International Journal of Biological Sciences
2009; 5(4):351-365

Research Paper

Characterization of the complete mitochondrial genome of the giant silkworm moth, *Eriogyna pyretorum* (Lepidoptera: Saturniidae)

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Received: 2009.02.21; Accepted: 2009.05.16; Published: 2009.05.22

Abstract

The complete mitochondrial genome (mitogenome) of *Eriogyna pyretorum* (Lepidoptera: Saturniidae) was determined as being composed of 15,327 base pairs (bp), including 13 protein-coding genes (PCGs), 2 rRNA genes, 22 tRNA genes, and a control region. The arrangement of the PCGs is the same as that found in the other sequenced lepidopteran. The AT skewness for the *E. pyretorum* mitogenome is slightly negative (–0.031), indicating the occurrence of more Ts than As. The nucleotide composition of the *E. pyretorum* mitogenome is also biased toward A + T nucleotides (80.82%). All PCGs are initiated by ATN codons, except for cytochrome c oxidase subunit 1 and 2 (*cox1* and *cox2*). Two of the 13 PCGs harbor the incomplete termination codon by T. All tRNA genes have a typical clover-leaf structure of mitochondrial tRNA, with the exception of *trnS1(AGN)* and *trnS2(UCN*). Phylogenetic analysis among the available lepidopteran species supports the current morphology-based hypothesis that Bombycoidea, Geometroidea, Notodontidea, Papilionoidea and Pyraloidea are monophyletic. As has been previously suggested, Bombycidae (*Bombyx mori* and *Bombyx mandarina*), Sphingoidae (*Manduca sexta*) and Saturniidae (*Antheraea pernyi*, *Antheraea yamamai*, *E. pyretorum* and *Caligula boisduvalii)* formed a group.

Key words: *Eriogyna pyretorum*; mitochondrial genome; Lepidoptera Saturniidae

1. Introduction

The giant silkworm moth, *E. pyretorum* is a member of the Saturniidae family (superfamily Bombycoidea), which spread over mainly in Burma, China, India, Malaysia and Vietnam. *E. pyretorum* has attracted a great deal of attention for its silk-producing character [1]. Economically important silk-producing insects of order Lepidoptera belong mainly to two families, Bombycidae and Saturniidae [2]. Non-mulberry feeding silkmoths belonging to the family Saturniidae are mostly wild silkmoths. Among Saturniids the most well-known species are *C. boisduvalii*, *E. pyretorum*, *A. pernyi* and *A. yamamai* belonging to the family Saturniidae, which are used for silk production and spread over mainly China, India, and Japan [3]. Phylogenetic relationships of silkmoths have been investigated using several molecular markers, such as of the nuclear arylphorin [4] and rRNA [5] genes, or single [6] and concatenated sets of mitochondrial genes [7], although more species must be evaluated in order to obtain a more complete picture of the relationships among the silkmoths [7].

Mitogenome of metazoan animals is a dou-

ble-stranded, circular molecule, ranging in size from 14 to 19 kb, which contains 37 genes including 13 PCGs (subunits 6 and 8 of the ATPase [*atp6* and *atp8*], cytochrome c oxidase subunits 1–3 [*cox1–cox3*], cytochrome B [*cob*], and NADH dehydrogenase subunits 1–6 and 4L [*nad1–6* and *nad4L*]), 2 ribosomal RNA genes (small- and large-subunit rRNAs [*rrnL* and *rrnS*]), and 22 tRNA genes. Additionally, it contains a control region of variable length known as the A+T-rich region in insects [8]. Mitogenomes are very important subject for different scientific disciplines including comparative and evolutionary genomics, molecular evolution, phylogenetics and population genetics [9].

To date, the complete or near-complete mitogenome have been sequenced from 120 insect species (http://www.ncbi.nlm.nih.gov). Within the order Lepidoptera, only 13 complete or near-complete mitogenomes were sequenced [2, 7, 10-19], and the covered taxon-sampling is extremely poor and limited to six superfamiles among the 45–48 known, and to 9 families of the recognized 120 (Table 1). A better understanding of the lepidopteran mitogenome requires an expansion of taxon and genome samplings. In the present study, we have sequenced the complete mitogenome of the giant silkworm moth *E. pyretorum* (accession number: FJ685653).

Table 1 List of complete mitochondrial genome of Lepidoptera

species	length (bp)	accession number	references
Bombyx mori	15, 656	AB070264	[2]
Japanese *Bombyx mandarina*	15, 928	NC_003395	[2]
Chinese *B. mandarina*	15, 682	AY301620	[10]
Caligula boisduvalii	15, 360	NC_010613	[7]
Antheraea pernyi	15, 575	AY242996	[11]
Antheraea yamamai	15, 338	EU726630	[12]
Maduca sexta	15, 516	EU286785	[13]
Ostrinia furnacalis	14, 536	NC_003368	[14]
Ostrinia nubilalis	14, 535	NC_003367	[14]
Artogeia melete	15, 140	EU597124	[19]
Adoxophyes honmai	15, 680	NC_008141	[15]
Coreana raphaelis	15, 314	NC_007976	[16]
Ochrogaste lunifer	15, 593	AM946601	[17]
Phthonandria atrilineata	15, 499	EU569764	[18]

In this study, we describe the complete mitogenome sequence of the *E. pyretorum*, that is compared with those of other lepidopteran species. Furthermore, the concatenated nucleotide and amino acids sequences of 13 PCGs of *E. pyretorum* were utilized to assess the phylogenetic relationships among lepidopteran superfamilies.

2. Materials and methods

Specimens sampling and mitochondrial DNA extraction

Larval of *E. pyretorum* was collected from the Sericultural Research Institute of Guangxi provice. DNA was extracted using the TaKaRa Genomic DNA Extraction Kit (TaKaRa Co., Dalian, China) in accordance with the manufacturer's instructions.

Primer design, PCR, cloning and sequencing

Twenty primers (Table 2) were synthesized (Shanghai Sangon Biotechnology Co., Ltd., Shanghai, China) to amplify the whole mitogenome of *E. pyretorum*. The primers 16SAA and 16SBB were designed followed the description by Hwang et al (2001) [20]. The other degenerate and specific primers were designed based on the conserved nucleotide sequences of the known mitochondrial sequences in Lepidoptera [2, 7, 10-19] or on the known sequence of fragments of the mitogenome of *E. pyretorum* that we have previously sequenced (accession number: EF206703 and DQ323517). The primers 16SAA and 16SBB were used for the amplification of the long fragment (14 kb) of the mitogenome [20], which were in turn used as template to amplify the fragments of ER1-10. The conditions for the amplification of the long fragment are as follows: an initial denaturation for 2 min at 96 °C, followed by 30 cycles of 10 s at 98 °C and 10 min at 58 °C, and a subsequent 10 min final extension at 72 °C.

The fragments of ER1-10 that range from 1.1 kb to 3.1 kb (Table 2) were amplified using the TaKaRa LA Taq (TaKaRa Co., Dalian, China) and the following PCR conditions: an initial denaturation for 2 min at 96°C, followed by 30 cycles of 10 s at 98 °C and 2 min at 58 °C, and a subsequent 10 min final extension at 72 °C. The above PCR products were separated by electrophoresis in a 1% agarose gel and purified using a DNA gel extraction kit (TaKaRa Co., Dalian, China). The purified PCR product was ligated into T-vector (TaKaRa Co., Dalian, China) and then transformed into XL-1 blue competent bacteria according to the method of Wei et al. (2008) [21]. The positive recombinant clone with an insert was sequenced using the dideoxynucleotide chain termination method (TaKaRa Co., Dalian, China) at least three times.

Table 2 The primers used in this study

Fragment	Region	Primer pair	Primer sequence (5′ →3′)	Size (kb)
ER1	*nad2→cox1*	ER 1F	TGATTTGGDTGTTGAATTGGHYTAGAA	1.6
		ER 1R	GCTCCTAAGATTGAAGAAATACCAGC	
ER 2	*cox1→cox2*	ER 2F	TGGAGCAGGAACAGGATGAAC	2.0
		ER 2R	GAGACCADTACTTGCTTTCAG	
ER 3	*cox2→cox3*	ER 3F	ATTTGTGGAGCTAATCATAG	1.1
		ER 3R	GGTCAGGGWCTATAATCYAC	
ER 4	*cox3→nad3*	ER 4F	TCGACCTGGAACTTTAGC	1.2
		ER 4R	TGGATCAAATCCACATTCA	
ER 5	*nad3→nad5*	ER 5F	GAAGCAGCTGCTTGATATTGACA	2.0
		ER 5R	GCAGCTATAGCCGCTCCTACTCCAGT	
ER 6	*nad5→nad4*	ER 6F	CCCCTGCAGTTACTAAAGTTGAAG	1.7
		ER 6R	CAACCTGAACGTGTACAAGCAGGAA	
ER 7	*nad4→cob*	ER 7F	GGAGCTTCTACATGAGCTTTTGG	2.0
		ER 7R	GTCCTCAAGGTAGAACATAACC	
ER 8	*cob→rrnL*	ER 8F	CGTACTCTTCATGCAAATGGAGC	2.2
		16SBB[a]	CTTATCGAYAAAAAAGWTTGCGACCTC GATGTTG	
ER 9	*rrnL→rrnL*	ER 9F	CGGTTTGAACTCAGA TCATGTAAG	1.1
		ER 9R	TATTGTATCTTGTGTATCAGAGTTTA	
ER 10	*rrnL→nad2*	16SAA[a]	ATGCTACCTTTGCACRGTCAAGATACYGCGGC	3.1
		ER 10R	TCAAAAATGGAAAGGGGCTGAACCTAT	

a. Primers from Hwang et al [20].

Sequence assembly and gene annotation

Protein-encoding genes (PCGs) of the *E. pyretorum* mitochondrial genes were identified by sequence similarity with *A. pernyi* [11]. Sequence annotation was performed using the DNAStar package (DNASTar Inc. Madison, USA) and the online blast tools available through the NCBI web site [22]. The nucleotide sequences of PCGs were translated on the basis of invertebrate mitogenome genetic code. Alignments of the PCGs for each of the available lepidopteran mitogenomes were made in MEGA ver 4.0 [23]. Composition skewness was calculated according to the formulas (AT skew=[A−T]/[A+T]; GC skew=[G−C]/[G+C]) [24]. Identification of tRNA genes was verified using the program tRNAscan-SE. The potential stem–loop secondary structures within these tRNA gene sequences were calculated using the tRNAscan-SE Search Server available online (http://lowelab.ucsc.edu/tRNAscan-SE/) [25]. The secondary structures of tRNA genes that could not be predicted using the tRNAscan-SE were analyzed by comparison with the nucleotide sequences of other insect tRNA sequences [2, 7, 10-19].

Phylogenetic analysis

To illustrate the phylogenetic relation of Lepidoptera, the other complete mitochondrial genomes were obtained from GenBank. *Drosophila yakuba* [26], *Drosophila melanogaster* [27], *Locusta migratoria* [28] and *Anopheles gambiae* [29] were used as outgroups. The alignment of the amino acid sequences of each 13 mitochondrial PCGs was aligned with Clustal X [23, 30] using default settings. Then, the alignment of the nucleotide sequences of 13 individual PCGs was performed using RevTrans ver.1.4 [31], which aligns coding sequences on the basis of protein alignment. These aligned sequences were subjected to GBlocks 0.91b [32] analysis under default conditions in order to select the best-aligned blocks. These were subsequently concatenated into an amino acid (3,507 sites in length, 30 gaps and 308 aligned positions were excluded) and a nucleotide sequence blocks (9,897 sites in length, 66 gaps and 1073 aligned positions were excluded). The concatenated set of nucleotide and amino acids sequences, from the 13 PCGs, was used in phylogenetic analyses performed according to Maximum Likelihood (ML) and Bayesian Inference (BI) methods. ML analyses and bootstrap resampling were performed using the set of programs in the PHYLIP package [33]. BI analyses were carried out using MrBayes 3.1 [34].

For ML analyses (PHYLIP package), the alignment of amino acids and nucleotides sequences were entered into the subprogram SEQBOOT (bootstrap sequence data sets) of the PHYLIP package to create 100 data sets by bootstrap resampling. These 100 data sets were used as input to generate the 100 most parsimonious trees using the subprogram PROML/DNAML, during which all the sequences were randomly entered into each data set 10 times, based on the random number 3. Thus, the resulting output was based on 100×10 runs of all the sequences, with *D. yakuba* [26], *D. melanogaster* [27], *L. migratoria* [28] and *A. gambiae* [29] used as outgroups. The output of these 100×10 runs of PROML/ DNAML was entered into the program CONSENSE to calculate a

majority-rules strict consensus tree with confidence intervals.

For BI analyses, substitution model selection was conducted via comparison of Akaike Information Criterion (AIC) scores [35], calculated using the programs Modeltest ver. 3.7 [36] for nucleotide sequence alignment and ProTest ver.1.4 for amino acid sequence alignment [37]. Bayesian inference (BI) of nucleotide and amino acid datasets were performed using the GTR +I+G [38] and MtRev +I+G [39] model, respectively. The BI analyses for both nucleotide sequences and amino acid were carried out using MrBayes ver. 3.1 [34] under the following conditions: 1,000,000 generations, four chains (one hot chain and three cold chains), and a burn-in step of the first 10,000. The confidence values of the BI tree are expressed as the Bayesian posterior probabilities in percent (BPP).

3. Results and discussion

Genome organization and base composition

The entire *E. pyretorum* mitogenome is 15, 327 bp long, similar to other sequenced lepidopteran mitogenomes (Table 3). The sequence analysis revealed the typical gene content observed in metazoan mitogenomes (Fig. 1): 13 PCGs (*cox1–cox3*, *cob*, *nad1–6* and 4L, *atp6* and 8), 22 tRNA genes (one for each amino acid, two for Leucine and Serine), the large and small rRNA (*rrnL* and *rrnS*) subunits, and a major non-coding region known as the A+T-rich region in insects, as has been detected in other insects [2, 7, 10-19].

The gene order of the lepidopteran mitogenomes are identical; including those of *E. pyretorum* (Fig. 1), however, it differs from the most common type found in a variety of insect orders, which was inferred to be ancestral for insects [40]. The difference between the two types is due the translocation of *trnM* to a position 5'-upstream of *trnI*, which results in an gene order of *trnM*, *trnI*, and *trnQ* (instead of *trnI*, *trnQ* and *trnM*) observed in the lepidopteran mtDNAs (Fig. 1). This suggests that the lepidopteran insects may have acquired such an orientation and gene order independently after their differentiation from the remaining insects [13].

Gene overlaps in the *E. pyretorum* mitogenome occur in seven locations (totally 41 bp), with the longest one (17 bp) observed between *trnF* and *nad5* (Table 3). Similarly-sized overlapping sequences are also detected between *trnF* and *nad5* in other sequenced lepidopteran species, such as *C. boisduvalii*, *A. pernyi*, *Ostrinia furnacalis* and *Ostrinia nubilalis*. The *atp8* and *atp6* of *E. pyretorum* are the only PCGs having a seven nucleotides overlap (Table 3). This feature is common to other sequenced lepidopteran mitogenomes [2, 7, 10-19] and is found in many animal mitogenomes [8].

The *E. pyretorum* mitogenome harbor a total of 186 bp intergenic spacer sequences and these are spread over 17 regions, ranging in size from 1 to 54 bp (Table 3). The longest one of these is present between *trnQ* and *nad2* (Table 3). The intergenic spacer sequences of *E. pyretorum* mitogenome is shorter than that of *Ochrogaste lunifer* (371 bp over 20 regions) and *C. boisduvalii* (194 bp over 16 regions), but longer than that of *M. sexta* (115 bp over 13 regions), *Artogeia melete* (118 bp over 10 regions) and *Coreana raphaelis* (178 bp over 17 regions).

The nucleotide composition of the *E. pyretorum* mitogenome is biased toward A + T nucleotides (80.82%) (Table 4), which is a lower percentage than that of *Phthonandria atrilineata* (81.02%), *B. mori* (81.36%), Japanese *B. mandarina* (81.68%), *M. sexta* (81.79%) and *C. raphaelis* (82.66%), but higher than other eight lepidopterans: *A. pernyi* (80.16%), *A. yamamai* (80.29%), *C. Boisduvalii* (80.62%), *O. nubilalis* (80.17%), *O. furnicalis* (80.37%), *A. honmai* (80.39%), *A. melete* (79.78%) and *O. lunifer* (77.84%). Within 13 protein-coding genes (PCGs) in the *E. pyretorum* mitogenome, the A + T composition is highest in the *atp8* gene (93.83%), and lowest in the *cox1* gene (72.16%).

The ATskew and GCskew [24] were calculated for all available complete mitogenome of lepidopterans and are presented in Table 4. The AT skewness for the *E. pyretorum* mitogenome is slightly negative (–0.031), indicating the occurrence of more Ts than As. Similar results are found in *C. raphaelis* (–0.047), *C. boisduvalii* (–0.024), *A. yamamai* (–0.022), *A. pernyi* (–0.021), *M. sexta* (–0.005) and *Adoxophyes honmai* (–0.001). In contrast, the AT skewness is slightly positive in *B. mori* (0.059), Chinese *B. mandarin*a (0.057), Japanese *B. Mandarina* (0.055), *O. nubilalis* (0.031), *O. furnicalis* (0.032), *O. lunifer* (0.030), *A. melete* (0.012) and *P. atrilineata* (0.007). When considering the 13 PCGs, the bias toward the use of Ts over As is more obvious in the *E. pyretorum* mitogenome, in which the AT skewness is –0.164. In all sequenced lepidopteran mitogenomes, the GC skewness values are negative (−0.158 to −0.318), meaning that there are more Cs than Gs, similar to the skewness values for dipteran and animal mitogenomes [41, 42]. In *E. pyretorum* rRNA, the GC skewness is −0.376. This bias is stronger than in the other genes, indicating that there is a heavy bias toward Cs and against Gs in the rRNA.

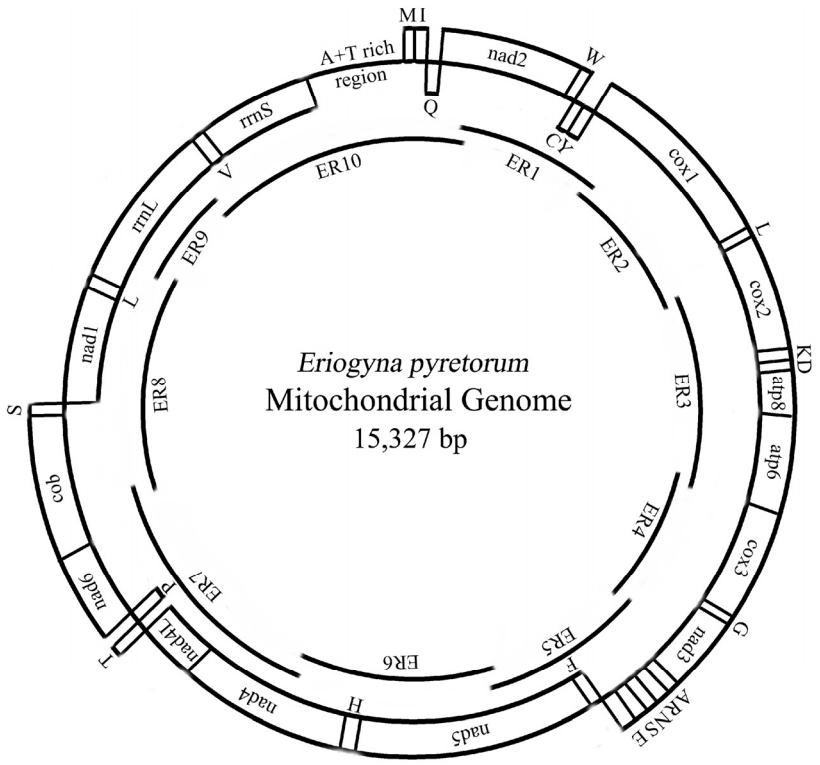


Fig. 1 Map of the mitochondrial genome of *E. pyretorum*. The abbreviations for the genes are as follows: *cox1*, *cox2*, and *cox3* refer to the cytochrome oxidase subunits, *cob* refers to cytochrome b, and *nad1–6* refers to NADH dehydrogenase components. tRNAs are denoted as one-letter symbol according to the IUPAC-IUB single-letter amino acid codes. The overlapping fragments ER1-10 are shown as single lines within a circle.

Table 3 Summary of mitogenome of *E. pyretorum*

Gene	Direction	Location	Size	Anticodon	Start codon	Stop codon	Intergenic nucleotides*
trnM	F	1—69	69	CAT			0
trnI	F	70—133	64	GAT			-3
trnQ	R	131—199	69	TTG			54
nad2	F	254—1267	987		ATT	TAA	8
trnW	F	1276—1343	68	TCA			-8
trnC	R	1336—1402	67	GCA			6
trnY	R	1409—1475	67	GTA			12
cox1	F	1488—3018	1531		CGA	T	0
trnL2(UUR)	F	3019—3086	68	TAA			0
cox2	F	3087—3768	682		GTG	T	0
trnK	F	3769—3839	71	CTT			23
trnD	F	3863—3934	72	GTC			0
atp8	F	3935—4096	162		ATT	TAA	-7
atp6	F	4090—4767	678		ATG	TAA	10
cox3	F	4778—5566	789		ATG	TAA	2
trnG	F	5569—5633	65	TCC			0
nad3	F	5634—5987	354		ATT	TAG	-2
trnA	F	5986—6052	67	TGC			6
trnR	F	6059—6124	66	TCG			-3
trnN	F	6122—6187	66	GTT			0
trnS1(AGN)	F	6188—6252	65	GCT			1
trnE	F	6254—6321	68	TTC			13
trnF	R	6335—6402	68	GAA			-17
nad5	R	6386—8143	1758		ATT	TAA	0

Gene	Direction	Location	Size	Anticodon	Start codon	Stop codon	Intergenic nucleotides*
trnH	R	8144—8209	66	GTG			1
nad4	R	8211—9551	1341		ATG	TAA	2
nad4L	R	9554—9844	291		ATG	TAA	8
trnT	F	9853—9917	65	TGT			0
trnP	R	9918—9981	65	TGG			0
nad6	F	9985—10521	570		ATA	TAA	-1
cob	F	10521—11672	1152		ATG	TAA	2
trnS2(UCN)	F	11675—11741	67	TGA			18
nad1	R	11760—12698	939		ATG	TAA	1
trnL1(CUN)	R	12700—12767	68	TAG			19
rrnL	R	12787—14124	1338				0
trnV	R	14125—14191	67	TAC			0
rrnS	R	14192—14969	778				0
CR		14970—15327	358				0

* Negative numbers indicate that adjacent genes overlap.

Table 4 Composition and skewness in the lepidopteran mitogenomes*

Species	Size (bp)	A%	G%	T%	C%	A+T %	ATskewness	GCskewness
Whole genome								
E. pyretorum	**15327**	**39.17**	**7.63**	**41.65**	**11.55**	**80.82**	**–0.031**	**–0.204**
A. pernyi	15566	39.22	7.77	40.94	12.07	80.16	–0.021	–0.216
A. yamamai	15338	39.26	7.69	41.04	12.02	80.29	–0.022	–0.220
C. boisduvalii	15360	39.34	7.58	41.28	11.79	80.62	–0.024	–0.217
B. mori	15656	43.06	7.31	38.30	11.33	81.36	0.059	–0.216
Japanese *B. mandarina*	15928	43.08	7.21	38.60	11.11	81.68	0.055	–0.213
Chinese *B. mandarina*	15682	43.11	7.40	38.48	11.01	81.59	0.057	–0.196
O. nubilalis [a]	14535	41.36	8.02	38.81	11.82	80.17	0.031	–0.192
O. furnicalis [a]	14536	41.46	7.91	38.92	11.71	80.37	0.032	–0.194
A. honmai	15680	40.15	7.88	40.24	11.73	80.39	–0.001	–0.178
C. raphaelis	15314	39.37	7.30	43.29	10.04	82.66	–0.047	–0.158
M. sexta	15516	40.67	7.46	41.11	10.76	81.79	–0.005	–0.181
A. melete	15140	40.38	7.87	39.41	12.35	79.78	0.012	–0.221
P. atrilineata	15499	40.78	7.67	40.24	11.31	81.02	0.007	–0.192
O. lunifer	15593	40.09	7.56	37.75	14.60	77.84	0.030	–0.318
PCG								
E. pyretorum	**11228**	**33.18**	**10.50**	**46.23**	**10.09**	**79.41**	**–0.164**	**0.020**
A. pernyi	11204	38.50	8.56	40.02	12.92	78.52	–0.019	–0.203
A. yamamai	11269	33.04	10.71	45.90	10.35	78.94	–0.163	0.017
C. boisduvalii	11227	38.83	8.32	40.31	12.53	79.15	–0.019	–0.202
B. mori	11187	42.91	8.15	36.68	12.26	79.58	0.078	–0.201
Japanese *B. mandarina*	11193	42.78	8.14	36.86	12.22	79.64	0.074	–0.200
Chinese *B. mandarina*	11196	42.83	8.26	37.04	11.87	79.87	0.072	–0.179
O. nubilali	11184	41.06	8.57	38.10	12.27	79.16	0.037	–0.178
O. furnicalis	11186	41.15	8.42	38.27	12.15	79.42	0.036	–0.181
A. honmai	11245	39.65	8.77	38.83	12.74	78.48	0.010	–0.181
C. raphaelis	11145	39.10	7.94	42.40	10.55	81.51	–0.04	–0.141
M. sexta	11207	40.39	8.20	39.95	11.46	80.34	0.005	–0.163
A. melete	11180	40.13	8.47	38.38	13.01	78.52	0.022	–0.211
P. atrilineata	11202	40.23	8.59	38.87	12.31	79.10	0.017	–0.178
O. lunifer	11266	32.47	12.08	43.26	12.19	75.73	–0.142	–0.004
tRNA								
E. pyretorum	**1424**	**42.59**	**10.61**	**39.35**	**7.45**	**81.94**	**0.039**	**0.174**
A. pernyi	1459	40.71	8.02	40.71	10.56	81.43	0	–0.137
A. yamamai	1473	41.07	8.08	40.26	10.59	81.33	0.010	–0.134
C. boisduvalii	1466	40.38	7.78	41.61	10.23	81.99	–0.015	–0.136

Species	Size (bp)	A%	G%	T%	C%	A+T %	ATskewness	GCskewness
B. mori	1461	42.20	7.80	39.70	10.47	81.72	0.031	–0.146
Japanese *B. mandarina*	1463	41.90	7.79	39.71	10.59	81.61	0.026	–0.152
Chinese *B. mandarina*	1472	41.78	7.81	39.95	10.46	81.73	0.022	–0.145
O. nubilalis	1425	42.11	7.86	39.58	10.46	81.68	0.031	–0.142
O. furnicalis	1424	42.21	8.01	39.12	10.67	81.32	0.038	–0.142
A. honmai	1474	41.11	8.41	39.89	10.58	81.00	0.015	–0.114
C. raphaelis	1516	40.90	7.72	42.15	9.23	83.05	–0.015	–0.089
M. sexta	1499	41.09	8.07	40.76	10.07	81.85	0.004	–0.110
A. melete	1509	41.42	11.33	38.070	8.55	80.12	0.034	0.142
P. atrilineata	1602	41.20	8.30	40.26	10.24	81.46	0.012	–0.105
O. lunifer	1666	41.78	7.32	39.86	11.04	81.63	0.023	–0.202
rRNA								
E. pyretorum	**2116**	**41.16**	**4.82**	**43.38**	**10.63**	**84.55**	**–0.026**	**–0.376**
A. pernyi	2144	40.86	4.90	43.10	11.15	83.96	–0.027	–0.390
A. yamamai	2156	40.68	5.06	43.46	10.81	84.14	–0.033	–0.363
C. boisduvalii	2165	40.60	5.13	43.93	10.44	84.53	–0.039	–0.341
B. mori	2162	43.73	4.58	41.09	10.60	84.82	0.031	–0.397
Japanese *B. mandarin*	2160	43.89	4.63	41.30	10.19	85.19	0.030	–0.375
Chinese *B. mandarina*	2134	43.86	4.78	41.05	10.31	84.91	0.028	–0.366
O. nubilalis	1773	42.41	5.13	41.79	10.66	84.21	0.007	–0.350
O. furnicalis	1774	42.39	5.07	42.05	10.48	84.44	0.004	–0.348
A. honmai	2166	40.49	4.94	43.72	10.85	84.21	–0.038	–0.374
C. raphaelis	2107	38.93	5.03	46.56	9.49	85.48	–0.089	–0.307
M. sexta	2168	41.37	4.84	44.05	9.73	85.42	–0.031	–0.335
A. melete	2096	40.65	5.25	43.56	10.54	84.21	–0.035	–0.335
P. atrilineata	2203	42.85	4.58	43.08	9.49	85.93	–0.003	–0.349
O. lunifer	2157	41.96	4.82	40.19	13.03	82.15	0.022	–0.460
A+T-rich region								
E. pyretorum	**358**	**42.18**	**2.51**	**50.00**	**5.31**	**92.18**	**–0.085**	**–0.358**
A. pernyi	552	41.12	4.17	49.28	5.43	90.40	–0.090	–0.127
A. yamamai	334	41.62	3.59	47.90	6.89	90.40	–0.070	–0.314
C. boisduvalii	330	42.12	2.12	49.39	6.36	91.52	–0.079	–0.500
B. mori	494	44.94	1.62	50.61	2.83	95.55	–0.059	–0.272
Japanese *B. mandarina*	747	45.52	2.41	49.67	2.41	95.18	–0.043	0
Chinese *B. mandarina*	484	46.49	2.69	47.93	2.89	94.42	–0.015	–0.036
A. honmai	489	48.47	2.86	45.81	2.86	94.27	0.028	0
C. raphaelis	375	44.27	1.33	49.87	4.53	94.13	–0.059	–0.545
M. sexta	324	45.00	1.54	50.31	3.29	95.37	–0.055	–0.334
A. melete	351	43.87	3.13	45.30	7.69	89.17	–0.016	–0.421
P. atrilineata	457	40.70	0.66	57.55	1.09	98.25	–0.172	–0.246
O. lunifer	319	44.5	1.6	48.9	5.0	93.4	–0.047	–0.524

* The overlapping regions are excluded in the protein-coding genes (PCGs) and tRNAs.

a, partial mitogenome lacking in the A+T-rich region, partial *trnM* and *rrnS* sequence.

Protein-coding genes

The mitogenome of *E. pyretorum* contains the full set of PCGs usually present in metazoan mitogenomes. 13 PCGs are arranged along the genome according to the standard order of insects (Fig. 1). The putative start codons of PCGs are those previously known for metazoan mitogenomes with the typical ATN codon (six with ATG, four with ATT and one with ATA) (Table 3), with the only exceptions represented by the CGA and GTG start codons observed in *cox1* and *cox2*, respectively. Some unusual codons for *cox1* have been proposed in lepidopteran mtDNAs. In *B. mori* [2], *B. mandarina* [2, 10], *A. pernyi* [11], *A. yamamai* [12] and *C. raphaelis* [16], the tetranucleotide, TTAG, has been designated as a *cox1*start codon; whereas in *Ostrina* species it has been proposed the hexanucleotide ATTTAG [14]. Similarly to *E. pyretorum,* CGA is also used as *cox1* start codon in *A. melete* [19], *C. boisduvalii* [7], *M. sexta* [13], *A. honmai* [15], *O.*

lunifer [17] and *P. atrilineata* [18].

Eleven of the thirteen PCGs harbor the complete stop codon TAA or TAG, whereas in *cox1* and *cox2* there are incomplete T stop codons for (Table 3). Incomplete stop codons are found in many lepidopteran mitogenomes sequenced to date [2, 7, 10-18] and more in general in many arthropod mitogenomes [8]. In *E. pyretorum, cox1* and *cox2* terminated with a single T residue which is directly adjacent to a tRNA gene and to a non-coding region, respectively. The common interpretation of this phenomenon is that TAA termini are created via post-transcriptional polyadenylation [43].

The analysis of the base composition at each codon position of the concatenated 13 PCGs of *E. pyretorum* mitogenome shows that each codon position has a different AT/GC bias (Table 5). The first codon positions of *E. pyretorum* are biased toward the use of T and G. The second and third codon positions are biased toward the use of T and C. The analysis of the base composition at each codon position of the concatenated 13 PCGs of *E. pyretorum* mitogenome demonstrates that the third codon position (91.80%) is considerably higher in A+T content than the first (75.80%) and second (73.20%) ones. The result is agreed with the previous analyses performed in other lepidopteran species (Table 5). A possible explanation for the observed differences in nucleotide composition is that the constraints on A+T content in the first and second codon positions are less relaxed than those in the third codon position, due to degenerated genetic code [44].

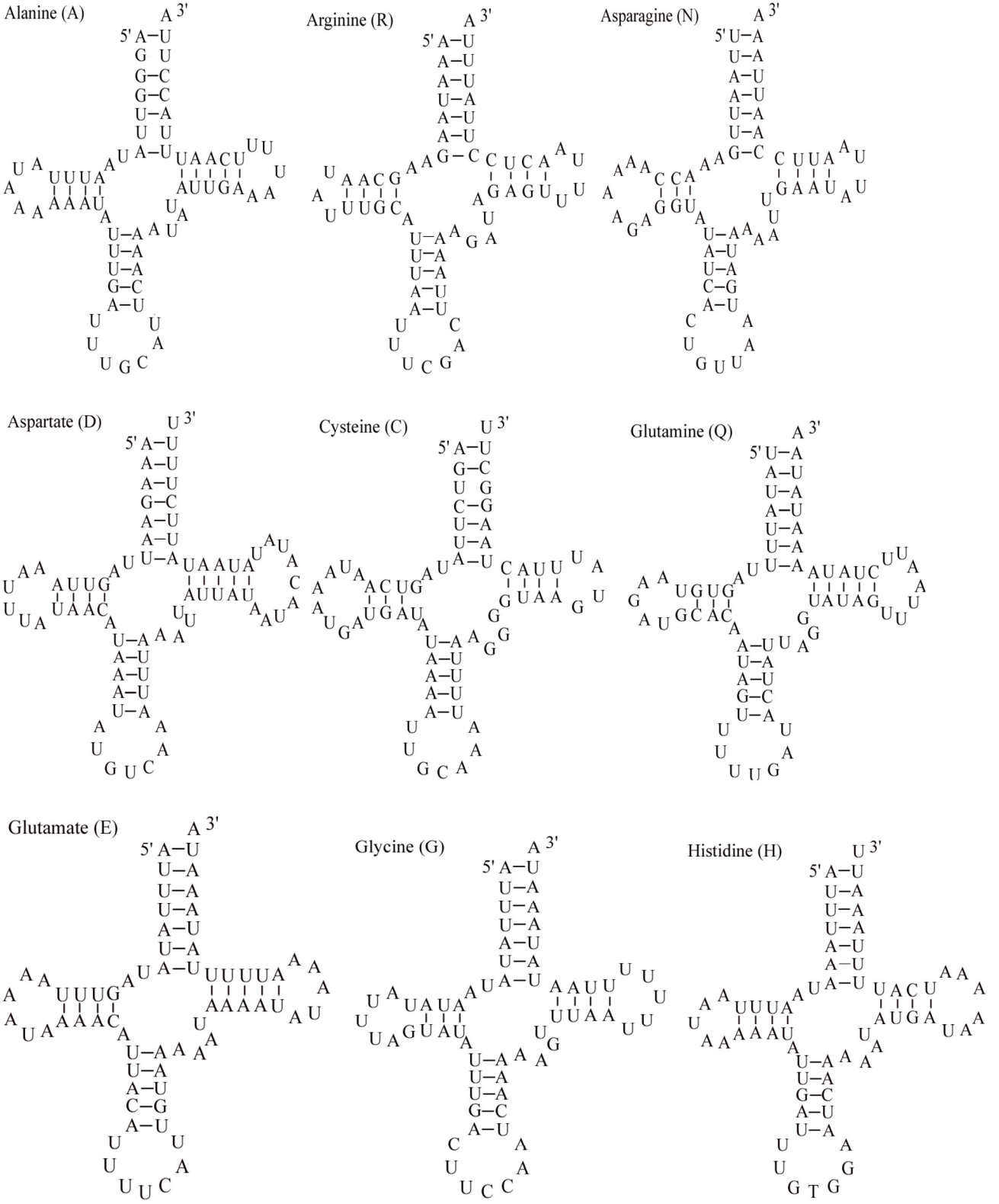
Table 5 Summary of base composition at each codon position of the concatenated 13 protein-coding genes in the lepidopteran mitogenomes

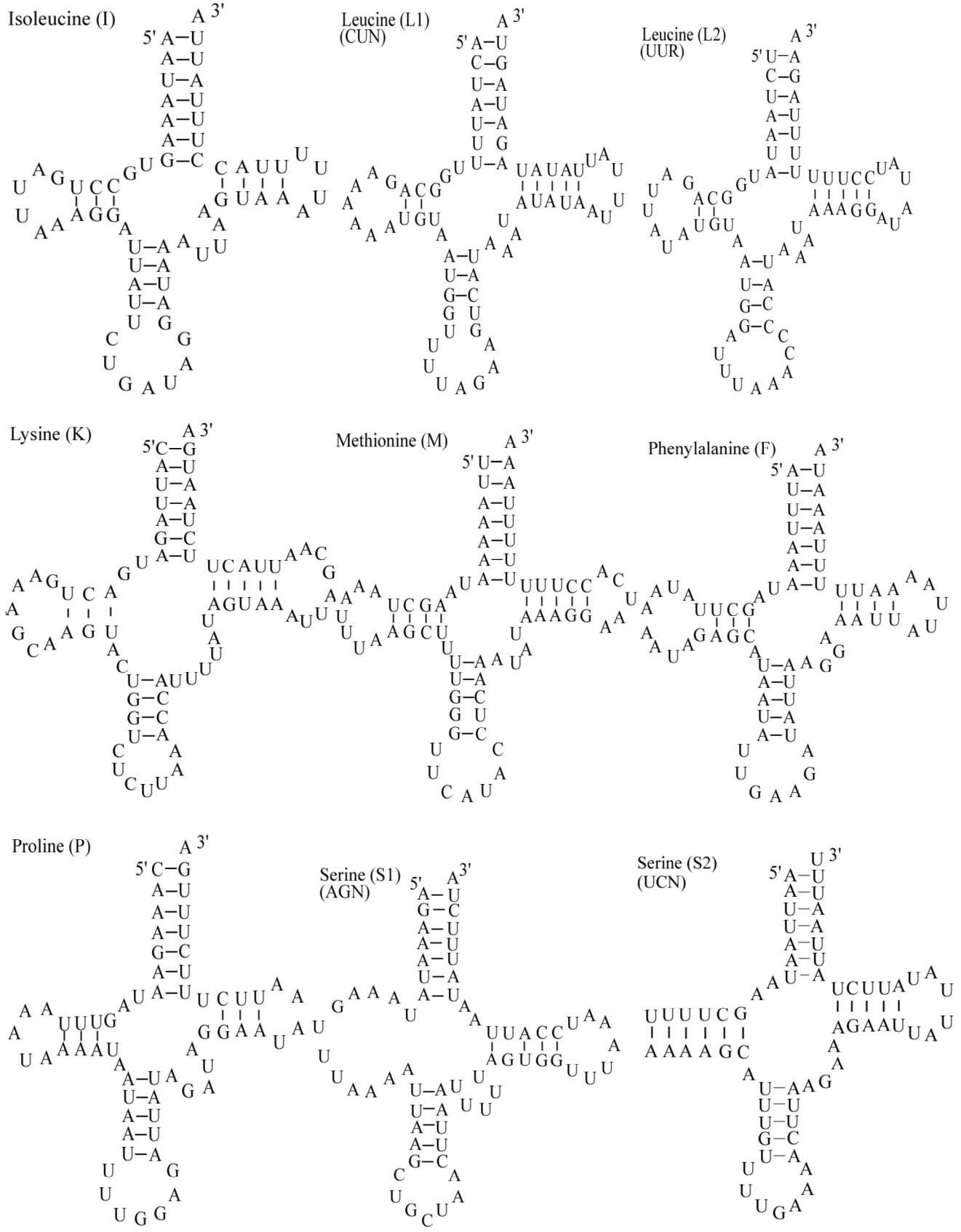
	1st codon position				2nd codon position				3rd codon position			
	%A	%T	% G	%C	% A	%T	%G	% C	%A	%T	% G	%C
E. pyretorum	**36.9**	**38.9**	**14.4**	**9.8**	**23.2**	**50.0**	**11.7**	**15.1**	**40.4**	**51.4**	**1.9**	**6.3**
A. pernyi	35.4	37.5	16.6	10.5	21.7	48.5	13.3	16.6	40.9	51.4	2.8	4.9
A. yamamai	35.6	37.2	16.5	10.7	21.6	48.7	13.2	16.5	41.4	51.9	2.6	4.1
C. boisduvalii	35.9	37.9	16.0	10.2	22.1	48.7	13.2	16.1	41.0	51.7	3.1	4.2
B. mori	37.3	37.6	9.4	15.7	22.2	48.6	15.9	13.3	43.6	49.2	4.5	2.7
Japanese *B. mandarina*	37.9	36.8	10.9	14.4	25.3	47.9	16.1	10.7	41.5	47.7	4.2	6.6
Chinese *B.mandarina*	38.2	36.9	16.1	8.8	22.9	50.7	13.4	13.0	40.6	52.7	3.4	3.3
O. nubilalis	35.8	38.3	15.1	10.8	26.0	48.6	11.2	14.2	41.0	47.6	6.3	5.1
O. furnicalis	37.7	36.8	16.1	9.4	21.6	48.7	13.3	16.3	43.7	49.6	2.7	4.0
A. honmai	36.4	36.6	16.5	10.5	22.4	48.0	13.4	16.3	41.2	50.7	3.3	4.8
C. raphaelis	38.5	37.9	14.8	8.8	22.0	49.2	13.1	15.7	45.7	51.1	1.2	2.0
M. sexta	37.1	37.7	15.7	9.5	22.2	48.8	13.1	16.0	43.7	51.3	2.5	2.6
A. melete	43.9	36.8	9.2	10.1	33.1	39.5	14.7	16.5	39.5	40.3	3.1	12.7
P. atrilineata	42.6	36.4	10.2	10.8	34.2	42.2	9.6	14.2	45.5	41.2	3.5	9.8
O. lunifer	37.2	36.2	15.2	11.4	23.2	48.9	12.3	15.6	38.7	46.6	5.2	9.5

Transfer and ribosomal RNA genes

The *E. pyretorum* tRNA genes structure was predicted using the tRNAscan-SE Search Server [25]. The predicted structure of the 22 *E. pyretorum* tRNAs is shown in Fig. 2. The tRNA genes size vary from 64 (*trnI*) to 72 *(trnD)* bp. All *E. pyretorum* tRNAs present the typical clover-leaf secondary structure previously found in mitochondrial tRNA genes except for *trnS1(AGN)* and *trnS2(UCN)*. Similarly to what has been observed in some insects, including lepidopteran species [2, 7, 10-19], and metazoan mitogenomes [8], the *E. pyretorum trnS1(AGN)* has an unusual shape, lacking a stable DHU arm (Fig. 2). Additionally, the *trnS2(UCN)* lacks a stable DHU arm (Fig. 2), an unique feature among lepidopteran mtDNAs. The anticodons of *E. pyretorum* tRNAs are all identical to those observed in lepidopterans [2, 7, 10-19].

A total of 24 unmatched base pairs occur in the *E. pyretorum* mitochondrial tRNA genes, 12 of them are G–U pairs, which form a weak bond. The *trnS2(UCN)* has two U–U mismatch in the anticodon stem, whereas *trnL1 (CUN)* and *trnA* contain an U–U mismatch in the acceptor stem,. The mismatches are scattered among 15 of the 22 *E. pyretorum* tRNA genes, including: *trnA*, *trnC*, *trnF*, *trnG*, *trnI*, *trnL1 (CUN)*, *trnL2 (UUR)*, *trnM*, *trnP*, *trnQ*, *trnS1 (AGN)*, *trnS2 (UCN)*, *trnT*, *trnV* and *trnW* (Fig. 2). Mismatches are located mostly in the acceptor, DHU and anticodon stems, with exception represented by *trnS1 (AGN)* and *trnP* that exhibits the G–U mismatch on the TΨC stem.


Alanine (A)
Arginine (R)
Asparagine (N)
Aspartate (D)
Cysteine (C)
Glutamine (Q)
Glutamate (E)
Glycine (G)
Histidine (H)


Isoleucine (I)
Leucine (L1) (CUN)
Leucine (L2) (UUR)
Lysine (K)
Methionine (M)
Phenylalanine (F)
Proline (P)
Serine (S1) (AGN)
Serine (S2) (UCN)

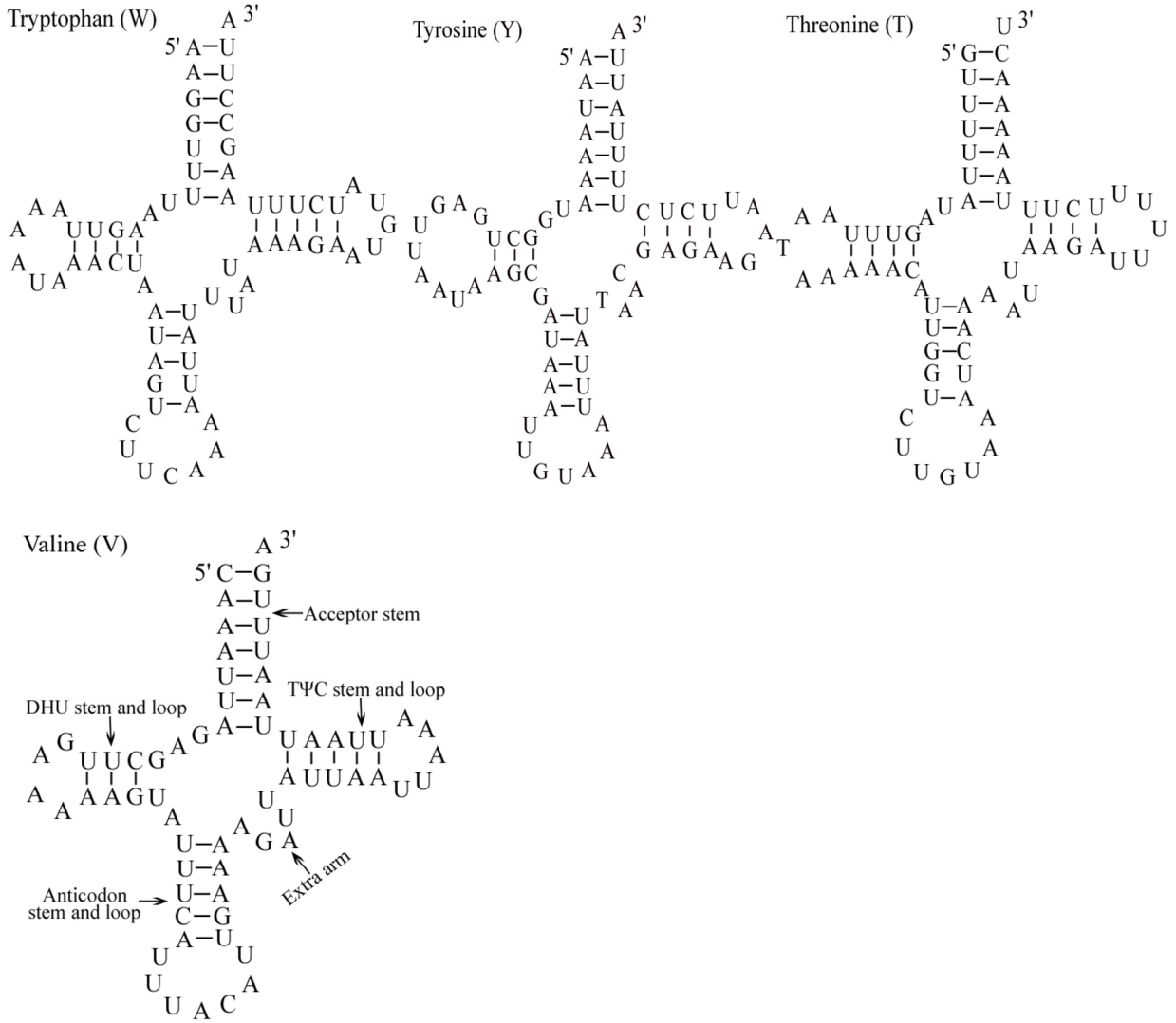


Fig. 2 Putative secondary structures for the tRNA genes of the *E. pyretorum* mitogenome.

As with all other insect mitogenome sequences, two rRNA (*rrnL* and *rrnS*) genes are present in the *E. pyretorum* mitogenome. These sequences are which located between *trnL1 (CUN)* and *trnV*, and between *trnV* and the A+T–rich region, respectively (Fig. 1). The lengths of *rrnL* and *rrnS* are1338 bp and 778 bp, respectively (Table 3), which is well within the size reported for other lepidopteran insects. The A + T content of the *rrnL* and *rrnS* genes of *E. pyretorum* mitogenome are 83.82 % and 84.45 %, respectively. These values are also well within the range reported with other lepidopteran insects (Table 4).

A+T-rich region

The *E. pyretorum* A+T-rich region extends over 358 bp and is located between *rrnS* and the *trnM-I-Q* tRNA cluster. This region is longer than that observed in *A. melete* (351 bp), *A. yamamai* (334 bp), *C. boisduvalii* (330 bp), *M. sexta* (324 bp) and *O. lunifer* (319 bp), but shorter than that of *C. raphaelis* (375 bp), *P. atrilineata* (457 bp), *A. honmai* (489 bp), *B. mori* (494 bp), *A. honmai* (489 bp), *A. pernyi* (552 bp) and *B. mandarina* (747 bp). The region contains the highest A+T content (92.18%), which is also well within the range reported for other lepidopteran insects (Table 4).

In the *E. pyretorum* A+T-rich region, there are some structures typical of other lepidopteran mitogenomes. From positions 14988 to 15011, there is a DNA fragment that includes the motif 'ATAGA' and a 19-bp poly-T stretch, which is widely conserved in lepidopteran mitogenomes [2, 7, 10-19] and has been suggested as the origin of the minority or light strand DNA replication [45]. A 10-bp poly-A is found immediately upstream of *trnM*. This poly-T element is a common feature of the A+T region of lepidopteram

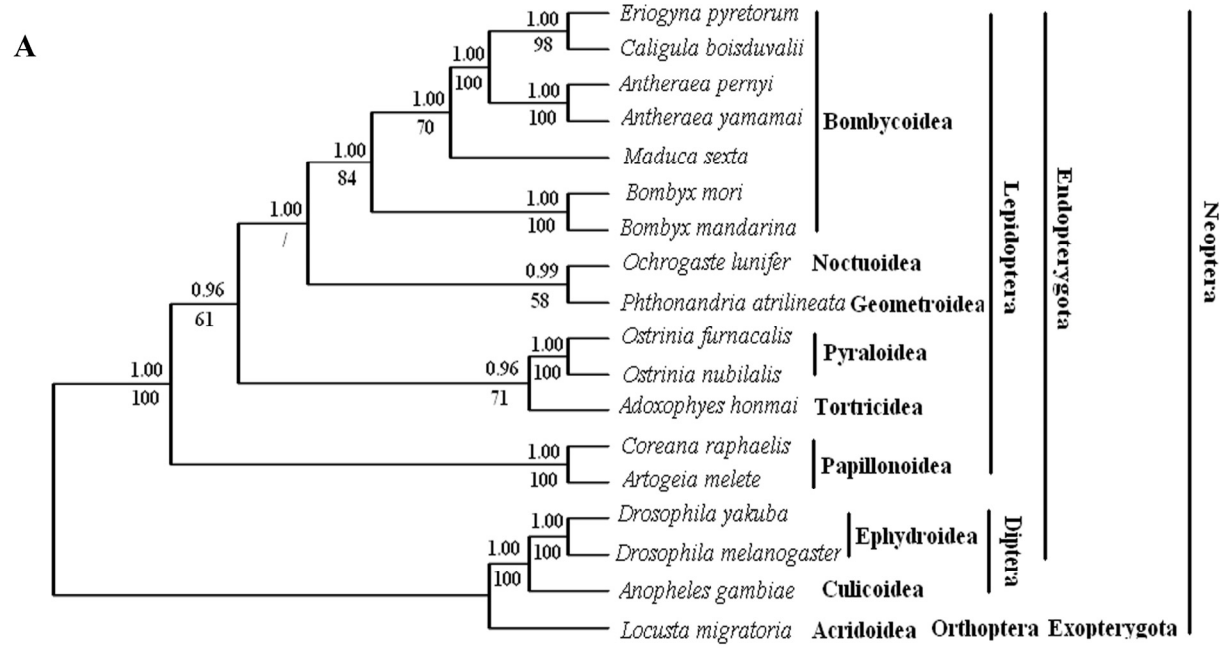
mtDNAs and is involved in controlling transcription and/or replication initiation or may have some other unknown functions [46, 47]. In *Antheraea* species (*A. pernyi*, *A. roylei* and *A. proylei*) A+T-rich region, there is a repeat unit that contain an approximately 20 bp core motif, flanked by 9 bp perfect inverted repeats [3]. This character can't be detected in the A+T-rich region of *E. pyretorum*.

Phylogenetic analyses

Phylogenetic analyses by BI and ML methods, of the concatenated nucleotide dataset (9,897 aligned sites, 66 gaps and 1073 excluded positions) and amino acid dataset (3,507 sites in length, 30 gaps and 308 excluded positions), produced similar tree topologies (Fig. 3 A and B). Both datasets yielded a topology with the relationship: Lepidoptera + (Diptera + Orthoptera). Lepidoptera as the sister clade of all other Neoptera is well supported by BI analysis and ML analysis with both nucleotide dataset (Fig. 3A) and amino acid dataset (Fig. 3B).

Among lepidopterans, the silk moths belong principally to two families, Bombycidae and Saturniidae, in the Bombycoidea superfamily. The phylogenetic analyse shows that Bombycidae (*Bombyx mori* and *Bombyx mandarina*), Sphingoidae (*Manduca sexta*) and Saturniidae (*Antheraea pernyi*, *Antheraea yamamai, E. pyretorum* and *Caligula boisduvalii)* formed a group (Fig. 3 A and B), this is in accordance with the traditional morphology-based classification [48].

These 14 sequences represent six lepidopteran superfamilies within the lepidopteran suborder: Bombycoidea, Noctuoidea, Pyraloidea, Tortricidea, Papillonoidea and Geometroidea. In our phylogenetic results, the two butterflies of Papillonoidea (*A. melete* and *C. raphaelis*) are sisters to the remaining lepidopteran superfamilies; Tortricoidea (*A. honmai*) is the sister group to the Pyraloidea (*O. furnacalis* and *O. nubilalis*) (Fig. 3A and B), which is different to the typical morphological analyses (Fig. 3C). According to the most recent consensus view of lepidopteran relationships in Kristensen & Skalski (1999) [48], Bombycoidea, Noctuoidea, Papillonoidea and Geometroidea are designated as the Macrolepidoptera; Pyraloidea together with Macrolepidoptera are designated as Obtectornera; Tortricoidea is the sisters to the remaining lepidopteran superfamilies included in the present study (Fig. 3C).



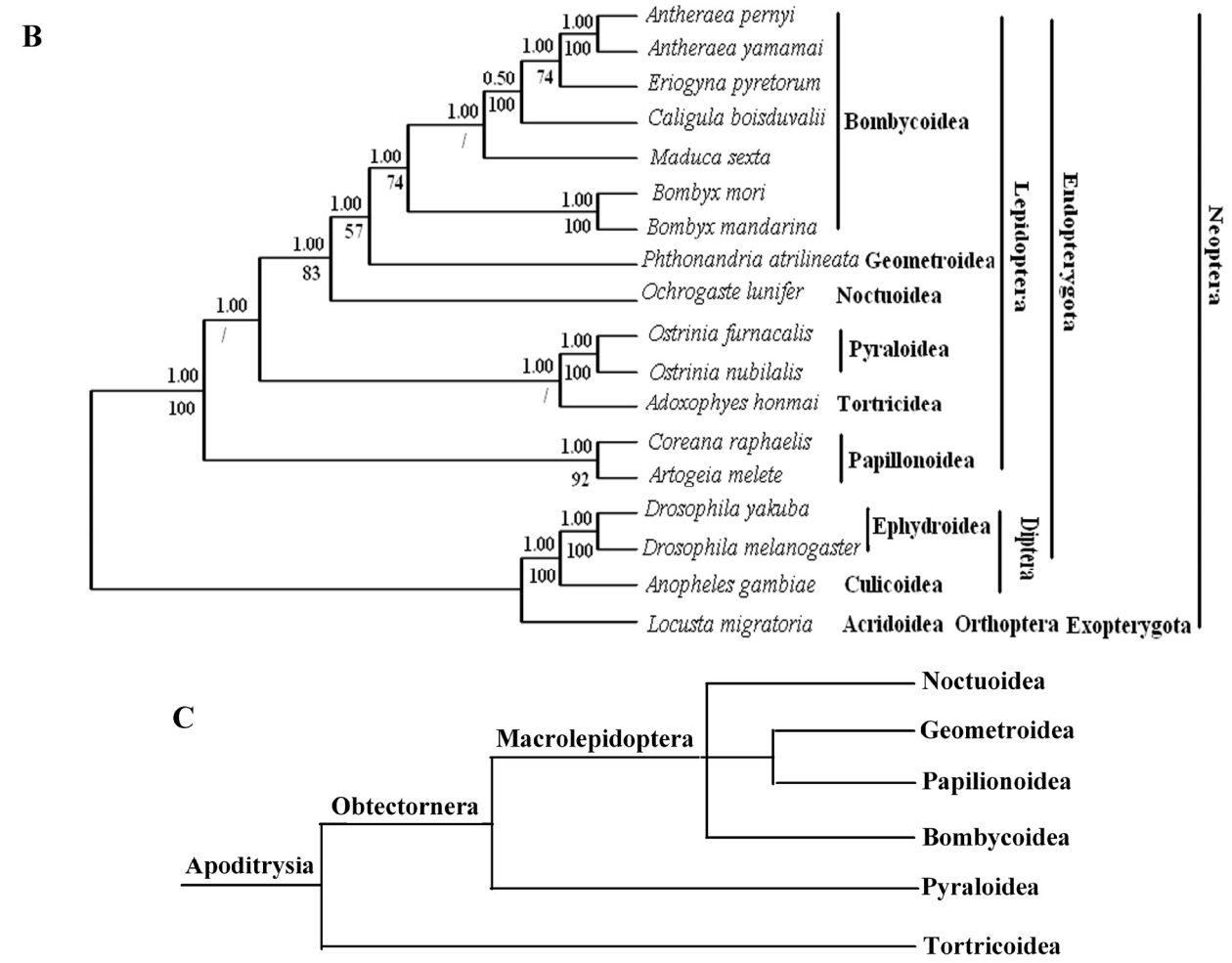


Fig. 3 Phylogeny of the lepidopteran species. Phylogenetic tree estimation from the mitogenome sequences of selected insects obtained with nucleotide dataset (A) and amino acid dataset (B) using BI analysis and ML analysis. (C) The most recent consensus view of lepidopteran relationships after Kristensen & Skalski (1999) [48]. *D. yakuba* [26], *D. melanogaster* [27], *L. migratoria* [28] and *A. gambiae* [29] were used as outgroups. The numbers above branches specify posterior probabilities from Bayesian inference (BI). The numbers under branches specify bootstrap percentages from maximum likelihood (ML,1000 replicates). /, denotes a value below 50% in the ML tree.

Acknowledgments

This study is supported by the Program for New Century Excellent Talents in University (NCET-07-0251), the grant from Science and Technological Fund of Anhui Province for Outstanding Youth (08040106803), the grant from the National Natural Science Foundation of China (30500374, 30800803), and the grant from the Natural Science Foundation of Anhui Province (KJ2008B044).

Conflict of Interest

The authors have declared that no conflict of interest exists.

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