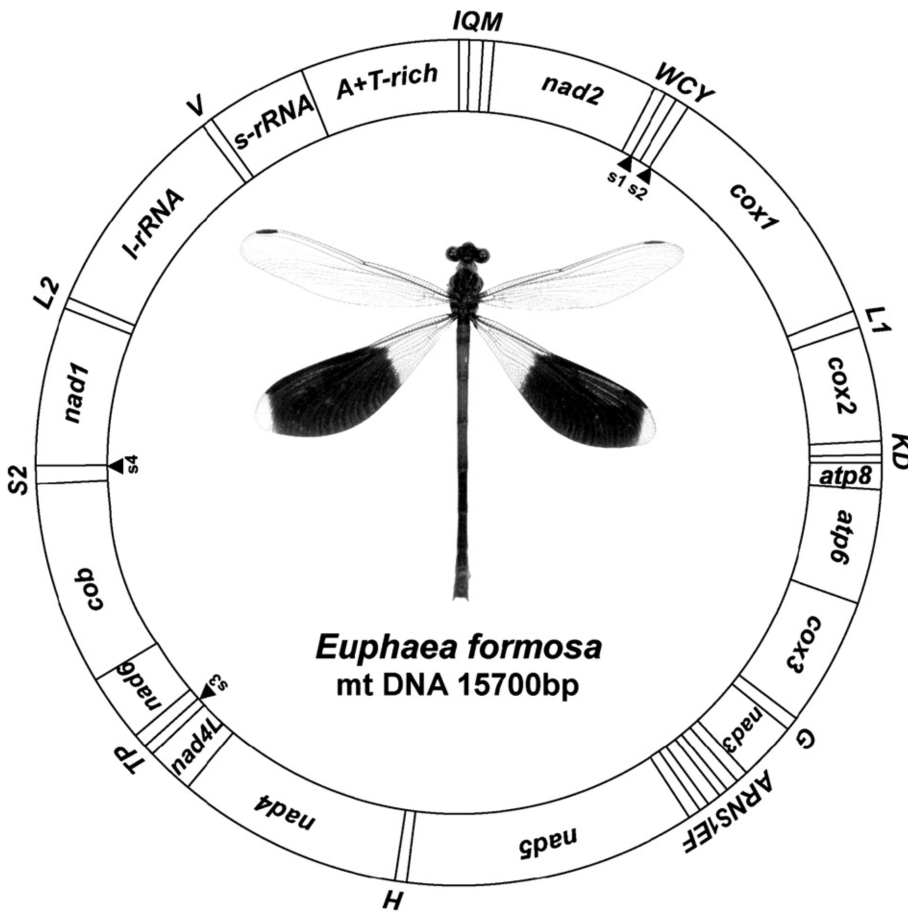

	Strand	Position	Length	A+T(%)	Start	Stop	inc
trnI	+	1..66	66	62.1			3
trnQ	-	70..137	68	70.5			-1
trnM	+	137..205	69	69.5			6
nad2	+	212..1201	990	72.3	ATA	TAG	1
trnW	+	1203..1272	70	75.7			0
s1		1273..1290	18	66.7			0
trnC	-	1291..1354	64	68.7			0
trnY	-	1355..1425	71	64.8			0
s2		1426..1460	35	80.0			0
cox1	+	1461..3008	1548	64.1	ATG	TAA	-5
trnL1	+	3004..3074	71	63.4			0
cox2	+	3075..3762	688	67.0	ATG	T(aa)	0
trnK	+	3763..3834	72	65.3			-1
trnD	+	3834..3900	67	83.6			0
atp8	+	3901..4059	159	76.7	ATT	TAA	-4
atp6	+	4056..4730	675	70.0	ATA	TAA	-1
cox3	+	4730..5516	787	64.7	ATG	T(aa)	0
trnG	+	5517..5584	68	77.9			0
nad3	+	5585..5938	354	71.7	TTG	TAA	-1
trnA	+	5938..6005	68	72.0			-1
trnR	+	6005..6068	64	73.4			2
trnN	+	6071..6137	67	73.1			-1
trnS1	+	6137..6207	71	64.8			1
trnE	+	6209..6275	67	82.1			-2
trnF	-	6274..6342	69	71.0			2
nad5	-	6345..8067	1723	70.6	ATT	T(aa)	1
trnH	-	8069..8133	65	67.7			2
nad4	-	8136..9497	1344	72.0	ATG	TAA	-7
nad4L	-	9473..9766	294	74.1	ATG	TAA	2
trnT	+	9769..9835	67	73.2			0
s3		9836..9848	13	38.5			0
trnP	-	9849..9914	66	77.3			1
nad6	+	9916..10413	498	73.5	ATC	TAA	-1
cob	+	10413..11546	1134	67.7	ATG	TAA	-2
trnS2	+	11545..11608	64	68.7			0
s4		11609..11624	16	75.0			0
nad1	-	11625..12575	951	69.5	TTG	TAG	1
trnL2	-	12577..12643	67	73.1			0
l-rRNA	-	12644..13931	1288	73.9			0
trnV	-	13932..14003	72	70.9			0
s-rRNA	-	14004..14781	778	70.8			0
A+T-rich		14782..15700	919	80.3			0

Fig. 2. Gene map and organization of the mitochondrial genome of *E. formosa*. Transfer RNA genes on the gene map are labeled by the one-letter amino acid code corresponding to the tRNA (*trn*) designation in the table on the right; inc, intergenic nucleotides. Negative inc values are overlapping nucleotide sequences of different genes. The incomplete stop codons are labeled with parentheses. The question mark represents doubtful start codon for *nad1* gene. *s1–s4*, intergenic spacers.

The number of codons used by the three odonate mtDNAs is similar and range from 3592 in *O. triangulare melania* to 3698 in *D. lunatus* (Fig. 3A). The overall codon families show a similar pattern among the three odonates, with the seven codon families (Phe, Gly, Leu2, Met, Ser2, Thr and Val) using over fifty codons per thousand codons (CDspT). The exception is that *O. triangulare melania* has a greatly reduced Leu1 (CDspT = 32) and highly elevated Leu2 (CDspT = 108) family. For all three odonates, the Phe and Cys family have the highest (94) and lowest (12) average value of CDspT, respectively. The RSCU results indicate the codon usage preference of A + T-rich over synonymous codon families (Fig. 3B). All codons ending with A or T outnumber those ending with C or G, except for the His family in *D. lunatus*, where the CAC is used more than the CAT codon (RSCU = 1.08 and 0.92, respectively). Six amino acid residues (Phe, Gly, Ile, Leu2, Met, and Val) with primarily hydrophobic side chains account for more than 45.53% (average = 46.81 ± 1.77%) of all residues in the 13 PCGs of the three odonates.

The tRNA sequences of *E. formosa* range in size from 64 bp in *trnC*, *trnR*, and *trnS2* to 72 bp in *trnK* and *trnV*. All twenty-two tRNA sequences can be folded into the characteristic clover leaf secondary structures, but *trnR*, *trnD*, *trnC*, and *trnH* lack the TΨC loop (Fig. 4). Findings show thirty-four mismatches of base pairs, with twenty-eight non-Watson–Crick interactions (27 G–T and 1 A–C). This study considers the other four A–G and two C–T base-pairings as mismatches in the stems of five different tRNAs (*trnA*, *trnN*, *trnC*, *trnE* and *trnL*). All A–G mismatches occur at the last base pairs near the end of the acceptor stem. The A + T-rich (control) region of *E. formosa* has 919 bp, which is shorter than that of *D. lunatus* (1066 bp) and contains two peculiar repeated DNA fragments of 159 bp length at the same direction (positions 14,941–15,099 and 15,100–15,258).

3.2. Phylogeny of basal Pterygota

Saturation test results indicated that both the pairwise transitional and transversional differences in the nt3 codon positions show marked saturation early in sequence divergence. They were excluded from the subsequent analyses of nucleotide sequences to remove the phylogenetic noise from saturated changes in the nt3 codon positions. Parsimony analyses of nt1 and nt2 codon positions found the most parsimonious tree (length = 25,151), which recovered the same relationships as the parsimony bootstrap consensus tree, except for *E. formosa* being sister to *D. lunatus* (Fig. 5A). Both the most parsimonious and bootstrap consensus tree indicated the monophyly of the Odonata (PB = 100%), and the Pterygota (PB = 87%) and basal position of the Odonata within the Pterygota (PB < 50%), followed by the Ephemeroptera + Plecoptera (PB = 100%). The resultant tree from the BI and ML analyses also showed strong support for a monophyletic Pterygota (BPP = 1, LB = 83%), with a basal Odonata (BPP = 1, LB = 95%) and a sister relationship of the Ephemeroptera + Plecoptera (BPP = 1, LB = 100%) (Fig. 5B). Protein sequence analyses revealed largely the same topology with high support values as that of nucleotide sequences (Fig. 5A and B), except that the most parsimonious trees of protein sequences did not recover a monophyletic Pterygota. Overall, the tree topologies of the parsimony and ML/BI analyses are similar except for the non-monophyletic Diplura (parsimony)

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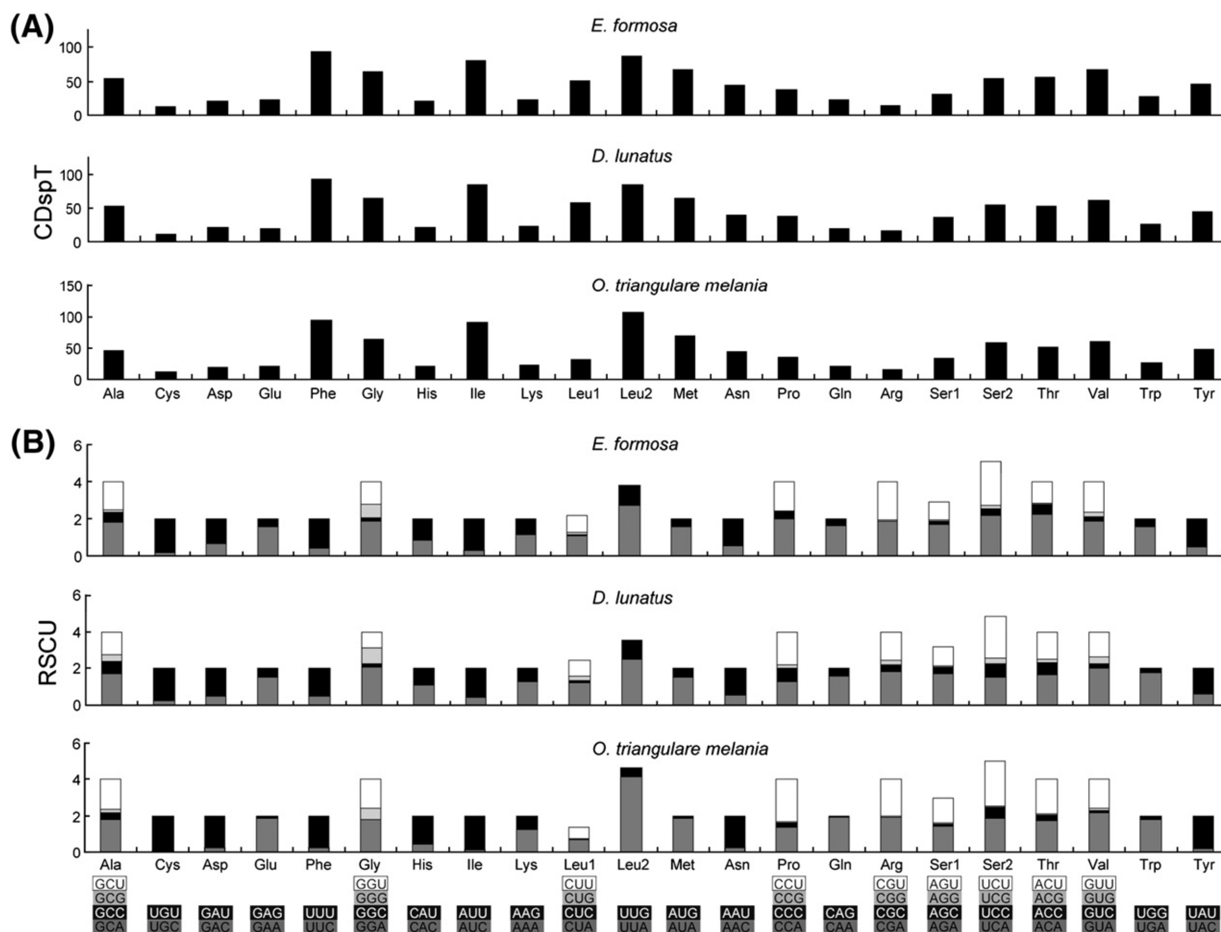

Fig. 3. Codon usage in the three odonate mtDNAs. A. Total codon distribution, x-axis shows the codon families and y-axis refers to the total number of the codon; CDspT, codons per thousand codons. B. The Relative Synonymous Codon Usage (RSCU), the codon families are listed on the x-axis.

and Zygentoma (ML/BI), and the placement of the Orthoptera. Compared with the ML/BI analyses, the parsimony analyses of protein sequences in general lack bootstrap supports. Therefore, the phylogenetic relationships of the basal Pterygota based on the ML/BI trees (Fig. 5B) are discussed since they were supported by higher bootstrap values and posterior probability. Table 2 summarizes the results of the phylogenetic hypotheses testing using the maximum likelihood and tree topology constraints. All tests (AU, KH, SH, WKH, and WSH) significantly supported tree topology resulting from this study, but failed to reject the Chiastomyaria hypothesis. Whereas the statistical analyses rejected the hypotheses of Palaeoptera and Metapterygota, except the SH test for the Palaeoptera (Table 2).

4. Discussion

4.1. Mitochondrial genomes of the Odonata

The newly sequenced *E. formosa* mt genome in the present study is similar in gene number, gene arrangement, and nucleotide composition compared to that of the other two available odonate species, as well as to the mtDNA of the presumed ancestral hexapod (Figs. 2 and 3) (Boore, 1999; Simon et al., 1994, 2006; Yamauchi et al., 2004). However, we found the presence of a unique spacer s1 in *E. formosa* (Zygoptera) and a s5 shared by *O. triangulare melania* and *D. lunatus* (Anisoptera) (Fig. 2), suggesting that the presence and absence of intergenic spacers may contain potentially useful phylogenetic markers in resolving suborder relationships within the Odonata (e.g., Salvato et al., 2008). To investigate whether these spacers are truly synapomorphies for the Zygoptera and Anisoptera, the phylogenetic range over which these intergenic spacers occur needs to be determined with additional odonate taxa, in particular the species within the Zygoptera and Anisozygoptera. Previous analyses of insect nuclear rRNA genes showed an excessively slow nucleotide substitution rate within the Odonata compared to other insect lineages, such as Diplura and Diptera (Kjer et al., 2006; Misof et al., 2007; Whitfield and Kjer, 2008). The present study found that the gene arrangement, nucleotide composition, and pattern of codon usage in mt genomes are similar across three odonate species of two suborders and three families (Fig. 3), suggesting the conservation of mitochondrial genome evolution within the Odonata. In contrast, other insect lineages frequently showed substantial intraordinal variation in gene number or order (e.g., Shao et al., 2001, 2003; Dowton et al., 2003; Cameron et al., 2007; reviewed in Cameron et al., 2006). The relatively slow rate of evolution in nuclear genes and conserved mt genome evolution in odonates as compared to other insect lineages are likely linked to lineage-specific purifying selective forces, life history characteristics, or demographic histories (Rand, 1994; Ballard and Rand, 2005). However, this analysis is preliminary due to the lack of mitochondrial genomes in other major odonate lineages.

4.2. Support for a Basal Odonata within the Pterygota

Resolving the long-standing "Palaeoptera problem" of the basal pterygotes is essential for interpreting the evolution of insect wings and their subsequent rapid diversification in winged insect lineages. The mitochondrial nucleotide and protein sequences of thirteen PCGs employed in this study provide useful characters for resolving phylogenetic relationships in the basal Pterygota. Our phylogenetic

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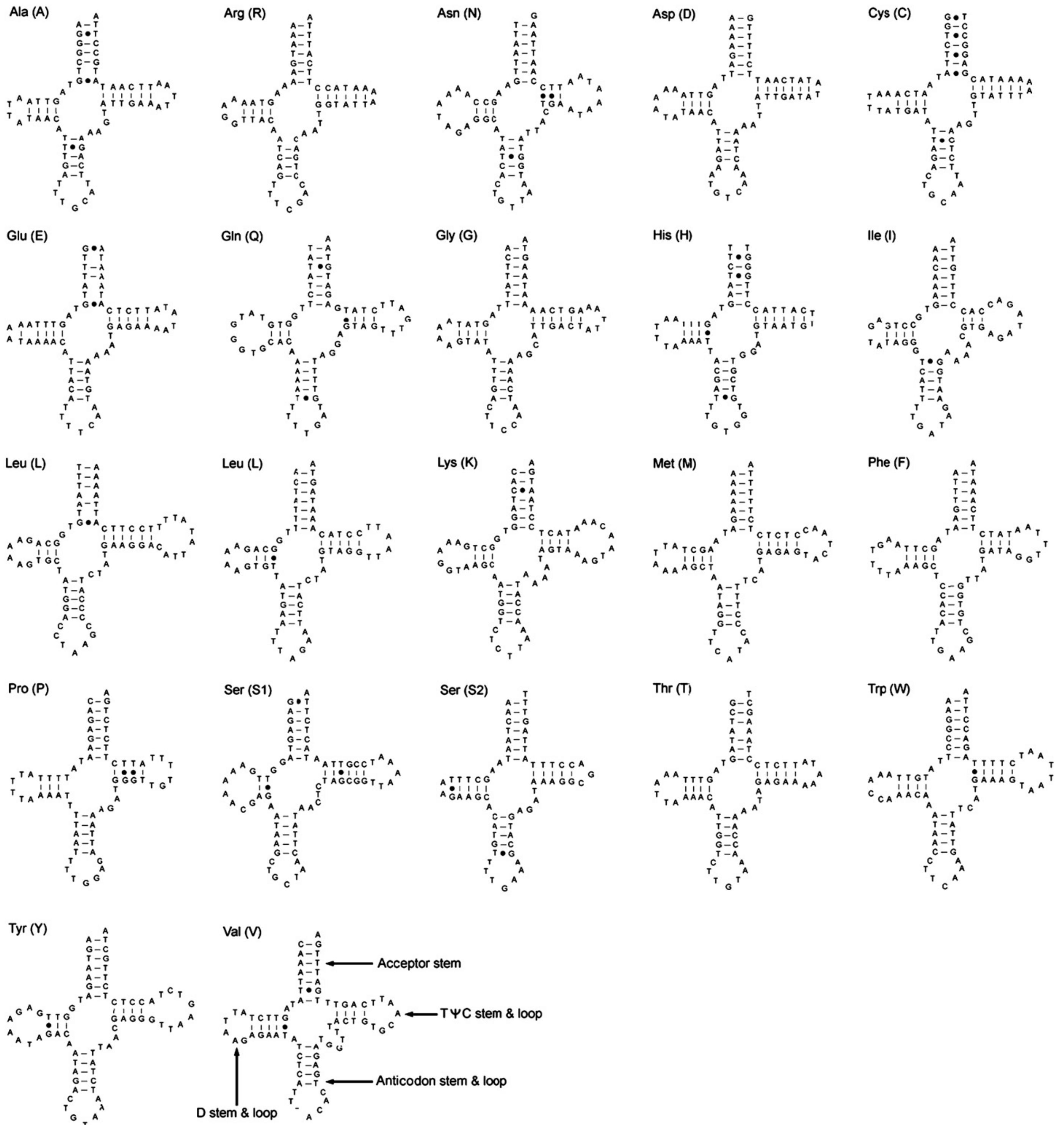

Fig. 4. Putative secondary structures for the 22 tRNA genes of the *E. formosa* mtDNA. Black dots indicate mismatches of base pairs.

and statistical analyses cannot support the three existing phylogenetic hypotheses of the basal Pterygota (Palaeoptera, Metapterygota and Chiastomyaria). In contrast, the results of the present data set indicate an alternative hypothesis of a basal Odonata and a sister relationship of the Ephemeroptera + Plecoptera (Figs. 1D and 5). The basal Odonata within the Pterygota is strongly supported by LB and BPP of both nucleotide and protein sequences, and is consistent with recent analyses of twenty-two basal hexapods based on twelve PCGs of mtDNAs (Zhang et al., 2009). However, our phylogenetic result differs from an earlier mitogenomic analysis of the basal pterygote insects that supported the Metapterygota (Zhang et al., 2008). Because the phylogenetic reconstruction methods used in our study and the study by Zhang et al., 2008 are comparable, the discrepancy between the two data sets is most likely a result of differences in sampled taxa or genes (twelve PCGs in Zhang et al., 2008). Our phylogenetic analyses also recovered a sister relationship between *Tricholepidion gertschi* (family Lepidotrichidae) and the Pterygota (Fig. 5B). This phylogenetic placement of *T. gertschi* implies a paraphyletic Zygentoma, a hypothesis suggested by analyses of 18S rDNA sequences (Yoshizawa and Johnson, 2005).

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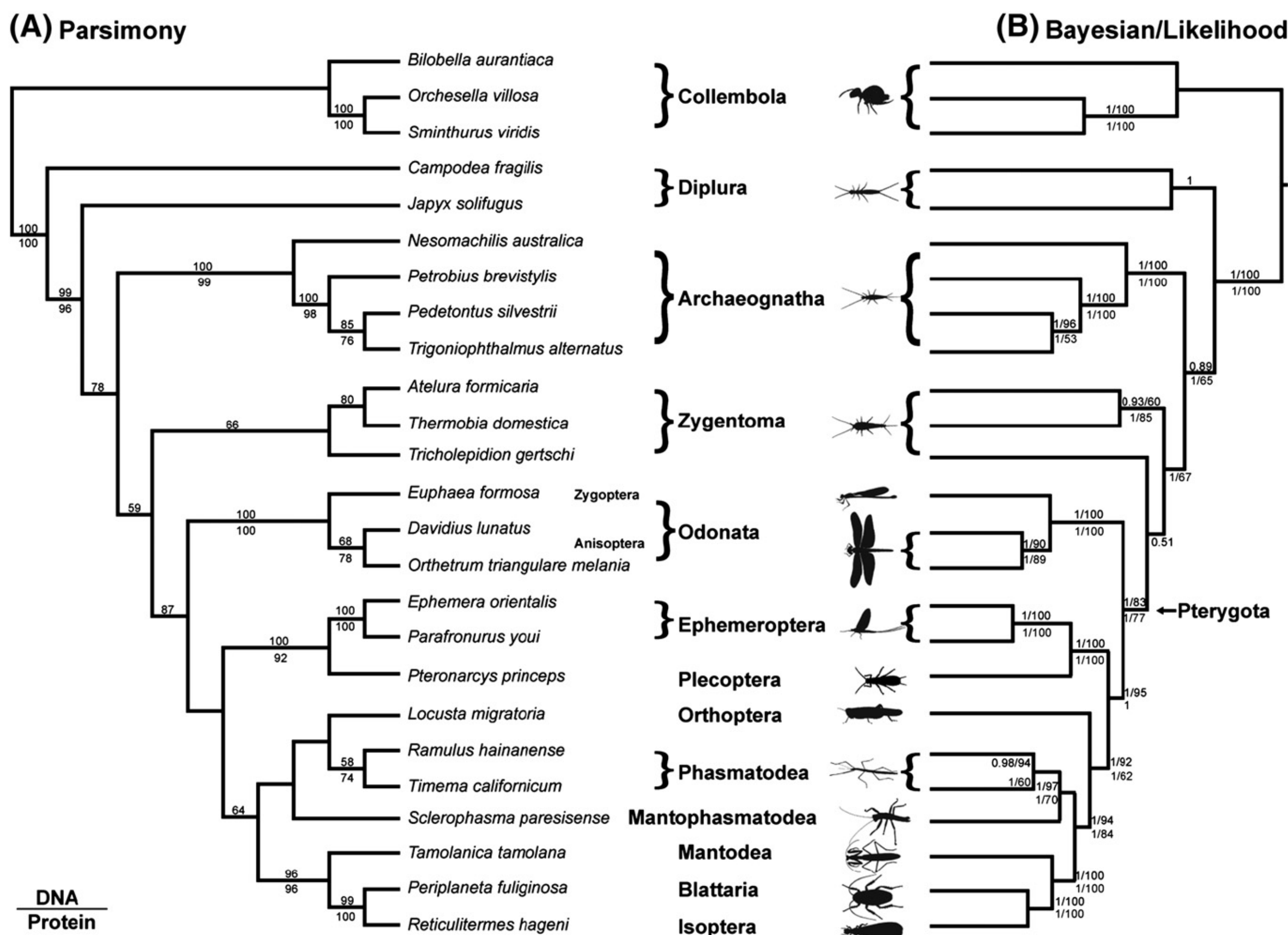

Fig. 5. Reconstructed phylogenies of basal Pterygota based on nt1 + nt2 nucleotide sequences and protein sequences of 13 mitochondrial PCGs. Numbers above and below the branches are support values of nucleotide and protein sequences, respectively (parsimony trees: MP bootstrapping values; Bayesian/likelihood trees: Bayesian posterior probability/ML bootstrapping values). Nodes without support values are those with values below 50%.

However, the present data set has low branch support for this node, which does not allow choosing between alternative hypotheses (i.e., a monophyletic Zygentoma, Kjer et al., 2006; Cameron et al., 2006; Comandi et al., 2009).

The phylogenomic hypothesis based on mtDNAs in this study has several important implications for early evolution of the winged insects, Pterygota. Firstly, most up to date data suggests the “Palaeoptera” as paraphyletic with respect to the Neoptera. The “palaeopterous condition”, a proposed synapomorphy, in which the wings cannot be folded over the abdomen, appears to be a plesiomorphic trait and has only retained in two extant lineages, the Odonata and the Ephemeroptera. Secondly, the direct mechanism of insect flight, where the flight musculature connecting directly to the base of the wings for the downward movement (Brodsky, 1994), has evolved twice independently, first in the Odonata, and later in the more derived Blattodea (cockroaches). By contrast, the downward movement of the indirect flight mechanism produced by contracting the dorsal longitudinal indirect muscles is likely a plesiomorphic trait, which has evolved early in the common ancestors of all pterygotes and now retained only in more derived Diptera (flies) and Hymenoptera (bees, wasps and ants). Other pterygote lineages, such as Orthoptera (grasshoppers and crickets) and Coleoptera (beetles), use a combination of the direct and indirect flight muscles for downward movement (Chapman, 1998). Finally, our phylogenetic results interpret the Odonata as the most basal lineage of extant winged insects, which shares the indirect sperm transfer mechanism with primitive wingless hexapods (Boudreaux, 1979). The reproductive mechanism of indirect sperm transfer is therefore a plesiomorphic trait, lost among the ancestral lineages of the Neoptera, and only regained in other lineages, such as the Orthoptera.

4.3. A sister Ephemeroptera + Plecoptera within the Neoptera?

In addition to a strongly supported basal Odonata within the Pterygota, our phylogenetic analyses show an unexpected monophyletic Ephemeroptera + Plecoptera clade sister to the other Neopteran taxa in the data set (Figs. 1D and 5B). A recent study of a cockroach's mitochondrial genome with similar taxon sampling also found this phylogenetic grouping (*Eupolyphaga sinensis*, Zhang et al., 2009). The phylogenetic clustering of the Ephemeroptera (mayflies) with Plecoptera (stoneflies) is intriguing, because the two taxa share aquatic life history of naiads, and a few studies have used stoneflies as models for understanding the origin of flight in early winged insects (e.g., Marden et al., 2000; Marden and Thomas, 2003). Therefore, a basal Plecoptera with respect to other Neopteran taxa reinforces the notion that stoneflies are early insect fliers, which retain a plesiomorphic aquatic immature stage shared with the Odonata and Ephemeroptera. Nevertheless, the extensive variation of gill types and their positions in Plecoptera, Ephemeroptera, and Odonata strongly suggest the independent origins of aquatic life history and associate morphological modifications (Grimaldi and Engel, 2005).

However, our phylogenetic trees cannot recover a monophyletic Neoptera (Fig. 5B), which is a widely accepted grouping based on extensive morphological and molecular studies (reviewed in Grimaldi

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and Engel, 2005; Whitfield and Kjer, 2008). In this data set, the tree branch leading to the monophyly of the Ephemeroptera and all Neopteran taxa has low LB values on the protein sequences (Fig. 5B), suggesting that the non-monophyly of the Neoptera cannot be strongly supported and needs further investigation. We suggest that the sister Ephemeroptera + Plecoptera clade recovered in this study may be a result of taxon sampling at the base of the pterygotes. Sufficient and broad taxon sampling is an important factor in phylogenetic analyses of insect mitochondrial genomes. One useful sampling approach is to increase the number of representative species within the target taxa, and to exclude the highly divergent genomes with variable genome rearrangements, elevated substitution rates, or base compositional bias, which likely cause long branch attraction (Cameron et al., 2006). The unexpected phylogenetic hypothesis of a sister Ephemeroptera + Plecoptera clade requires extended analyses with additional mitochondrial genome sampling not only within the targeted Ephemeroptera and Plecoptera, but also at the base of the Neoptera, particularly the missing intermediate mt genomes of the Polyneopteran, such as the Dermaptera (earwigs), Embioptera (web spinners), and Zoraptera (angel insects). Earlier studies of these insect taxa have shown their phylogenetic affinity with the Plecoptera (Yoshizawa and Johnson, 2005; Kjer et al., 2006; Misof et al., 2007).

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