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Complete mitochondrial genome of the laced fritillary *Argyreus hyperbius* (Lepidoptera: Nymphalidae)

WANG Xiao-Can[1], SUN Xiao-Yan[2], SUN Qian-Qian[1], ZHANG Da-Xiu [1],
HU Jing[1], YANG Qun[2,*], HAO Jia-Sheng[1,2,*]

(1. *College of Life Science, Anhui Normal University, Wuhu* 241000, *China;*
2. *LPS, Institute of Geology and Paleontology, the Chinese Academy of Sciences, Nanjing* 210008, *China*)

Abstract: We investigated the complete mitochondrial genome (mitogenome) of *Argyreus hyperbius*. The 15156 bp long genome harbored the gene content (13 protein coding genes, 22 tRNA genes, 2 rRNA genes and an A+T-rich region) and the gene arrangement was identical to all known lepidopteran mitogenomes. Mitogenome sequence nucleotide organization and codon usage analyses showed that the genome had a strong A+T bias, accounting for A+T content of 80.8%, with a small negative AT skew (−0.019). Eleven intergenic spacers totaling 96 bp, and 14 overlapping regions totaling 34 bp were scattered throughout the whole genome. As has been observed in other lepidopteran species, 12 of the 13 protein-coding genes (PCGs) were initiated by ATN codons, while the COI gene was tentatively designated by the CGA codon. A total of 11 PCGs harbored the complete termination codon TAA, while the COI and COII genes ended at a single T residue. All of the 22 tRNA genes showed typical clover structures except that the $tRNA^{Ser(AGN)}$ lacks the dihydrouridine (DHU) stem which is replaced by a simple loop. The intergenic spacer sequence between the $tRNA^{Ser(AGN)}$ and ND1 also contained the ATACTAA motif, which is conserved in all other lepidopterans as well. Additionally, the 349 bp A+T-rich region was not comprised of large tandem repetitive sequences, but harbored a few structures common to other lepidopteran insects, such as the motif ATAGA followed by a 20 bp poly-T stretch, a microsatellite-like $(AT)_9$ element preceded by the ATTTA motif, and a 5 bp poly-A site present immediately upstream of $tRNA^{Met}$. The mitochondrial genomic sequence features found in this study not only contribute to genetic diversity information of the group, but also are useful in future studies of the endangered nymphalid butterfly in population genetic dynamics, species conservation, phylogeography and evolution.

Key words: *Argyreus hyperbius*; Nymphalidae; Lepidoptera; Mitochondrial genome

斐豹蛱蝶线粒体基因组全序列的测定和分析

王晓灿[1], 孙晓燕[2], 孙倩倩[1], 张大秀[1], 胡 静[1], 杨 群[2,*], 郝家胜[1,2,*]

（1. 安徽师范大学 生命科学学院分子进化与生物多样性研究室, 安徽 芜湖 241000;
2. 中国科学院南京地质古生物研究所 现代古生物学与地层学国家重点实验室, 江苏 南京 210008）

摘要：该研究对斐豹蛱蝶（*Argyreus hyperbius*）（鳞翅目：蛱蝶科）线粒体基因组全序列进行了测定和初步分析。结果表明：斐豹蛱蝶线粒体基因全序列全长为 15156bp, 包含 13 个蛋白质编码基因、22 个 tRNA 和 2 个 rRNA 基因以及 1 个非编码的 A+T 富集区, 基因排列顺序与其它鳞翅目种类一致; 线粒体全序列核苷酸组成和密码子使用显示出明显的 A+T 偏好（80.8%）和轻微的 AT 偏移（AT skew, −0.019）。基因组中共存在 11 个 2～52 bp 不等的基因间隔区, 总长 96 bp; 以及 14 个 1～8 bp 不等的基因重叠区, 总长 34 bp。除 COI 以 CGA 作为起始密

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* Corresponding authors (通信作者), E-mail: qunyang@nigpas.ac.cn; jshaonigpas@sina.com
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码子外, 13 个蛋白质编码基因中的其余 12 个基因是以 ATN 作为起始密码子。除 COI 和 COII 基因是以单独的一个 T 为终止密码子, 其余 11 个蛋白质编码基因都是以 TAA 结尾的。除了缺少 DHU 臂的 $tRNA^{Ser(AGN)}$, 其余的 tRNA 基因都显示典型的三叶草结构。$tRNA^{(AGN)}$ 和 ND1 之间的基因间隔区包含一个 ATACTAA 结构域, 这个结构域在鳞翅目中是保守的。A+T 富集区没有较大的多拷贝重复序列, 但是包含一些微小重复结构：ATAGA 结构域下游的 20 bp poly-T 结构, ATTTA 结构域后的$(AT)_9$重复, 以及位于 $tRNA^{Met}$ 上游的 5 bp poly-A 结构等。这项研究所揭示的斐豹蛱蝶的线粒体基因组特征, 不仅为认识蛱蝶科的遗传多样性贡献数据, 而且对于该物种的保护生物学、群体遗传学、谱系地理及演化研究等具有重要意义。

关键词：斐豹蛱蝶; 蛱蝶科; 鳞翅目; 线粒体基因组
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The laced fritillary, *Argyreus hyperbius* Linnaeus, is an oriental nymphalid butterfly species distributed in areas of south-east Asia, India, and north-east Africa. In recent decades, mainly owing to habitat destruction, numerous local populations have shown a sharp decline, and thus this species is considered endangered in some countries including China. Known as the “flying flower”, *A. hyperbius* was once wide-spread but is now rarely found in any large cities, such as Nanjing (Wu, 2008). To date, however, this once widely distributed species has received little attention. Detailed research focusing on aspects such as population genetic divergence, phylogeography and other relevant areas are required; thus, our study was conducted to assist in the protection and better understanding of this butterfly species.

Animal mitochondrial genomes are generally a circular molecule, ranging from 15−20 kb in size, and with a few exceptions, they all encode 37 genes: 13 protein-coding genes (PCGs), 2 ribosomal RNA genes (lrRNA and srRNA), and 22 transfer RNA genes and non-coding control elements regulating the transcription and replication of the mitochondrial genome (Taanman, 1999). Maternally inherited mtDNA is simple and stable in structure. These genes are predominantly encoded on both strands and are compactly arranged, with coding segments separated by none or only very short (a few base pairs) non-coding spacers, and in rare cases, a few genes overlap. Therefore, mitochondrial genes or genomes have been used as potential tools in studies of phylogenetics, phylogeography, phylogenetic chronology, and molecular diagnostics (Nardi et al, 2005; Simonsen et al, 2006) especially with the aid of PCR methodologies (Kocher et al, 1989; Yamauchi et al, 2004).

Within the Lepidoptera order, the butterflies (Rhopalocera) account for nearly 16000 species, and its largest subgroup (Nymphalidae) contain approximately 5000 species (DeVries, 2001). Despite this large taxonomic diversity, information about the nymphalid butterfly mitogenome is still limited, and to the best of our knowledge, only a few complete or nearly complete mitogenomes of nymphalid species are currently available on GenBank (Tab.1). Thus, newly added mitogenome sequences of nymphalid species can provide

Tab. 1 Mitochondrial genomes employed in this study

Family	Subfamily	Species	GenBank Acc. No.	Reference
Papilionidae	Papilioninae	*Papilio maraho*	NC_014055	From GenBank
Papilionidae	Papilioninae	*Teinopalpus aureus*	NC_014398	From GenBank
Papilionidae	Papilioninae	*Papilio xuthus*	EF621724	Feng et al (2010)
Papilionidae	Parnassiinae	*Parnassius bremeri*	FJ871125	Kim et al (2009)
Pieridae	Pierinae	*Pieris melete*	NC_010568	Hong et al (2009)
Pieridae	Pierinae	*Pieris rapae*	HM156697*	Mao et al (2010)
Nymphalidae	Heliconiinae	*Acraea issoria*	NC_013604	Hu et al (2010）
Nymphalidae	Argynninae	*Argyreus hyperbius*	JF439070*	This study
Nymphalidae	Apaturinae	*Sasakia charonda*	NC_014224	Unpublished
Nymphalidae	Calinaginae	*Calinaga davidis*	HQ658143*	Xia et al (2011)
Nymphalidae	Satyrinae	*Melanitis leda*	JF905446*	Unpublished
Nymphalidae	Satyrinae	*Eumenis autonoe*	GQ868707	Kim et al (2010)
Nymphalidae	Danainae	*Euploea mulciber*	HQ378507*	Unpublished
Nymphalidae	Libytheinae	*Libythea celtis*	HQ378508*	Unpublished
Lycaenidae	Theclinae	*Coreana raphaelis*	DQ102703	Kim et al (2006)

* Unreleased mitochondrial genomes determined by our laboratory.

further insights into their diversity and evolution. In this study, we sequenced the entire mitogenome of the nymphalid butterfly *Argyreus hyperbius* and analyzed its nucleotide organization and major characteristics compared with those of other butterfly species to increase of understanding of mitogenomes and phylogenies of correlative butterflies.

1 Materials and Methods

1.1 Sample and DNA extraction

Adult *A. hyperbius* individuals were collected on Huangshan Mountain in Anhui Province, China, on August 2006 (specimen voucher ZWX09). After collection, the fresh materials were preserved in 100% ethanol immediately and stored in a −20 °C refrigerator before genomic DNA extraction.

Whole genomic DNA was extracted and purified by the modified glass powder method, whereby rice-sharp thorax muscle taken and put into one 10 mL Eppendorf tube, washed twice with ddH_2O, soaked for about 2−3 h, and then incubated with 500 μL DNA liquid (5 mmol/L of NaCl, 0.5% SDS, 15 mmol/L of EDTA, 10 mmol/L of Tris-HCl, pH 7.6) and 40 μL of Proteinase-K (20 mg/ml), After this, the muscle was bathed at 55 °C for 10−12 h and centrifuged at 4 000 rpm for 2 min. The liquid supernatant was diverted into a new 10 mL Eppendorf tube, to which 500 μL of 8 mol/L GuSCN and 40 μL of 50% clean glass liquid mixture was added and the solution was then bathed at 37 ℃ for 1−2 h, rocked for 1 h, and centrifuged at 4 000 r/min for 1 min. The supernatant was then removed and the sediments were twice cleaned with 75% alcohol and once with acetone, and dried thoroughly in a vacuum dryer at 45 °C. Then 60 μL of TE (10 mmol/L Tris-Cl, 1 mmol/L EDTA, pH 8.0) was added into the Eppendorf tube with powder, and bathed at 56 °C for 30 min, then finally speed up slowly till 4 000 r/min and centrifuged for 1 min. The supernatant containing the genomic DNA was then transferred into a clean 1.5 mL Eppendorf tube and preserved at −20 °C till use (Hao et al, 2007).

1.2 PCR amplification and sequencing

Some universal primers for short fragment amplifications of 12S rRNA, COI, Cyt b genes were used for PCR (Simon et al, 1994; Simons & Weller, 2001). Long primers and some short ones including COIII and ND5 were designed by the multiple sequence alignments of all the available complete lepidopteran mitochondrial genomes (Tab. 1) using ClustalX1.8 (Thompson et al, 1997) and Primer Premier 5.0 software (Singh et al, 1998).

Long PCRs were performed using TaKaRa LA Taq polymerase with the following cycling parameters: initial denaturation for 5 min at 95 °C, followed by 30 cycles at 95 °C for 50 s, 50 °C for 50 s, 68 °C for 2 min and 30 s; and a final extension step of 68 °C for 10 min. Short fragments were amplified with TaKaRa Taq polymerase: initial denaturation for 5 min at 94 °C, followed by 35 cycles at 94 °C for 1 min, 45−53 °C for 1 min, 72 °C for 2 min, and a final extension step of 72 °C for 10 min. The PCR products were detected via electrophoresis in 1.2% agarose gel, purified using the 3S Spin PCR Product Purification Kit and sequenced directly with an ABI-3730 automatic DNA sequencer.

1.3 Sequence analysis

The determined sequences were checked firstly with the NCBI Internet BLAST search function. Raw sequence files were proof read and assembled in BioEdit version 7.0 (Hall, 1999) as well as ClustalX 1.8 (Thompson et al, 1997). Transfer RNA gene analysis was conducted using tRNAscan-SE software v.1.21 (Lowe & Eddy, 1997). Putative tRNA genes not found by tRNAscan-SE were confirmed by sequence comparison between *A. hyperbius* and other lepidopterans. Both PCGs and ribosomal RNA genes were identified by ClustalX1.8 software, and the PCGs nucleotide sequences were translated on the basis of the Invertebrate Mitochondrial Genetic Code. Nucleotide composition skewness (AT skew=(A−T)/(A+T), GC skew=(G−C)/(G+C) (Irwin et al, 1991)) and codon usage were calculated in MEGA 4.0 software (Kumar et al, 2004). The *A. hyperbius* mitogenome sequence data were deposited into the GenBank database under the accession number JF439070.

2 Results

2.1 Genome organization

The mitogenome of *A. hyperbius* was 15 156 bp in length (Fig. 1) and encoded 37 genes totally 13 PCGs (ATP6, ATP8, COI-III, ND1-6, ND4L, Cyt b), 2 ribosomal RNA genes for small and large subunits (srRNA and lrRNA), and 22 transfer RNA genes) and a non-coding A+T-rich region (the control region) (Tab. 2). Among these, 14 genes were encoded on the N strand, including 4 PCGs (ND1, ND4, ND4L, ND5), 2 ribosomal RNA genes for small and large subunits, and 8 transfer RNA genes ($tRNA^{Gln}$, $tRNA^{Cys}$, $tRNA^{Tyr}$,

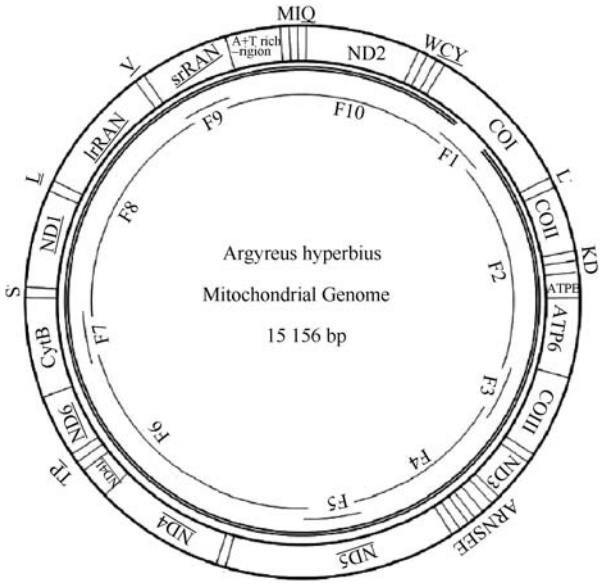


Fig. 1 Circular map of the *Argyreus hyperbius* mitochondrial genome

tRNA^{Phe}, tRNA^{His}, tRNA^{Pro}, tRNA$^{Leu(CUN)}$, tRNA^{Val}). The remaining 22 genes and A+T-rich region were encoded on the J strand. Eleven intergenic spacers totaling 96 bp, and 14 overlapped regions totaling 34 bp were scattered throughout the whole genome.

2.2 PCGs, tRNA and rRNA genes, A+T-rich region

Twelve of the 13 PCGs were initiated by ATN codons, while the COI gene was tentatively designated by the CGA codon; eleven PCGs harbored the complete termination codon TAA, while the COI and COII genes ended at a single T residue.

Results showed *A. hyperbius* harbored the typical set of 22 tRNA genes ranging from 61 to 71 bp in size. All the predicted secondary structures of the *A. hyperbius* tRNAs are shown in Fig. 2. Some 22 tRNA genes showed typical clover structures except that the tRNA$^{Ser(AGN)}$ lacked the dihydrouridine (DHU) stem, which was replaced by a simple loop. Seventeen tRNA genes has a total of 26 pair mismatches in their stems, among which, seven were in the DHU stems, nine in the amino acid acceptor stems, one in the TΨC stem, and nine in the anticodon stems, respectively.

Based on the mitogenomes of the other insects, two rRNA genes (lrRNA and srRNA) were present in *A. hyperbius*. The 1 330 bp lrRNA and 778 bp srRNA were located between tRNA^{Leu}(CUN) and tRNA^{Val}, and between tRNA^{Val} and the A+T-rich region, respectively.

The 349 bp A+T-rich region was not comprised of large tandem repetitive sequences, but harbored a few structures common to other lepidopteran insects, such as motif ATAGA followed by a 20 bp poly-T stretch, a microsatellite-like $(AT)_9$ element preceded by the ATTTA motif, and a 5 bp poly-A site present immediately upstream of tRNA^{Met}.

2.3 Sequence variation and codon usage

The A+T content of the *A. hyperbius* was 80.8%, and the whole mitogenome showed obvious A+T bias (Tab. 3). The relative synonymous codon usage (RSCU) in the *A. hyperbius* mitochondrial PCGs was investigated and the results are summarized in Tab. 4. The four most frequently used codons were TTA (leucine, Leu), ATT (isoleucine, Ile), TTT (phenylalanine, Phe), and ATA (methionine, Met), accounting for 40.4% of all the codons in the *A. hyperbius* mitogenome. These four codons were composed of A or T nucleotides, indicating their biased usage. The total number of non-stop codons (CDs) of the *A. hyperbius* mitochondrial PCGs was 3 718. Among these amino acid codons, the Leu (14.20%), Ile (12.80%), Phe (10.27%), and Ser (8.50%) were the most frequently used.

3 Discussion

3.1 Genome organization

The size of the mitogenome was congruent with the sizes of other known lepidopteran mitogenomes, ranging from 15122 bp in *Melanitis leda* (unpublished, GenBank accession number JF905446) to 16 094 bp in *Papilio maraho* (unpublished, NC_014055). The gene content of the *A. hyperbius* mitogenome was the same as the typical animal mitogenome, and the gene order and orientation were identical to the already determined lepidopteran mitogenomes. Compared with other lepidopterans, however, the *A. hyperbius* mitogenome was relatively more compacted, with a total of only 96 bp intergenic spacers ranging from 2−52 bp in length. Additionally, a total of 34 bp overlapped regions were scattered throughout the whole genome. Its tRNA cluster existing ahead of NADH dehydrogenase subunit 2 (ND2) was arranged in M-I-Q order, which means the tRNA^{Met}(M) was followed by tRNA^{Ile}(I) and tRNA^{Gln}(Q), which was similar to lycaenid *Coreana raphaelis* (Kim et al, 2006) and the noctuid *Ochrogaster lunifer* (Salvato et al, 2008). As far as we know, all determined lepidopteran genomes, including that of *A. hyperbius*, share the same order of gene arrangement but differ from that of hypothesized ancestral insects. This confirms the suggestion proposed by Boore et al (1998) that the Lepidoptera may have diverged from other insect orders for a certain period of time, forming an independent evolutionary lineage.

Tab. 2 Organization of the *Argyreus hyperbius* mitochondrial genome

Gene	Nucleotide[a] No.	Size	Anticodon	IGNc[b]	Start code	Stop code
tRNA^{Met}	1−68	68	32−34 CAT	−2		
tRNA^{Ile}	67−131	65	97−99 GAT	−3		
tRNA^{Gln}	129−197	69	165−167 TTG	52		
ND2	250−1 263	1 014		−2	ATT	TAA
tRNA^{Trp}	1 262−1 330	69	1 294−1 296 TCA	−8		
tRNA^{Cys}	1 323−1 384	62	1 353−1 355 GCA	−1		
tRNA^{Tyr}	1 384−1 448	65	1 415−1 417 GTA	3		
COI	1 452−2 982	1 531		0	CGA	T-tRNA^{Leu}
tRNA$^{Leu(UUR)}$	2 983−3 049	67	3 013−3 015 TAA	0		
COII	3 050−3 725	676		0	ATG	T-tRNA^{Lys}
tRNA^{Lys}	3 726−3 796	71	3 756−3 758 CTT	−1		
tRNA^{Asp}	3 796−3 861	66	3 826−3 828 GTC	0		
ATP8	3 862−4 023	162		−7	ATT	TAA
ATP6	4 017−4 694	678		−1	ATG	TAA
COIII	4 694−5 482	789		2	ATG	TAA
tRNA^{Gly}	5 485−5 549	65	5 515−5 517 TCC	0		
ND3	5 550−5 903	354		8	ATT	TAA
tRNA^{Ala}	5 912−5 977	66	5 943−5 945 TGC	2		
tRNA^{Arg}	5 980−6 043	64	6 006−6 008 TCG	0		
tRNA^{Asn}	6 044−6 109	66	6 075−6 077 GTT	−2		
tRNA$^{Ser(AGN)}$	6 108−6 169	61	6 129−6 131 GCT	0		
tRNA^{Glu}	6 170−6 234	65	6 200−6 202 TTC	3		
tRNA^{Phe}	6 238−6 302	67	6 268−6 270 GAA	−1		
ND5	6 302−8 023	1 722		15	ATA	TAA
tRNA^{His}	8 039−8 105	67	8 073−8 075 GTG	−1		
ND4	8 105−9 445	1 341		2	ATG	TAA
ND4L	9 448−9 732	285		2	ATG	TAA
tRNA^{Thr}	9 735−9 798	64	9 765−9 767 TGT	0		
tRNA^{Pro}	9 799−9 865	67	9 831−9 833 TGG	4		
ND6	9 870−10 397	528		−1	ATA	TAA
Cyt b	10 397−11 551	1 155		−2	ATG	TAA
tRNA$^{Ser(UCN)}$	11 550−11 613	64	11 579−11 581 TGA	−2		
ND1	11 612−12 565	954		3	ATA	TAA
tRNA$^{Leu(CUN)}$	12 569−12 634	66	12 603−12 605 TAG	0		
lrRNA(16S)	12 635−13 964	1 330		0		
tRNA^{Val}	13 965−14 029	65		0		
srRNA(12S)	14 030−14 807	778		0		
D-loop	14 808−15 156	349				

[a] Underlines denote that the genes encoded on the N strand.

[b] IGN: intergenic nucleotides; negative numbers indicate overlapping nucleotides between adjacent genes.

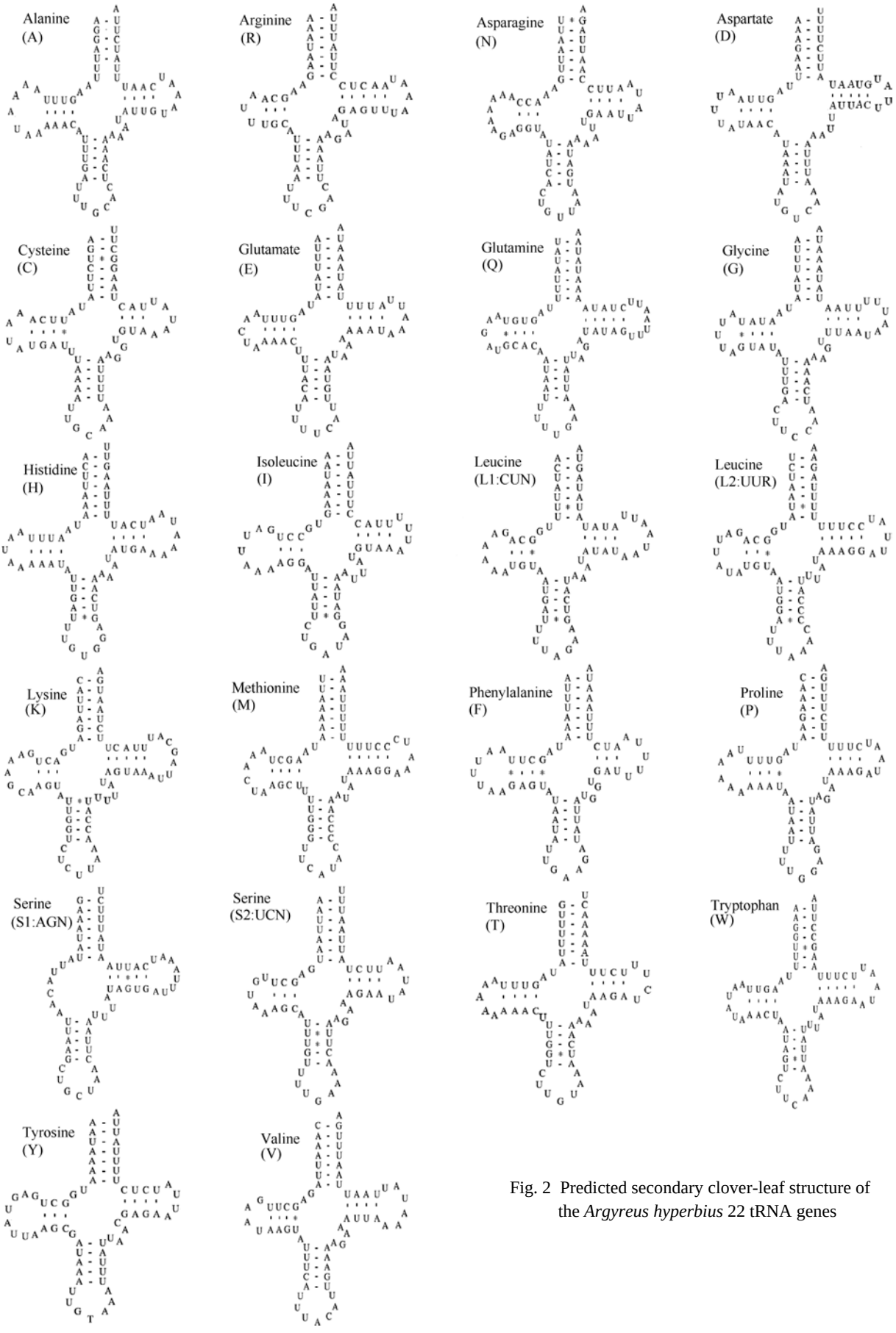


Fig. 2 Predicted secondary clover-leaf structure of the *Argyreus hyperbius* 22 tRNA genes

Tab. 3 Nucleotide composition and skewness in different regions of the *Argyreus hyperbius* mitogenome

	Size (bp)	Nucleotide composition (%)				
		T	C	A	G	AT
All gene	15 156	41.4	11.7	39.4	7.5	80.8
Genes on J-strand	6 887	44.2	12.7	33.7	9.4	77.9
Genes on N-strand	4 302	47.7	6.1	34.3	11.9	82.0
First codon positions	3 718	39.6	9.9	37.6	15.5	74.5
Second codon positions	3 718	48.3	16.1	22.5	13.1	70.8
Third codon positions	3 718	51.4	4.5	41.5	2.6	92.9
rRNA	2 108	45.4	10.1	39.4	5.2	84.7
Control region	349	50.1	3.7	45.3	0.9	95.4

Tab. 4 The codon number and RSCU in the *Argyreus hyperbius* mitochondrial PCGs

Codon	Num.	RSCU	Codon	Num.	RSCU	Codon	Num.	RSCU	Codon	Num.	RSCU
UUU(F)	357	1.87	UCU(S)	113	2.86	UAU(Y)	183	1.80	UGU(C)	31	1.94
UUC(F)	25	0.13	UCC(S)	12	0.30	UAC(Y)	20	0.20	UGC(C)	1	0.06
UUA(L)	439	4.99	UCA(S)	76	1.92	UAA(*)	0	0.00	UGA(W)	91	1.96
UUG(L)	21	0.24	UCG(S)	2	0.05	UAG(*)	0	0.00	UGG(W)	2	0.04
CUU(L)	42	0.48	CCU(P)	65	2.20	CAU(H)	60	1.74	CGU(R)	16	1.21
CUC(L)	4	0.05	CCC(P)	14	0.47	CAC(H)	9	0.26	CGC(R)	3	0.23
CUA(L)	22	0.25	CCA(P)	37	1.25	CAA(Q)	60	1.97	CGA(R)	30	2.26
CUG(L)	0	0.00	CCG(P)	2	0.07	CAG(Q)	1	0.03	CGG(R)	4	0.30
AUU(I)	440	1.85	ACU(T)	95	2.47	AAU(N)	238	1.85	AGU(S)	35	0.89
AUC(I)	36	0.15	ACC(T)	7	0.18	AAC(N)	19	0.15	AGC(S)	3	0.08
AUA(M)	266	1.83	ACA(T)	51	1.32	AAA(K)	97	1.81	AGA(S)	74	1.87
AUG(M)	25	0.17	ACG(T)	1	0.03	AAG(K)	10	0.19	AGG(S)	1	0.03
GUU(V)	60	2.02	GCU(A)	68	2.23	GAU(D)	62	1.85	GGU(G)	46	0.93
GUC(V)	1	0.03	GCC(A)	10	0.33	GAC(D)	5	0.15	GGC(G)	0	0.00
GUA(V)	53	1.78	GCA(A)	42	1.38	GAA(E)	66	1.81	GGA(G)	139	2.82
GUG(V)	5	0.17	GCG(A)	2	0.07	GAG(E)	7	0.19	GGG(G)	12	0.24

3.2 Protein-coding genes

All protein-coding sequences except COI gene use standard ATN start codon in *A. hyperbius* (Tab. 2). Three PCGs (ND5, ND1 and ND6) were initiated by ATA (Met); six PCGs (COII, ATP6, COIII, ND4, ND4L and Cyt b) were initiated by ATG (Met), and three PCGs (ND2, ATP8 and ND3) were initiated by ATT (Ile), respectively. However, the COI gene generally uses non-canonical initial codons across different insect groups. The use of non-canonical initial codons for the COI gene has been reported in a number of other insect species. For example, Junqueira et al (2004) and Friedrich & Muqim (2003) proposed AAA or TCG as the initial site for COI in dipteran *Chrysomya chloropyga* and in coleopteran *Tribolium castanaeum*, respectively. Other studies have determined that TTG is the initiation codon for COI in some invertebrates such as *Anopheles quadrimaculatus* (Mitchell et al, 1993), *Pyrocoelia rufa* (Bae et al, 2004), *Caligula boisdnvalii* (Hong et al, 2008) and *Acraea issoria* (Hu et al, 2010). In addition, the tetranucleotide TTAG in *Coreana raphaelis* (Kim et al, 2006), the hexanucleotide TATTAG in *Ostrinia nubilalis* and *Ostrinia furnicalis* (Coates et al, 2005), TTTTAG in *Bombyx mori* (Yukuhiro et al, 2002), ATTACG in *Papilio xuthus* (Feng et al, 2010), and TTAAAG in *Pieris rapae* (Mao et al, 2010) have also been proposed as the COI start codon. In the case of *A. hyperbius*, we tentatively presumed CGA as the start codon for COI, which was congruent with *Parnassius bremeri* (Kim et al, 2009), *Eumenis autonoe* (Kim et al, 2010), and *Hyphantria cunea* (Liao et al, 2010). Besides ATN, GTN has also been reported in Heterocera as the initiation codon for some PCGs. For instance, GTG has been reported as the start codon for COII in *Caligula boisduvalii* (Hong et al, 2008) and *Eriogyna pyretorum* (Jiang et al, 2009), and for ND1 in *Ochrogaster lunifer* (Salvato et al, 2008). Furthermore, ND4 and ND4L in *Ochrogaster lunifer* use GTT as their initiation codon.

Eleven of the 13 protein-coding genes had the common stop codon (TAA), while COI and COII terminated with a single T residue in the *A. hyperbius* mitogenome. Similar cases have been found in most insect mitogenomes including all known lepidopteran mitogenomes. For example, a single T residue has been deemed the stop codon for COI, COII, ND5 and Cyt b, and a dinucleotide residue TA has been deemed the stop

codon for ATP6, ND4, ND4L, ND6 in *Coreana raphaelis* (Kim et al, 2006); similarly, a single T has been considered the stop codon for COI, COII and ND4, while TA residue is considered the stop codon for ATP6 in *Hyphantria cunea* (Liao et al, 2010). Incomplete stop codons produce functional stop codons in polycistronic transcription cleavage and polyadenylation processes (Ojala et al, 1981).

Three of the 13 PCGs (ATP8, ATP6, ND6) in *A. hyperbius* were flanked by other PCGs at the 3' end: ATP8-ATP6, ATP6-COIII, and ND6-Cyt b were overlapped by seven (ATGATAA), one (A) and one (A) nucleotide, respectively. The 3' end region of these three genes had the potential to form hairpin-like structures, which are crucial for precise mRNA cleavage to generate mature PCGs (Kim et al, 2006; Fenn et al, 2007).

Those genes encoded by the N strand are underlined. The tRNA genes are designated by single letter amino acid codes. L* and S* denote the $tRNA^{Leu(UUR)}$ and $tRNA^{Ser(UCN)}$, respectively.

3.3 Transfer RNA and ribosomal RNA genes

All the tRNA genes showed typical clover structure, with the exception of the $tRNA^{Ser(AGN)}$ gene which lacks the dihydrouridine (DHU) stem and was replaced by a simple loop. This phenomenon has also been detected in other insect groups (Wolstenholme, 1992) including lepidopterans (Hong et al, 2008; Kim et al, 2006; Salvato et al, 2008; Liao et al, 2010). Seventeen tRNA genes had a total of 26 pair mismatches in their stems, among which eighteen G·U, seven U·U, and one A·C were present. These mismatches found in tRNAs can be corrected through RNA-editing mechanisms (Lavrov et al, 2000). To date, however, these modifications in insect tRNA genes are not well understood in light of their mechanism, although some researchers propose there to be a connection with rapid species evolution of insects (Takashi et al, 1991; Watanabe & Watanabe , 1994).

Two rRNA genes were in the observed size range of known lepidopteran mitogenomes. For example, the 1 330 bp lrRNA was well within the range of other known lepidopterans (from 1319 bp in *A. melete* (Hong et al, 2009) to 1426 bp in *H. cunea* (Liao et al, 2010)). The case was similar with srRNA, in which size was also within the observed size range of other lepidopteran insects (from 434 bp in *Ostrinia nubilalis* (Coates et al, 2005) to 808 bp in *H. cunea*)).

3.4 Intergenic spacer sequences

Because of their rapid evolutionary rates, intergenic spacer sequences (IGS) show remarkable differences even among closely related insect species. Except for the A+T-rich region, the *A. hyperbius* mitogenome in this study was interleaved with 11 intergenic spacers totaling 96 bp and ranging in size from 2−52 bp (Fig. 1). The longest spacer (52 bp) located between the $tRNA^{Gln}$ and ND2 genes is a common feature to all lepidopteran mitogenomes, but has not yet been detected in non-lepidopteran species. This spacer showed a relatively high level of homology (62%) with its ND2 gene, which is similar to the 70% detected in *Parnassius bremeri* (Kim et al, 2009) but significantly different from the 32% in *Sasakia charonda* (unpublished, NC_014224). Accordingly, this spacer is thought to have originated from a partial duplication of the ND2 gene and undergone rapid sequence divergence for their non-coding nature among even closely related taxa (Kim et al, 2009). The other IGS more than 10 bp was present between the ND5 and $tRNA^{His}$, and this 15 bp long intergenic spacer exists in 15 of the 27 determined lepidopteran mitogenomes. Furthermore, a relatively conservative element of the nucleotides ATTTT was present within this spacer, which has also been found in determined insect species in the overwhelming majority of conditions. The IGS between $tRNA^{Ser}$(UCN) and ND1 is common among lepidopteran insects, spanning from 9 bp in *Diatraea saccharalis* (unpublished, NC_013274) to 38 bp in *Ostrinia nubilalis* (Coates et al, 2005). In the present study, however, it wsa nearly absent in *A. hyperbius* with only a 2 bp overlap, which is similar to findings on *Acraea issoria* (Hu et al, 2010), *Sasakia charonda* (unpublished, NC_014224), and *Calinaga davidis* (Xia et al, HQ658143) with 2-, 1-, 1- overlaps respectively. The conserved ATACTAA motif is regarded as a possible recognition site for the transcription termination peptide (mtTERM protein) and is usually located in the IGS between the $tRNA^{Ser}$(UCN) and ND1 genes. However, this motif was detected within the NDI genes of *A. hyperbius*. This is same as *S. charonda* and *C. davidis*, but it is present within the $tRNA^{Ser}$(UCN) in *Eumenis autonoe* (Kim et al, 2010) and absent in the *Sasakia charonda kuriymaensis* (unpublished, NC_014223).

3.5 A+T-rich region

The A+T-rich region harbors the origin sites for transcription and replication (Taanman, 1999). In *Drosophila* species, this region includes the replication origin for mtDNA heavy-strands and minor-strands

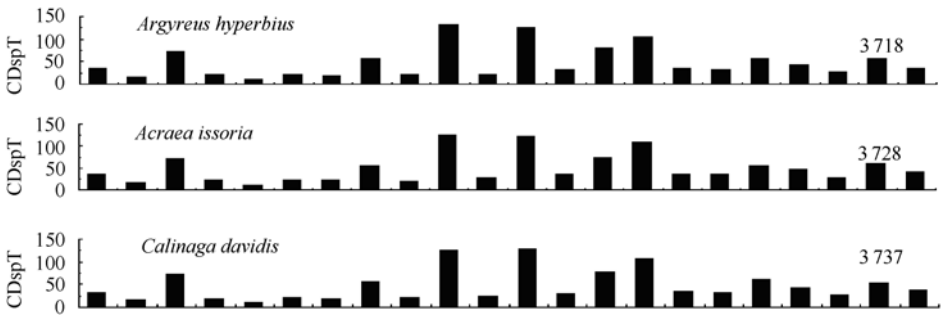
(Clary & Wolstenholme, 1987). Saito et al (2005) precisely determined that the replication origin site for mtDNA minor-strand was located in this region in *Bombyx mori* (Yukuhiro et al, 2002). In the present study, the A+T-rich region of the *A. hyperbius* mitochondrial genome was located between the srRNA and $tRNA^{Met}$ genes (Tab. 2) and was 349 bp in length. This was well within the range observed in the completely sequenced lepidopteran insects from 317 bp in *Melanitis leda* (unpublished, by our lab) to 747 bp in *Bombyx mandarina* (Liao et al, 2010). The A+T-rich region exhibited a remarkably high A+T content (95.41%) and did not contain macrorepeat units. However, it included some microsatellite-like repeats (e.g. polyT, $(AT)_9$, $(TA)_8$ and poly-A), as seen in other insect species. For example, the polyT stretch (20 bp), which is considered the structural signal for recognizing proteins in the mtDNA minor-strand initiation (Kim et al, 2009), was located 24 bp downstream from srRNA preceded by the motif ATAGA, which is conserved across the lepidoptera orders as well. The microsatellite-like repeat $(AT)_9$ element, located 235 bp downstream from srRNA, was preceded by the conserved motif ATTTA, which is similar to ATTTA$(TA)_8$ in *Manduca sexta* (Cameron et al, 2008), ATTTA$(AT)_8$ in *Hyphantria cunea* (Liao et al, 2010), ATTTA$(AT)_7$ in *Coreana raphaelis* (Kim et al, 2006), and ATTTA$(AT)_9$ in *Pieris rapae* (Mao et al, 2010). Thus, this phenomenon may be characteristic of the insect AT-rich regions. Additionally, another microrepeat unit $(TA)_8$ and a 5 bp long poly-A stretch were situated at the 284 bp site downstream from srRNA, and immediately upstream $tRNA^{Met}$, respectively.

3.6 Sequence variation and codon usage

The AT-skewness values of the J strand (majority or heavy strand) and N strand (minority or light strand) were −0.135 and −0.163, respectively, indicating the occurrence of more Ts than As in both the J and N strands; whereas, the GC skewness about the J and N strands were −0.149 and 0.322, respectively, suggesting a contrary condition of Gs and Cs.

For the 13 PCGs, the A+T content at the third codon position (92.9%) was higher than the first (74.5%) and second position (70.8%). The value of the A+T content of PCGs was 79.4% with a strong A+T bias. This result has been observed in other insects species, for examples, the AT contents of *Sasakia charonda*, *Coreana raphaelis*, *Parnassius bremeri* and *Helicoverpa armigera* PCGs have been reported to be 78.2%, 81.5%, 80.1% and 79.4%, respectively.

The relative synonymous codon usage (RSCU) analysis showed that TTA, ATT, TTT, and ATA were the four most frequently used codons, accounting for 40.4% of all codons in the *A. hyperbius* mitogenome. These four codons were all composed of A or T nucleotides, which indicated their biased usage. Such results have also been detected in other sequenced lepidopteran insects. For example, these four codons account for 39.1% in *Teinopalpus aureus*, 44.1% in *Coreana raphaelis*, and 40.7% in *Helicoverpa armigera*. For amino acids, the Leu, Ile, Phe, and Ser were the most frequently used in the *A. hyperbius* mitogenome PCGs, which is in agreement with findings for other lepidopteran insects (Fig. 3). The total number of non-stop codons (CDs) for the *A. hyperbius* mitochondrial PCGs was 3718, which accords with the range for other known butterfly species, from 3695 in *Sasakia charonda* to 3737 in *Calinaga davidis*. The codons per thousands codons(CDspT) of the Ile, Leu2 and Phe were more than 100, the CDspT of Met, Asn (asparagine), Gly (glycine), Ser2 and Tyr (tyrosine) were more than 50, and the Arg (arginine), Asp (aspartic acid), Glu (glutamic acid), Gln (glutamine), His (histidine) and Leu1 were below 20, with Cys (cysteine) the lowest at 8.61 in *A. hyperbius* mitochondrial PCGs. Both the CDs and CDspT of the *A. hyperbius* in this study shared similar patterns with those of other Papilionoidea butterfly species (Fig. 3).



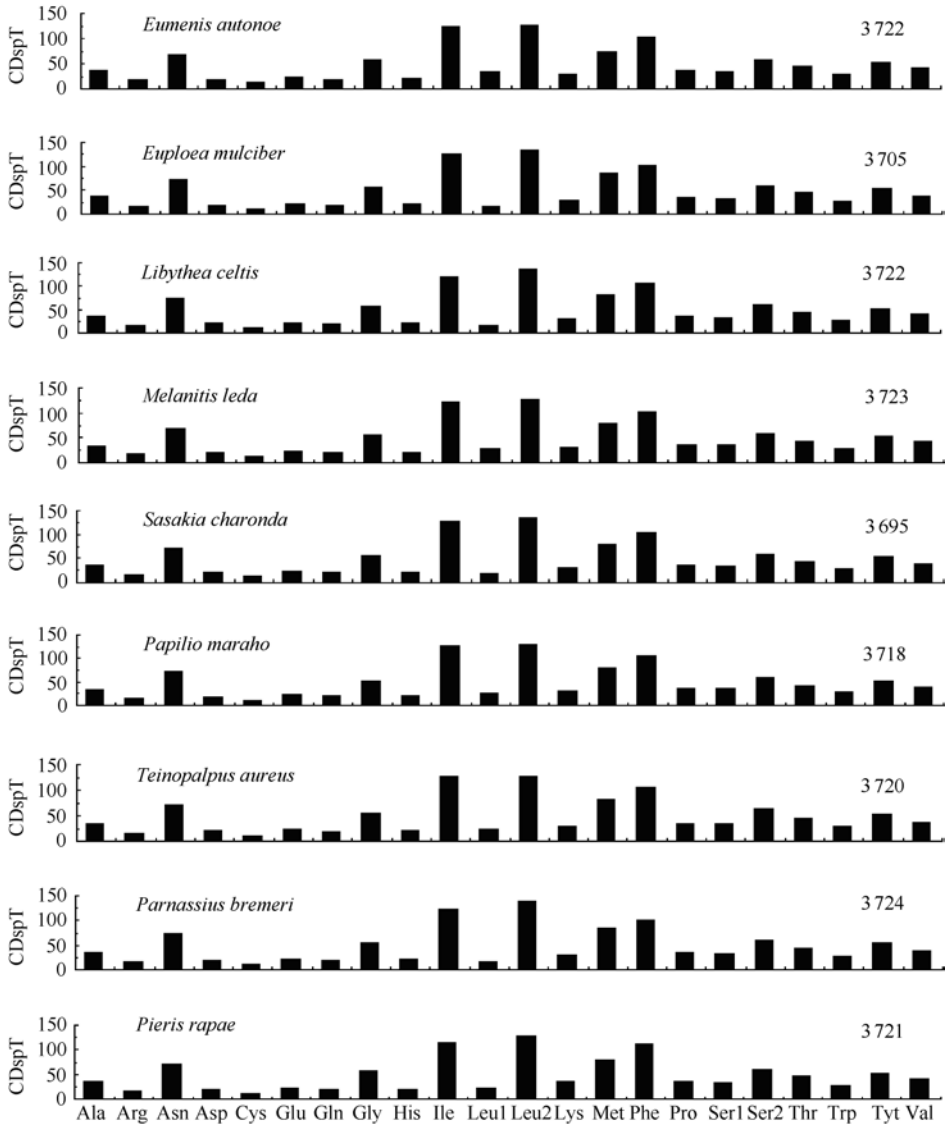


Fig. 3 Codon distribution in Papilionoidea mtDNAs

The ND5, ND4, ND4L, ND1 genes converted to the same orientation as the rest of the protein-coding genes. Numbers to the right refer to the total number of codons (CDs); the scale to the left refers to codons per thousands codons (CDspT). Codon families are provided on the x axis. The Leu1, Leu2, Ser1 and Ser2 are defined on the basis of their anti-codon, namely, Leu1 (CUN), Leu2 (UUR), Ser1 (AGN) and Ser2 (UCN).

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