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ORIGINAL ARTICLE

Characterization of the complete mitochondrial genome of *Spilarctia robusta* (Lepidoptera: Noctuoidea: Erebidae) and its phylogenetic implications

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Abstract. The complete mitochondrial genome (mitogenome) of *Spilarctia robusta* (Lepidoptera: Noctuoidea: Erebidae) was sequenced and analyzed. The circular mitogenome is made up of 15,447 base pairs (bp). It contains a set of 37 genes, with the gene complement and order similar to that of other lepidopterans. The 12 protein coding genes (PCGs) have a typical mitochondrial start codon (ATN codons), whereas cytochrome c oxidase subunit 1 (*cox1*) gene utilizes unusually the CAG codon as documented for other lepidopteran mitogenomes. Four of the 13 PCGs have incomplete termination codons, the *cox1*, *nad4* and *nad6* with a single T, but *cox2* has TA. It comprises six major intergenic spacers, with the exception of the A+T-rich region, spanning at least 10 bp in the mitogenome. The nucleotide composition of the genome is greatly A+T biased (81.09%), with a negative AT skewness (–0.007), indicating the presence of fewer As than Ts, similar to other Noctuoidea. The A+T-rich region is 343 bp long, and contains some conserved regions, including an "ATAGA" motif followed by a 19 bp poly-T stretch, a microsatellite-like $(AT)_9$ and a poly-A element, a characteristic shared with other lepidopteran mitogenomes. Phylogenetic analysis, based on 13 PCGs using Maximum likelihood methods revealed that *S. robusta* belongs to the superfamily Noctuoidea.

INTRODUCTION

The insect mitochondrial genome is a circular molecule, ranging in size from 15 to 19 kb (Jiang et al., 2009). It contains a set of 37 genes that are typically similar in all insects sequenced to date. On the basis of their physiological functions, they are divided into 13 protein coding (two ATPase genes [*atp6* and *atp8*], seven NADH dehydrogenase [*nad1-nad6* and *nad4L*], a cytochrome b [*cob*], three cytochrome c oxidase [*cox1-cox3*]), 22 transfer RNAs and two ribosomal RNAs (*rrnL* and *rrnS*) genes (Shadel & Clayton, 1993; Cameron, 2014). In addition, it has a control region of variable length (A+T-rich region) (Wolstenholme, 1992). The mitochondrial DNA (mtDNA) is extremely conserved and is maternally inherited. Moreover, it is non-recombinant and undergoes reductive evolution. Therefore, the study of the mitogenome is considered to be important for understanding molecular evolution, comparative and evolutionary genomics, phylogenetics and population genetics (Boore, 1999; Babbucci et al., 2014; Cameron, 2014).

Lepidoptera (moths and butterflies) is the second largest order in the class Insecta, containing greater than 155,000 described species that are classified into 45–48 superfamilies. Noctuoidea is a highly diverse superfamily, with approximately 42,400 species distributed worldwide (Hao et al., 2012; Zhang, 2013). Despite, this enormous taxonomic diversity, knowledge on the mitochondrial genome (mitogenome) of the Noctuoidea is uneven (Table 1). In particular, information is scanty on six families (Oenosandridae, Notodontidae, Erebidae, Euteliidae, Nolidae and Noctuidae) of Noctuoidea, and interestingly only a few lineages support the relationship of Erebidae (Zahiri et al., 2012). Arctiinae upgraded to family from Erebidae (Lafontaine & Fibiger, 2006) has been recently revised by Zahiri et al. (2011). Moreover, it also has a complex evolutionary relationship with plant and fungal chemistry (Zaspel et al.,

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Table 1. Details of the lepidopteran mitogenomes used in this study.

Superfamily	Family	Species	Size (bp)	GenBank accession no.	Reference
Bombycoidea	Bombycidae	*Bombyx mandarina*	15,682	NC_003395	Yukuhiro et al., 2002
		Bombyx mori	15,643	NC_002355	direct submission
	Saturniidae	*Actias selene*	15,236	NC_018133	Liu et al., 2012
		Antheraea pernyi	15,566	AY242996	Liu et al., 2008
		Antheraea yamamai	15,338	NC_012739	Kim et al., 2009b
	Sphingidae	*Manduca sexta*	15,516	NC_010266	Cameron & Whiting, 2008
		Sphinx morio	15,299	NC_020780.1	Kim et al., 2013
Noctuoidea	Lymantriidae	*Lymantria dispar*	15,569	NC_012893	unpublished
	Erebidae	***Spilarctia robusta***	15,447	KX753670	**present study**
		Lemyra melli	15,418	NC_026692	unpublished
		Amata formosae	15,463	KC513737	Lu et al., 2013
		Hyphantria cunea	15,481	NC_014058	Liao et al., 2010
	Notodontidae	*Phalera flavescens*	15,659	NC_016067	Sun et al., 2012
		Ochrogaster lunifer	15,593	NC_011128	Salvato et al., 2008
	Noctuidae	*Agrotis ipsilon*	15,377	KF163965	Wu et al., 2015
		Eutelia adulatricoides	15,360	KJ185131	Yang et al., 2015
		Mythimna separata	15,329	KM099034.1	Liu et al., 2015
	Nolidae	*Gabala argentata*	15,337	KJ410747	Yang et al., 2015
Geometroidea	Geometridae	*Apocheima cinerarium*	15,722	KF836545	Liu et al., 2014
		Biston panterinaria	15,517	NC_020004	Yang et al., 2013
		Phthonandria atrilineata	15,499	NC_010522	Yang et al., 2009
		Biston thibetaria	15,484	KJ670146.1	unpublished
		Biston suppressaria	15,628	KP278206	Chen et al., 2016
		Jankowskia athleta	15,534	KR822683	Xu et al., 2015
Pyraloidea	Crambidae	*Chilo suppressalis*	15,395	NC_015612	Chai et al., 2012
		Diatraea saccharalis	15,490	NC_013274	Li et al., 2011
	Pyralidae	*Ephestia kuehniella*	15,327	KF305832.2	Lammermann et al., 2016
Tortricoidea	Tortricidae	*Spilonota lechriaspis*	15,368	NC_014294	Zhao et al., 2011
		Grapholita molesta	15,717	NC_014806	Gong et al., 2012
Papilionoidea	Papilionidae	*Parnassius bremeri*	15,389	NC_014053	Kim et al., 2009a
		Papilio maraho	16,094	NC_014055	Wu et al., 2010
		Teinopalpus aureus	15,242	NC_014398	unpublished
	Nymphalidae	*Apatura ilia*	15,242	NC_016062	Chen et al., 2012
		Apatura metis	15,236	NC_015537	Zhang et al., 2012
		Fabriciana nerippe	15,140	NC_016419	Kim et al., 2011
		Argynnis hyperbius	15,156	NC_015988	Kim et al., 2011
Yponomeutoidea	Plutellidae	*Plutella xylostella*	16,179	JF911819	Wei et al., 2013
	Lyonetiidae	*Leucoptera malifoliella*	15,646	NC_018547	Wu et al., 2012
Hepialoidea	Hepialidae	*Thitarodes renzhiensis*	16,173	NC_018094	Cao et al., 2012
		Ahamus yunnanensis	15,816	NC_018095	Cao et al., 2012

2014). Therefore, this taxonomic group has attracted the attention of researchers all over the world, and their main focus is to establish relationships within the group as well as with other taxonomic categories.

Spilarctia robusta (Lepidoptera: Noctuoidea: Erebidae: Arctiinae) is a major arthropod pest of trees. This species largely infests forests, but also damages roadside and garden trees in urban areas. The economic losses are increasing at an alarming rate (Liao et al., 2010). Therefore, considering its economic importance as well as the lack of information on its evolutionary relationships, we designed the present study, in order to sequence and annotate the complete mitogenome of *S. robusta*. Moreover, we compared it with other Lepidoptera that have been sequenced in order to highlight their evolution, particularly the phylogenetic relationships of Noctuoidea and Erebidae.

MATERIALS AND METHODS

Experimental insects and DNA extraction

Spilarctia robusta (moth) specimens were collected from Anhui Agricultural University (AHAU), Anhui Province, China. These specimens were identified as *S. robusta* by a taxonomist (Department of Entomology, AHAU). Total DNA was extracted using a Genomic DNA Extraction Kit (Aidlab Co., Beijing, China) according to the manufacturer's instructions.

PCR amplification, cloning and sequencing

We designed twelve pairs of primers from the conserved nucleotide sequences of known mitochondrial genomes of Lepidoptera to determine the sequence characteristics of the *S. robusta* mitogenome (Liu et al., 2013; Dai et al., 2015). The complete list

Table 2. Details of the primers used to amplify the mitogenome of *S. robusta*.

Primer pair	Primer sequences (5'-3')
F1	TAAAAATAAGCTAAATTTAAGCTT
R1	TATTAAAATTGCAAATTTTAAGGA
F2	AAACTAATAATCTTCAAAATTAT
R2	AAAATAATTTGTTCTATTAAAG
F3	TGGAGCAGGAACAGGATGAAC
R3	GAGACCADTACTTGCTTTCAG
F4	ATTTGTGGAGCTAATCATAG
R4	GGTCAGGGACTATAATCTAC
F5	TCGACCTGGAACTTTAGC
R5	GCAGCTATAGCCGCTCCTACT
F6	TAAGCTGCTAACTTAATTTTTAGT
R6	CCTGTTTCAGCTTTAGTTCATTC
F7	CCTAATTGTCTTAAAGTAGATAA
R7	TGCTTATTCTTCTGTAGCTCATAT
F8	TAATGTATAATCTTCGTCTATGTAA
R8	ATCAATAATCTCCAAAATTATTAT
F9	ACTTTAAAAACTTCAAAGAAAAA
R9	TCATAATAAATTCCTCGTCCAATAT
F10	GGAGCTTCTACATGAGCTTTTGG
R10	GTTTGCGACCTCGATGTTG
F11	GGTCCCTTACGAATTTGAATATATCCT
R11	AAACTAGGATTAGATACCCTATTAT
F12	CTCTACTTTGTTACGACTTATT
R12	TCTAGGCCAATTCAACAACC

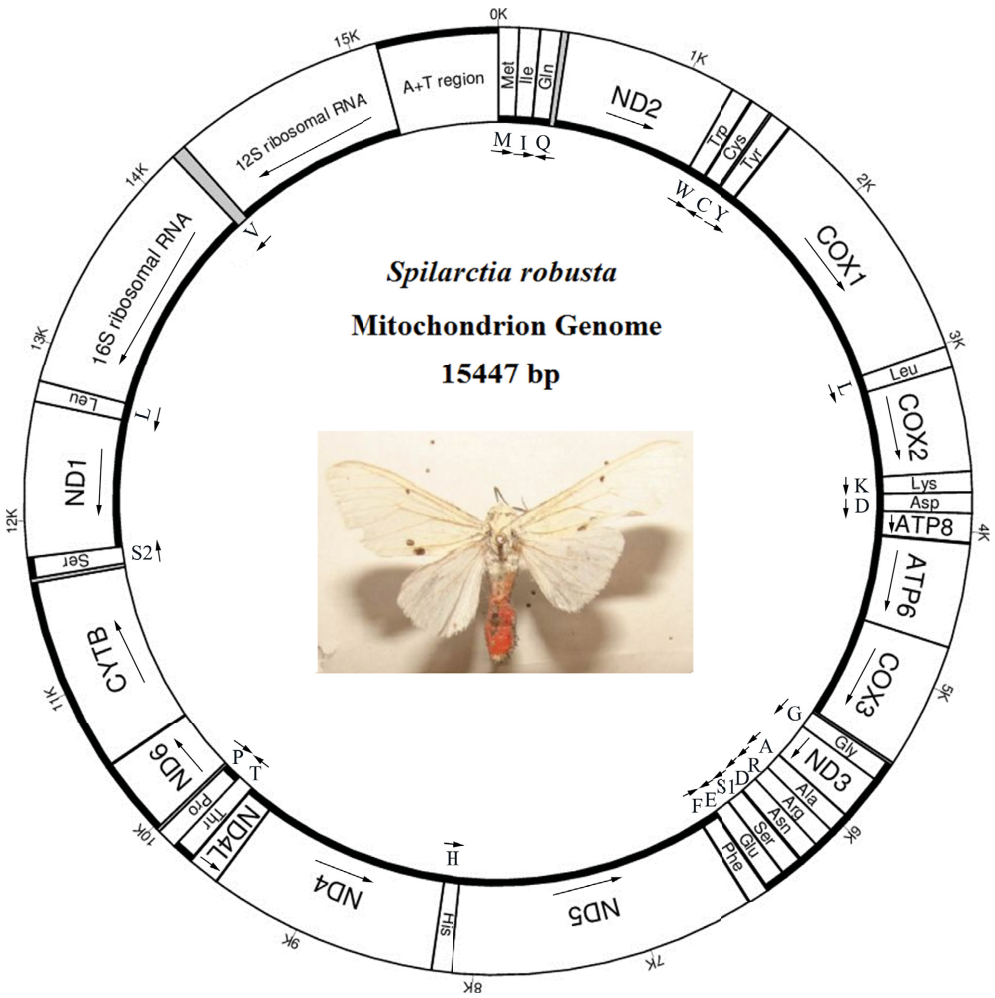

Fig. 1. Map of the mitogenome of *S. robusta*. The tRNA genes are labelled according to the IUPAC-IUB single-letter amino acids: *cox1*, *cox2* and *cox3* refer to the cytochrome c oxidase subunits; *cob* cytochrome b; *nad1-nad6* NADH dehydrogenase components; *rrnL* and *rrnS* ribosomal RNAs. Genes named above the bar are located on major strands, while the others are located on minor strands.

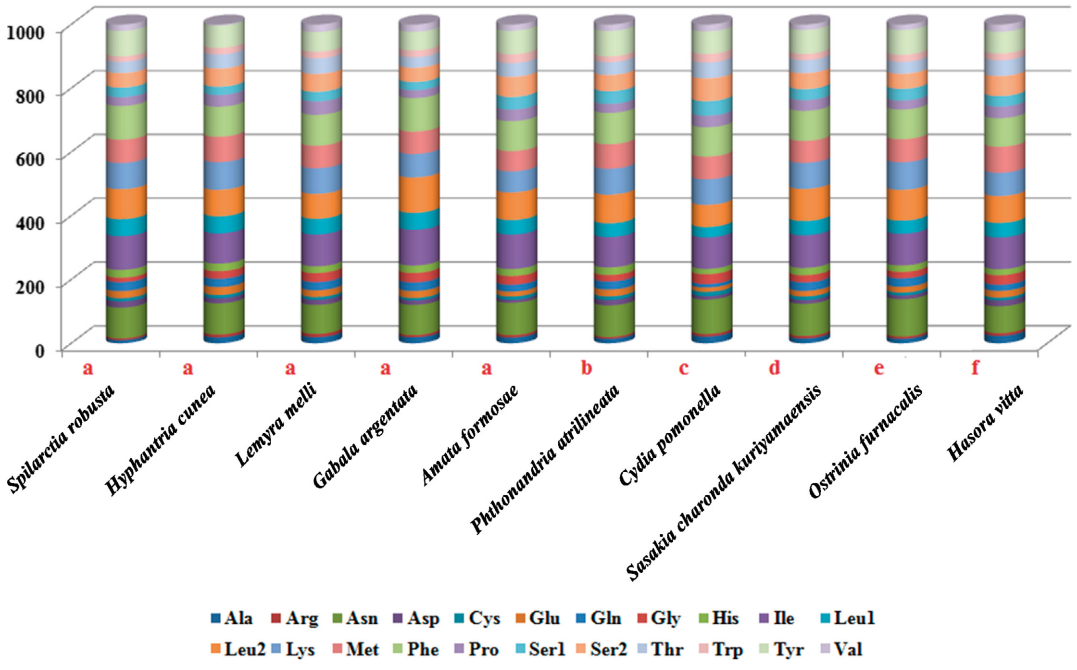

Fig. 2. Comparison of the size of each codon within the mitochondrial genome of different species of Lepidoptera. Lowercase letters (a, b, c, d, e and f) above species name indicate the superfamily to which the species belong (a – Noctuoidea; b – Geometroidea; c – Tortricoidea, d – Papilionoidea; e – Pyraloidea; f – Hesperioidea).

Table 3. List of the annotated mitochondrial genes of *S. robusta*.

Gene	Product	Direction	Location	Size	Anti codon	Start codon	Stop codon	Intergenic Nucleotides
trnM	$tRNA^{Met}$	F	1–67	67	CAT	—	—	0
trnI	$tRNA^{Ile}$	F	68–132	65	GAT	—	—	2
trnQ	$tRNA^{Gln}$	R	135–203	69	TTG	—	—	37
nad2	NADH2	F	241–1273	1033		ATT	TAA	–2
trnW	$tRNA^{Trp}$	F	1272–1340	69	TCA	—	—	–8
trnC	$tRNA^{Cys}$	R	1333–1398	66	GCA	—	—	14
trnY	$tRNA^{Tyr}$	R	1413–1477	65	GTA	—	—	6
cox1	COX1	F	1484–3017	1534		CGA	T	0
trnL2	$tRNA^{Leu(UUR)}$	F	3018–3084	67	TAA	—	—	0
cox2	COX2	F	3085–3766	682		ATG	TA	0
trnK	$tRNA^{Lys}$	F	3767–3837	71	CTT	—	—	–1
trnD	$tRNA^{Asp}$	F	3837–3902	66	GTC	—	—	0
atp8	ATP8	F	3903–4064	162		ATT	TAA	–7
atp6	ATP6	F	4058–4736	679		ATG	TAA	5
cox3	COX3	F	4742–5538	797		ATG	TAA	2
trnG	$tRNA^{Gly}$	F	5541–5605	65	TCC	—	—	0
nad3	NADH3	F	5606–5969	364		ATT	TAA	2
trnA	$tRNA^{Ala}$	F	5972–6038	67	TGC	—	—	0
trnR	$tRNA^{Arg}$	F	6039–6100	62	TCG	—	—	0
trnN	$tRNA^{Asn}$	F	6101–6166	66	GTT	—	—	9
trnS1	$tRNA^{Ser(AGN)}$	F	6176–6241	66	GCT	—	—	3
trnE	$tRNA^{Glu}$	F	6245–6313	69	TTC	—	—	9
trnF	$tRNA^{Phe}$	R	6323–6387	64	GAA	—	—	–6
nad5	NADH5	R	6382–8127	1746		ATA	TAA	0
trnH	$tRNA^{His}$	R	8128–8194	66	GTG	—	—	0
nad4	NADH4	R	8195–9530	1336		ATG	T	3
nad5	NADH4L	R	9534–9821	288		ATG	TAA	5
trnT	$tRNA^{Thr}$	F	9827–9891	65	TGT	—	—	0
trnP	$tRNA^{Pro}$	R	9892–9957	65	TGG	—	—	18
nad6	NADH6	F	9976–10504	529		ATA	T	6
cytb	CYTB	F	10511–11672	1158		ATG	TAA	25
trnS2	$tRNA^{Ser(UCN)}$	F	11698–11762	65	TGA	—	—	17
nad1	NADH1	R	11780–12716	937		ATG	TAA	1
trnL	$tRNA^{Leu(CUN)}$	R	12718–12785	67	TAG	—	—	0
rrnL	16S rRNA	R	12786–14206	1421	—	—	—	0
trnV	$tRNA^{Val}$	R	14207–14271	65	TAC	—	—	14
rrnS	12S rRNA	R	14285–15101	816	—	—	—	2
A+T-rich Region			15104–15447	343				

of successful primers is given in Table 2 (Sangon Biotech Co., Shanghai, China). All amplifications were performed on an Eppendorf Mastercycler and Mastercycler gradient in 50 μL reaction volumes, including 35 μL sterilized distilled water, 5 μL 10 × *Taq* buffer (Mg^{2+} plus), 4 μL dNTP (25 mM), 1.5 μL extracted DNA as a template, forward and reverse primers 2 μL each (10 μM) and 0.5 μL (1 unit) *Taq* (Takara Co., Dalian, China). The PCR amplification conditions were as follows: an initial denaturation cycle at 94°C for 4 min followed by 38 cycles, one cycle at 94°C for 30 s, one cycle at 48–59°C for 1–3 min (depending on putative length of the fragments), and a final extension step of one cycle at 72°C for 10 min. The PCR products were detected using electrophoresis in agarose gel (1%, w/v), purified using a DNA gel extraction kit (Transgen Co., Beijing, China) and sequenced with the PCR primers.

Sequence assembly and gene annotation

Sequence annotation was performed using the blast tools available on NCBI (http://blast.ncbi.nlm.nih.gov/Blast) and the SeqMan II program in the Lasergene software package (DNAStar Inc., Madison, USA). The protein-coding sequences were translated into putative proteins on the basis of the Invertebrate Mitochondrial Genetic Code. The skewness was measured using the method of Junqueira et al. (2004) and base composition of nucleotide sequences was described as: AT skew = [A−T]/[A+T], GC skew = [G−C]/[G+C]. The Relative Synonymous Codon Usage (RSCU) values were calculated using MEGA 5.0 (Tamura et al., 2011).

Transfer RNA genes were determined using tRNAscan-SE software (http://lowelab.ucsc.edu/tRNAscan-SE/) (Lowe & Eddy, 1997), or predicted by sequence features of being capable of folding into the typical cloverleaf secondary structure with legitimate anticodon. The tandem repeats in the A+T-rich region were found using the Tandem Repeats Finder program (http://tandem.bu.edu/trf/trf.html) (Benson, 1999).

Phylogenetic analysis

To reconstruct the phylogenetic relationships of Lepidoptera, 38 complete or partially complete mitogenomes were downloaded from the GenBank database (Table 1). The mitogenomes of *Drosophila melanogaster* (U37541.1) (Lewis et al., 1995) and *Locusta migratoria* (NC_001712) (Flook et al., 1995) were used as an outgroup. The amino acid sequences of each of the 13 mitochondrial PCGs were aligned with Clustal X using default settings and concatenated (Thompson et al., 1997). Later a concatenated set of amino acid sequences from the 13 PCGs was used for phylogenetic analyses, which were performed using the Maximum Likelihood (ML) method with the MEGA version 5.1 program (Tamura et al., 2011). The method was used to infer phylogenetic trees based on 1000 bootstrap replicates.

RESULTS

Genome structure, organization and composition

The complete mitogenome of *S. robusta* is a closed circular molecule, 15,447 bp long (Fig. 1). The complete mitogenome is deposited in NCBI GenBank database under accession number KX753670. It contains the entire set of 37 genes (22 tRNA genes, 13 PCGs [*nad1-6*, *nad4L*, c*ox1-3*, *cob*, *atp6* and *atp8*], two rRNAs [*rrnS* and *rrnL*]. In addition, there is one major non-coding A+T-rich region

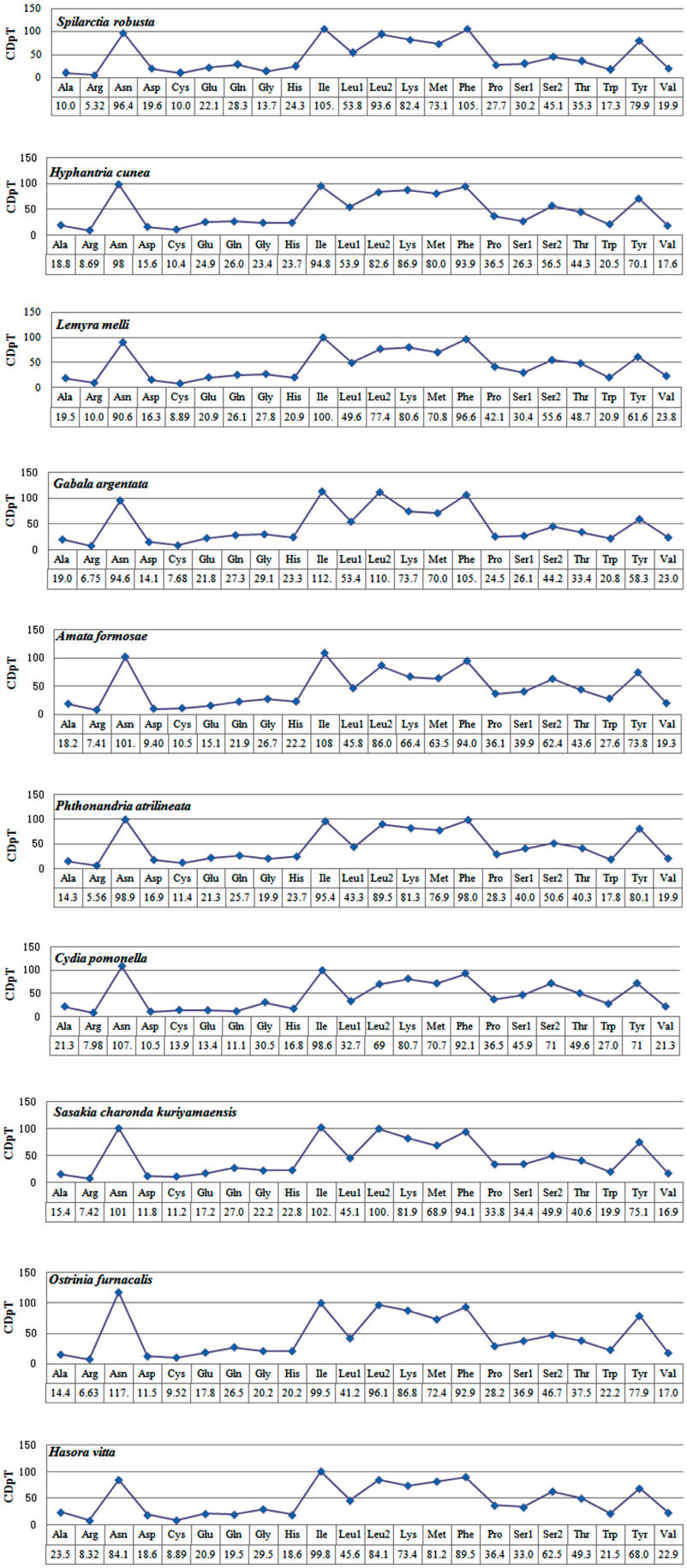

Fig. 3. Distribution of the codons in different species of Lepidoptera. CDspT – codons per thousand codons.

Table 4. Composition and skewness of different lepidopteran mitogenomes.

Species	Size (bp)	A%	G%	T%	C%	A+T%	AT skewness	GC skewness
Whole genome								
S. robusta	15,447	40.28	7.57	40.81	11.34	81.09	–0.007	–0.199
L. melli	15,418	39.38	8.72	39.29	13.06	78.67	0.001	–0.199
H. cunea	15,481	40.58	7.55	39.81	12.06	80.39	0.010	–0.230
A. formosae	15,463	38.67	7.53	40.83	12.98	79.49	–0.027	–0.266
G. argentata	15,337	39.64	7.56	42.05	10.75	81.69	0.030	–0.174
S. morio	15,299	40.64	7.58	40.53	11.23	81.17	0.001	–0.194
C. pomonella	15,253	39.92	7.88	40.21	11.99	80.13	–0.004	–0.207
P. atrilineata	15,499	40.78	7.67	40.24	11.31	81.02	0.007	–0.192
S. c. kuriyamaensis	15,222	39.68	7.86	40.21	12.25	79.89	–0.007	–0.218
O. furnacalis	14,536	41.46	7.91	38.92	11.71	80.37	0.032	–0.194
H. vitta	15,282	39.58	7.81	40.34	12.27	79.92	–0.010	–0.222
B. mandarina	15,682	43.11	7.40	38.48	11.01	81.59	0.057	–0.196
A. pernyi	15,566	39.22	7.77	40.94	12.07	80.16	–0.021	–0.216
M. sexta	15,516	40.67	7.46	41.11	10.76	81.79	–0.005	–0.181
C. suppressalis	15,395	40.64	7.39	40.03	11.94	80.67	0.007	–0.235
A. ipsilon	15,377	40.38	7.71	40.87	11.04	81.25	–0.006	–0.178
PCG								
S. robusta	11,120	39.31	7.65	41.47	11.57	80.77	–0.027	–0.204
L. melli	11,120	38.47	9.17	38.17	14.19	76.64	0.004	–0.215
H. cunea	11,198	39.98	8.35	38.61	13.06	78.59	0.017	–0.220
A. formosae	11,217	38.18	8.28	39.62	13.92	77.80	–0.019	–0.254
G. argentata	10,303	38.10	8.61	41.88	11.41	79.98	–0.047	–0.140
S. morio	11,179	40.28	8.27	39.56	11.89	79.84	0.009	–0.180
C. pomonella	11,199	39.55	8.69	39.00	12.76	78.55	0.007	–0.190
P. atrilineata	11,203	40.23	8.59	38.87	12.31	79.10	0.017	–0.178
S. c. kuriyamaensis	10,795	39.39	8.62	38.92	13.08	78.30	0.006	0.997
O. furnacalis	11,194	41.16	8.43	38.26	12.14	79.42	0.037	–0.180
H. vitta	11,202	38.76	8.61	39.43	13.20	78.19	–0.009	–0.210
B. mandarina	11,196	42.83	8.26	37.04	11.87	79.87	0.072	–0.179
A. pernyi	11,204	39.22	7.77	40.94	12.07	80.16	–0.021	–0.216
M. sexta	11,185	40.41	8.23	39.88	11.48	80.30	0.007	–0.165
C. suppressalis	11,230	40.42	8.16	38.48	12.95	78.90	0.025	–0.227
A. ipsilon	11,226	39.69	8.44	40.14	11.72	79.83	–0.006	–0.163
tRNA								
S. robusta	1,473	41.00	8.21	40.19	10.59	81.19	0.010	–0.127
L. melli	1,486	40.58	8.55	40.24	10.63	80.82	0.004	–0.109
H. cunea	1,463	41.83	7.86	39.99	10.32	81.82	0.022	–0.135
A. formosae	1,457	40.43	7.96	40.36	11.26	80.78	0.001	–0.172
G. argentata	1,468	41.35	8.24	40.19	10.22	81.54	0.014	–0.107
S. morio	1,462	40.63	8.21	40.97	10.19	81.60	–0.004	–0.107
C. pomonella	1,464	41.19	7.92	40.23	10.66	81.42	0.012	–0.147
P. atrilineata	1,476	41.4	8.2	40.04	10.37	81.44	0.017	–0.117
S. c. kuriyamaensis	1,459	40.85	8.09	40.37	10.69	81.22	0.006	–0.138
O. furnacalis	1,433	42.22	8.03	39.01	10.75	81.23	0.040	–0.145
H. vitta	1,456	41.41	8.04	39.84	10.71	81.25	0.019	–0.142
B. mandarina	1,472	41.78	7.81	39.95	10.46	81.73	0.022	–0.145
A. pernyi	1,459	39.22	7.77	40.94	12.07	80.16	–0.021	–0.217
M. sexta	1,554	40.99	7.92	41.06	10.04	82.05	–0.001	–0.118
C. suppressalis	1,482	40.89	7.89	40.89	10.32	81.78	0.000	–0.133
A. ipsilon	1,465	41.23	8.12	40.48	10.17	81.71	0.009	–0.112
rRNA								
S. robusta	2,238	42.14	4.65	43.3	9.92	85.43	–0.014	–0.362
L. melli	2,233	42.23	4.93	41.96	10.88	84.19	0.003	–0.376
H. cunea	2,234	42.08	4.92	42.75	10.25	84.83	–0.008	–0.351
A. formosae	2,163	38.93	4.72	44.85	11.51	83.77	–0.071	–0.418
G. argentata	2,165	40.6	4.76	45.13	9.52	85.73	–0.053	–0.333
S. morio	2,152	41.73	4.83	43.08	10.36	84.8	–0.016	–0.364
C. pomonella	2,147	40.48	5.03	43.92	10.57	84.4	–0.041	–0.355
P. atrilineata	2,203	42.85	4.58	43.08	9.49	85.93	–0.003	–0.349
S. c. kuriyamaensis	2,086	39.98	5.18	44.53	10.31	84.52	–0.054	–0.331
O. furnacalis	1,774	42.39	5.07	42.05	10.48	84.44	0.004	–0.348
H. vitta	2,194	41.43	4.88	43.25	10.44	84.69	–0.021	–0.363
B. mandarina	2,134	43.86	4.78	41.05	10.31	84.91	0.033	–0.366
A. pernyi	2,144	39.22	7.77	40.94	12.07	80.16	–0.021	–0.217
M. sexta	2,168	41.37	4.84	44.05	9.73	85.42	–0.031	–0.336
C. suppressalis	2,171	41.27	4.97	43.67	10.09	84.94	–0.028	–0.340
A. ipsilon	2,162	41.58	5	43.57	9.85	85.15	–0.023	–0.327
A+T-rich region								
S. robusta	344	45.35	0.58	50	4.07	95.35	–0.049	–0.751
L. melli	338	43.2	1.48	51.18	4.14	94.38	–0.085	–0.473
H. cunea	357	45.66	1.12	49.3	3.92	94.96	–0.038	–0.556
A. formosae	482	42.95	2.9	49.79	4.36	92.74	–0.074	–0.201
G. argentata	340	43.24	1.47	52.06	3.24	95.29	–0.093	–0.376
S. morio	316	44.3	2.53	48.42	4.75	92.72	–0.044	–0.305
C. pomonella	351	43.3	1.14	52.42	3.13	95.73	–0.095	–0.466
P. atrilineata	457	40.7	0.66	57.55	1.09	98.25	–0.172	–0.246
S. c. kuriyamaensis	380	44.74	3.68	47.11	4.47	91.84	–0.026	–0.097
H. vitta	255	45.88	2.75	48.24	3.14	94.12	–0.025	–0.066
B. mandarina	484	46.49	2.69	47.93	2.89	94.42	–0.015	–0.036
A. pernyi	552	39.22	7.77	40.94	12.07	80.16	–0.021	–0.216
M. sexta	324	45.06	1.54	50.31	3.09	95.37	–0.005	–0.335
C. suppressalis	348	42.24	0.29	53.16	4.31	95.4	–0.114	–0.874
A. ipsilon	332	46.08	1.51	48.8	3.61	94.88	–0.029	–0.41

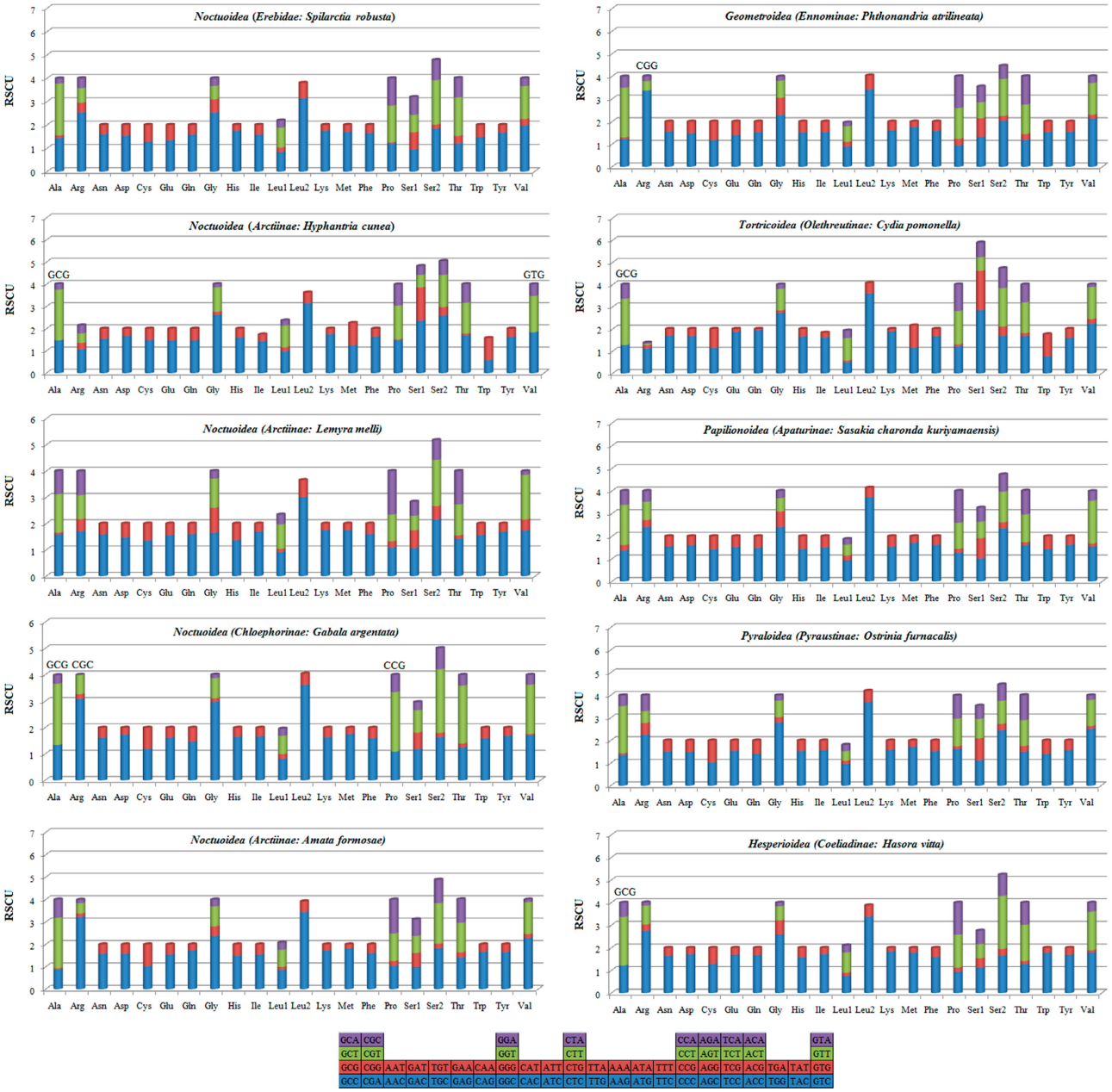

Fig. 4. The Relative Synonymous Codon Usage (RSCU) of the mitochondrial genome in six super families of Lepidoptera. Codon families are plotted on the x-axis. Codons indicated above the bar are not present in the mitogenomes.

(Table 3). The gene arrangement and orientation is *trnM-trnI-trnQ*. The nucleotide composition of the major strand is 40.28% A, 40.81% T, and 7.57% G and: 11.34% C, with a total of 81.09% A+T content (Table 4). The AT skewness and GC skewness are –0.007 and –0.199, respectively.

Protein-coding genes and codon usage

Twelve of the 13 PCGs of *S. robusta* use ATN (ATT, ATG and ATA) as an initiation codon. Of which, the ATG is the most frequent initiation codon as the *cox2*, *cox3*, *atp6*, *nad4*, *nad4L*, *cytb* and *nad1* begin with it. Whereas *cox1* has a CGA start codon. Four of the 13 PCGs (*cox1*, *cox2*, *nad6* and *nad4*) terminate with incomplete stop codons, either TA or T nucleotide, and the remainder of the PCGs terminate with the canonical stop codon TAA. The *cox1*, *nad4* and *nad6* have a single T as a stop codon, while the *cox2* has TA. We analyzed the codon usage of ten lepidopteran species, of which five belonged to Noctuoidea and one each to Geometroidea, Tortricoidea, Papilionoidea, Pyraloidea and Hesperioidea (Fig. 2). The analysis reveals that Asn, Ile, Leu2, Lys, Phe, Tyr and Met are the most frequently utilized amino acids, of which, the hydrophobic amino acid Leu2 family is the most and the Arg codon family the least frequent. The codon distribution of five Noctuoidea species is consistent, and the content of each amino acid is similar in the different species (Fig. 3).

The Relative Synonymous Codon Usage (RSCU) in the six lepidopteran superfamilies with known mitogenomes reveals that *S. robusta* PCGs are relatively similar to those of *L. melli*, *A. formosae*, *S. charonda kuriyamaensis* and *O. furnacalis*, and different from the species, which lack GCG

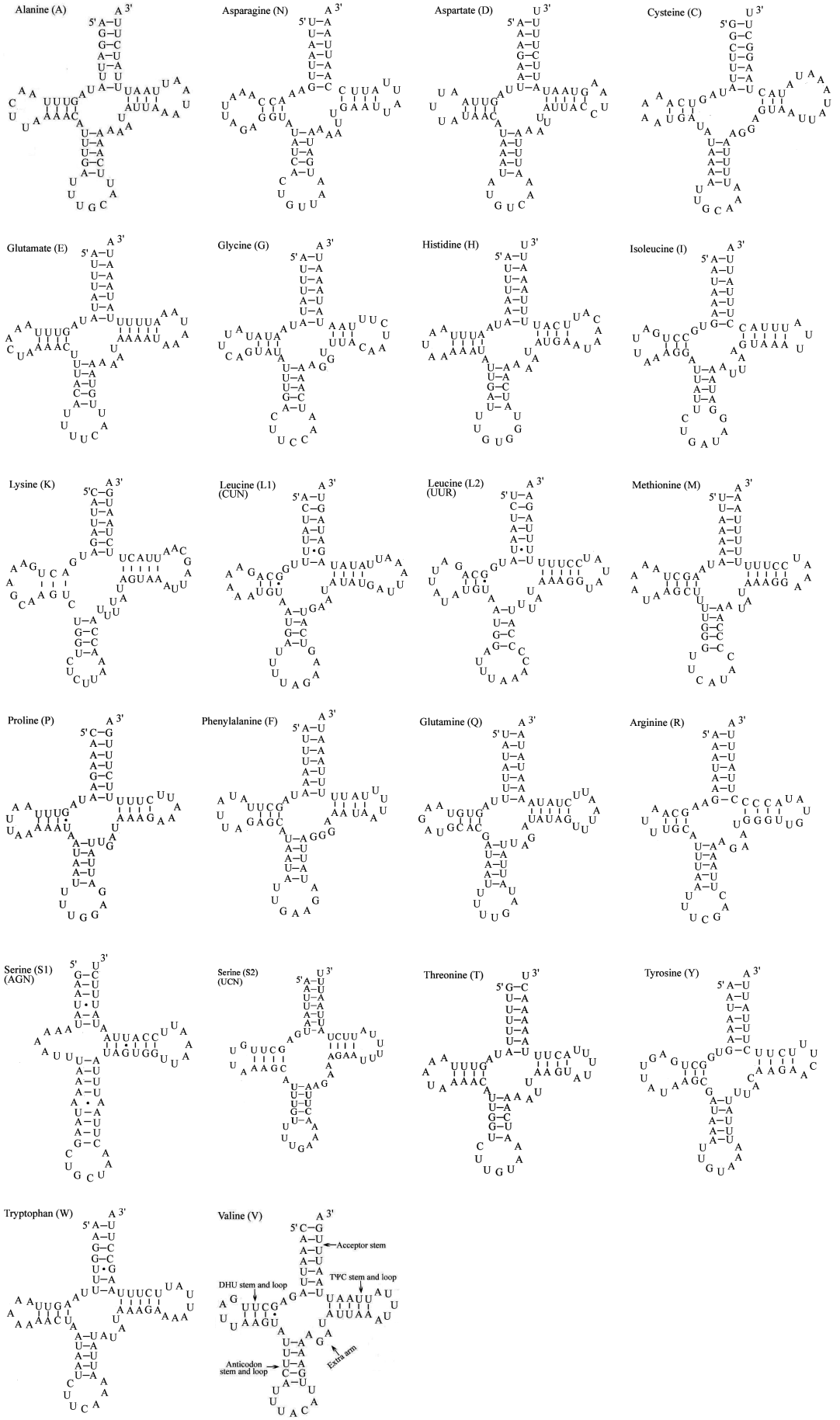

Fig. 5. Putative secondary structures of the 22 tRNAs of the mitogenome of *S. robusta*.

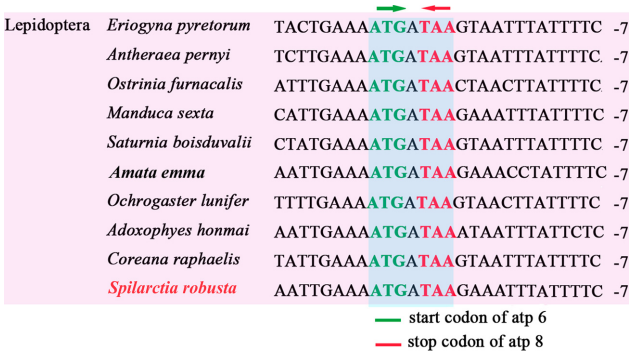

Fig. 6. Alignment of the overlapping region between *atp8* and *atp6* in Lepidoptera and other insects. The numbers on the right refer to the number of intergenic nucleotides.

& GTG (*H. cunea*), GCG & CGC & CCG (*G. argentata*), CGG (*P. atrilineata*), GCG (*C. pomonella*) and GCG (*H. vitta*) Codons (Fig. 4).

Ribosomal and tRNA genes

As in other Lepidoptera, *S. robusta* has two rRNA genes. The *rrnL* gene (1421 bp) is at the junction between $tRNA^{Leu}$ (CUN)-$tRNA^{Val}$ and the *rrnS* gene (816 bp), which is located between $tRNA^{Val}$ and the A+T-rich region (Table 3), and the A+T content is 83.81%. The value of the A+T content is well within the range of 80.16% (*B. mandarina*) to 85.93% (*P. atrilineata*) recorded for Lepidotera. Both the AT skewness (–0.014) and GC skewness (–0.362) are negative.

Fourteen of the 22 tRNA genes on the H-strand and eight on the L-strand were identified. The length of tRNA genes ranges from 62 bp to 71 bp, which is similar to that of most Lepidoptera sequenced. It is highly A+T (81.19%) biased, and exhibits positive AT-skewness (0.010) and negative GC skewness (–0.127) (Table 4). All the tRNAs fold into the expected secondary cloverleaf structure, with the exception of $tRNA^{Ser(AGN)}$ (Fig. 5). It forms an unusual secondary structure lacking a stable stem-loop structure in the DHU arm. There are a total of 11 mismatches in *S. robusta* tRNA genes. The G-U wobble pairs are scattered throughout the 6 tRNA genes, (two in the acceptor stem, four in DHU and one in TψC) and there is one A-A mismatch in the anticodon stem of *trnS1* and three U-U mismatches in the acceptor stem of *trnA*, *trnL2* and *trnS1* (Fig. 5).

Overlapping and intergenic spacer regions

There are five overlapping regions, with a total of 24 bp in the mitogenome of *S. robusta*. On the basis of their location, they are categorized into three types: tRNA and tRNA (*trnW* and *trnC*, *trnK* and *trnD*), tRNA and protein (*nad2* and *trnW*, *trnF* and *nad5*) and protein and protein (*atp6* and *atp8*). The length of these sequences varies from 1 bp to 8 bp. The largest overlapping region (8 bp), located between *trnW* and *trnC*, the rest of 6 bp, 2 bp, and 1 bp overlaps located between *trnF* and *nad5*, *nad2* and *trnW*, and *trnK* and *trnW*, respectively (Table 3). In addition, there is a 7 bp overlap located at the junction of *atp8*-*atp6*. Further we recorded intergenic nucleotides between *atp8* and *atp6* in ten species of Lepidoptera (Fig. 6).

The intergenic spacers in the mitogenome of *S. robusta* are spread over 18 regions and ranged in size from 1 bp to 37 bp, with a total of 168 bp in length. Of which there are six major intergenic spacers of at least 10 bp in length (Table 3). The largest intergenic spacer (37 bp) is present between *trnQ* and *nad2* and has an extremely high A+T content. Further we identified a 17 bp intergenic spacer between *trnS2* (UCN) and *nad1*, which contains the "ATACTAA" motif (Fig. 7A).

The A+T-rich region

With a length of 344 bp, the A+T-rich region in the mitogenome of *S. robusta* is located at the junction *rrnS*-*trnM* (Table 4). This region has the highest A+T (95.35%) content, and most negative AT skewness (–0.049) and GC skewness (–0.751) (Table 4). We recorded four short repeating sequences located on both sides of the A+T-rich region, the motif "ATAGA" and a 19 bp poly-T stretch downstream from the *rrnS* gene, while the microsatellite-

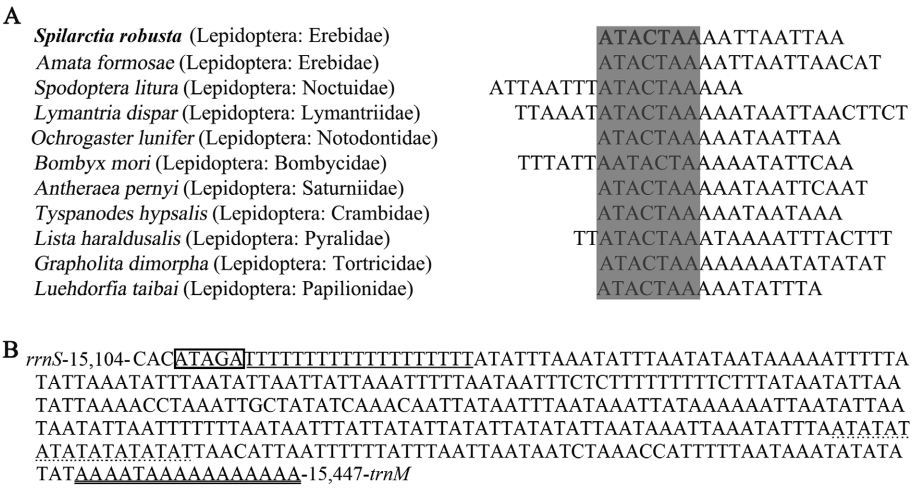

Fig. 7. (A) Alignment of the intergenic spacer region between *trnS2* (UCN) and *nad1* in several species of Lepidoptera. The shaded "ATACTAA" motif is conserved in Lepidoptera. (B) Features present in the A+T-rich region in *S. robusta*. The sequence is shown in the reverse strand. The ATATG motif is shaded. The poly-T stretch is underlined, while the poly-A stretch is double underlined. The single microsatellite T/A repeats sequence are indicated by dotted underlining.

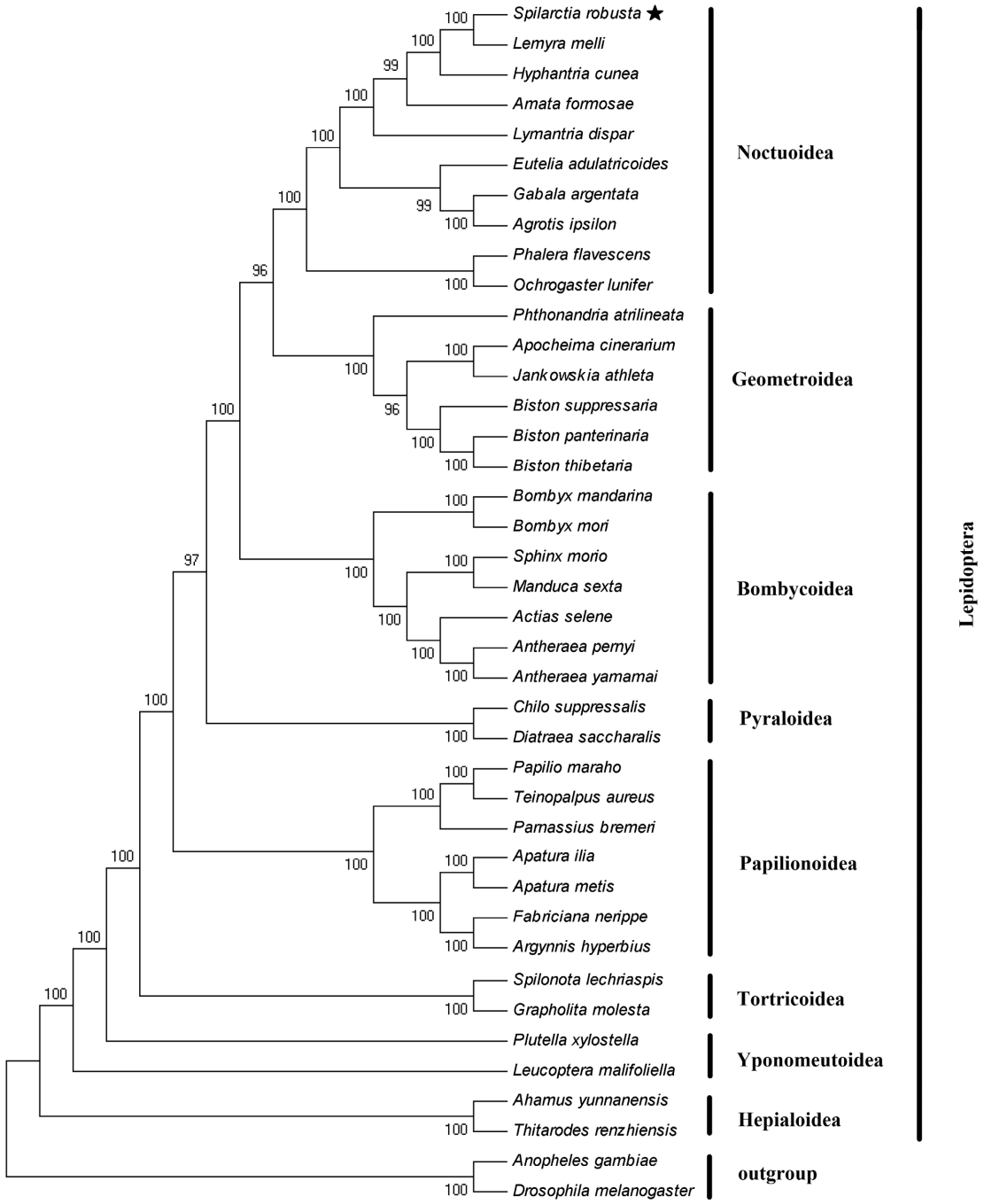

Fig. 8. Tree showing the phylogenetic relationships of Lepidoptera, constructed using the Maximum Likelihood method. Bootstrap values (1000 repetitions) of the branches are indicated. *Anopheles gambiae* (L20934.1) and *Drosophila melanogaster* (U37541.1) were used as outgroups.

like element $(AT)_9$ and a poly-A element are located upstream of the *trnM* gene (Fig. 7B).

Phylogenetic relationships

We reconstructed the phylogenetic relationships using the ML method based on the concatenated nucleotide sequences of the 13 PCGs of the related lepidopteran superfamilies. The phylogenetic analysis reveals that the superfamilies Noctuoidea, Geometroidea, Bombycoidea, Pyraloidea, Papilionoidea, Tortricoidea, Yponomeutoidea and Hepialoidea are monophyletic (Table 1), and Noctuoidea is most closely related to the superfamilies Geometroidea and Bombycoidea. Different species of the same family form a single cluster and *S. robusta* is closely related to *L. melli* in the Erebidae (Fig. 8).

DISCUSSION

In the present study, the size of the newly sequenced *S. robusta* (15,447 bp) mitogenome falls within the range of those recorded for other species of Lepidoptera sequenced; *Artogeia* (15,140 bp) has the shortest and *B. mandarina* (15,928) the longest. The variation in size is primarily due to differences in the number of repeats in the control regions (Pan et al., 2008; Hong et al., 2009). The gene number and

nucleotide composition is similar to that of Metazoa, however their arrangement and orientation (*trnM-trnI-trnQ*) is different from the ancestral gene order *trnI-trnQ-trnM* (Boore, 1999). The AT skewness (–0.007) of the mitogenome studied indicates the presence of less As than Ts. This remarkable feature is also reported for several arthropod species including *A. formosae* (–0.027), *C. pomonella* (–0.004), *H. vitta* (–0.010) and *A. pernyi* (–0.021). Interestingly, the GC skewness (−0.362) of the rRNA is much lower than in previously sequenced animals and further reveals that the mitgenome is more biased toward Cs than Gs (Jiang et al., 2009; Liu et al., 2012).

Twelve of the 13 protein-coding genes have the standard ATN (ATT, ATG and ATA) start codon, while *cox1* has CGA. Most of *S. robusta* PCGs (*cox2*, *cox3*, *atp6*, *nad4*, *nad4L*, *cytb* and *nad1*) have ATG as the initiation codon (Dai et al., 2016, Liu et al., 2016). Four PCGs (*cox1*, *cox2*, *nad6* and *nad4*) have incomplete stop codons, either TA or T, while the remaining end with TAA. Partial stop codons are reported in many lepidopteran mitogenomes. In Lepidoptera there seems to be a high degree of conservation of incomplete stop codons (Liao et al., 2010; Liu et al., 2013).

The comparative analysis of the different codons in ten species of Lepidoptera (Fig. 2) reveals that *Asn*, *Ile*, *Leu2*, *Lys*, *Phe*, *Tyr* and *Met* are the most frequent amino acids, of which the *Leu2* family (hydrophobic amino acid) the most frequent. The composition of amino acid might be related to the function of the chondriosome in encoding several transmembrane proteins (Lu et al., 2013). Furthermore, the relative codon usage (RSCU) recorded for *S. robusta* is similar to that for *L. melli*, *A. formosae*, *S. charonda kuriyamaensis* and *O. furnacalis*, but different from that recorded for the codons of species that lack GCG&GTG (*H. cunea*), GCG&CGC&CCG (*G. argentata*), CGG (*P. atrilineata*), GCG (*C. pomonella*) and GCG (*H. vitta*). It is likely there are fewer codons with a high GC, as this feature seems to be conserved in insects (Lu et al., 2013; Dai et al., 2015).

Five overlaps, with a total length of 24 bp were recorded in the mitogenome of *S. robusta*. The largest 8 bp overlap is between *trnW* and *trnC* (Table 3) as documented for other species of Lepidoptera, for instance *B. mandarina* (Li et al., 2010) and *B. mori* Dazao (Liu et al., 2013). An interesting aspect of the present study is the finding that there is an overlapping sequence of 7 bp (ATGATAA) at the junction of the *atp8-atp6* genes. This overlap seems to be conserved in the Lepidoptera currently sequenced (Liu et al., 2008; Zhu et al., 2013). The overall organization of the mitogenome of *S. robusta* is compact, with only 168 bp intergenic spacers dispersed in 18 regions and ranging in size from 1 to 37 bp (Table 3). The longest intergenic spacer (37 bp) is located at the junction *trnQ-nad2*, with an extremely high A+T content, which is frequently recorded in the mitogenomes of Lepidoptera (He et al., 2015). The intergenic spacers in the mitogenome studied is longer than that in *A. selene* (137 bp over 13 regions), but shorter than that in *O. lunifer* (371 bp over 20 regions) (Salvato et al., 2008; Liu et al., 2012). The 17 bp spacer between *trnS2* (UCN) and *nad1* contains the "ATACTAA" motif (Fig. 7A), which is a highly conserved region in most insect mtDNAs and is proposed as a possible mitochondrial transcription termination peptide-binding site (mtTERM protein) (Taanman, 1999).

The AT-rich region in the mitogenomes of arthropods is a non-coding stretch, usually located between the *trnI-trnQ-trnM* gene cluster and the *rrnS* gene. The occurrence of different copy numbers of tandemly repeated elements is documented as one of the remarkable features of the A+T-rich region in insects. The A+T-rich region in *S. robusta* extends over 344 bp (15,104–15,447) and is located between *rrnS* and *trnM*. The region is highly A+T (95.35%) biased compared to the mitogenome as a whole (81.09%). The first two repeat regions, the motif "ATAGA" and the 19 bp poly-T stretch are located on the *rrnS* gene side of the A+T-rich region, while the microsatellite-like $(AT)_9$ and poly-A element are located upstream of the *trnM* gene (Fig. 7B). Comparison with other previously sequenced lepidopterans revealed that the A+T-rich region of the mitogenome studied is longer than that in *L. melli* (338 bp), *G. argentata* (340 bp), *S. morio* (316 bp), *H. vitta* (255 bp), *M. sexta* (324 bp) and *A. ipsilon* (332 bp), but shorter than that in *H. cunea* (357 bp), *A. formosae* (482 bp), *C. pomonella* (351 bp), *P. atrilineata* (457 bp), *B. mandarina* (484 bp), *A. pernyi* (552 bp) and *C. suppressalis* (348 bp) (Table 4). The length of the poly-T stretch varies from species to species (Lu et al., 2013; Dai et al., 2015) and the ATAGA region is conserved in Lepidoptera (Cameron & Whiting, 2008).

The phylogenetic analyses revealed that the different species from the same family clustered together. These results are consistent with the conclusion of many authors, e.g. Liu et al. (2015) and Lammermann et al. (2016). Results of further analyses strongly support a close relationship between *S. robusta* and *L. melli* (Erebidae).

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