

The complete nucleotide sequence of the mitochondrial genome of *Phthonandria atrilineata* (Lepidoptera: Geometridae)

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Abstract Using long-polymerase chain reaction (Long-PCR) method, we determined the complete nucleotide sequence of the mitochondrial genome (mitogenome) of *Phthonandria atrilineata*. The complete mtDNA from *P. atrilineata* was 15,499 base pairs in length and contained 13 protein-coding genes (PCGs), 2 rRNA genes, 22 tRNA genes, and a control region. The *P. atrilineata* genes were in the same order and orientation as the completely sequenced mitogenomes of other lepidopteran species. The nucleotide composition of *P. atrilineata* mitogenome was biased toward A + T nucleotides (81.02%), and the 13 PCGs show different A + T contents that range from 73.25% (*cox1*) to 92.12% (*atp8*). *Phthonandria* had the canonical set of 22 tRNA genes, that fold in the typical cloverleaf structure described for metazoan mt tRNAs, with the unique exception of trnS(AGN). The phylogenetic relationships were reconstructed with the concatenated sequences of the 13 PCGs of the mitochondrial genome, which confirmed that *P. atrilineata* is most closely related to the superfamily Bombycoidea.

Keywords Mitochondrial genome · Lepidoptera · Geometridae · *Phthonandria atrilineata*

Introduction

Insect mitochondrial DNA (mtDNA) is a closed-circular molecule ranging in size from 14 to 20 kb, which encodes 37 genes, including 13 protein-coding genes, two ribosomal RNA genes, and 22 tRNA genes [1]. Additionally, it contains at least one sequence of variable length known in insect mtDNA as the adenine (A) + thymine (T)-rich region, which contains initiation sites for transcription and replication [2]. The mtDNA of metazoan present the strand specific bias in nucleotide composition, where one strand is G rich, whereas the other strand is G poor, and because they show different buoyant densities in a cesium chloride gradient, they are respectively called heavy (H) and light (L) strands [3, 4]. To date, the complete or near-complete mitogenome have been sequenced from 73 insect species [5], including eight species from the order Lepidoptera: four species of *Bombyx mori*, *Bombyx mandarina* [6], *Maduca sexta* [5] and *Antheraea pernyi* (GenBank AY242996) belonging to the superfamily Bombycoidea, two species of *Ostrinia furnacalis* and *Ostrinia nubilalis* belonging to the superfamily Pyraloidea [7], two species of *Coreana raphaelis* [8] and *Artogeia melete* (GenBank EU597124) belonging to the superfamily Papilionoidea, and one specie belonging to the superfamily Tortricoidea, *Adoxophyes honmai* [9]. This study extends the description of the complete mitogenome to *Phthonandria atrilineata*, a new member of another superfamily of Lepidoptera, the Geometroidea. According to the most recent consensus view of lepidopteran relationships, the expected relationships of these species would be (((Bombycoidea) + Papillioidea + Geometroidea) + Pyraloidea) + Tortricoidea) [10].

The *P. atrilineata* is widely distributed in China, Korea, and Japan, and is a major pest to mulberry leaves. In the present study, we sequenced the complete sequence of

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mitochondrial genome of *P. atrilineata*. The gene order, the nucleotide composition of PCGs and the secondary structures of tRNA genes of mitochondrial genome of *P. atrilineata* were analyzed and compared with other lepidopteran sequences. In addition, a phylogenetic analysis of the lepidopteran superfamilies was performed.

Materials and methods

DNA samples extraction

Larvae of *P. atrilineata* were collected from mulberry leaves in Hefei, China. According to the manufacturer's instructions, total DNA was isolated from single specimens using the Takara Genomic DNA Extraction Kit (Takara Co., Dalian, China), and the DNA of the larvae of *P. atrilineata* was used for amplification of the fragments of the complete genome.

Primers design, PCR amplification, and sequencing

In order to amplify the whole mitochondrial genome of *P. atrilineata*, 24 primers were designed and synthesized

(Shanghai Sangon Biotechnology Co., Ltd., Shanghai, China). According to the One-Step PCR Amplification of Complete Arthropod Mitochondrial Genomes method [11], two degenerate primers 16SAA and 16SBB (Table 1) were used for amplification of the long fragment of the mitochondrial genome. The other degenerate and specific primers were designed based on the conserved nucleotide sequences of the known mitochondrial sequences in Lepidoptera [5–9] or the known sequences of fragments of mitochondrial genome of *P. atrilineata* that we have previously sequenced (GenBank: DQ235691 and DQ132998).

The fragment of PF1 (about 15 kb) was obtained by long-polymerase chain reaction (Long-PCR) using Takara LA Taq (Takara Co., Dalian, China): 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 long PCR product was used as a template to amplify the overlapping fragments PF2 to PF12 (Table 1).

The fragments that range from 1.3 to 2.3 kb (Table 1) were amplified using Takara LA Taq (Takara Co., Dalian, China) by PCR: 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,

Table 1 Primers used for amplification of the *Phthonandria atrilineata* mitochondrial genome

Fragment	Primer	Primer sequence	Size (kb)
PF1	16SAA	ATGCTACCTTTGCACRGTCAAGATACYGCGGC	15
	16SBB	CTTATCGAYAAAAAAGWTTGCGACCTCGATGTTG	
PF2	16SAA	ATGCTACCTTTGCACRGTCAAGATACYGCGGC	1.3
	PF2R	ACTAGGATTAGATACCCTAT	
PF3	PF3F	TCCAGTACCT CTA CTTTGT T ACG	1.8
	PF3R	TCA(A/G)AAATG(A/G)AA(G/T/A)GG(G/T)GCTGATCCTAT	
PF4	PF4F	TGATTTGG(T/A/G)TGTTGAATTGG(A/C)(T/C)TAGAA	1.6
	PF4R	GCTCCTAAAATTGAAGAAATCCAGC	
PF5	PF5F	TGGTGCAGGAACAGGATGAAC	1.9
	PF5R	GAGACCA(G/T)TACTTGCTTTCAG	
PF6	PF6F	ATTTGTGGAGCTAATCATAG	1.1
	PF6R	GGTCAGGG(T/A)CTATAATC(T/C)AC	
PF7	PF7F	TCGACCTGGAACCTTTCAGC	1.2
	PF7R	TGGATCAAATCCACATTCA	
PF8	PF8F	AGCAGCAGCTTGGTATTGAC	2.3
	PF8R	TATT(A/G)GG(A/T)TG(A/G)GATGGTTTAGG	
PF9	PF9F	GCTGGTAA(T/C)CAAGA AACTAAA(A/T)GGA	2.1
	PF9R	GTTTGTGA(G/A)GGT(G/T)CTTTAGG	
PF10	PF10F	CCTGAAAC(A/T)GG(A/G)GC(C/T)TCAACATGAGC	2.3
	PF10R	CAATCCTAGAGGATTATTAGAACCTG	
PF11	PF11F	TTTGAGGGGGTTTGCAGTAGA	1.0
	PF11R	GAATATAGAAGAGGGGGATTTCG	
PF12	PF12F	CATAACCTCCTAAAATAAAC	1.1
	16SBB	CTTATCGAYAAAAAAGWTTGCGACCTCGATGTTG	
PF13	PF13F	CGGTT TGA AACTCAGA TCATGTAAG	1.1
	PF13R	TATTGTATCTTGTGTATCAGAGTTTA	

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 [12]. The positive recombinant clone with an insert was sequenced using the dideoxynucleotide chain termination method (Takara Co., Dalian, China) at least 3 times.

Gene identification and sequence analysis

Protein-encoding genes of the *P. atrilineata* mitochondrial genome were identified by sequence similarity of other lepidopteran species [5–9]. 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/>) [13]. Alignments of the protein-encoding genes for each of the available lepidopteran mitogenomes were made in MEGA ver4.0 [14]. All regions involving ambiguity for the position of the gaps were excluded from the analyses to avoid erroneous hypotheses of primary homology [4]. The reduced alignment of mt sequences consists of 11,450 nucleotides and 3,802 amino acids. Based on the concatenated amino acids and nucleotide data sets from the 13 PCGs, the phylogenetic analyses were performed using maximum likelihood (ML), maximum parsimony (MP) and neighbor-joining (NJ) methods. MP and NJ analyses were carried out using MEGA ver4.0 [14]. ML analyses and bootstrap resampling were performed using the set of programs in the PHYLIP package [15]. The mitochondrial genomes of *Drosophila melanogaster* [16] and *Locusta migratoria* [17] were used as outgroups.

For ML analyses (PHYLIP package), the concatenated amino acids /nucleotide sequence of *P. atrilineata* mitochondrial genome was aligned with the sequences of other species, using CLUSTALX 1.83 [18]. These aligned sequences were entered into the subprogram SEQBOOT (bootstrap sequence data sets) of the PHYLIP package to create 100 datasets by bootstrap resampling. These 100 datasets were used as input to generate the 100 most parsimonious trees using the subprogram PROML/DNAML. During the creation of the 100 most parsimonious trees, all the sequences were randomly entered into each dataset 10 times, based on the random number 3. Thus, the resulting output was based on 100 × 10 runs of all the sequences, with *D. melanogaster* [16] and *L. migratoria* [17] used as the 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 [13]. For NJ and MP analyses, a distance matrix was constructed from the aligned sequences using Kimura two-parameter formula [19]. The bootstrap test (1,000 repeats) was used to determine the reliability of the different branches in the NJ dendrogram [14].

Results and discussion

Genome organization and base composition

The mitogenome of *P. atrilineata* is a closed-circular molecule of 15,499 bp (GenBank EU569764) (Fig. 1), the size of which is comparable to other lepidopteran mitogenomes, which range from 15,314 bp in *Coreana raphaelis* [8] to 15,928 bp in *Bombyx mandarina* [6]. The gene content is typical of other metazoan mitochondrial genomes: 13 PCGs (*cox1-3*, *nad1-6*, *nad4L*, *cob*, *atp6* and *atp8*), 22 tRNA genes (one for each amino acid, two for Leucine and Serine), and two for mitochondrial ribosomal RNAs (*rrnS* and *rrnL*). There is a 457 bp A + T-rich region between *rrnS* and *trnI*, and it was deemed homologous to the control region (CR) by positional homology, general structure, and base content. Gene order follows the other Lepidoptera arrangement. When searching tRNA

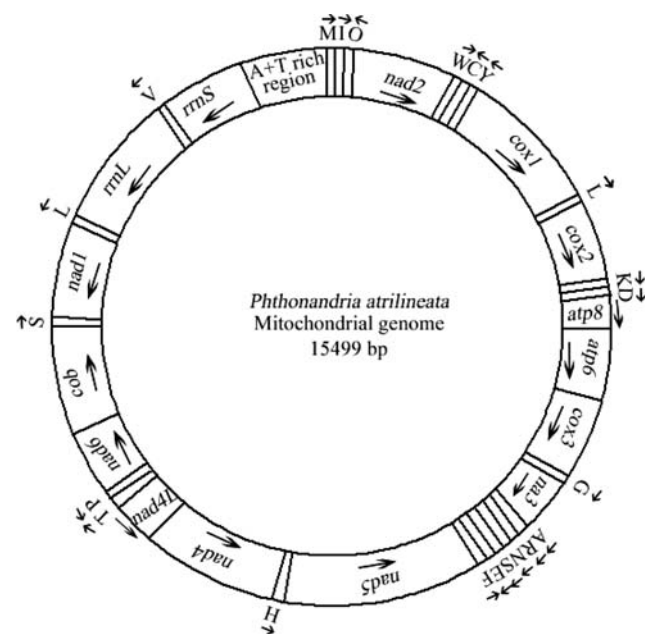


Fig. 1 Circular map of the mitochondrial genome of *Phthonandria atrilineata*. The abbreviations for the genes are as follows: *cox1*, *cox2*, and *cox3* refer to the cytochrome oxidase subunits, *cob* refers to cytochrome *b*, and *nad1-6* refers to NADH dehydrogenase components. tRNAs are denoted as one-letter symbol according to the IUPAC-IUB single-letter amino acid codes. Direction of gene transcription is indicated with an arrow

Table 2 A + T contents of 13 protein-coding genes from Lepidoptera mitogenome

	Pha	Mas	Adh	Anp	Bma	Bom	Osf	Osn	Cor	Arm	Drm	Lom
Genome	81.02	81.79	80.39	80.15	81.68	81.32	80.37	80.17	82.66	79.77	82.16	75.32
atp6	79.20	80.97	77.97	78.61	79.35	79.79	78.96	78.67	81.48	79.56	76.59	74.63
atp8	92.12	89.70	89.51	91.67	90.12	87.65	93.21	92.59	90.57	89.51	83.33	83.65
cox1	73.25	73.68	71.31	70.98	72.35	72.75	71.48	71.03	74.51	71.44	70.90	69.01
cox2	76.51	77.97	76.36	76.36	77.24	76.21	77.09	76.95	78.98	77.73	73.83	71.05
cox3	73.26	75.76	72.50	71.74	73.38	74.52	74.12	74.24	76.55	73.61	70.72	68.94
cob	76.39	77.58	75.48	75.98	77.89	76.22	76.09	76.27	78.30	76.13	74.05	71.14
nad1	77.81	79.17	77.53	77.74	78.94	78.99	77.96	77.63	81.85	77.56	77.74	75.80
nad2	84.53	85.60	83.88	82.15	86.41	85.87	85.63	85.83	86.88	85.31	82.85	74.83
nad3	81.07	84.18	80.23	81.64	82.91	83.19	83.05	82.34	83.90	79.10	80.79	77.59
nad4	80.78	81.95	80.51	81.21	81.43	81.08	80.99	80.85	83.96	79.37	80.27	76.93
nad4L	84.54	85.07	85.22	85.22	83.85	83.51	85.03	84.35	87.02	84.88	82.13	76.19
nad5	81.46	83.04	81.50	81.71	81.68	82.20	82.79	82.21	84.06	80.41	78.86	76.69
nad6	87.78	87.57	85.80	85.66	86.63	87.76	86.96	86.41	88.76	83.90	85.33	81.03

Pha, *Phthonandria atrilineata* (EU569764); Cor, *Coreana raphaelis* (NC_007976); Adh, *Adoxophyes honmai* (NC_008141); Mas, *Maduca sexta* (EU286785); Anp, *Antheraea pernyi* (AY242996); Bma, *Bombyx mandarina* (NC_003395); Bom, *Bombyx mori* (AB070264); Osf, *Ostrinia furnacalis* (NC_003368); Osn, *Ostrinia nubilalis* (NC_003367); Arm, *Artogeia melete* (EU597124); Lom, *Locusta migratoria* (NC_001712); Drm, *Drosophila melanogaster* (NC_001709)

genes, tRNA-Scan-SE failed to find a copy of *trnS*(UCN) and *trnL*(UUR), which was determined by sequence comparison of the region in which this gene occurs in other insect species.

Likewise to other insect mtDNA sequences, the nucleotide composition of the *P. atrilineata* mitogenome is also biased toward A + T nucleotides (81.02%), which is lower than *M. sexta* (81.79%), *B. mandarina* (81.68%), *B. mori* (81.2%) and *C. raphaelis* (82.66%), and higher than the other five lepidopterans. Within 13 protein-coding genes (PCGs), the A + T composition in the *atp8* gene is the highest (92.12%), and 73.25% for the *cox1* gene, with this value the lowest (Table 2).

Protein-coding genes

The mitochondrial genome of *P. atrilineata* encodes the regular set of 13 PCGs (Fig. 1) found, with few exceptions, in all insect mitochondrial genomes. Reading frames for protein-coding genes were determined by comparison with alignments of other insect mitochondrial genes and by looking at start and stop codons.

The start and stop codons of the 13 protein-coding genes in the *P. atrilineata* mitochondrial genome are shown in Table 3. Six protein-coding genes share the start codon ATG (*cox2*, *atp6*, *cox3*, *nad4*, *nad4L* and *cob*), four genes start with ATA (*nad1*, *nad2*, *nad6*, *nad8* and *atp8*), and *nad3* and *nad5* start with ATT, while *cox1* starts with CGA (Table 3). For *cox1* none of the lepidopterans start with a typical ATN codon, rather all start with CGA and *D. melanogaster* starts with TCG. The atypical start codon

of mitochondrial *cox1* gene had also been observed and extensively discussed in other insect and arthropod species; suggestions include one hexanucleotide (ATTTAA) [20] and some tetranucleotides (ATAA, TTAA, and ATTA) [16, 17]. Eight of the protein-coding genes stop with TAA; *nad4L* uses TAG instead of TAA as a stop codon. Four of the PCGs had incomplete stop codons consisting of just a T-nucleotide, including *cox1*, *cox2*, *nad4* and *nad5* (Table 3). There is also a high degree of conservation of incomplete stop codons across the order; *cox1* and *cox2* have incomplete stop codons in all lepidopteran species and *nad5* has one in all species with the exception of *Bombyx* [5–9].

Transfer RNA and ribosomal RNA genes

Phthonandria had the typical set of 22 tRNA genes, which are interspersed between ribosomal RNAs and protein-encoding regions, range in size from 64 to 71 nucleotides. About 14 tRNAs are codified by the H-strand and eight are codified by the L-strand, which similar to the organization in other lepidopterans. All *P. atrilineata* tRNAs have the typical cloverleaf structure of mt tRNAs (Fig. 2), except for the *trnS*(AGN), wherein the dihydrouridine (DHU) arm forms a simple loop (Fig. 2) as seen in many metazoan mtDNAs [21, 22]. The anticodons of *P. atrilineata* tRNAs were all identical to their counterpart tRNAs of the sequenced lepidopterans [5–9]. As reported previously, the *trnM* is quite well conserved, showing high identity with the sequence found in *M. sexta* [5], *B. mandarina*, *B. mori* [6] and *C. raphaelis* [8].

Table 3 Summary of the mitogenome of *Phthonandria atrilineata*

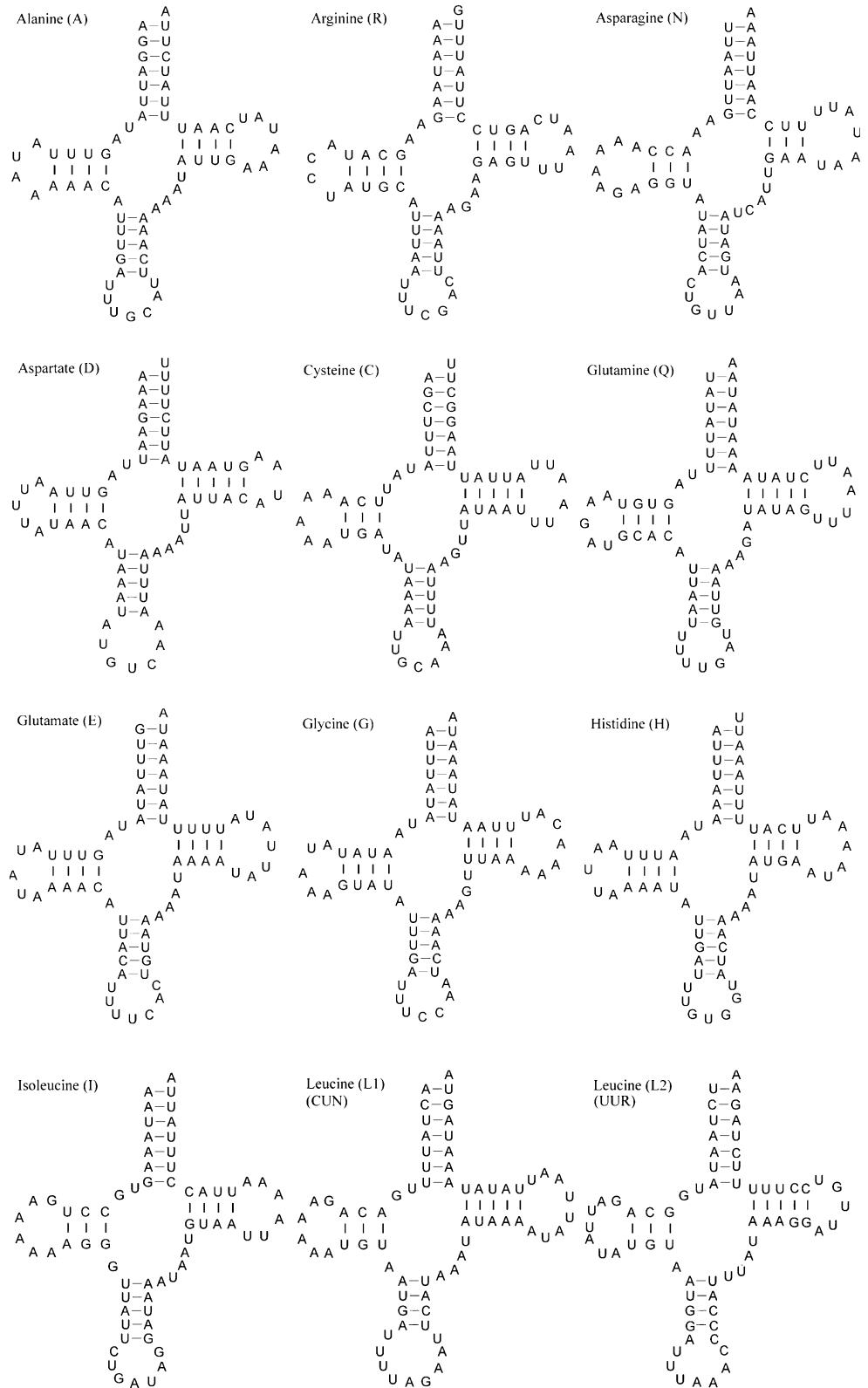
Gene	Direction	Location	Size	Anticodon	Start codon	Stop codon
trnM	F	1–68	68	CAT32-34	–	–
trnI	F	69–134	66	GAT98-100	–	–
trnQ	R	200–132	69	TTG170-168	–	–
nad2	F	264–1265	1002	–	ATA	TAA
trnW	F	1277–1346	70	TCA1309-1311	–	–
trnC	R	1406–1339	68	GCA1376-1374	–	–
trnY	R	1483–1418	66	GTA1452-1450	–	–
cox1	F	1487–3017	1531	–	CGA	T
trnL2(UUR)	F	3018–3084	67	TAA3048-3050	–	–
cox2	F	3085–3766	682	–	ATG	T
trnK	F	3767–3837	71	CTT3797-3799	–	–
trnD	F	3838–3903	66	GTC3868-3870	–	–
atp8	F	3904–4068	165	–	ATA	TAA
atp6	F	4062–4739	678	–	ATG	TAA
cox3	F	4749–5537	789	–	ATG	TAA
trnG	F	5550–5616	67	TCC5580-5582	–	–
nad3	F	5617–5970	354	–	ATT	TAA
trnA	F	5974–6038	65	TGC6003-6005	–	–
trnR	F	6038–6102	65	TCG6067-6069	–	–
trnN	F	6107–6172	66	GTT6137-6139	–	–
trnS1(AGN)	F	6172–6239	68	GCT6198-6200	–	–
trnE	F	6239–6305	67	TTC6269-6271	–	–
trnF	R	6370–6304	65	GAA6337-6335	–	–
nad5	R	6356–8087	1732	–	ATT	T
trnH	R	8172–8106	67	GTG8142-8140	–	–
nad4	R	8173–9526	1354	–	ATG	T
nad4L	R	9563–9853	291	–	ATG	TAG
trnT	F	9856–9919	64	TGT9886-9888	–	–
trnP	R	9984–9920	65	TGG9954-9952	–	–
nad6	F	9987–10526	540	–	ATA	TAA
cob	F	10528–11679	1152	–	ATG	TAA
trnS2(UCN)	F	11679–11747	68	TGA11710-11712	–	–
nad1	R	11766–12698	933	–	ATA	TAA
trnL1(CUN)	R	12773–12705	69	TAG12744-12742	–	–
rrnL	R	12774–14173	1400	–	–	–
trnV	R	14239–14174	66	TAC14210-14208	–	–
rrnS	R	14240–15042	803	–	–	–
D-loop		15043–15499	457	–	–	–

The *rrnL* and *rrnS* genes in *P. atrilineata* are 1,400 and 803 nucleotides long, respectively. Both ribosomal RNA genes show great sequence similarity to other lepidopterans. The base composition of the *rrnL* is 42.92% A, 9.51% C, 4.72% G, and 42.85% T. The *rrnS* composition is 42.82% A, 9.36% C, 4.37% G, and 43.45% T. The A + T content of *rrnL* and *rrnS* gene are 85.77% and 86.27%, respectively. These values are high but within the range of those found in the insect species sequenced to date.

Phylogenetic analysis

In the present study, the sequences of the 13 PCGs of the mitochondrial genome were concatenated, rather than analyzed separately, to reconstruct the phylogenetic relationships, which may result in a more complete analysis [23]. All the ML, MP and NJ analyses clustered *P. atrilineata*, *B. mandarina*, *B. mori*, *A. pernyi*, and *M. sexta* in one branch (Fig. 3a–f). The phylogenetic relationships

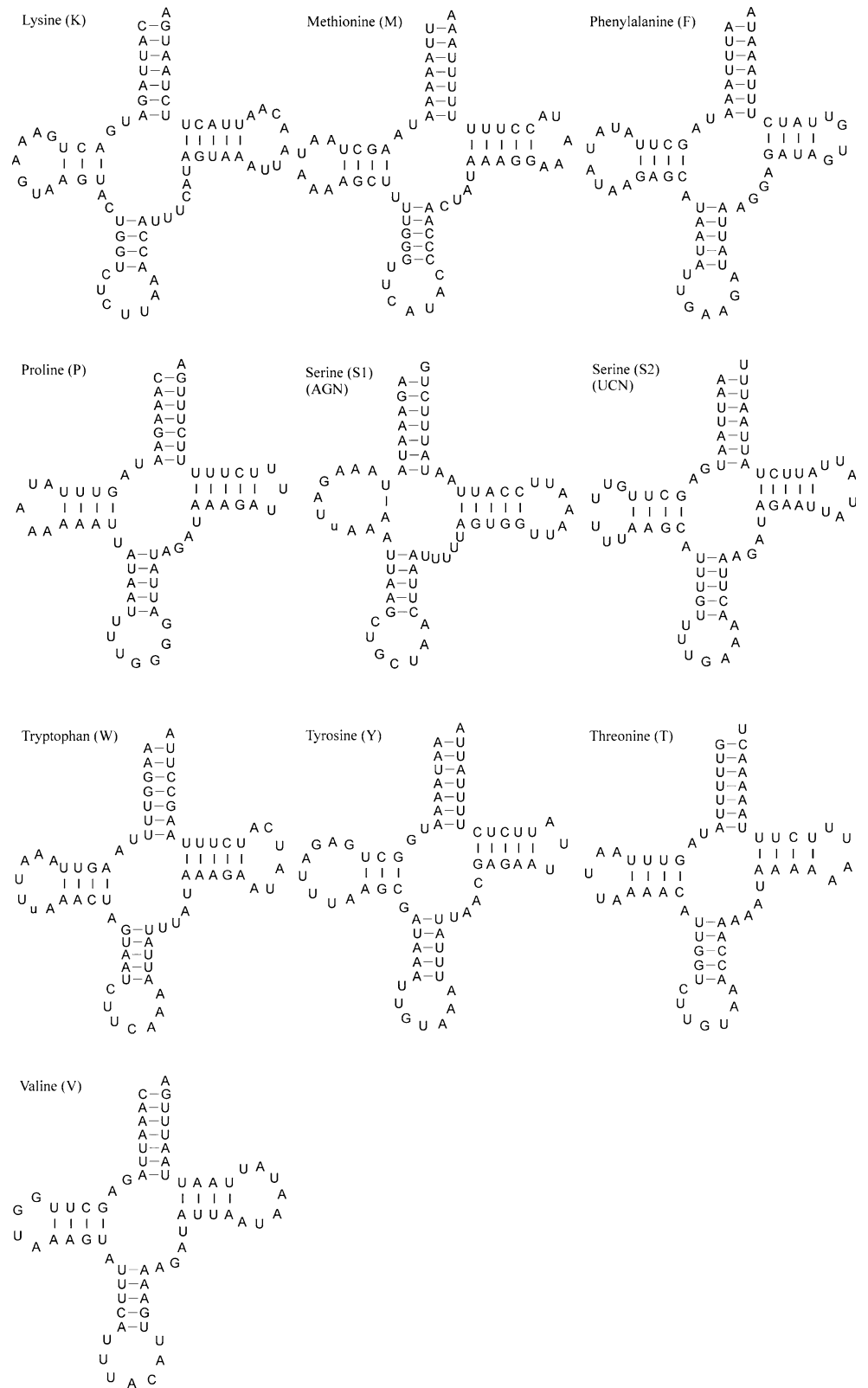
Fig. 2 Predicted secondary cloverleaf structure for the tRNA genes of *Phthonandria atrilineata*



confirmed that *P. atrilineata* is most closely related to the superfamily Bombycoidea, which was in accordance with the phylogeny of lepidopteran superfamilies proposed by Kristensen and Skalski [10].

Exception of the ML tree based on the concatenated amino acid sequences, the other five trees showed similar topology (Fig. 3b–f). The 10 species of Lepidoptera was divided into three clades: two species of the superfamily Papilionoidea (I);

Fig. 2 continued



two species of the superfamily Pyraloidea and *A. honmai* of Tortricoidea (II); four species of the superfamily Bombycoidea and *P. atrilineata* of Geometroidea (III). The ML tree

based on amino acid sequences (Fig. 3a) showed that *A. honmai* was not clustered to the two species of the superfamily Pyraloidea firstly. Thus we divided *A. honmai* as a

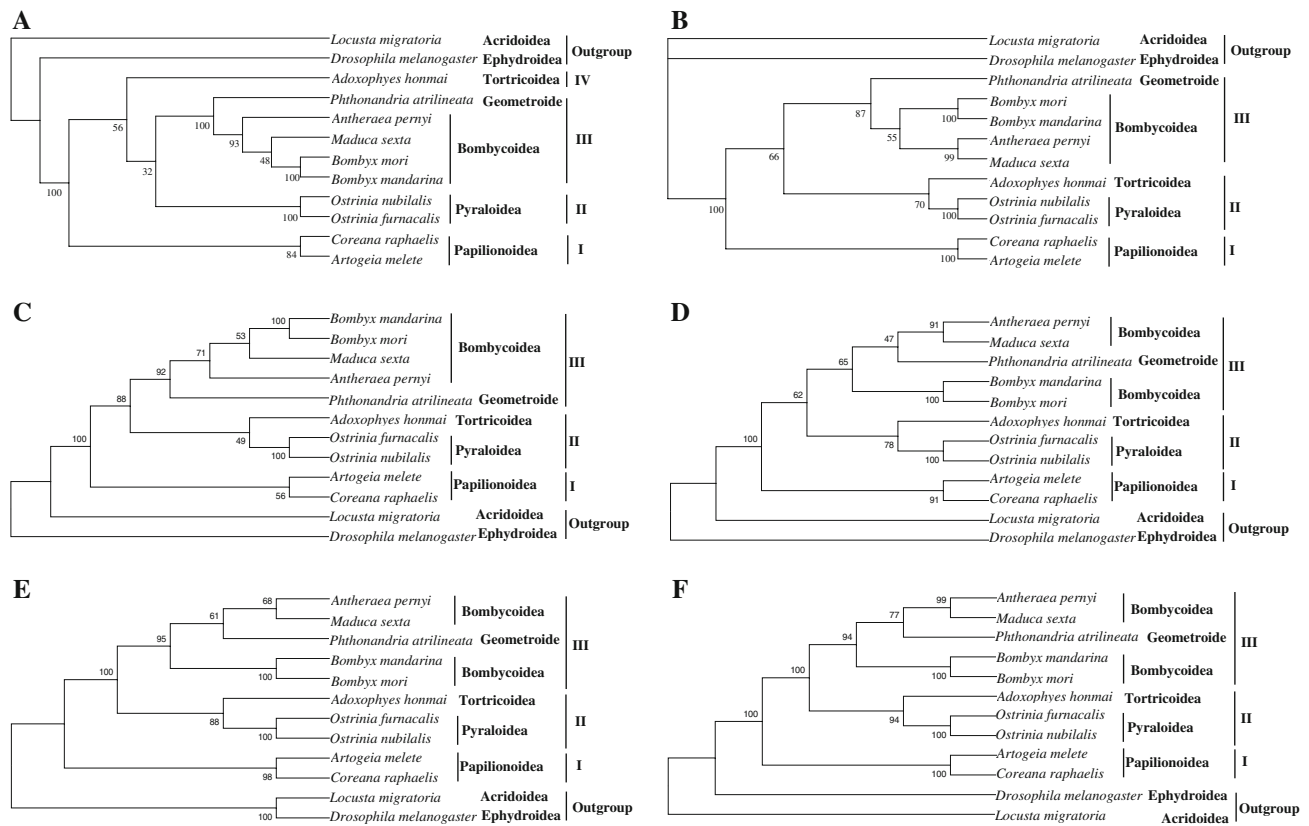


Fig. 3 Phylogeny of the lepidopteran species. Phylogenetic analysis were performed by maximum likelihood (**a, b**), maximum parsimony (**c, d**) and neighbor-joining methods (**e, f**), using the nucleotide (**b, d** and **f**) and amino acid sequences (**a, c** and **e**) of the concatenated 13 PCGs. The sequences of *Locusta migratoria* (NC_001712) and *Drosophila melanogaster* (NC_001709) were used as outgroups. *Phthonandria*

atrilineata (EU569764); *Coreana raphaelis* (NC_007976); *Adoxophyes honmai* (NC_008141); *Maduca sexta* (EU286785); *Antheraea pernyi* (AY242996); *Bombyx mandarina* (NC_003395); *Bombyx mori* (AB070264); *Ostrinia furnacalis* (NC_003368); *Ostrinia nubilalis* (NC_003367); *Artogeia melete* (EU597124)

separate clade (IV). In addition, the bootstrap score of the clades were different when reconstructed based on the two type sequences, or using different methods.

In our results, the two butterflies (*A. melete* and *C. raphaelis*) are the sister to the remaining lepidopteran superfamilies (Fig. 3a–f); *A. honmai* is the sister group to the crambids (*Ostrinia*) (Fig. 3b–f), which was different to the lepidopteran relationships in Kristensen and Skalski [10]. Regier et al. [24] demonstrated that Tortricoidae was the sister to the other 5 superfamilies included in the present study when their phylogenetic relationships were reconstructed using the period gene [24]. In contrast to our findings, butterflies (Papilionoidea) are typically considered to be very highly derived lepidopterans, not the sister of the remaining macrolepidopteran families; *Adoxophyes* would be expected to be the sister to all other families based on previous work, not the sister group to the crambids (*Ostrinia*) [10].

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