

ORIGINAL ARTICLE

The complete mitochondrial genome of *Parnassius actius* (Lepidoptera: Papilionidae: Parnassinae) with the related phylogenetic analysis

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Abstract In this study, the complete mitochondrial genome of *Parnassius actius* (Lepidoptera: Papilionidae: Parnassinae) was reported. Meanwhile, the phylogenetic position of this Apollo species was inferred from this sequence combined with other available related sequence data. The results showed that the whole mitogenome is 15,386 bp in length, containing 13 typical protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), two ribosomal RNA genes (rRNAs) and a noncoding control region (A+T-rich region). The mitogenomic gene orientation and arrangement are the same with other sequenced *Parnassius* species. Thirteen protein coding genes is 11,195 bp in size, encoding 3,720 amino acids totally; the lrRNA and srRNA genes are 1,351 bp and 779 bp, respectively. Eleven intergenic spacers ranging from 1 to 40 bp (129 bp in total), 10 overlapping regions from 1 to 8 bp (26 bp in total), and a 504 bp A+T-rich region harboring some *Parnassius*-specific sequences are scattered throughout the whole mitogenome. The maximum likelihood and Bayesian phylogenetic analyses showed that the genus *Parnassius* of this study contained five major clades which can be categorized into the eight subgenera reported by the previous molecular studies; additionally, the *P. actius* was shown to be more closely related to *P. nomion* than other *Parnassius* species, suggesting that the *P. actius* stands as an effective species with its phylogenetic position awaiting further studies.

Key words Lepidoptera, Parnassinae, *Parnassius actius*, mt DNA, phylogenetic analysis.

1 Introduction

The Actius Apollo butterfly, *Parnassius actius* is a member of the snow Apollo genus *Parnassius* in the swallowtail family of Papilionidae. This species is usually distributed in mountainous areas of the Europe and Central Asia. Mainly due to the habitat destruction and environmental degradation, this species is declining rapidly in number and thus considered to be a vulnerable species nowadays (Fang *et al.*, 2010). Especially, some controversies are still existed about its taxonomic status and phylogenetic position (Chou, 1998; Wu, 2001).

The insect mitochondrial genomes are usually circular molecules about 15–16 kb long, and contains 37 genes, including 13 protein coding genes (PCGs), 22 tRNA genes, 2 ribosome RNA (lrRNA and srRNA) genes and a non-coding A+T-rich region harboring the initiation sites for transcription and replication (Wolstenholme, 1992; Boore, 1999). With the rapid

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progress of PCR and techniques in its product sequencing, the complete mitogenomic data of many animal groups including insects is much easier to obtain and has been very popularly used in the studies of comparative and evolutionary genomics, molecular evolution, phylogenetics, and population genetics (Zakharov *et al.*, 2004; Hurst & Jiggins, 2005; Cameron & Whiting, 2008; Footitt, 2008; Papanicolaou *et al.*, 2008; Wei, 2010; Hao *et al.*, 2012; Cameron, 2014).

Up to the present, about 150 complete or nearly complete mitogenomes of true butterflies (superfamily Papilionoidea), including 22 from the Papilionidae species have been sequenced and deposited in the GenBank. However, for the subfamily Parnassiinae, only eight complete mitogenome sequences have been reported (KM373898, FJ871125, HQ259122, NC023938, NC025587, KF746065, KU360130, NC026457). Thus, addition of more complete mitogenomic data of the *Parnassius* species are necessary for the further phylogenetic and evolutionary studies.

In this study, we reported the complete sequence of *P. actius* mitogenome compared with other determined Papilionidae species. Meanwhile, we conducted a more detailed *Parnassius* phylogenetic analysis incorporated with other available sequence data, in order to further clarify the phylogenetic position of this obscure Apollo butterfly species.

2 Materials and methods

2.1 Sample collection and DNA extraction

An adult specimen of *P. actius* was collected in Tianshan Moutains, Xinjiang Province, China, in August 2014. The fresh tissues were preserved in 100% ethanol and stored at -20°C until DNA extraction. Total genomic DNA was extracted from the thoracic muscle of an adult individual using a DNA extraction kit (Sangon Biotech, Shanghai, China) after the manufacture's instruction.

2.2 PCR amplification and sequencing

To sequence the full-length mitogenome of *P. actius*, 14 pairs of primers for the amplification of 7 short fragments and 7 long fragments were used (Fig. 1). Four short fragments (SF2, SF3, SF5, SF6) and four long fragments (LF1, LF4, LF5, LF7) were amplified by using the universal PCR primers from Simmons & Weller (2001), Simon *et al.* (2006), Zhao *et al.* (2013) and Chen *et al.* (2014). Other primers including that for the amplification of A+T-rich region were designed by the multiple sequence alignments of the known mitochondrial sequences of the lepidopteran species, using Clustal X 1.83 (Thompson *et al.*, 1997) and Primer Premier 5.0 softwares (Singh *et al.*, 1998) (Table 1). All primers were synthesized by the Sangon Biotechnology Co. Ltd., Shanghai, China.

Short fragments were amplified under the following condition: 5 min at 95°C; followed by 35 cycles of 50 s at 94°C, 50 s at 46°C–52°C and 1.5 min at 72°C; a final elongation for 10min at 72°C. Long fragment PCR was implemented using LA Taq DNA polymerase (TakaraBio, Otsu, Shiga, Japan) with the cycling parameters: 5 min at 95°C; followed by 30 cycle of 55s at 95°C, 2 min at 47°C–53°C and 2.5 min at 68°C; final elongation for 10 min at 68°C. The PCR products was detected by 1.2% agarose gel electrophoresis and purified using the DNA gel extraction kit (TaKaRa). All PCR products were directly sequenced after purification with the QIA quick PCR purification Kit reagents (QIAGEN). All of the long PCR fragments were sequenced using the primer walking strategy.

2.3 Data analysis

Sequence from overlapping fragments were initially assembled via the alignment of neighboring fragments using the BioEdit 7.0 (Hall, 1999) and ClustalX 1.83 (Thompson *et al.*, 1997). PCGs and rRNA genes were identified using NCBI Internet BLAST search function and subsequently by alignment with other butterflies using Clustal X 1.83 (Thompson *et al.*, 1997) and MEGA 6.0 (Tamura *et al.*, 2013) softwares. Both the lrRNA and srRNA predicted secondary structures were drawn according to models proposed for these genes in other insects (Gillespie *et al.*, 2006; Cameron & Whiting, 2008). The secondary structures of most of the tRNA genes were predicted with tRNAscan-SE1.21 (Lowe & Eddy, 1997) using invertebrate codon predictors, but some tRNAs not found by tRNAscan-SE were identified through with other lepidopteran tRNA genes. All tRNA were drawn by hand, using tRNAscan-SE output as template when possible.

The base composition, codon usage, and nucleotide substitution were analyzed with Mega 6.0 (Tamura *et al.*, 2013). AT-skew=(A-T)/(A+T) and GC-skew=(G-C)/(G+C) were used to measure the base-compositional difference between genes (Perna & Kocher, 1995). The tandem repeats in the control region were predicted using the Tandem Repeats Finder (Benson, 1999).

2.4 Phylogenetic analysis

Parnassius phylogeny were reconstructed with the maximum likelihood (ML) and Bayesian inference (BI) methods based on the four partial mitochondrial sequences (1rRNA, COI, ND1, ND5) available from GenBank and the corresponding sequence of *Parnassius actius* we determined (Table 2), using the three Papilionidae species *Byasa alcinous* (KJ476729), *Troides aeacus* (EU625344) and *Sericinus montela* (HQ259122) as the outgroups. The matrix data used here contain 3,198 nucleotides sites totally (1rRNA, 522 bp; COI, 1419 bp; ND1, 471 bp; ND5, 786 bp), and the softwares PAUP* version 4.0b10 (Swofford, 2002) and MrBayes 3.2.5 (Ronquist *et al.*, 2012) software packages were used for the ML and Bayesian analyses respectively. Model selection was conducted using Modeltest 3.7 (Posada & Crandall, 1998) and the optimal model (GTR+I+G) was selected according to the Akaike information criterion. In the ML analysis, 1,000 bootstrap replicates were conducted to obtain the support values of each node of the tree (Felsenstein, 1985), while in the Bayesian inference, two simultaneous chains were run for 1,000,000 generations with the first 25% discarded as the burn-in samples, and the resultant posterior probability was obtained as the support values of each tree node.

3 Results and Discussion

3.1 Genome organization

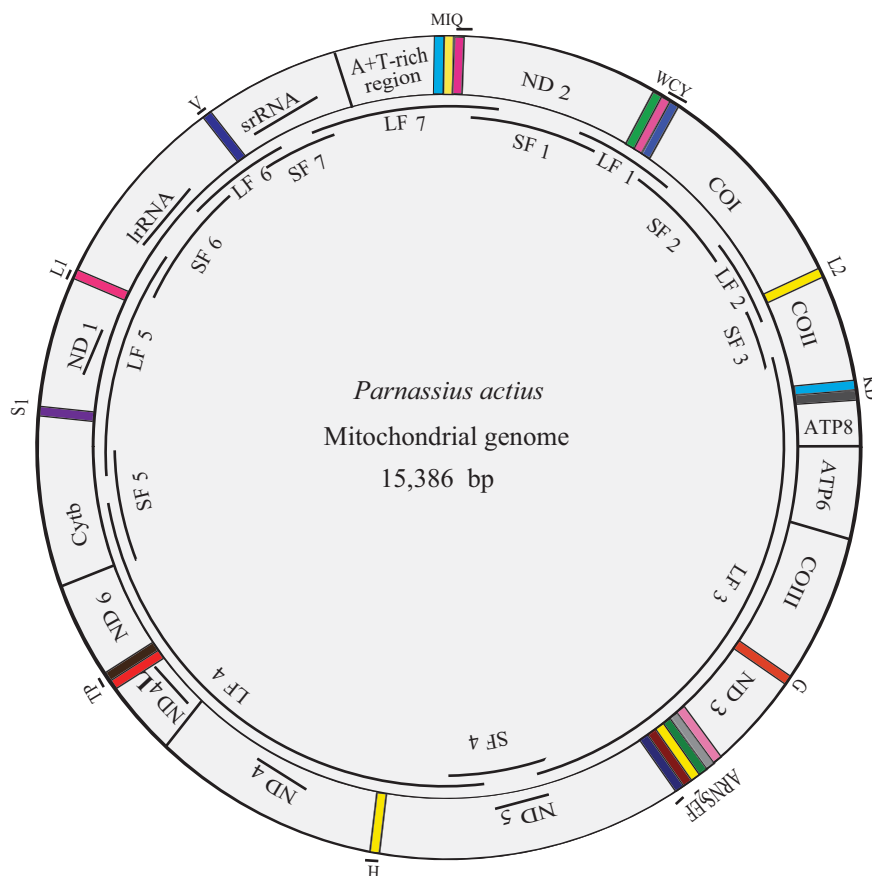


Figure 1. Circular map of the mitochondrial genome of *P. actius*. The abbreviations for the genes are as follows: COI, COII and COIII refer to the cytochrome oxidase subunits; Cytb refers to cytochrome B; ATP6 and ATP8 refer to subunits 6 and 8 of ATPase; ND1–6 refers to components of NADH dehydrogenase. The tRNAs are indicated by the IUPAC-IUB single letter amino acid codes, while L1, L2, S1, S2 denote tRNA^{Leu}(CUN), tRNA^{Leu}(UUR), tRNA^{Ser}(AGN) and tRNA^{Ser}(UCN), respectively. Gene names that are not underlined indicate the direction of transcription from left to right, and with underline indicates right to left. The *P. actius* mitogenome was sequenced by using 7 short fragments (SF1–SF7) and 7 long fragments (LF1–LF7) as templates, shown as single lines within a circle.

The *P. actius* mitogenome contains 37 genes (13 protein coding genes, 22 tRNA, 2 rRNA genes) and one non-coding A+T-rich region (control region), with its structure and organization identical to those of other determined lepidopterans (Fig. 1, Table 3). The complete mitogenome sequences of the *Parnassius actius* is 15,386 bp in size, fallen within the range of other Papilionidae butterflies determined from 15,158 bp in *Papilio machaon* (HM243594) to 16,094 bp in *Papilio maraho* (FJ810212) (Table 4). Four of the 13 PCGs (ND5, ND4, ND4L, ND1), 8 tRNAs (trnQ, trnC, trnY, trnF, trnH, trnP, trnL, trnV) and 2 rRNAs (rrnL and rrnS) are coded with the minority-strand, while the rest 23 genes are encoded by the majority-strand. The gene order and arrangement are identical to all other sequenced lepidopterans, with typical the tRNA cluster tRNA^{Met}-tRNA^{Ile}-tRNA^{Gln} (M-I-Q) order, which is different from the proposed ancestral I-Q-M order of insects (Taylor *et al.*, 1993; Boore *et al.*, 1998; Crease, 1999; Cao *et al.*, 2012) (Fig. 1).

Like the other Papilionidae species, the nucleotide composition of the entire mitogenome is significantly AT biased, with the relatively higher A+T contents of whole mitogenome (81.3%) (Table 4). The overall AT-skew and GC-skew in the whole genome of *P. actius* are -0.016 and -0.193, respectively, showing that more Ts and more Cs than As and Gs are used in the mitogenome (Table 5). A similar situation has been found in other determined Papilionidae species, which have negligible AT-skew values (-0.047 to 0.006) and moderate GC-skew values (-0.276 to -0.183). Additionally, the AT-skew of tRNA (0.006), the GC-skew of PCGs (0.025) and first codon positions (0.159) shows clearly that more As than Ts in tRNA and more Gs than Cs in the first codon position are used in the mitogenome (Table 5). The complete mitogenome of *P. actius* has been deposited in GenBank under accession number KY072796.

Table 1. List of primer used for the amplification of *P. actius* mitogenome.

Fragment*	Primer**	Sequence(5'-3')	Annealing temperature (°C)	Reference
SF1	ND2-F	AATTAAGCTATTAGGTTTCATACCC	52.8	This study
	ND2-R	GGCTGAATAATAAGCGATAAATTGTAAA		
SF2	COI-F	GTAAAWTAACTAATARTCTTCAAA	51.4	Simon <i>et al.</i> (2006)
	COI-R	GCTCGTGTATCAATATCTATWCC		
SF3	CO3-F	TATTTCAATGATGACGAGAT	47.8	Simon <i>et al.</i> (2006)
	CO3-R	CAAATCCAAAATGGTGAGT		
SF4	ND5-F	GGAACACAATCCTCATCAT	50.1	This study
	ND5-R	TTGCTTTGTCTACTTTAAGACA		
SF5	CytB-F	TACGTTTACCATGAGGTCAAATATC	52.1	Simmons & Weller (2001)
	CytB-R	ACTTCTTTTCTTATGTTTTCAAAAC		
SF6	lrRNA-F	CTGTACAAAGGTAGCATA	48.5	Zhao <i>et al.</i> (2013)
	lrRNA-R	GCCAAAACCTTTAGTCTAG		
SF7	srRNA-F	AAGAGCGACGGGCGATGTGT	55	This study
	srRNA-R	AAACTAGGATTAGATACCCTATTAT		
LF1	ND2-COI-F	CCCTTTCATTTCTGATTCC	51.1	Chen <i>et al.</i> (2014)
	ND2-COI-R	ACTGTTCGTCCTGTTCTCCT		
LF2	COI-COIII-F	TTTGTAGAAATGCCAA	41.0	This study
	COI-COIII-R	TCCAGATAAGGGTCA		
LF3	COIII-ND5-F	TAAGTGTCTACTGATGTGATT	48.5	This study
	COIII-ND5-R	AGCTGGGTTTTATTCTAAG		
LF4	ND5-CytB-F	AATTATACCAGCACATAT	47.1	Zhao <i>et al.</i> (2013)
	ND5-CytB-R	TTATCGACTGCAAATC		
LF5	CytB-lrRNA-F	TCCTGCTAACCCCTTAGTCA	50.9	Chen <i>et al.</i> (2014)
	CytB-lrRNA-R	GAGTATTTTGTGGGGT		
LF6	lrRNA-srRNA-F	CTGGGGTCTTCTCGTCT	51.5	This study
	lrRNA-srRNA-R	GCAATAAGTTGGCGGTA		
LF7	srRNA-ND2-F	GAAACACTTTCCAGTACCT	49.7	Chen <i>et al.</i> (2014)
	srRNA-ND2-R	CTAAACCAATTCAACATCC		

*SF—short fragment; LF—long fragments

**F—forward direction of transcription; R—reverse direction of transcription

Table 2. General informatics of the *Parnassius* species used in this study.

Species	Accession number			
	IrRNA	COI	ND1	ND5
<i>Parnassius smintheus</i>	EF473768	EF473777	EF473852	AB095653
<i>Parnassius phoebus</i>	AB186139	JN204959	AB186173	AB095654
<i>Parnassius jaquemontii</i>	EF473774	EF473782	AJ972111	AB095647
<i>Parnassius honrathi</i>	EF473750	EF473785	AJ972129	AB096091
<i>Parnassius tianschanicus</i>	EF473752	EF473787	EF473858	AB095648
<i>Parnassius nomion</i>	AB186141	EF473781	AB186175	AB095609
<i>Parnassius maharaja</i>	AJ971980	EF473817	AB186150	AB095615
<i>Parnassius szechenyii</i>	EF473759	EF473821	EF473850	AB095642
<i>Parnassius acco</i>	AB186145	EF473823	AB186145	AB095652
<i>Parnassius schultei</i>	AJ971976	EF473824	AB186149	AB095619
<i>Parnassius hardwickii</i>	AB186144	EF473822	AB186144	AB094969
<i>Parnassius andreji</i>	EF473766	EF473813	EF473855	AB095643
<i>Parnassius simo</i>	AJ971971	EF473815	EF473854	AB095640
<i>Parnassius boedromius</i>	AJ971970	EF473814	EF473856	AB095629
<i>Parnassius simonius</i>	DQ351093	EF473816	EF473857	AB095649
<i>Parnassius ariadne</i>	AB186160	EF473794	AB186194	AB094970
<i>Parnassius mnemosyne</i>	AJ971955	EU093018	AB186159	AB095626
<i>Parnassius glacialis</i>	AB186165	EF485045	AB186165	AB063353
<i>Parnassius stubbendorffii</i>	AJ971956	EF473800	EF473836	AB013141
<i>Parnassius clodius</i>	DQ351094	EF473795	EF473838	AB095624
<i>Parnassius eversmanni</i>	AB186163	EF473797	AB186197	AB094971
<i>Parnassius nordmanni</i>	AB186161	EF473799	AB186161	AB094968
<i>Parnassius acdestis</i>	AB186156	EF473792	AB186156	AB095621
<i>Parnassius autocrator</i>	AB186158	EF473788	AB186158	AB095634
<i>Parnassius loxias</i>	EF473763	EF473791	EF473840	AB096090
<i>Parnassius charltonius</i>	AB186154	EF473789	AB186155	AB095630
<i>Parnassius inopinatus</i>	EF473762	EF473790	EF473841	AB095641
<i>Parnassius cardinal</i>	EF473755	EF473803	EF473847	AB095644
<i>Parnassius staudingeri</i>	AJ972000	EF473810	EF473846	AB095637
<i>Parnassius hide</i>	AB186153	EF473807	AB186153	AB095613
<i>Parnassius stenosemus</i>	EF473758	EF473811	EF473845	AB095656
<i>Parnassius stoliczkanus</i>	AJ971992	EF473812	EF473844	AB095650
<i>Parnassius delphius</i>	AB186151	EF473804	AB186151	AB095632
<i>Parnassius maximinus</i>	EF473772	EF473805	EF473848	AB095651
<i>Parnassius patricius</i>	AB186152	EF473806	AB186152	AB095620
<i>Parnassius arcticus</i>	EF473764	EF473826	EF473843	AB095639
<i>Parnassius tenedius</i>	DQ351092	EF473825	AJ972063	AB095658
<i>Parnassius apollo</i>	KF746065			
<i>Parnassius bremeri</i>	FJ871125			
<i>Parnassius epaphus</i>	KM373898			
<i>Parnassius cephalus</i>	KP100655			
<i>Parnassius imperator</i>	KM507326			
<i>Parnassius choui</i>	KY072797			
<i>Parnassius actius</i>	This study			

Table 3. Basic components of the *P. actius* mitogenome.

Gene	Direction	Location	Size (bp)	Intergenic length*	Anticodon	Start codon	Stop codon
tRNA ^{Met}	F	1–69	69	0	33–35 CAT	-	-
tRNA ^{Ile}	F	70–133	64	0	99–101 GAT	-	-
tRNA ^{Gln}	R	131–199	69	-3	167–169 TTG	-	-
ND2	F	240–1253	1014	40	-	ATT	TAA
tRNA ^{Trp}	F	1254–1319	66	0	1284–1286 TCA	-	-
tRNA ^{Cys}	R	1312–1377	66	-8	1346–1348 GCA	-	-
tRNA ^{Tyr}	R	1382–1445	64	4	1413–1415 GTA	-	-
COI	F	1448–2978	1531	0	-	CGA	T-tRNA
tRNA ^{Leu} (UUR)	F	2979–3045	67	0	3009–3011 TAA	-	-
COII	F	3046–3727	682	0	-	ATG	T-tRNA
tRNA ^{Lys}	F	3728–3798	71	0	3758–3760 CTT	-	-
tRNA ^{Asp}	F	3798–3864	67	-1	3729–3831 GTC	-	-
ATP8	F	3865–4029	165	0	-	ATT	TAA
ATP6	F	4023–4700	678	-7	-	ATG	TAA
COIII	F	4700–5488	789	-1	-	ATG	TAA
tRNA ^{Gly}	F	5492–5558	67	3	5522–5524 TGC	-	-
ND3	F	5559–5912	354	0	-	ATT	TAA
tRNA ^{Ala}	F	5912–5977	66	-1	5942–5944 TGC	-	-
tRNA ^{Arg}	F	5977–6042	66	-1	6006–6008 TCG	-	-
tRNA ^{Asn}	F	6043–6106	64	0	6073–6075 GTT	-	-
tRNA ^{Ser} (AGN)	F	6110–6170	61	3	6131–6133 GCT	-	-
tRNA ^{Glu}	F	6210–6275	66	39	6241–6243 TTC	-	-
tRNA ^{Phe}	R	6274–6339	66	-2	6304–6306 GAA	-	-
ND5	R	6341–8074	1734	1	-	ATT	TAA
tRNA ^{His}	R	8075–8138	64	0	8106–8109 GTG	-	-
ND4	R	8138–9478	1341	-1	-	ATG	TAA
ND4L	R	9478–9768	291	-1	-	ATG	TAA
tRNA ^{Thr}	F	9771–9837	67	2	9803–9805 TGT	-	-
tRNA ^{Pro}	R	9838–9902	65	0	9870–9872 TGG	-	-
ND6	F	9905–10432	528	2	-	ATT	TAA
Cytb	F	10450–11599	1150	17	-	ATG	TAA
tRNA ^{Ser} (UCN)	F	11600–11666	67	0	11629–11631 TGA	-	-
ND1	R	11683 – 12621	939	16	-	ATG	TAG
tRNA ^{Leu} (CUN)	R	12624 – 12691	68	2	12650 – 12652 TAA	-	-
lrRNA	R	12692 – 14042	1351	0	-	-	-
tRNA ^{Val}	R	14043 – 14107	65	0	14079 – 14081 TAC	-	-
srRNA	R	14108 – 14886	779	0	-	-	-
A+T-rich region		14887 – 15386	500	0	-	-	-

*The negative indicates the overlapping base pairs, and the positive indicates the interval base pairs.

3.2 Protein-coding genes

The 13 PCGs of the *Parnassius actius* is 11,195 bp in size and totally contains 3,720 codons, excluding termination codons. The overall A+T content of PCGs is 80.2%, which is the highest in all Pailionidae species determined, with the third codon position (92.4%) being higher than that of the first (73.8%) and the second (73.9%) (Table 5). All PCGs are initiated by ATN codons, except for COI that is initiated by the CGA codon. The nonoverlapping CGA codon is highly conserved across most lepidopteran insect groups (Cameron & Whiting, 2008; Kim *et al.*, 2009; Hao *et al.*, 2013). All PCGs are terminated with TAA or TAG, except for COI and COII which use the incomplete termination codon T. This

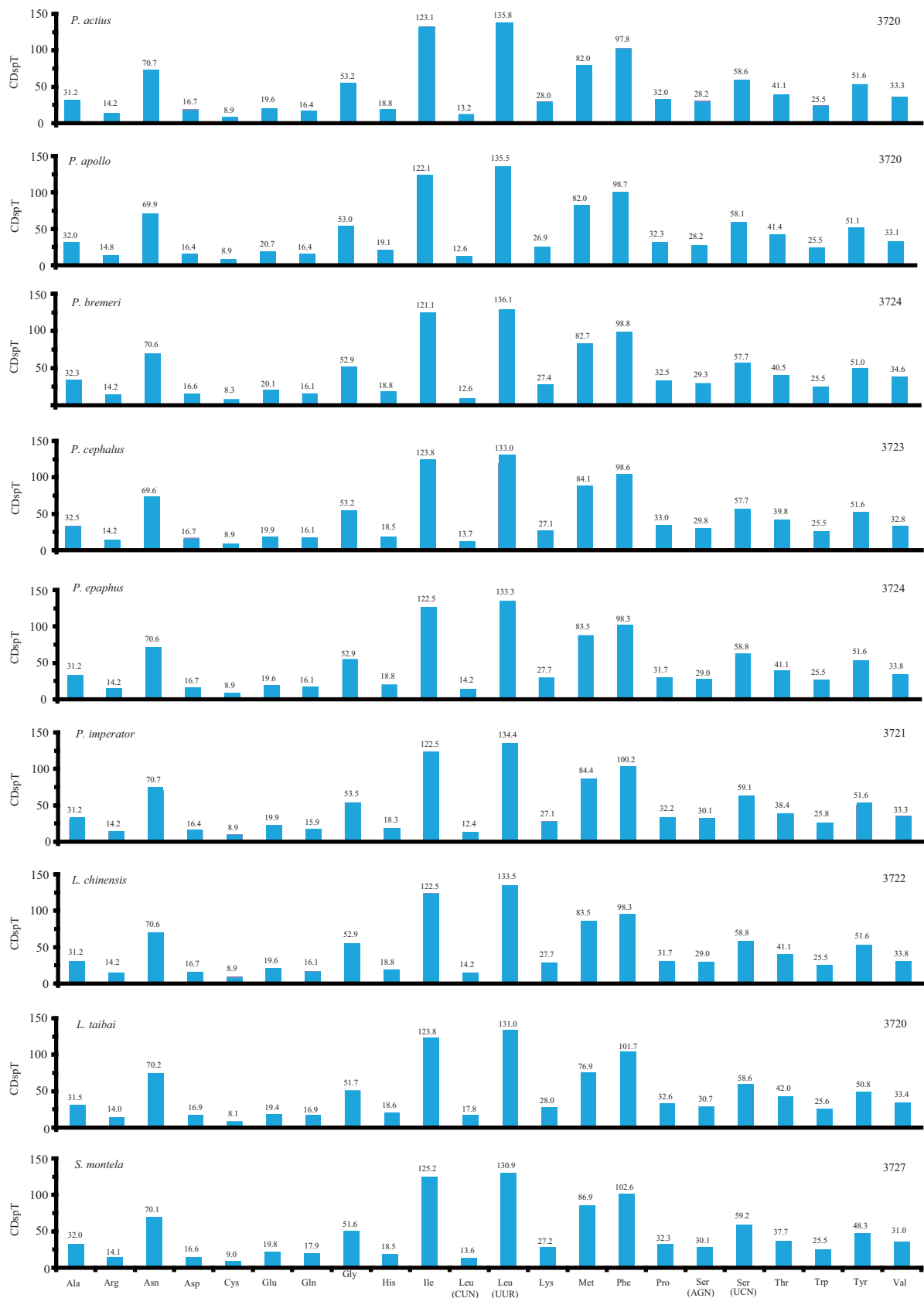


Figure 2. Codon distribution in nine Parnassiinae mitogenome (Numbers to the left refer to the total number of codons; CDspT—codons per thousand codons; codon families are provided on the x axis).

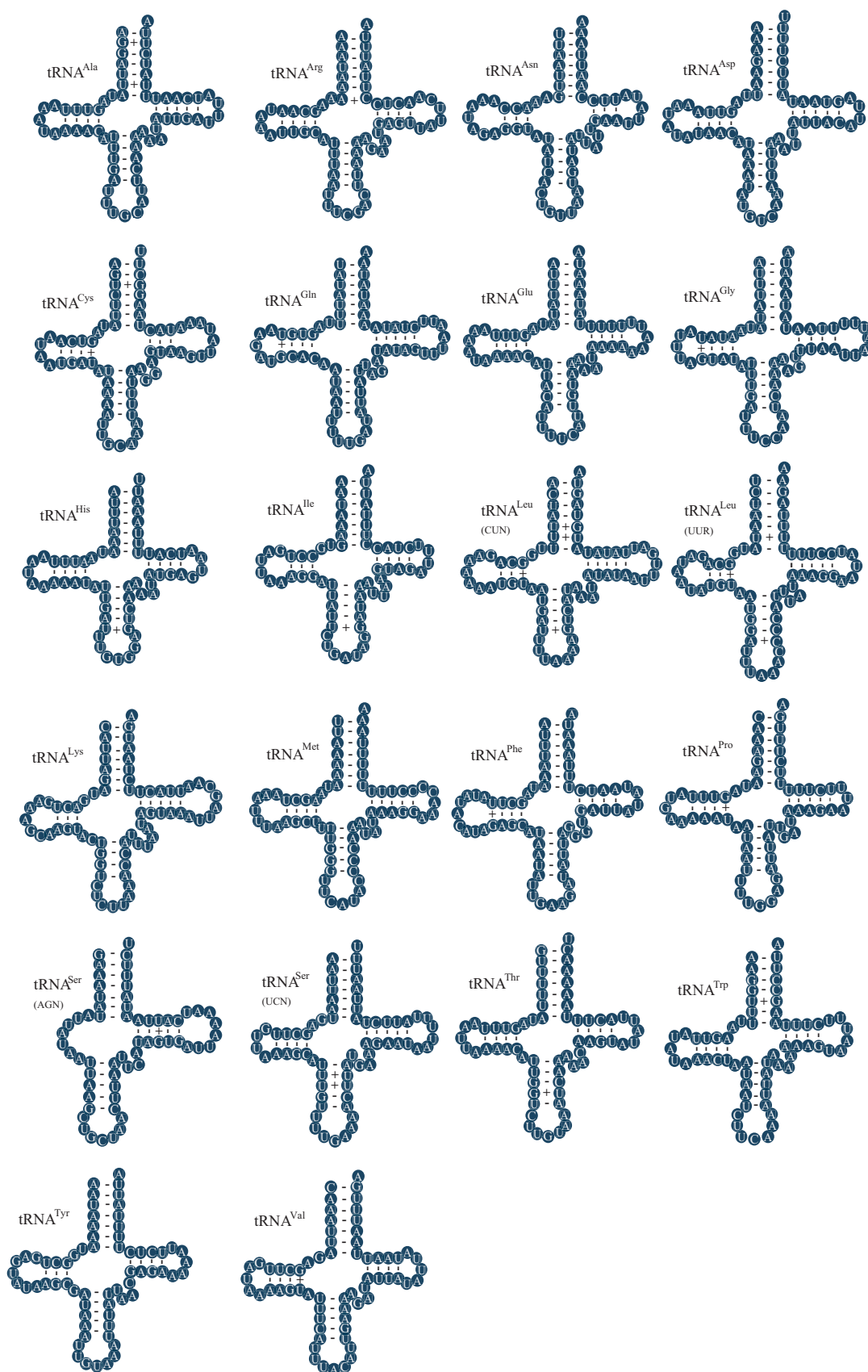


Figure 3. Predicted secondary clover-leaf structure of the *P. actius* 22 tRNA genes. Watson-Crick pairs are joined by dashes (-), other interactions are joined by plus signs (+).

Table 4. Characteristics of the Papilionidae mitogenomes.

Taxon	Mitogenome(majority strand)				PCG* NO codons**	AT (%)	tRNA		rRNA		A+T-rich region		Genbank accession no.
	Size (bp)	AT (%)	AT-skew	GC-skew			Size (bp)	AT (%)	Size (bp)	AT (%)	Size (bp)	AT (%)	
Papilionidae													
Papilioninae													
<i>Papilio bianor</i>	15,340	80.6	-0.015	-0.21	3719	79	1453	81.4	2097	84.2	498	94	NC018040
<i>Papilio maraho</i>	16,094	80.5	0.006	-0.262	3717	78.1	1442	80.7	2112	84.4	1270	94.3	FJ810212
<i>Papilio maackii</i>	15,357	80.7	-0.014	-0.212	3721	79.2	1452	81.4	2100	84.3	514	92.8	NC021411
<i>Papilio machaon</i>	15,158	80.3	-0.031	-0.198	3720	79	1446	81.4	2092	83.8	362	92.5	HM243594
<i>Triodes aeacus</i>	15,263	80.2	-0.04	-0.232	3724	79	1472	80.6	2018	83.9	419	89.8	EU625344
<i>Teinopalpus aureus</i>	15,242	79.9	-0.005	-0.238	3719	78.3	1455	81.2	2101	83.7	395	93.2	HM563681
<i>Graphium timur</i>	15,226	80.4	-0.008	-0.196	3714	79.7	1448	81.6	2112	84.7	403	95.8	NC024098
<i>Papilio syfanius</i>	15,359	80.6	-0.019	-0.205	3721	79.1	1439	81.4	2100	84.3	514	92.8	NC023978
<i>Lamproptera curius</i>	15,227	80.5	-0.008	-0.193	3717	79.3	1455	81.5	2119	83.6	470	89.8	NC023953
<i>Papilio dardanus</i>	15,337	79.2	-0.004	-0.276	3713	77.1	1441	80.7	2097	83.5	514	94.2	JX313686
<i>Papilio polytes</i>	15,256	81.1	-0.023	-0.207	3720	79.8	1443	81.9	2143	84.3	428	95.1	KM014701
<i>Atrophaneura alcinous</i>	15,266	81.1	-0.047	-0.217	3725	79.7	1451	81.7	2155	84.9	405	95.3	KJ540880
<i>Papilio helenus</i>	15,349	80.1	-0.005	-0.25	3739	78.4	1449	81.3	1808	84.5	490	94.3	NC025757
<i>Papilio xuthus</i>	15,359	80.5	-0.014	-0.231	3719	79	1440	81.1	2071	84.2	504	94	NC029244
<i>Papilio demoleus</i>	15,249	80.9	-0.028	-0.183	3713	79.5	1450	81.1	2147	84.4	403	95.3	NC027506
<i>Papilio glaucus</i>	15,306	80.9	-0.006	-0.22	3720	79	1444	80.9	2099	84.1	735	88.8	NC027252
<i>Teinopalpus imperialis</i>	15,229	79.6	-0.005	-0.235	3941	77.9	1454	81.4	2110	83.6	401	93.6	NC027108
<i>Graphium chirondies</i>	15,235	80.4	-0.015	-0.214	3727	78.8	1451	81.8	2121	84.6	419	95.7	KP159289
Parnassiinae													
<i>Parnassius epaphus</i>	15,458	81.4	-0.017	-0.193	3724	80.2	1458	81.3	2119	84.6	496	91.8	KM373898
<i>Parnassius bremeri</i>	15,389	81.3	-0.011	-0.191	3724	80.2	1462	80.9	2117	84.4	504	93.7	FJ871125
<i>Sericinus montela</i>	15,242	80.9	-0.009	-0.221	3691	79.8	1457	81.6	2097	83.9	408	94.1	HQ259122
<i>Luehdorfia taibai</i>	15,553	81.5	-0.009	-0.202	3717	79.7	1399	82.3	1805	84	939	94.6	NC023938
<i>Parnassius imperator</i>	15,424	81.1	-0.012	-0.207	3720	79.7	1460	81.3	2127	84.5	491	95.5	NC025587
<i>Parnassius apollo</i>	15,404	81.3	-0.016	-0.187	3720	80.1	1460	81.5	2113	84.5	504	93.8	KF746065
<i>Parnassius actius</i>	15,386	81.3	-0.016	-0.193	3720	80.2	1440	81.1	2130	84.6	500	93.8	KY072796
<i>Luehdorfia chinensis</i>	15,550	81.4	-0.01	-0.211	3719	79.7	1458	81	2190	84.6	474	96.8	KU360130
<i>Parnassius cephalus</i>	15,343	81.4	-0.012	-0.193	3721	80.2	1459	81.4	2118	84.3	487	95.2	NC026457

*Protein coding genes

**Termination codons were excluded in total codon count

Table 5. The nucleotide composition of *P. actius* mitogenome.

Feature	T (U)	C	A	G	AT%	AT skew	GC skew
Whole genome	41.3	11.1	40	7.5	81.3	-0.016	-0.193
Protein-coding genes	45.8	9.7	34.4	10.2	80.2	-0.142	0.025
First codon position	39	10.8	34.8	14.9	73.8	-0.056	0.159
Second codon position	49	14.2	24.9	11.8	73.9	-0.326	-0.092
Third codon position	49	4.1	43.4	3.7	92.4	-0.061	-0.051
tRNA genes	40.3	10.7	40.8	8.2	81.1	0.006	-0.132
rRNA genes	43	10.3	41.6	5.1	84.6	-0.016	-0.337
Cotrol region	47.6	4.2	46.2	2	93.8	-0.015	-0.355

Table 6. Relative synonymous codon usage (RSCU) and the codons per thousand codons (CDspT) in the *P. actius* PCGs.

Amino acid	Codon	RSCU	CDspT	Amino acid	Codon	RSCU	CDspT	Amino acid	Codon	RSCU	CDspT	Amino acid	Codon	RSCU
F	TTT	1.87	91.67	S2	TCT	2.75	29.8	Y	TAT	1.9	48.9	C	TGT	2
	TTC	0.13	6.18		TCC	0.35	3.76		TAC	0.1	2.69		TGC	0
L	TTA	5.36	133.06		TCA	2.3	25	H	CAT	1.86	17.47	W	TGA	1.94
	TTG	0.11	2.69		TCG	0	0		CAC	0.14	1.34		TGG	0.06
L2	CTT	0.31	7.79	P	CCT	2.45	19.62	Q	CAA	1.9	15.6	R	CGT	1.21
	CTC	0	0		CCC	0.4	3.23		CAG	0.1	0.81		CGC	0.15
	CTA	0.22	5.38		CCA	1.14	9.14	N	AAT	1.86	65.86		CGA	2.57
	CTG	0	0		CCG	0	0		AAC	0.14	4.84		CGG	0.08
I	ATT	1.94	119.35	T	ACT	2.3	23.66	K	AAA	1.9	26.61	S	AGT	0.59
	ATC	0.06	3.76		ACC	0.29	2.96		AAG	0.1	1.34		AGC	0.1
M	ATA	1.9	77.69		ACA	1.39	14.25	D	GAT	1.84	15.32		AGA	1.91
	ATG	0.1	4.3		ACG	0.03	0.27		GAC	0.16	1.34		AGG	0
V	GTT	1.84	15.32	A	GCT	2.26	20.7	E	GAA	1.84	18.01	G	GGT	1.05
	GTC	0.06	0.54		GCC	0.03	0.27		GAG	0.16	1.61		GGC	0.1
	GTA	1.97	16.4		GCA	1.24	9.68						GGA	2.32
	GTG	0.13	1.08		GCG	0.07	0.54						GGG	0.53

phenomenon of partial termination codons is observed in all sequenced lepidopteran insects, with the view of a probable logic of minimizing the sizes of intergenic spacers and overlaps in the evolutionary economic perspective (Kim *et al.*, 2009; Shi *et al.*, 2012; Hao *et al.*, 2013; Chen *et al.*, 2014).

The codon distribution analysis show that the five codon families (Leu (UUR), Ile, Phe, Met and Asn) are the most frequently used, each with at least 69 CDs per thousand CDs, totally accounting for about 50% of all the PCG codons. The three codon families with at least 100 CDs per thousand (Leu (UUR), Ile and Phe) are only found in the *P. imperator*, *L. taibai* and *S. montela* mitogenomes, whereas the rarest used codon family Cys is detected in all the nine mitogenomes (Fig. 2). Additionally, the highest usage frequency of hydrophobic amino acid Leu2 may be related to the function of encoding many transmembrane proteins of chondriosome (Gillespie *et al.*, 2006). Moreover, the relative synonymous codon usage (RSCU) analysis show that NNT and NNA are higher than 1.0, indicating a strong As or Ts bias in the third codon position (Table 6). In general, codon bias is positively correlated with the AT bias of the codon position for the insect mitogenomes (Salvato *et al.*, 2008; Kim *et al.*, 2009; Wang *et al.*, 2011).

3.3 Transfer RNA genes and ribosomal RNA genes

The *P. actius* mitogenome bears the typical set of 22 tRNA genes, ranging from 61 bp to 71 bp nucleotides in size (Table 3). Eight of them are encoded by the L-strand and the remaining 14 by the H-strand. All tRNAs are shown to be folded into the cloverleaf secondary structures, except for the tRNA^{Ser}(AGN) which lacks the DHU loop (Fig. 3). The special secondary structure of tRNA^{Ser}(AGN) has been found widely in most lepidopteran insects (Kim *et al.*, 2006; Yang *et al.*, 2009). A total of 23 unmatched base pairs are detected in the 22 tRNAs, and 17 of them are G-U pairs, which form a weak bond in the secondary structures, the remaining 6 are atypical pairs, including 4 U-U pairs, and 2 A-C pairs (Fig. 3). A post-transcriptional RNA editing mechanism maintaining the function of these tRNAs has been proposed to correct the errors of dislocation, such as the bulged nucleotides (U) on the stems, abnormal loops and arms (Lavrov *et al.*, 2004; Zhang *et al.*, 2008; Song *et al.*, 2013).

As is the case with all other Papiolionidae mitogenome sequences, two rRNAs genes (1,351 bp lrRNA and 779 bp srRNA) are found in *P. actius* mitogenome. These two rRNAs were located between tRNA^{Leu}(CUN) and tRNA^{Val}, and

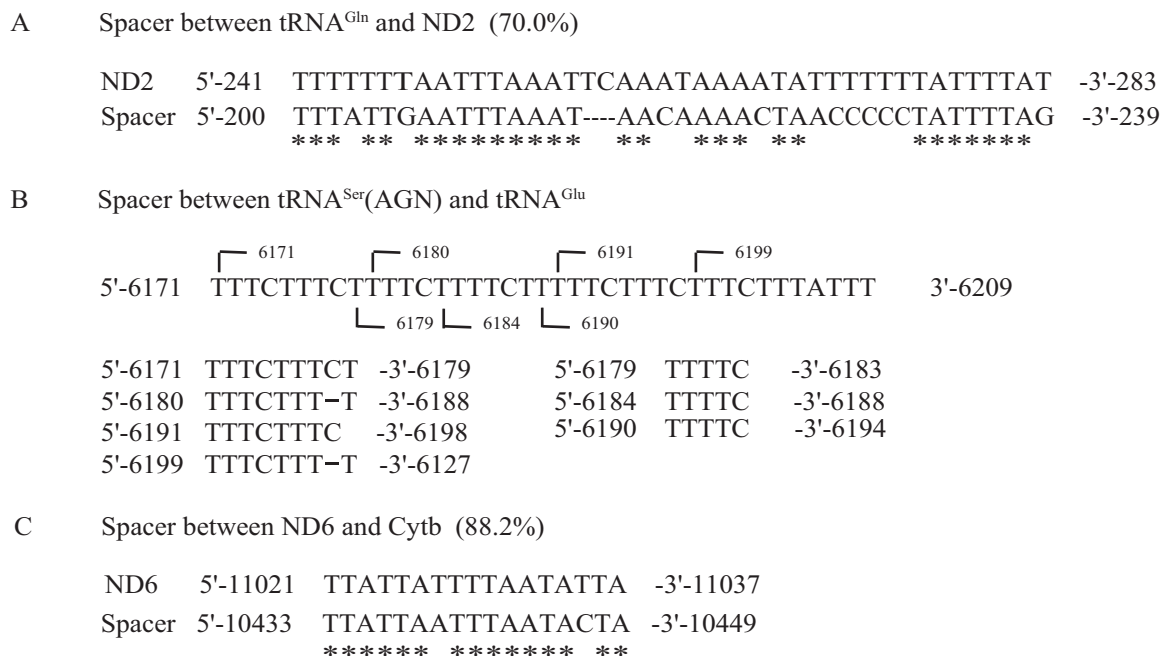


Figure 4. Sequences of two relatively large intergenic spacers of the *P. actius* mitogenome. A. Alignment of the spacer sequence located between tRNA^{Gln} and ND2 gene and the neighboring partial ND2 gene of *P. actius* (Asterisks indicate consensus sequences between the spacer sequence and the ND2 gene, sequence homology is shown on the right side of the alignment). B. The intergenic spacer sequence detected between the tRNA^{Ser}(AGN) and tRNA^{Glu} of *P. actius* mitogenome (39 bp), and the alignment of repeat sequences detected within the intergenic spacer sequence (The nucleotide position is indicated at the beginning and end sites of the sequence). C. Alignment of the spacer sequence located between ND6 and Cytb gene (sequence homology is shown on the right of the alignment).

The *P. actius* mitogenome includes a total of 129 bp and contains 11 intergenic spacers, ranging from 1 to 42 bp. The four spacers longer than 16 bp are located respectively between tRNA^{Gln} and ND2 (40 bp), tRNA^{Ser} (AGN) and tRNA^{Glu} (39 bp), ND6 and Cytb (17 bp), tRNA^{Ser} (UCN) and ND1 (16 bp) (Table 3). The first intergenic spacer (40 bp) is inserted between tRNA^{Glu} and ND2, which has been detected in most lepidopteran insects with the sizes ranging from 40 to 72 bp. This sequence is 70% homologous to its neighboring ND2 gene, indicating that this sequence maybe derived from ND2 (Fig. 4). The second spacer (39 bp) is located between tRNA^{Ser} and tRNA^{Glu}, which appears to be the result of a 4-fold repetition

srRNA -ATATAAAATTTTCTCAT **ATAGATTTTTTTTTTTTTTTT** ATATTAATATATTT
poly-T 13-bp repeat element
ATTAAATAATATAAATAAAATTTAAAATATTATTTTTTATACTAATATCAATA
TAAAAATATGAA **TATATATATATATATATA** ATTTATAAAATATATTAATATAT
(TA)₁₀ 13-bp repeat element
AATTATATATATATTAAATATTTAATATATAAAAAAATAAGTATATAAAGTAT
TTAATATCAATTATTAATATTGAATAATTTCTCTCTTTTTTTTTTTCATAATAT
TGAATTAAATACCTAAATTGCTATTTAAATTTTTATAATTCAAATAAATTATAT
TAAATTAATATAATTAATAATTATATAAGTATATTTAATATATTAATATATAAT
TTTAATATATTAAT **ATTTAATATATATATATATATATAT** GCTATACCAAATTAATGT
tRNA^{Trp}-like sequence (AT)₉
AATTTTCATTTAATTATTTTCATTAAACCATTTTTTAATTTTTTTCATATATAAAATAA
AAAAAAAAA **ATTTAT** AAAAAAAAAAAAAAAAAAAAAA TCTAAAATATTATTTTTTATACT
poly-A
AATATCAT -tRNA^{Met}

5'-'3' 3'-'5'

anticodon 5'-'3'

tRNA_{Gly}-like sequence

Figure 5. A. The structure of the A+T-rich region of *P. actius* mitogenome. B. Secondary structures of the tRNA^{Gly}-like sequence.

of TTTCTTTCT motif or a 3-fold repetition of TTTTC motif (Fig. 4). The third one (17 bp) is located between the ND6 and Cytb, with 88.2% homologous to its neighboring ND6 gene, suggesting its origination from ND6 duplication (Fig. 4). Finally, the last one (16 bp) is located between tRNA^{Ser} (UCN) and ND1 genes, harboring an ATACTAA motif which is the possible binding site for mtTERM (the transcription termination peptide) (Taanman, 1999; Cameron & Whiting, 2008).

3.5 A+T-rich region

The A+T-rich region (504 bp) of the *P. actius* mitogenome is located between srRNA and tRNA^{Met}, with a A+T content of 93.8% which is fallen within the range of 88.8% in *P. glaucus* (NC027252) to 96.8% in *L. chinensis* (KU360130) (Tables 4). Most of the region is composed some typical structures characteristic of other butterfly mitogenomes: the putative O_N (Origin of minority or light strand replication) located 17 bp upstream of the srRNA gene, which include the motif ATAGA followed by an 18 bp poly-T stretch; additionally a microsatellite-like repeat (AT)₉ preceded by the ATTTA motif (Fig. 7), and the (TA)₁₀ located 134 bp upstream of the srRNA, are also found in the A+T-rich region as in other *Parnassius* species (Chen *et al.*, 2014). A 16 bp poly-A interrupted by T characteristic of lepidopterans is also detected immediately upstream of the tRNA^{Met} (Wang *et al.*, 2013). Moreover, an 18 bp poly-A element located 31 bp upstream of the srRNA is also detected in the A+T-rich region as in most of the other lepidopterans (Fig. 5).

In this study, the A+T-rich region of *P. actius* harbors 13 repeat units (Fig. 5). A series of repetitive elements are also found in the A+T-rich regions of other insect groups (Cameron & Whiting, 2007), for example, the *S. charonda* harbors 17 bp repeat units, while the *A. metis* have 27 bp repeat units.

In *P. actius* A+T-rich region, one tRNA^{Gly}-like (67 bp) is detected, as is the case in its congeneric species *P. bremeri*

A Alignment of partial A+T-rich region

<i>P. actius</i>	5'---	TATAAAGTATTTAATATCAATTATTTAAATATTGAAT	---3'
<i>P. cephalus</i>	5'---	TATAAAATATTTAATATTAATTATTTAAATATTGAAT	---3'
<i>P. epaphus</i>	5'---	TATAAAATATTTA--TATCA--CTATTAAATATTGAAT	---3'
<i>P. imperator</i>	5'---	TATAAAATATTTAATATTAATTATTTAAATATTGAAT	---3'
<i>P. apollo</i>	5'---	TATAAAATATTTAATATCAATTATTTAAACATTGAAT	---3'
<i>P. bremeri</i>	5'---	TATAAAATATTTAATATCAATTATTTAAACATTGAAT	---3'

B stem-loop structures

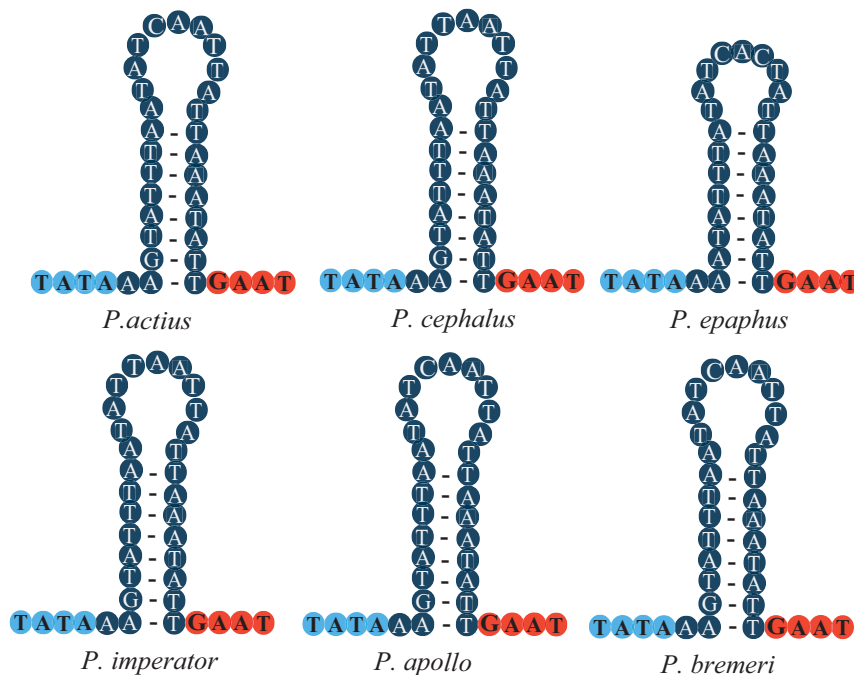


Figure 6. A. Sequence alignment of partial mitogenomic A+T-rich regions of *Parnassius* species. B. The putative stem-loop structures found in the control regions of *Parnassius* species. The light blue and red indicates highly conserved flanking sequences.

and *P. apollo* and *P. epaphus* (Kim *et al.*, 2009; Chen *et al.*, 2014; Wang *et al.*, 2015). Additionally, some other insect groups are detected to have the similar tRNA sequences also, such as the hymenopteran *Bombus ignites* (Cha *et al.*, 2007), the dipteran *Simosyrphus grandicornis* (Cameron & Whiting, 2007), the coleopteran *Chrysochroa fulgidissima* (Hong *et al.*, 2009), the heteropteran *Eusthenes cupreus* (Song *et al.*, 2013).

Additionally, one sequence stretches (36 bp) potential to form stem-loop structures are also found in all sequenced *Parnassius* species (Fig. 6). The stem loop structures with a 5'-consensus of 'TATA' and 3' consensus of 'G(A)nT' was proposed as the initiation site of the secondary strand replication (Clary & Wolstenholme, 1987; Zhang *et al.*, 1995; Zhang & Hewitt, 1997; Schultheis *et al.*, 2002).

3.6 Phylogenetic analysis

The phylogenetic trees inferred by BI and ML methods are highly congruent and most of the tree internal nodes are strongly supported (BPP >0.95 and BP >85% for most nodes). The 45 *Parnassius* species are shown to be assigned into the

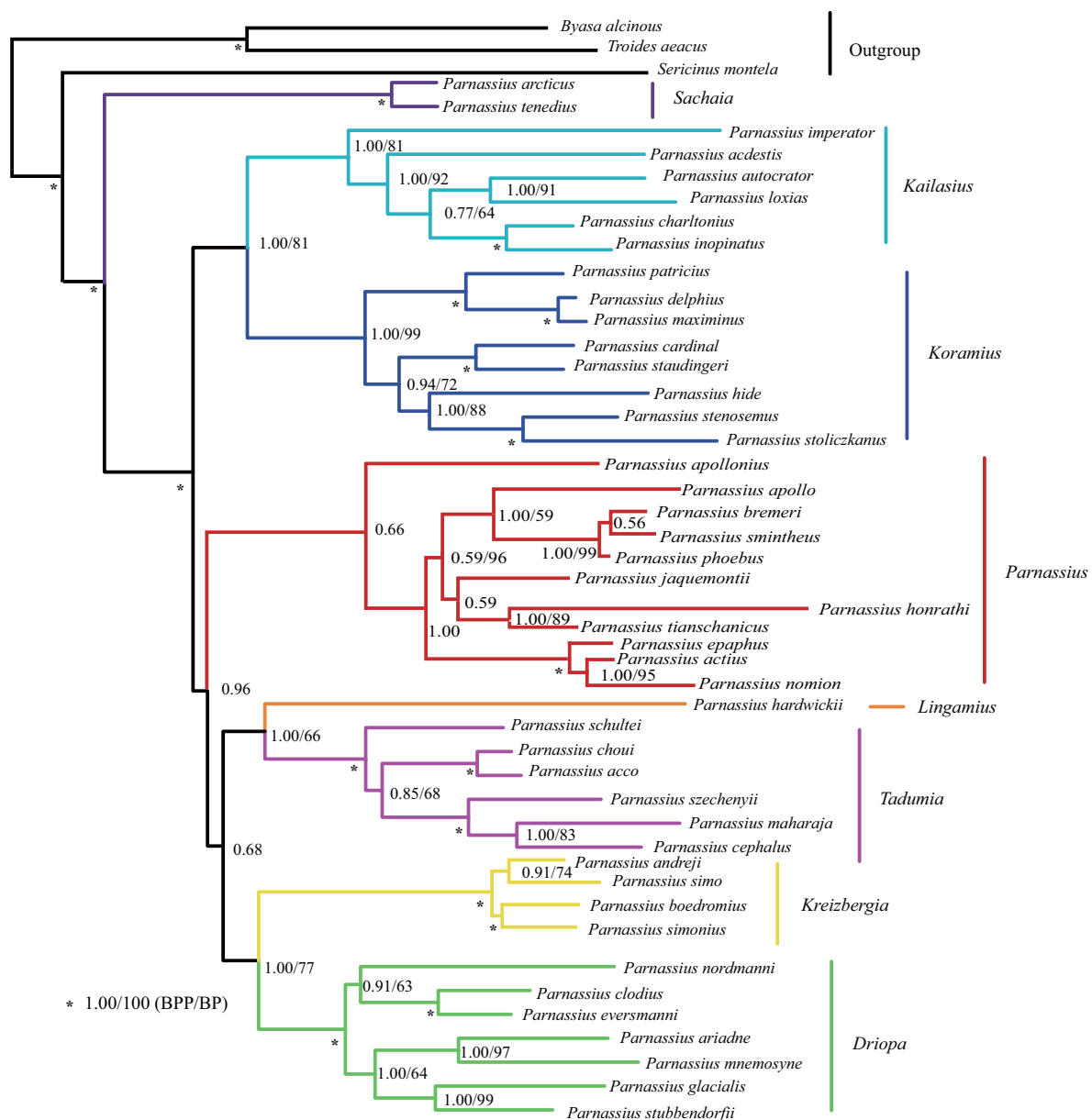


Figure 7. Bayesian Inference and maximum likelihood phylograms of the 45 *Parnassius* species in this study (Numbers on each node correspond to the posterior probability values of the BI analysis (left) and the ML bootstrap percentage values for 1000 replicates (right), posterior probability values below 0.5 or bootstrap percentage values below 50% was not shown on the diagram).

eight major subgenera as reported in previous studies (Omoto *et al.*, 2004, 2009; Michel *et al.*, 2008), namely the subgenera *Driopa*, *Kailasius*, *Koramius*, *Kreizbergia*, *Lingamius*, *Parnassius*, *Sachaia* and *Tadumia*. Obviously, the *Parnassius* species of this study contained five major clades (clade I is the subgenus *Parnassius*, clade II is the subgenera *Tadumia* plus *Lingamius*, clade III contains the *Kreizbergia* and *Driopa*, clade IV contains the *Kailasius* and *Koramius*, and clade V is the subgenus *Sachaia*). This molecular grouping is generally compatible with their morphological features, for example, on the trailing edge of hindwing, the *Parnassius*, *Kreizbergia* and *Driopa* do not harbor obvious spots, while the *Sachaia* harbors a series of black spots, the *Tadumia*, *Lingamius*, *Kailasius* and *Koramius* have some blue spots with black circle outside the spots; the *Tadumia* + *Lingamius* harbors two or more red spots, while the *Kailasius* + *Koramius* only harbors black spots on the forewing.

The inferred molecular phylogenetic relationships of this study (Fig. 7) are somewhat in conflict with the results of previous molecular studies, for instance, Michel *et al.* (2008) reconstructed the *Parnassius* phylogeny based on the combined one nuclear (18S rRNA) plus three mitochondrial (ND1, COI, ND5) gene sequence data using ML and Bayesian methods, the results showed that the subgenus *Parnassius* was sister to the grouping of all other subgenera; Omoto *et al.* (2004, 2009) conducted the *Parnassius* phylogenetic analyses based on partial mitochondrial ND5 sequences with NJ, MP and ML method respectively, and the results was nearly the same with that obtained by Michel *et al.* (2008). Generally speaking, the deep relationships of the *Parnassius* are still in state of controversies and awaits further studies with more molecular and morphological criteria.

As for the *Parnassius actius*, its phylogenetic position within genus *Parnassius* remains uncertain until now. In morphological view, it was suggested to be more closely related to *P. nomion* than to other *Parnassius* species owing to its nearly the same wing pattern and its relatively smaller body size compared with *P. nomion* (Chou, 1999). However, some scholars proposed that it is probably the subspecies of *P. tianschanicus* due to their same genitalia characters and nearly the same body size (Wu, 2001, personal communications), and this suggestion was somewhat supported by some previous molecular studies (Michel *et al.*, 2008; Omoto *et al.*, 2009). Our study showed that *P. actius* was obviously the closest relative of *Parnassius nomion* in both the ML and Bayesian trees with strong supports, and this result is congruent with the results obtained by Omoto *et al.* (2004) and Rebourg *et al.* (2006). Considering the morphological difference is usually relatively small existed between closely related species of some alpine butterfly groups, such as the genera *Aporia*, *Colias*, *Parnassius* and others, it is astonishingly difficult to identify species and infer their relationships upon their morphological and structural characters, it is reasonable herein to propose that the *P. actius* is an effective species with its phylogenetic position awaiting further studies upon more molecular data.

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