International Journal of Biological Sciences
2010; 6(2):172-186

Research Paper

The complete mitochondrial genome of the fall webworm, *Hyphantria cunea* (Lepidoptera: Arctiidae)

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Received: 2010.02.03; Accepted: 2010.03.26; Published: 2010.03.29

Abstract

The complete mitochondrial genome (mitogenome) of the fall webworm, *Hyphantria cunea* (Lepidoptera: Arctiidae) was determined. The genome is a circular molecule 15 481 bp long. It presents a typical gene organization and order for completely sequenced lepidopteran mitogenomes, but differs from the insect ancestral type for the placement of $tRNA^{Met}$. The nucleotide composition of the genome is also highly A + T biased, accounting for 80.38%, with a slightly positive AT skewness (0.010), indicating the occurrence of more As than Ts, as found in the Noctuoidea species. All protein-coding genes (PCGs) are initiated by ATN codons, except for *COI*, which is tentatively designated by the CGA codon as observed in other lepidopterans. Four of 13 PCGs harbor the incomplete termination codon, T or TA. All tRNAs have a typical clover-leaf structure of mitochondrial tRNAs, except for $tRNA^{Ser}(AGN)$, the DHU arm of which could not form a stable stem-loop structure. The intergenic spacer sequence between $tRNA^{Ser}(AGN)$ and *ND1* also contains the ATACTAA motif, which is conserved across the Lepidoptera order. The *H. cunea* A+T-rich region of 357 bp is comprised of non-repetitive sequences, but harbors several features common to the Lepidoptera insects, including the motif ATAGA followed by an 18 bp poly-T stretch, a microsatellite-like $(AT)_8$ element preceded by the ATTTA motif, an 11 bp poly-A present immediately upstream $tRNA^{Met}$. The phylogenetic analyses support the view that the *H. cunea* is closerly related to the *Lymantria dispar* than *Ochrogaster lunifer*, and support the hypothesis that Noctuoidea (*H. cunea*, *L. dispar*, and *O. lunifer*) and Geometroidea (*Phthonandria atrilineata*) are monophyletic. However, in the phylogenetic trees based on mitogenome sequences among the lepidopteran superfamilies, Papillonoidea (*Artogeia melete*, *Acraea issoria*, and *Coreana raphaelis*) joined basally within the monophyly of Lepidoptera, which is different to the traditional classification.

Key words: Fall webworm; *Hyphantria cunea*; Mitochondrial genome; Lepidoptera; Arctiidae; Phylogeny

1. Introduction

The fall webworm, *Hyphantria cunea* Drury (Lepidoptera: Arctiidae), is a severe invasive and quarantine pest which has a wide range of habitats. It is a polyphagus pest that feeds on about 160 species of broad leaf trees. The preferred host plants include mulberry, oak, hickory, pecan, walnut, elm, alder,

willow, sweetgum, and poplar. This insect has caused serious damage to forests throughout its range and appears to be continuing to spread. It also damages the roadside and garden trees around urban areas. The species was introduced from North America to Central Europe and Eastern Asia in the early 1940s [1, 2]. In China, this species was first found in Dandong (124°N/40°E) of Liaoning Province in 1979, and now has spread southwards to Shanghai (129°N/31°E) and westwards to Xianyang (108°N/34°E) of Shanxi Province. The southern populations in China may complete three generations in one year, while in the north the fall webworm completes only one life cycle. Many studies have been done on aspects of adaptability, sex pheromones, host preference and natural enemies of the fall webworm [3]. As *H. cunea* is a devastating invasive species, the mitochondrial genome (mitogenome) information of the species may provide fundamental information for future phylogenetic analyses and evolutionary biology.

Insect mitochondrial DNA (mtDNA) is a circular DNA molecule with 14-20 kb in size and has a remarkably conserved set of 37 genes, including 13 protein-coding genes (PCGs; subunits 6 and 8 of the F_0 ATPase [*ATP6* and *ATP8*]; cytochrome oxidase subunits 1-3 [*COI–III*]; cytochrome b [*Cytb*]; NADH dehydrogenase subunits 1-6 and 4L [*ND1–6* and *ND4L*]), two ribosomal RNA genes (large and small ribosomal RNAs [*lrRNA* and *srRNA*]), and 22 tRNA genes [4, 5]. It additionally contains a control region of variable length, known as the adenine (A) + thymine (T)-rich region in insect mtDNA, which is involved in the regulation and initiation of mtDNA replication and transcription [6]. The mitochondrial genes and genomes have been widely used as an informative molecular marker for diverse evolutionary studies of animals, including phylogenetics and population genetics [7-9], with the development of long range PCR for amplification of partial sequence of mtDNA genes and whole mitochondrial genome [10].

At present, the complete or nearly complete mitogenome sequences from more than 100 species of insects have been determined. However, only 19 complete or nearly complete mitogenomes are currently available in the GenBank for lepidopteran species (Table 1). The *Ostrinia* sequences each lack the sequence information of the A+T-rich region, partial $tRNA^{Met}$ and *srRNA* sequence. The *L. chinensis* and *P. xuthus* sequences each lack the sequence information of the partial *srRNA*, A+T-rich region, $tRNA^{Met\text{-}Ile\text{-}Gln}$, and partial *ND2*. Within the insects, the Lepidoptera order accounts for more than 160 000 species. Despite this huge taxonomic diversity the existing information on lepidopteran mtDNA is very limited and limited to six superfamiles among the 45-48 known and to 13 families of the recognized 120. Newly added lepidopteran mitogenomes can provide further insights into our understanding of diversity of lepidopteran mitogenomes and evolution.

Table 1 List of the complete mitogenome of Lepidoptera

Superfamily / Family	Species	Acc. number	Reference
Noctuoidea			
Arctiidae	*Hyphantria cunea*	GU592049	This study
Lymantriidae	*Lymantria dispar*	FJ617240	Zhu et al. unpublished
Notodontidae	*Ochrogaster lunifer*	AM946601	[11]
Geometroidea			
Geometridae	*Phthonandria atrilineata*	EU569764	[12]
Bombycoidea			
Saturniidae	*Antheraea pernyi*	AY242996	[13]
Saturniidae	*Antheraea yamamai*	EU726630	[14]
Saturniidae	*Caligula boisduvalii*	EF622227	[15]
Saturniidae	*Eriogyna pyretorum*	FJ685653	[16]
Bombycidae	*Bombyx mori*	AB070264	[17]
Bombycidae	Chinese *Bombyx mandarina*	AY301620	[18]
Bombycidae	Japanese *Bombyx mandarina*	NC_003395	[17]
Sphingidae	*Manduca sexta*	EU286785	[19]
Pyraloidea			
Crambidae	*Diatraea saccharalis*	FJ240227	Li and Yue, unpublished
Crambidae	*Ostrinia furnicalis*	NC_003368	[20]
Crambidae	*Ostrinia nubilalis*	NC_003367	[20]
Tortricoidea			
Tortricidae	*Adoxophyes honmai*	DQ073916	[21]
Papilionoidea			
Nymphalidae	*Acraea issoria*	NC_013604	[22]
Pieridae	*Artogeia melete*	EU597124	[23]
Lycaenidae	*Coreana raphaelis*	DQ102703	[24]
Papilionidae	*Luehdorfia chinensis*	EU622524	Liu et al. unpublished
Papilionidae	*Papilio xuthus*	EF621724	Feng et al. unpublished

The *H. cunea* mitochondrial *COI*, *COIII* and *Cytb* have been utilized for biological identification as DNA barcode and phylogenetic studies [25, 26], but the genetic information on the complete mtDNA of the species remains largely unknown. In this study, we describe the complete mitogenome sequence of the fall webworm, *H. cunea*, and compare its sequence with other available lepidopteran mitogenomes. Furthermore, the mitogenome sequence of *H. cunea* was used to provide further insight into the phylogenetic relationships among lepidopteran superfamilies.

2. Materials and Methods

Insect and total DNA extraction

The *H. cunea* larvae were collected on the mulberry trees on the campus of the Shenyang Agricultural University, Shenyang, China. The larvae were then fed on the leaves of mulberry trees in room until pupation. The fresh pupae were directly frozen and kept in the laboratory at – 80 °C. A single pupa was used to extract the total DNA using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instruction.

PCR amplification and sequencing

The full mitogenome of *H. cunea* was amplified in four overlapping fragments by PCR amplification using universal primers and specific primers designed for this study (Fig. 1; Table 2). All PCR reactions were performed in a 50 µl volume with 1 U of LA Taq (TaKaRa Co., Dalian, China), 1 µl (about 20 ng) of DNA, 5 µl 10 × LA Taq buffer (Mg^{2+} plus), 200 µM dNTPs, and 10 pmol each primer. Initially, the *H. cunea COI* gene of ~600 bp was amplified using the primer set LYQ1/LYQ2 as previously reported [25], and the *Cytb* gene of ~400 bp was amplified using the primer set LYQ5/LYQ6 as previously reported [27]. The PCR amplification was performed under the following procedure: 2 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 30 sec at 50 °C, and 1 min at 72 °C, with a subsequent 10 min final extension at 72 °C. After purification with TIANgel Midi Purification Kit (TIANGEN, Beijing, China), the PCR fragments were directly sequenced with the PCR primers.

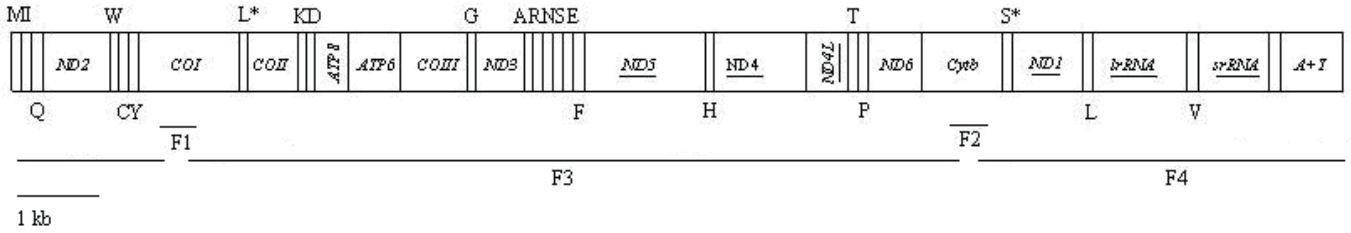


Fig. 1 Linear map of the mitogenome of *Hyphantria cunea*. The tRNAs are labeled according to the IUPAC-IUB single letter amino acid codes above the bar indicating coding sequence on major strand or below the bar showing on minor strand. One-letter symbol L, L*, S and S* denote codon $tRNA^{Leu}(CUN)$, $tRNA^{Leu}(UUR)$, $tRNA^{Ser}(AGN)$, and $tRNA^{Ser}(UCN)$, respectively. Underlined PCGs or rRNA genes are located on minor strand and PCGs that are not underlined are located on major strand. Overlapping lines (F1-F4) under the map denote four overlapping PCR fragments amplified for sequencing. The line at the lower left represents the map scale.

Table 2 Primers used to amplify the *Hyphantria cunea* mitogenome

Primer	Sequence (5′- 3′)	Fragment	Reference
LYQ1	GGTCAACAAATCATAAAGATATTGG	F1	[25]
LYQ2	TAAACTTCAGGGTGACCAAAAAATCA		
LYQ5	TATGTACTACCATGAGGACAAATATC	F2	[27]
LYQ6	ATTACACCTCCTAATTTATTAGGAAT		
LYQ29	CTTTTCTATTACTTCTTTCTCTTCCTGTTTTA	F3	This study
LYQ32	TAAAATAATAAATGGTAATAAAAAATGAAATG		
LYQ30	TAAAACAGGAAGAGAAAGAAGTAATAGAAAAG	F4	This study
LYQ31	CATTTCATTTTTTATTACCATTTATTATTTTA		

On the basis of the information from the determined fragments, two new primer pairs LYQ29/LYQ32 and LYQ30/LYQ31 were designed to amplify the remaining longer fragments of the mitogenome of *H. cunea* (F3 and F4 in Fig. 1 and Table 1). The two fragments were amplified with denaturation at 94 °C for 2 min, followed by 35 cycles of 1 min at 94 °C, 10 min at 65 °C, with a subsequent 10 min final extension at 72 °C. These PCR products were then utilized to construct a shotgun sequencing library. In brief, DNAs were sheared into 1-3 kb fragments using DNase I, and the DNA fractions were collected with a Chromaspin TE 1000 column. The DNA fractions were then cloned into the pGEM-T easy vector (Promega, USA), and each of the resultant plasmid DNAs was isolated with a Wizard Plus SV Minipreps DNA Purification System (Promega, USA). DNA sequencing was conducted using the ABI PRISM BigDyeTerminator v3.1 Cycle Sequencing Kit and the ABI PRISMTM 3100 Genetic Analyzer (PE Applied Biosystems, USA). All fragments were sequenced from both strands. The number of clones sequenced was sufficient to fulfill the six times coverage of the mitogenome.

Sequence analysis

The sequence alignment was carried out using Clustal X [28]. The PCGs and rRNA genes were determined by BLAST on NCBI Entrez Database and by comparing them with homologous regions in other lepidopteran mitogenome sequences. The PCG nucleotide sequences were translated on the basis of the Invertebrate Mitochondrial Genetic Code. The tRNA genes and its secondary structure were predicted using the tRNAscan-SE Search [29]. The two $tRNA^{Ser}$ secondary structure not found by tRNAscan-SE Search was developed using the constraints proposed by Steinberg and Cedergren [30]. Composition skew analysis was carried out to describe the base composition of nucleotide sequences, which measures the relative number of As to Ts (AT skew=[A–T]/[A+T]) and Gs to Cs (GC skew=[G–C]/[G+C]) [31]. Codon usage was calculated using the Countcodon program version 4 (http://www.kazusa.or.jp/codon/countcodon.html). The entire A+T-rich region was subjected to a search for the tandem repeats using Tandem Repeats Finder program [32]. The sequence data has been deposited in GenBank under accession No. GU592049.

Phylogenetic analysis

To illustrate the phylogenetic relationship of Lepidoptera, the other complete mitogenomes were obtained from GenBank. The *L. chinensis* and *P. xuthus* sequences lacking more sequence information were excluded. The mitogenomes of *Drosophila yakuba* (NC_001322) [33] and *Anopheles gambiae* (NC_002084) [34] were used as outgroups. The alignment of the amino acid sequences of each 13 mitochondrial PCGs was aligned with Clustal X [28] using default settings and concatenated. As for the *ND4* genes, the insertion of A nucleotide in *O. furnacalis* (position 8211 bp) and *O. nubilalis* (position 8206 bp) resulted in transcript frameshifts [13], the amino acid sequences of which were therefore revised for further phylogenetic analyses. The concatenated set of amino acids sequences from the 13 PCGs was used in phylogenetic analyses, which was performed using maximum parsimony (MP) and Nerghbor-joining (NJ) methods by using MEGA ver 4.0 [35].

3. Results and Discussion

Genome organization

The *H. cunea* mitogenome presents the typical gene content observed in metazoan mitogenomes (Table 3, Fig. 1): 13 PCGs, 22 tRNA genes, two rRNA subunits, and a major non-coding region known as the A+T-rich region in insects [5]. The complete mitogenome of *H. cunea* consists of 15 481 bp, which is well within the range observed in the completely sequenced lepidopteran insects, with size ranging from 15 140 in *A. melete* to 15 928 in Japanese *B. mandarina* (Table 4). The gene order and orientation of the *H. cunea* mitogenome are identical to the completely sequenced lepidopteran mitogenomes. By the translocation of $tRNA^{Met}$ to a position 5′ upstream of $tRNA^{Ile}$, the lepidopteran arrangement differs from that of *D. yakuba*, the hypothesized ancestral gene order of insects [36]. This suggests that the mitochondrial gene arrangement in lepidopteran insects evolved independently after splitting from its stem lineage [24].

Genome composition and skewness

The genome composition of the major strand of the *H. cunea* mitogenome is heavily biased toward As and Ts, accounting for 80.38%: A 40.58%, G 7.55%, T 39.80% and C 12.07%, as is the case with other insect sequences (Table 4). The bias value is similar to the completely sequenced lepidopteran insects, with the range from 77.84% in *O. lunifer* to 82.66% in *C. raphaelis*. The A+T content in the sequence of the A+T-rich region is 94.96%, also within the range observed in the completely sequenced lepidopteran insects, with the value from 89.17% in *A. melete* to 98.25% in *P. atrilineata*.

Table 3 Annotation and gene organization of the *Hyphantria cunea* mitogenome

Gene	Strand	Nucleotide no.	Size(bp)	Anticodon	Non	OL	Start codon	Stop codon
tRNA^{Met}	J	1-67	67	CAT				
tRNA^{Ile}	J	68-136	69	GAT	11			
tRNA^{Gln}	N	148-216	69	TTG	50			
ND2	J	267-1277	1011		18		ATT	TAA
tRNA^{Trp}	J	1 296-1 364	69	TCA		8		
tRNA^{Cys}	N	1 357-1 419	63	GCA	4			
tRNA^{Tyr}	N	1 424-1 489	66	GTA	11			
COI	J	1 501-3 034	1534				CGA	T-tRNA
tRNA^{Leu}(UUR)	J	3 035-3 100	66	TAA				
COII	J	3 101-3 782	682				ATG	T-tRNA
tRNA^{Lys}	J	3 783-3 853	71	CTT		1		
tRNA^{Asp}	J	3 853-3 918	66	GTC				
ATP8	J	3 919-4 080	162			7	ATA	TAA
ATP6	J	4 074-4 750	677		5		ATG	TA-COIII
COIII	J	4 756-5 547	792		9		ATG	TAA
tRNA^{Gly}	J	5 557-5 621	65	TCC				
ND3	J	5 622-5 975	354				ATT	TAA
tRNA^{Ala}	J	5 976-6 042	67	TGC	7			
tRNA^{Arg}	J	6 050-6 116	67	TCG	5			
tRNA^{Asn}	J	6 122-6 188	67	GTT	9			
tRNA^{Ser}(AGN)	J	6 198-6 263	66	GCT	21			
tRNA^{Glu}	J	6 285-6 352	68	TTC		2		
tRNA^{Phe}	N	6 351-6 418	68	GAA	3			
ND5	N	6 422-8 167	1746				ATA	TAA
tRNA^{His}	N	8 168-8 235	68	GTG				
ND4	N	8 236-9 574	1339				ATG	T-tRNA
ND4L	N	9 575-9 862	288		5		ATG	TAA
tRNA^{Thr}	J	9 868-9 932	65	TGT				
tRNA^{Pro}	N	9 933-9 997	65	TGG	7			
ND6	J	10 005-10 535	531		13		ATT	TAA
Cytb	J	10 549-11 697	1149		17		ATA	TAA
tRNA^{Ser}(UCN)	J	11 715-11 782	68	TGA	34			
ND1	N	11 817-12 755	939		1		ATG	TAA
tRNA^{Leu}(CUN)	N	12 757-12 824	68	TAG				
lrRNA	N	12 825-14 250	1426					
tRNA^{Val}	N	14 251-14 316	66	TAC				
srRNA	N	14 317-15 124	808					
A+T-rich region		15 125-15 481	357					

J-strand, majority-coding strand; N-strand, minority-coding strand; Non, non-coding region; OL, overlapping region.

Table 4 Composition and skewness in the major strand of lepidopteran mitogenomes

Species	size (bp)	A%	G%	T%	C%	A+T %	AT	GC
Whole genome								
Hyphantria cunea	15 481	40.58	7.55	39.80	12.07	80.38	0.010	−0.230
Lymantria dispar	15 569	40.58	7.57	39.30	12.55	79.88	0.016	−0.247
Ochrogaster lunifer	15 593	40.09	7.56	**37.75**	**14.60**	**77.84**	0.030	**−0.318**
Phthonandria atrilineata	15 499	40.78	7.67	40.24	11.31	81.02	0.007	−0.192
Antheraea pernyi	15 566	39.22	7.77	40.94	12.06	80.16	−0.021	−0.216
Antheraea yamamai	15 338	39.26	7.69	41.04	12.02	80.29	−0.022	−0.219
Caligula boisduvalii	15 360	39.34	7.58	41.28	11.79	80.62	−0.024	−0.217
Eriogyna pyretorum	15 327	39.17	7.63	41.65	11.55	80.82	−0.030	−0.204
Bombyx mori	15 656	43.06	7.31	38.30	11.33	81.36	**0.059**	−0.216
Chinese *Bombyx mandarina*	15 682	**43.11**	7.40	38.48	11.01	81.59	0.057	−0.196
Japanese *Bombyx mandarina*	**15 928**	43.08	7.21	38.60	11.11	81.68	0.055	−0.213

Manduca sexta	15 516	40.67	7.46	41.11	10.76	81.79	−0.005	−0.181
Diatraea saccharalis	15 490	40.87	7.42	39.15	12.56	80.02	0.021	−0.257
**Ostrinia nubilalis*	14 535	41.36	**8.02**	38.81	11.82	80.17	0.031	−0.192
**Ostrinia furnicalis*	14 536	41.46	7.91	38.92	11.71	80.37	0.032	−0.194
Adoxophyes honmai	15 680	40.15	7.88	40.24	11.73	80.39	−0.001	−0.178
Acraea issoria	15 245	**38.94**	7.74	40.81	12.50	79.76	−0.023	−0.235
Artogeia melete	**15 140**	40.38	7.87	39.41	12.35	79.78	0.012	−0.222
Coreana raphaelis	15 314	39.37	**7.30**	**43.29**	**10.04**	**82.66**	**−0.047**	**−0.158**
**Luehdorfia chinensis*	13 860	40.07	7.74	40.44	11.75	80.51	−0.005	−0.206
**Papilio xuthus*	13 964	39.53	7.85	40.45	12.17	79.98	−0.012	−0.216
A+T-rich region								
Hyphantria cunea	357	45.66	1.12	49.30	3.92	94.96	−0.038	−0.556
Lymantria dispar	435	45.29	1.61	50.50	2.30	96.09	−0.054	−0.176
Ochrogaster lunifer	**319**	44.51	1.57	48.90	5.02	93.42	−0.047	−0.524
Phthonandria atrilineata	457	40.70	0.66	57.55	**1.09**	**98.25**	**−0.171**	−0.246
Antheraea pernyi	552	**41.12**	**4.17**	49.28	5.43	90.40	−0.090	−0.127
Antheraea yamamai	334	41.62	3.59	47.90	**6.89**	89.52	−0.070	−0.315
Caligula boisduvalii	330	42.12	2.12	49.39	6.36	91.52	−0.079	−0.500
Eriogyna pyretorum	358	42.18	2.51	50.00	5.31	92.18	−0.085	−0.358
Bombyx mori	494	44.94	1.62	50.61	2.83	95.55	−0.059	−0.272
Chinese *Bombyx mandarina*	484	46.49	2.69	47.93	2.89	94.42	−0.015	−0.036
Japanese *Bombyx mandarina*	**747**	45.52	2.41	49.67	2.41	95.18	−0.043	**0**
Manduca sexta	324	45.00	1.54	50.31	3.29	95.37	−0.055	−0.334
Diatraea saccharalis	335	43.28	**0.60**	**51.64**	4.48	94.93	−0.088	**−0.764**
Adoxophyes honmai	489	**48.47**	2.86	45.81	2.86	94.27	**0.028**	**0**
Acraea issoria	430	45.81	1.40	50.23	2.56	96.05	−0.046	−0.293
Artogeia melete	351	43.87	3.13	**45.30**	7.69	**89.17**	−0.016	−0.421
Coreana raphaelis	375	44.27	1.33	49.87	4.53	94.13	−0.059	−0.545

* partial mitogenome lacking of the A+T-rich region.

The lepidopteran AT skewness values vary from –0.047 in *C. raphaelis* to 0.059 in *B. mori* (Table 4). The AT skewness for the major strand of the *H. cunea* mitogenome is slightly positive (0.010), indicating the occurrence of more As than Ts. This case is also found in *L. dispar* (0.016), *O. lunifer* (0.030), *P. atrilineata* (0.007), *B. mori* (0.059), Japanese *B. mandarina* (0.055), Chinese *B. mandarina* (0.057), *A. melete* (0.012), *D. saccharalis* (0.021), *O. nubilalis* (0.031), and *O. furnicalis* (0.032). In contrast, the AT skews are negative in the other lepidopteran mitogenomes. When considering the A+T-rich region, however, the bias toward the use of Ts over As is more obvious in the analyzed lepidopteran mitogenomes with the *H. cunea* mitogenome exhibiting a slightly value (–0.038). The only one exception is represented in *A. honmai* where the A+T-rich region exhibits a slightly positive AT skewness (0.028).

In all sequenced lepidopteran mitogenomes, the GC skewness values vary from –0.158 in *C. raphaelis* to –0.318 in *O. lunifer* with the *H. cunea* mitogenome exhibiting a moderate skewness value (–0.230), referring to the occurrence of more Cs than Gs in the lepidopteran mitogenomes.

Protein-coding genes

All of the PCGs in the *H. cunea* mitogenome are initiated by typical ATN codons (six with ATG, three with ATT, and three with ATA), except for *COI* (Table 3). The open reading frame of the *H. cunea COI* gene also starts at a CGA codon for arginine as found in all lepidopteran insects (Fig. 2). The typical ATN initiator for mitochondrial PCGs is also not found at the start site for *H. cunea COI* or near the $tRNA^{Tyr}$. The plausible translation initiator for *H. cunea COI* is ATA, located within the $tRNA^{Tyr}$ gene, overlapping 19 bp with the $tRNA^{Tyr}$; however, a codon following this triplet has a TAG-stop codon before the CGA codon. This ATA sequence is unlikely to be the start site for *H. cunea COI*, and there are no other probable start codons for *H. cunea COI*. Thus, the *COI* gene must use an atypical start site. In the previous studies, some tetranucleotide (ATAA, TTAA, GTAA and ATTA) and a hexanucleotide (ATTTAA) have been proposed as an initiator of *COI* for Diptera insects including mosquitoes and *Drosophila* [34, 37-41]. Among the completely sequenced lepidopteran insects, including *A. pernyi*, *A. yamamai*, *B. mori*, *B. mandarina*, and *C. raphaelis*, the TTAG has been designated as an initiator for *COI* [13, 14, 17, 18], however, *Ostrina* species were designated

as ATTTAG [20], and *A. issoria* and *C. boisduvalii* were designated as TTG [15, 22], and six species (*A. honmai*, *A. melete*, *E. pyretorum*, *M. sexta*, *P. atrilineata*, and *O. lunifer*) were designated as CGA [11, 12, 16, 19, 21, 23]. A recent study by analysis of the transcript information from the cDNA sequence of the mtDNA-encoded protein gene revealed that the translation initiation codon for the *COI* gene is TCG (Serine), rather than those atypical and longer codons in Diptera [41] Therefore, we tentatively designated the CGA as the *COI* start codon although no mRNA expression data for *H. cunea* are available until now.

Nine of 13 PCGs in *H. cunea* harbor the usual termination codon TAA, but the remaining four possess the incomplete termination codons T for *COI*, *COII*, *ND4*, and TA for *ATP6* (Table 3). The *COI*, *COII*, and *ND4* terminate with T exactly adjacent to tRNAs, and *ATP6* terminate with TA immediately followed by the ATG translation initiation codon of *COIII*. These incomplete stop codons are commonly found in metazoan mitochondrial genes [33]. The common interpretation of this phenomenon is that TAA termini are created via posttranscriptional polyadenylation [42].

The Relative Synonymous Codon Usage (RSCU) in PCGs was investigated and the results are summarized in Table 5. In PCGs of the *H. cunea* mitogenome, the codons CCG, GCG, TGC, CGC, AGC, and AGG are not represented. The genome-wise A+T bias is also reflected in the codon usage of *H. cunea* mitogenome. The codons TTA (Leu), ATT (Ile), TTT (Phe), and ATA (Met) are the four most frequently used codons in the *H. cunea* mitogenome, accounting for 39.2%. These codons are all composed of A or T nucleotides, thus indicating the biased usage of A and T nucleotides in the *H. cunea* PCGs. These four codons were also most frequently used in the sequenced lepidopteran insects. Leucine (14.82%), isoleucine (11.99%), phenylalanine (9%), and serine (8.52%) are the most frequent amino acids in *H. cunea* mitochondrial proteins, accounting for 44.33%. These amino acids are also the most frequently represented in other insects, averaging 45.08% [43].

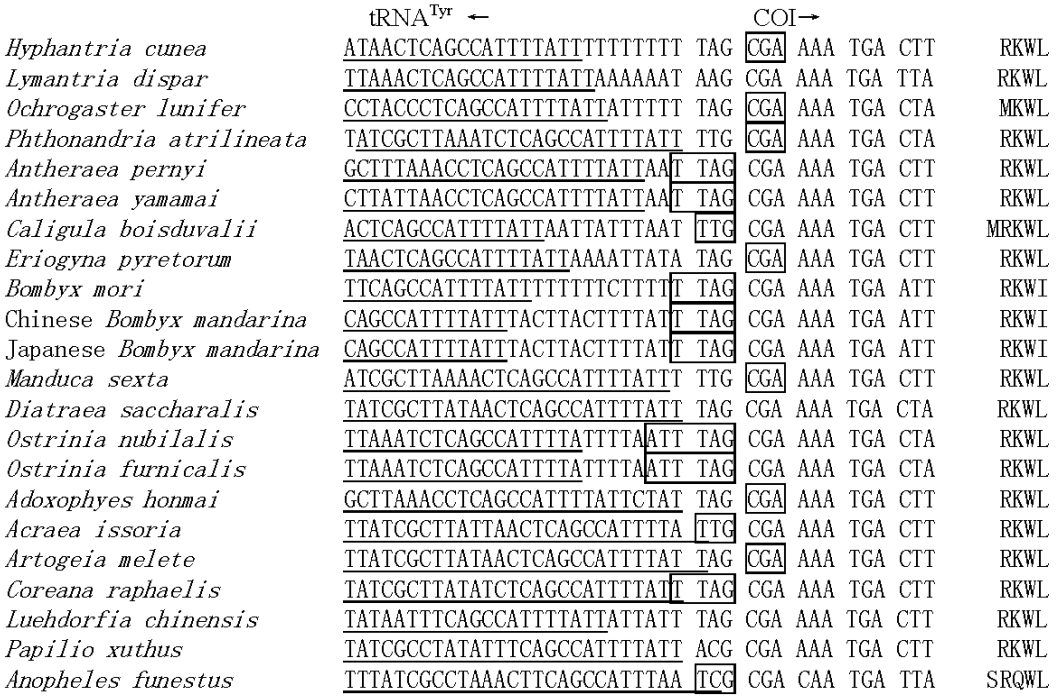


Fig. 2 Alignment of initiation region for the cytochrome oxidase subunit I (*COI*) genes of lepidopteran insects. The Diptera insect *Anopheles funestus* was included due to fact that the translation initiation codon for the *COI* gene was determined by analysis of the transcript information analysis [41]. The first four or five codons and their amino acids are shown on the right-hand side of the figure. Boxed nucleotides are the presumed translation initiators, which have been postulated as the initiation codon for *COI* in each species. Underlined nucleotides indicate the adjacent partial sequence of *tRNA^{Tyr}*. Arrows indicate the direction of transcription.

Table 5 Codon usage of the protein-coding genes in *Hyphantria cunea* mitogenome*

Codon (aa)	n	%	RSCU	Codon (aa)	n	%	RSCU	Codon (aa)	n	%	RSCU
UUU(F)	**332**	8.95	1.99	UCU(S)	113	3.05	2.86	UAU(Y)	180	4.85	1.82
UUC(F)	2	0.59	0.01	UCC(S)	10	0.27	0.25	UAC(Y)	18	0.49	0.18
UUA(L)	**449**	0.10	4.85	UCA(S)	81	2.18	2.05	UAA(*)	—	—	—
UUG(L)	35	0.94	0.38	UCG(S)	2	0.05	0.05	UAG(*)	—	—	—
CUU(L)	43	1.16	0.47	AGU(S)	28	0.75	0.71	CAU(H)	56	1.51	1.65
CUC(L)	1	0.03	0.01	AGC(S)	0	0	0	CAC(H)	12	0.32	0.35
CUA(L)	20	0.54	0.22	AGA(S)	82	2.21	2.08	CAA(Q)	60	1.62	1.97
CUG(L)	2	0.05	0.02	AGG(S)	0	0	0	CAG(Q)	1	0.03	0.03
AUU(I)	**410**	11.05	1.84	ACU(U)	73	1.97	1.93	AAU(N)	215	5.79	1.69
AUC(I)	35	0.94	0.16	ACC(U)	14	0.38	0.37	AAC(N)	39	1.05	0.31
AUA(M)	**265**	7.14	1.91	ACA(U)	62	1.68	1.64	AAA(K)	100	2.69	1.82
AUG(M)	12	0.32	0.09	ACG(U)	2	0.05	0.05	AAG(K)	10	0.27	0.18
GUU(V)	90	2.43	2.37	GCU(A)	84	2.26	2.63	GGU(G)	68	1.83	1.35
GUC(V)	5	0.13	0.13	GCC(A)	6	0.16	0.19	GGC(G)	3	0.08	0.06
GUA(V)	50	1.35	1.32	GCA(A)	38	1.02	1.19	GGA(G)	108	2.91	2.14
GUG(V)	7	0.19	0.18	GCG(A)	0	0	0	GGG(G)	23	0.62	0.26
UGU(C)	28	0.75	2.00	CGU(R)	14	0.38	1.08	CCU(P)	75	2.02	2.36
UGC(C)	0	0	0	CGC(R)	0	0	0	CCC(P)	11	0.30	0.35
UGA(W)	92	2.48	1.88	CGA(R)	36	0.97	2.77	CCA(P)	41	1.10	1.29
UGG(W)	6	0.16	0.12	CGG(R)	2	0.05	0.15	CCG(P)	0	0	0
GAU(D)	59	1.59	1.84	GAA(E)	66	1.78	1.74				
GAC(D)	5	0.13	0.16	GAG(E)	10	0.27	0.26				

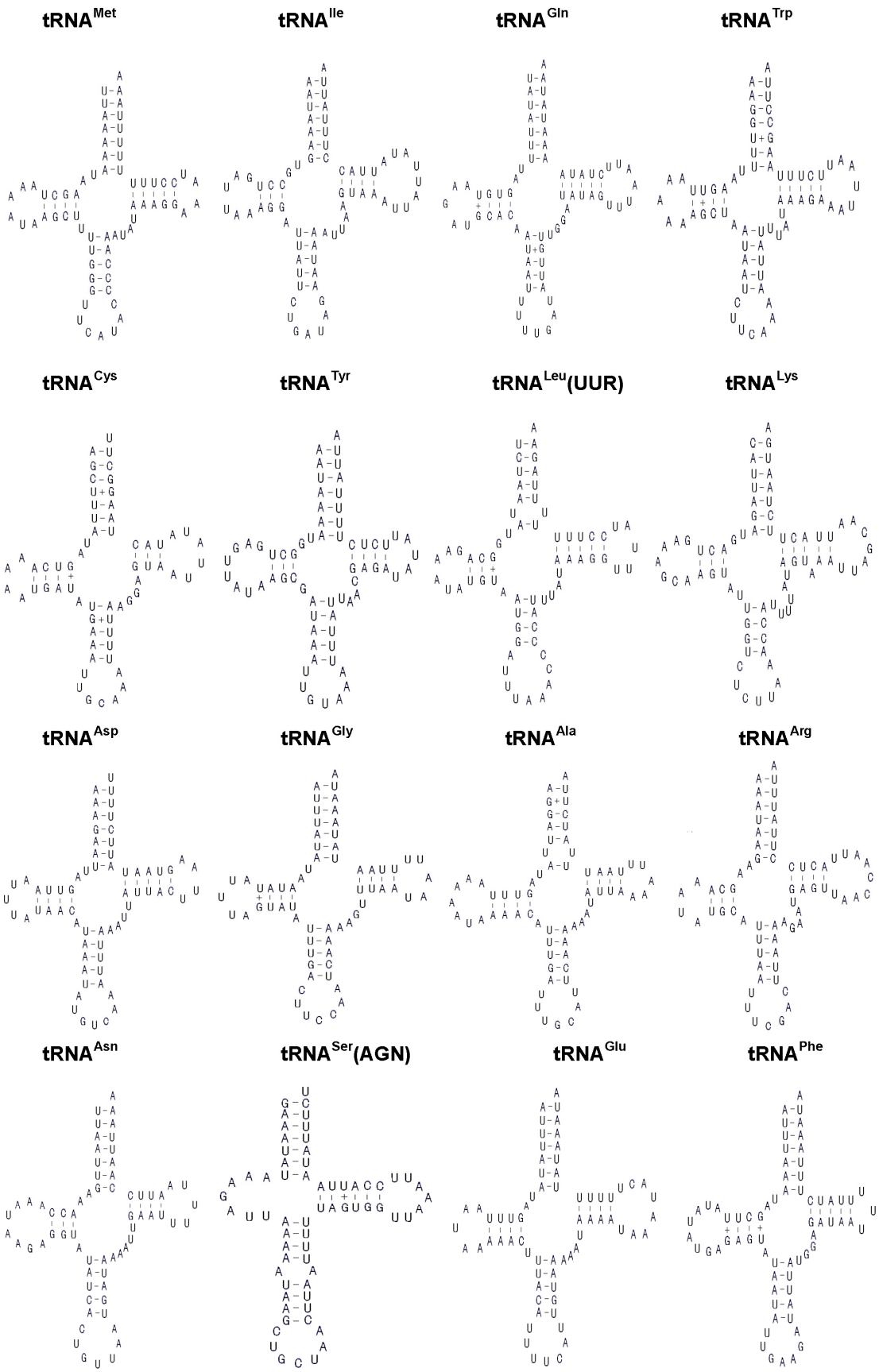
*A total of 3711 codons were analyzed excluding the initiation and termination codons. RSCU, relative sunonymous codon usage.

rRNA genes and tRNA genes

As in all other insect mitogenome sequences, two rRNA genes are present in *H. cunea*. They are located between $tRNA^{Leu}(CUN)$ and $tRNA^{Val}$, and between $tRNA^{Val}$ and the A+T-rich region, respectively. The length of the *H. cunea lrRNA* is 1 426 bp, which is the longest among the available completely sequenced lepidopteran insects, with the size range from1 412 bp in *D. saccharalis* to 1 319 bp in *A. melete*. The length of the *H. cunea srRNA* is 808 bp, which is well within the range observed in the available completely sequenced lepidopteran insects, with the size range from 806 bp in *O. lunifer* to 774 bp in *C. boisduvalii*.

The *H. cunea* mitogenome harbors 22 tRNA genes, which are scattered around the molecule (Table 2**;** Fig. 1). The predicted secondary structure of the *H. cunea* tRNAs are shown in Fig. 3. Twenty tRNA genes were identified by tRNAscan Search [29]. These 20 tRNA genes vary from 63 bp ($tRNA^{Cys}$) to 71 bp ($tRNA^{Lys}$) in size, and present a typical clover-leaf secondary structure of previously published mitochondrial tRNA genes. The *H. cunea* $tRNA^{Ser}(AGN)$ and $tRNA^{Ser}(UCN)$ genes not identified by tRNAscan-SE Search were determined to be 66 bp and 68 bp in size, respectively. Their sizes were determined by comparing the conserved relative genome position and sequence similarity with other lepidopteran mitogenome sequences. The $tRNA^{Ser}(UCN)$ also shows a typical clover-leaf secondary structure. However, the $tRNA^{Ser}(AGN)$ presents an unusual secondary structure lacking a stable stem-loop structure in the DHU arm, which has been observed in several other metazoan species including insects [4]. The anticodons are identical to those observed in other lepidopteran insects.

A total of 23 unmatched base pairs occurred in the *H. cunea* tRNA genes. Twelve of 22 tRNA genes, including $tRNA^{Gln}$, $tRNA^{Trp}$, $tRNA^{Cys}$, $tRNA^{Phe}$, $tRNA^{Gly}$, $tRNA^{Ala}$, $tRNA^{Leu}(CUN)$, $tRNA^{Leu}(UUR)$, $tRNA^{His}$, $tRNA^{Pro}$, $tRNA^{Thr}$, and $tRNA^{Val}$, were found to have 18 G-U mismatches in their secondary structures, which forms a weak bond. Three U-U mismatches were found in the amino acid acceptor stem of $tRNA^{Ala}$, $tRNA^{Leu}(CUN)$, and $tRNA^{Leu}(UUR)$. The $tRNA^{Thr}$ gene was proposed to contain an A-A mismatch in the TψC stem, and the $tRNA^{Ser}(AGN)$ gene contained an A-A mismatch in the anticodon stem. Moreover, the $tRNA^{His}$ and $tRNA^{Lys}$ genes were found to contain an extra nucleotide A in the TψC stem, respectively. Mismatches observed in tRNAs can be corrected through RNA-editing mechanisms that are well known for arthropod mtDNA [44]. The number of mismatches in the *H. cunea* is similar to those observed in other available lepidopteran insects: 24 in *A. pernyi* [13], and 24 in *E. pyretorum* [16]; but lower than in *O. lunifer* where 35 mismatches were found [11]. No mechanism, however, has been deduced for such high numbers of mismatches in insect mitochondrial tRNAs.


tRNA Met
tRNA Ile
tRNA Gln
tRNA Trp
tRNA Cys
tRNA Tyr
tRNA Leu(UUR)
tRNA Lys
tRNA Asp
tRNA Gly
tRNA Ala
tRNA Arg
tRNA Asn
tRNA Ser(AGN)
tRNA Glu
tRNA Phe

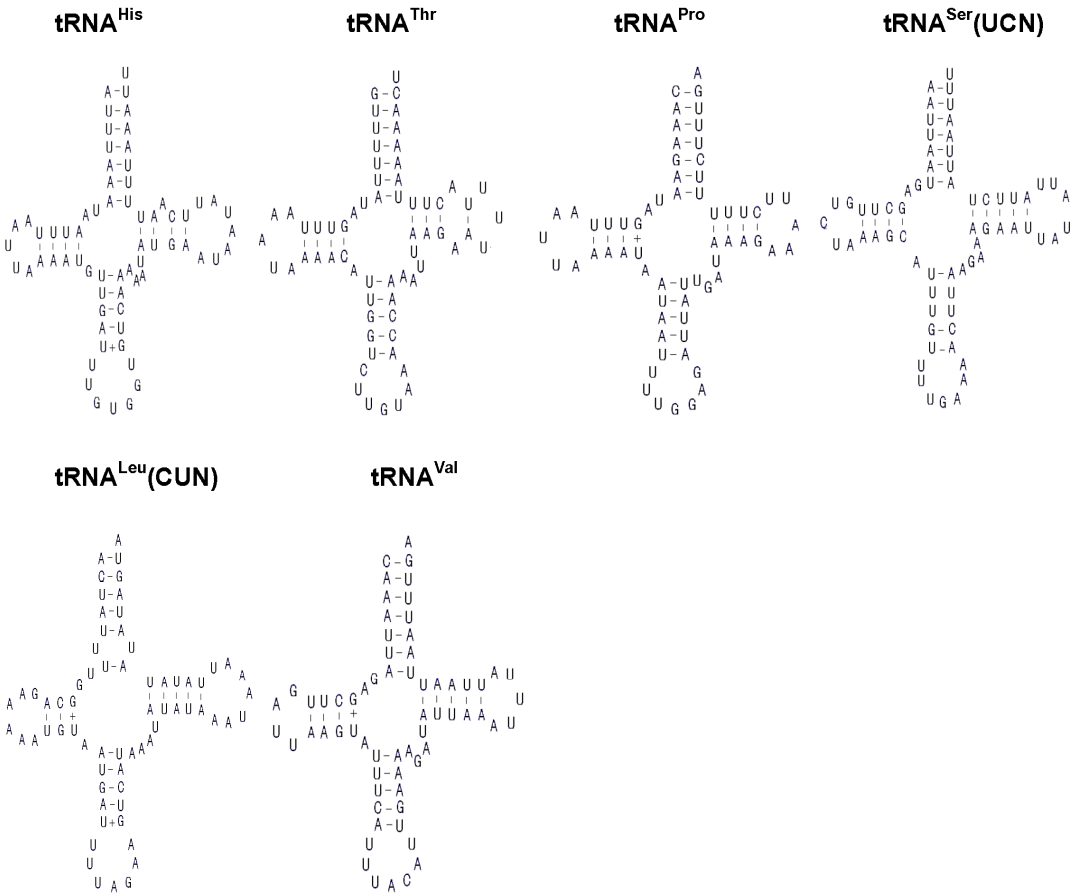


Fig. 3 Inferred secondary structure of the 22 tRNAs of the *Hyphantria cunea* mitogenome. +, GT pairs; –, AT/GC pairs. The tRNAs are labeled with the abbreviations of their corresponding amino acids. Arms of tRNAs (clockwise from top) are the amino acid acceptor arm, the TψC arm, the anticodon arm, and the dihydrouridine arm.

Noncoding and overlapping regions

The *H. cunea* mitogenome harbors a total of 230 bp intergenic spacer sequences, which are spread over 18 regions ranging in size from 1 to 50 bp (Table 3). The largest intergenic spacer sequence is present between *tRNA^{Gln}* and *ND2* gene, with an extreme richness in A and T nucleotides (92%). This spacer is not found in non-lepidopteran insect species [19], but is found to be a feature common to the 21 lepidopteran mitogenomes which have been sequenced to date. By alignment analysis, whilst invariant between each of the congeneric species-pairs which have been examined (*O. furnicalis* and *O. nubilalis*; *B. mori* and *B. mandarina*; *A. pernyi* and *A. yamamai*), this region showed limited sequence conservation between even closely related lepidopteran groups such as within Bombycoidea or between bombycoids and other macrolepidopterans [11, 19], indicating it would imply no functional significance or might not serve as another origin of replication [15].

Additionally, two other intergenic spacer sequences of more than 20 bp are present between *tRNA^{Ser}(AGN)* and *tRNA^{Glu}* (21 bp), and between *tRNA^{Ser}(AGN)* and *ND1* (34 bp), respectively. The spacer region of 34 bp also contains the ATACTAA motif [19], which is conserved across the Lepidoptera order (Fig. 4). This 7 bp motif is possibly fundamental to site recognition by the transcription termination peptide (mtTERM protein) [45]. This region is present in most insect mtDNAs even if the nucleotide sequence can be quite divergent [19].

Eighteen base pairs were identified as overlapping sequences varying from 1 to 8 bp in four regions (Table 3). The longest overlap is 8 bp between *tRNA^{Trp}* and *tRNA^{Cys}*. Similarly sized overlaps are also observed in other sequenced lepidopteran species [14]. The 7 bp overlap with the reading frame involving the *ATP8/ATP6* genes was found. This feature is common to other sequenced lepidopteran mitogenomes, and was found in many animal mitogenomes [5]. As for the two remaining overlaps, one is 1 bp between *tRNA^{Lys}* and *tRNA^{Asp}*, the other is 2 bp between *tRNA^{Glu}* and *tRNA^{Phe}*.

Fig. 4 Alignment of the intergenic spacer region between *tRNA^{Ser}(UCN)* and *ND1* of lepidopteran insects. The shaded ATACTAA motif [19] was conserved across the Lepidoptera order.

A+T- rich region

The *H. cunea* A+T-rich region is located between the *srRNA* gene and *tRNA^{Met}* (Table 3; Fig. 1), which includes the origin sites for transcription and replication [45]. This region was identified to be 357 bp in length, which is well within the range observed in the completely sequenced lepidopteran insects, with size ranging from 319 bp in *O. lunifer* to 747 bp in Japanese *B. mandarina* (Table 4). The A+T-rich region shows the highest A+T content (94.96%) of any region of the *H. cunea* mitogenome.

The presence of extra tRNA-like structures in the A+T-rich region has been reported in the lepidopteran insects. In the case of Chinese *B. mandarina*, one *tRNA^{Ser}*(*TGA*)-like sequence is located within the A+T-rich region forming a structure with four stem-loops and one big loop [18]. In *A. yamamai* A+T-rich region, two tRNA-like structures are present: *tRNA^{Ser}*(UCN)-like sequence and *tRNA^{Phe}*-like sequence, which possess the proper anticodon and form a clover-leaf structure, indicating they may be functional although there are many mismatches in both aminoacyl and anticodon stem regions [14]. However, no tRNA-like structure was detected in the *H. cunea* A+T-rich region.

The presence of varying copy numbers of tandemly-repeated elements has been reported to be one of the characteristics of the insect A+T-rich region [7]. Some lepidopteran insects have been observed to possess the repeat element in the A+T-rich region. In the case of *Antheraea*, the *A. pernyi* A+T-rich region harbors a repeat element of 38 bp tandemly repeated six times [13], whereas the *A. roylei* A+T-rich region has five repeat elements [9, 13]. In Japanese *B. mandarina*, the A+T-rich region harbors a tandem triplication of a 126 bp repeat unit, whereas in *B. mori* and Chinese *B. mandarina* the A+T-rich region has only one repeat element [17, 18]. It has also been reported that *Arethusana arethusa* (Nymphalidae: Satyrinae), *Leptidea sinapis* (Pieridae), and *Parnassius apollo* (Papilionidae) have a longer A+T-rich region (~500–700 bp), due mainly to an increase in the size and copy number of repeat units [46]. However, the *H. cunea* A+T-rich region is comprised of non-repetitive sequences, but harbors several features (Fig. 5) common to the Lepidoptera A+T-rich region [11, 19]. In *B. mori*, the O_N (origin of minority or light strand replication) is located 21 bp downstream from *srRNA* gene, which contains the motif ATAGA followed by an 18 bp poly-T stretch [47]. A very similar pattern occurs in *H. cunea* where the ATAGA motif is located 20 bp downstream from *srRNA* gene and is followed by an 18 bp poly-T stretch. A microsatellite-like $(AT)_8$ element preceded by the ATTTA motif is present in the 3' end of the *H. cunea* A+T-rich region. The presence of a microsatellite preceded by the ATTTA motif is common in the control regions of insect mitogenomes, and

has been found in all other lepidopteran species which have been sequenced [19]. Finally, an 11 bp poly-A is present immediately upstream *tRNA*^{Met}. This poly-A element is still a common feature of the A+T-rich region in Lepidoptera [19, 46]. This sequence has been suggested to be involved in the control of transcription and/or replication initiation in other insects or have some other unknown functional role [7].

Phylogenetic relationships

To place the *H. cunea* mtDNA sequence in perspective relative to other lepidopteran insect mitogenomes and to probe into the phylogenetic relationships among the lepidopteran superfamilies, a data set containing the concatenated amino acid sequences of 13 PCGs was generated. The sequences of the 13 PCGs were concatenated, rather than analyzed separately, to reconstruct the phylogenetic relationships, which may result in a more complete analysis [48]. The final alignment resulted in 3838 amino acid sites for the 19 ingroup and two outgroup taxa, including gaps. Of these sites, 1606 were conserved, 2170 were variable, and 1555 were informative for parsimony. The MP and NJ analyses generate overall similar topology except for the branching superfamily among *A. honmai*, and Papillonoidea species (Fig. 6). The phylogenetic analyses support a close relationship between *H. cunea* and *L. dispar* with 100% bootstrapping value, which is consistent with the morphological classification. The phylogenetic analyses also support a sister relationship between Noctuoidea (*H. cunea*, *L. dispar*, and *O. lunifer*) and Geometroidea (*P. atrilineata*).

The *M. sexta* is sometimes placed in their own superfamily Sphingidea. However, phylogenetic analyses based on the complete mitogenome in this study strongly support the placement within the superfamily Bombycoidea, which is consistent with the previous findings by morphological analysis [49] and by molecular analysis based on some nuclear genes [50].

These 19 sequences represent six superfamilies within the lepidopteran suborder: Bombycoidea, Geometroidea, Noctuoidea, Papillonoidea, Pyraloidea, and Tortricidea. Based on morphological analysis, Bombycoidea, Noctuoidea, Papillonoidea and Geometroidea are designated as the Macrolepidoptera; Pyraloidea together with Macrolepidoptera are designated as Obtectornera; Tortricoidea is the sister to the remaining lepidopteran superfamilies covered in the present study (Fig. 6A) [49]. In the phylogenetic trees constructed (Fig. 6B and C), the butterflies of Papillonoidea (*A. melete*, *A. issoria*, and *C. raphaelis*) are sisters to the remaining lepidopteran superfamilies, also showing a basal position within the monophyly of Lepidoptera [16]. This result is different to the traditional morphological analyses (Fig. 6A). Also, recent phylogenetic analyses of 123 species representing 27 superfamilies of Ditrysia based on five protein-coding nuclear genes (6.7 kb total) provide sufficient information to conclusively demonstrate that several prominent features of the current morphology-based hypothesis, including the position of the butterflies, need revision [51]. These results present in this study suggest that the complete insect mitogenome sequence has a power to resolve the majority of family relationships within superfamilies, however, the deeper nodes among superfamilies need more efforts.

ttatatatattatttctaatATAGATTTTTTTTTTTTTTTTTTatatttaaatatttaatataataaaaatttttatattaaatgtttaatattaatt
attaaacaattaataatttctctttttttctttttataatattaatattaaaacctaaattgctatataaacaattataatttattaaattataaaaaattaatataattaatat
taatttttttcaataatttaatgtattatatatatatattaataaattaaatATTTAATATATATATATATATATaattaattaaatttaaatttaattaattat
ataaaccatttctaataattttacaataaatAAAAAAAAAAAA

Fig. 5 The features present in the A+T-rich region of *Hyphantria cunea*. The sequence is shown in the N strand.

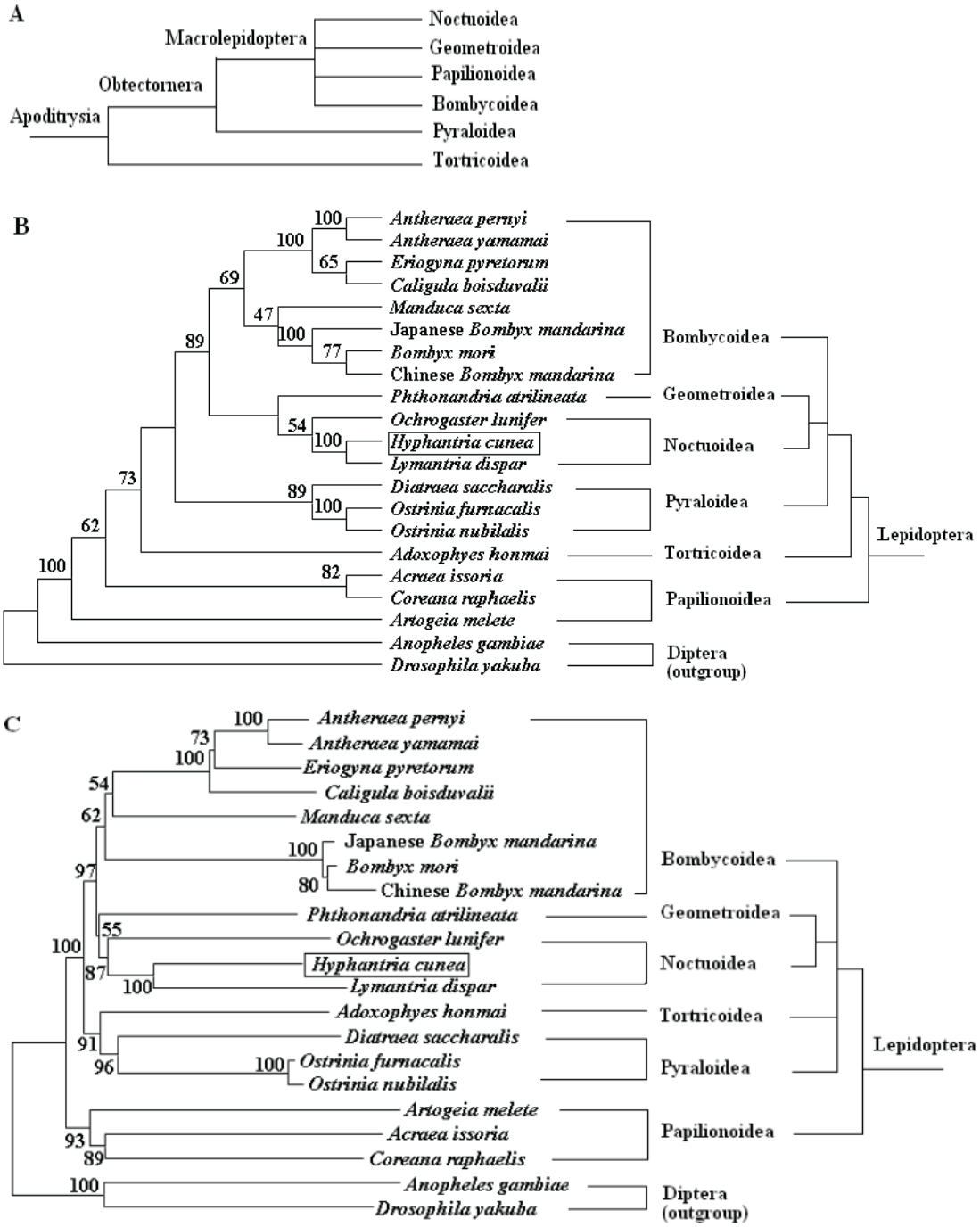


Fig. 6 Phylogeny of lepidopteran insects. (A) Current hypothesis of lepidopteran superfamily relationships after Kristensen and Skalski (1999) [50]. Phylogenetic trees inferred from amino acid sequences of 13 PCGs of the mitogenome by using MP analysis (B) and NJ analysis (C). *Drosophila yakuba* [33] and *Anopheles gambiae* [34] were used as outgroups. The numbers above branches specify bootstrap percentages (1000 replicates).

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant 30800803), the National 863 program of China (grant 2007AA06Z323), the National Key Technology R&D Program in the 11th Five year Plan of china (grant 2006BAK10B06), and the Key Technology R&D Program of Tianjin City (grant 06YFGZNC00700).

Conflict of Interest

The authors have declared that no conflict of interest exists.

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