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RESEARCH ARTICLE

Complete mitochondrial genome of *Ampittia dioscorides* (Lepidoptera: Hesperiidae) and its phylogenetic analysis

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Abstract **The complete mitochondrial genome of *Ampittia dioscorides* (Lepidoptera: Hesperiidae) was determined. The sequenced genome is a circular molecule of 15313 bp, containing 13 protein-coding genes (PCGs), 22 tRNA genes, two rRNA genes, and an A + T-rich region. The gene arrangements and transcribing directions are identical to those in most of the reported lepidopteran mitogenomes. The base composition of the whole genome and genes or regions are also similar to those in other lepidopteran species. All the PCGs are initiated by typical ATN codons; the exception being *COI*, which begins with a CGA codon. Eight genes *(ND2, ATPase8, ATPase6, COIII, ND5, ND4L, ND6, and Cytb)* end with a TAA stop codon, and two genes *(ND1 and ND3)* end with TAG. The remaining three genes (*COI* and *COII*, which end with TA-, and *ND4*, which ends with T-) have incomplete stop codons. All tRNAs have the typical clover-leaf structure of mitochondrial tRNAs, with the exception of tRNA^{Ser}(AGY). On the basis of the concatenated nucleotide and amino acid sequences of the 13 PCGs and *wingless* gene of 22 butterfly species, maximum parsimony (MP) and Bayesian inference (BI) trees were constructed, respectively. Both MP and BI trees had the same topological structure: ((((Nymphalidae + Danaidae) + Lycaenidae) + Pieridae) + Papilionidae) + Hesperiidae). The results provide support for Hesperiidae as a superfamily-level taxon.**

Keywords *Ampittia dioscorides*, mitochondrial genome, phylogeny

Introduction

In traditional taxonomic studies based on morphology, butterflies are divided into two superfamilies and five families: the Hesperiidae are generally designated as an independent superfamily (Hesperioidea), whereas all species in the other four families (Papilionidae, Pieridae, Lycaenidae, and Nymphalidae) are placed within the superfamily Papilionoidea (Harvey,1991). However, phylogenetic studies based on molecular data have led to controversy regarding the phylogenetic relationship between Papilionoidea and Hesperioidea.

Weller and Pashley (1995) explored the phylogenetic relationships of butterflies and their related species in the macrolepidopteran superfamilies based on three integrated gene sequences (*ND1*, *18S rRNA*, and *28S rRNA*) and 43 morphological characteristics. The results showed that Hesperioidea and Papilionoidea comprise a monophyletic group. Wahlberg et al. (2005) sequenced DNA fragments of substantial length (3258 bp) from three genes (*COI*, *EF-1α*, and *wingless*) and analyzed these using 99 morphological characters of 57 taxa. The results also showed that Hesperioidea and Papilionoidea are a monophyletic group. Some recent studies have also supported the contention that Hesperioidea and Papilionoidea are sister groups (Warren et al., 2008, 2009; Zou et al., 2009). However, other studies have indicated that Hesperiidae appears to be a sister group of families included in Papilionoidea, thereby invalidating the superfamily status of Hesperioidea (Regier et al., 2008; Mutanen et al., 2010; Kim et al., 2014).

Owing to its maternal inheritance, lack of recombination, and accelerated nucleotide substitution rates compared to those of nuclear DNA, the mitochondrial genome has in recent years been routinely used in studies on phylogenetics, comparative and evolutionary genomics, population genetics,

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and molecular evolution. However, as a semi-autonomous organelle, the development of mitochondria has been controlled by both mitochondrial and nuclear genes. Therefore, nuclear genes may have an impact on the evolution of mitochondrial genes. Moreover, the biologic information contained in the nuclear genome is more abundant than that in the mitochondrial genome. Accordingly, to more accurately reflect the phylogenetic relationship among species, it would be preferable to use the data of concatenated mitochondrial and nuclear genes in phylogenetic analyses. Indeed, it is now common practice among insect molecular systematists to combine one or more mitochondrial genes with one or more nuclear genes, as the two types of data are unlinked and evolve under different constraints (Hsu et al., 2001; Lin and Danforth, 2004; Pena et al., 2006; Silva-Brandao et al., 2008; Kim et al., 2010).

The *wingless* gene (nuclear gene) is a single-copy gene of the *WNT* gene family, which plays an important role in the formation of wing shape. To date, the gene has been widely used in the phylogenetic analyses of Lepidoptera (Brower, 2000; Campbell et al., 2000; Pena et al., 2006; Silva-Brandão et al., 2008).

Ampittia dioscorides is a representative species of the Hesperiidae (Lepidoptera: Hesperioidea), which is widely distributed in South-eastern Asian areas, including Sri Lanka, India, Malaysia, and China. In this study, we determined the complete mitochondrial genome and *wingless* nuclear gene sequence of *A. dioscorides*.

To date, there have been few studies on the phylogenetic analysis of a butterfly based on a combination of the complete mitochondrial genome and nuclear genes. In the present study, based on the respective characteristics of the mitochondrial genome and nuclear genes, we sequenced the mitochondrial genome and nuclear *wingless* gene of *A. dioscorides*. Using these sequences, together with the corresponding sequences generated in previous studies on 21 other species of true butterflies, we evaluated previous phylogenetic hypotheses regarding the Papilionoidea and Hesperioidea.

Materials and Methods

Specimen collection and DNA extraction

An adult specimen of *A. dioscorides* was collected from Lingui, Guangxi Province, China. The specimen was immediately preserved in 100% ethanol and then stored at −20°C before genomic DNA extraction. Total DNA was isolated from the muscles of the thorax or leg using a routine phenol/chloroform method (Zhou et al., 2007).

Primer design, PCR, and sequencing

The primers used in this study for the amplification of the complete mitogenome and *wingless* gene were based on the sequences listed in Table 1. A few universal PCR primers for short-fragment amplifications of the *cox1*, *cox2*, *nd5*, *cytb*, and *12S* genes were synthesized based on sequences from a previous study (Simon et al., 1994). The remaining primers were designed based on sequence alignments of the available complete lepidopteran mitogenomes using Primer Premier 5.0 software (Singh et al., 1998). The entire mitogenome of *A. dioscorides* was amplified in nine fragments (*nd2-cox1*, *cox1-cox2*, *cox2-cox3*, *nd3-nad5*, *cox3-nd5*, *nad5-nad4*, *nad4-cytb*,

Table 1 List of primers used to amplify and sequence the mitogenome of *Ampittia dioscorides*

Primer 1	Sequence (5′→3′)	Primer 1	Sequence (5′→3′)	Fragment
1A	GCTAAATTAAGCTTTTGGGTTCA	1B	CCCGGTAAAATTAAAATATAAACTTC	ND2-COI
2A	GGTCAACAAATCATAAAGATATTG	2B	TAAACTTCAGGGTGACCAAAAAAT	COI
3A	GGATCACCTGATATAGCATTCCC	3B	GAGACCATTACTTGCTTTCAGTCATC	COI-COII
4A	AGAGCCTCTTTAATAGAACA	4B	GAGACCATTACTTGCTTTCAGTCATC	COII
5A	GAAATTTGTGGAGCTAATCATAG	5B	TCAACAAAATGTCAATATCA	COII-COIII
6A	GGTTTACGATGAGGAATAATTT	6B	TTACAATGAAAATGTAATG	COIII-ND3
7A	CATTACATTTTCATTGTAA	7B	TTCTGCTTTGGTTCATTCT	ND3-ND5
8A	GGTTTACGATGAGGAATAATTT	8B	TTAGGTTGAGATGGGTTAGG	COIII-ND5
9A	GCTAATTATGAATTTGATT	9B	GATACTCTTCATCATATA	ND5
10A	ATAATAACTCCAGCACAT	10B	GCTTATTCTTCAGTTGCTCA	ND5-ND4
11A	GAAGGAGGAGCTGCTATATTAG	11B	CCTCAAAATGATATTTGACCTC	ND4- Cytb
12A	TACGTTTTACCATGAGGTCAAATATC	12B	ACTTCTTTTCTTATGTTTTCAAAAC	Cytb
13A	CCGACCTGTTGAAGATCCTTAT	13B	TCAGATCAAGATGCCGATT	Cytb-12
14A	GACGGGCGATRTGTRCATA	14B	CCAGCAGTCGCGGTTATAC	12S
15A	AGGGTATCTAATCCTAGTTT	15B	TGGGGTATGAACCCAAAAGC	12S-ND2
WG1	GARTGYAARTGYCAYGGYATGTCTGG	WG2	ACTICGCRCACCARTGGAATGTRCA	Wingless

Note: Characters of degenerate primer are as follow: Y(C,T), R(A,G), I (A,T,C,G) *cytb-12s*, and *12s-nd2*) using long-PCR techniques with TaKaRa LATaq polymerase under the following cycling conditions: initial denaturation for 5 min at 95°C, followed by 30 cycles of 95°C for 50 s, 45–50°C for 50 s, 68°C for 2 min and 30 s; and a final extension step of 68°C for 10 min. The PCR products were visualized by electrophoresis on a 1.0% agarose gel, then purified using a QIAGEN PCR purification kit (QIAGEN, Düsseldorf, Germany), and sequenced directly with an ABI 3730 DNA Analyzer using a BigDye chemistry kit (Applied Biosystems, Inc., Carlsbad, CA, USA), in which the same PCR primers were used. All PCR products were sequenced from both strands. The resultant mitogenome sequence and *wingless* gene data were deposited into the GenBank database under the accession numbers KM102732 and KP153245, respectively.

Sequence analysis and annotation

Sequence annotation was performed using the blast tools from the NCBI website (http://blast. ncbi.nlm.nih.gov/Blast) and DNAStar package (DNAStar Inc., Madison, USA). The secondary structures of most of the tRNA genes were predicted with tRNAscan-SE 1.21 (Lowe and Eddy, 1997) using invertebrate codon predictors; however, some [e.g., tRNA^{Ser}(AGN)] were drawn by hand based on the nucleotide sequences of the tRNA genes of other butterflies. The protein-coding genes (PCGs) and rRNAs were confirmed by sequence comparison with ClustalX1.8 software and the NCBI BLAST search function (Altschul et al., 1990). Nucleotide composition and codon usage were calculated with DAMBE software (Xia and Xie, 2001).

Phylogenetic analysis

To evaluate the phylogenetic relationships among butterflies, we used the complete mitogenomes of *A. dioscorides* and 21 other butterfly species (obtained from the GenBank database) (Table 2). The phylogenetic trees were constructed base on complete mitogenomes and *wingless* genes using Bayesian Inference (BI) and Maximum parsimony (MP) algorithms. The amino acid sequences of 13 PCGs and *wingless* from the 22 sequenced butterflies were aligned, together with those of two Bombycoidea species, *Bombyx mori* and *Bombyx mandarina,* used as an outgroup.

Table 2 Related information on the complete mitogenome used in the phylogenetic analyses

Superfamily/Family	Species	GenBank Accession No.	Wingless Accession No.
Papilionoidea			
Nymphalidae	*Kallima inachus*	JN857943	AY788769
	Acraea issoria	NC_013604	EU275636
	Argynnis hyperbius	NC_015988	GU372617
	Issoria lathonia	NC_018030	DQ922886
	Melitaea cinxia	NC_018029	AY788536
	Junonia orithya	NC_022697	EU53389
Danaidae	*Danaus plexippus*	NC_021452	KJ496142
	Euploea midamus	KJ866207	KP153246
	Tirumala limniace	KJ784473	GQ864909
	Euploea mulciber	NC_016720	GU365941
Papilionidae	*Lamproptera curius*	NC_023953	KP153247
	Papilio bianor	KF859738	GU372632
	Papilio polytes	KM014701	AK402256
	Papilio machaon	NC_018047	GU372637
	Sericinus montela	HQ259122	GU372642
Lycaenidae	*Curetis bulis*	JX_262888	JQ786749
Pieridae	*Eurema hecabe*	NC_022685	DQ018876
	Pieris rapae	NC_015895	GU372646
	Leptidea morsei	NC_022686	GU372654
	Delias hyparete	NC_020428	DQ082831
	Aporia crataegi	NC_018346	AY870529
Hesperioidea			
Hesperiidae	*Ampittia dioscorides*	KM102732	KP153245
Bombycoidea			
Bombycidae	*Bombyx mandarina*	NC_003395	EU033068
	Bombyx mori	NC_002355	EU141241

Table 3 Summary of the mitogenome of *Ampittia dioscorides*

Gene	Direction	Location	Size (bp)	Spacer(+) Overlap(−)	Anti-codon	Start codon	Stop codon
tRNA^{Met}	F	1-68	68	+ 6	CAT		
tRNA^{Ile}	F	75-138	64	− 3	GAT		
tRNA^{Gln}	R	136-204	69	+ 74	TTG		
ND2	F	279-1300	1022	− 2		ATT	TAA
tRNA^{Trp}	F	1299-1365	67	− 9	TCA		
tRNA^{Cys}	R	1357-1421	65	+ 1	GCA		
tRNA^{Tyr}	R	1423-1487	65	+ 4	GTA		
COI	F	1492-3024	1533			CGA	T–
tRNA^{Leu}(UUR)	F	3025-3093	69		TAA		
COII	F	3094-3769	676			ATG	T–
tRNA^{Lys}	F	3770-3840	71	+ 11	CTT		
tRNA^{Asp}	F	3852-3918	67		GTC		
ATPase8	F	3919-4080	162	− 7		ATT	TAA
ATPase6	F	4074-4760	687	− 5		ATG	TAA
COIII	F	4756-5542	787	+ 2		ATT	TAA
tRNA^{Gly}	F	5545-5608	64		TCC		
ND3	F	5609-5962	354	+ 7		ATA	TAG
tRNA^{Ala}	F	5970-6036	67	− 1	TGC		
tRNA^{Arg}	F	6036-6098	63		TCG		
tRNA^{Asn}	F	6099-6164	66	+ 13	GTT		
tRNA^{Ser}(AGY)	F	6178-6234	57	+ 13	GCT		
tRNA^{Glu}	F	6248-6313	66	− 2	TTC		
tRNA^{Phe}	R	6312-6379	68	− 65	GAA		
ND5	R	6315-8117	1803			ATG	TAA
tRNA^{His}	R	8118-8185	68		GTG		
ND4	R	8186-9533	1348	+ 2		ATG	TA-
ND4L	R	9536-9821	286	+ 4		ATG	TAA
tRNA^{Thr}	F	9826-9890	65		TGT		
tRNA^{Pro}	R	9891-9955	65	− 2	TGG		
ND6	F	9954-10494	541	− 4		ATT	TAA
Cytb	F	10491-11634	1144	+ 6		ATG	TAA
tRNA^{Ser}(UCN)	F	11641-11705	65	+ 22	TGA		
ND1	R	11728-12666	939			ATG	TAG
tRNA^{Leu}(CUN)	R	12667-12734	68	+ 6	TAG		
16SrRNA	R	12741-14056	1316	+ 4			
tRNA^{Val}	R	14061–14125	65		TAC		
12SrRNA	R	14126-14846	721	+ 78			
A + T-rich region		14925-15313	389				

tRNA abbreviations follow the IUPAC-IUB three letter code.

Results and discussion

Genome organization

The complete mitochondrial DNA (mtDNA) sequence of *A. dioscorides* is 15,313 bp in length (Table 3) and consists of two rRNAs, 22 tRNAs, 13 PCGs, and one major non-coding A + T-rich region. As shown in Fig. 1, and consistent with the mitogenomes of many insect, the major strand of the DNA codes for a higher number of genes (nine PCGs and 14 tRNAs) than the minor strand (four PCGs, eight tRNAs, and two rRNA genes).

Almost all PCGs in the *A. dioscorides* mitogenome are initiated by typical ATN codons (seven with ATG, four with ATT, and one with ATA); the single exception being the *COI* gene, which was tentatively designated to have a CGA start codon (Table 3). Among the stop codons of 13 PCGs, three types of codon were found in *A. dioscorides*: TAA (*ND2,*

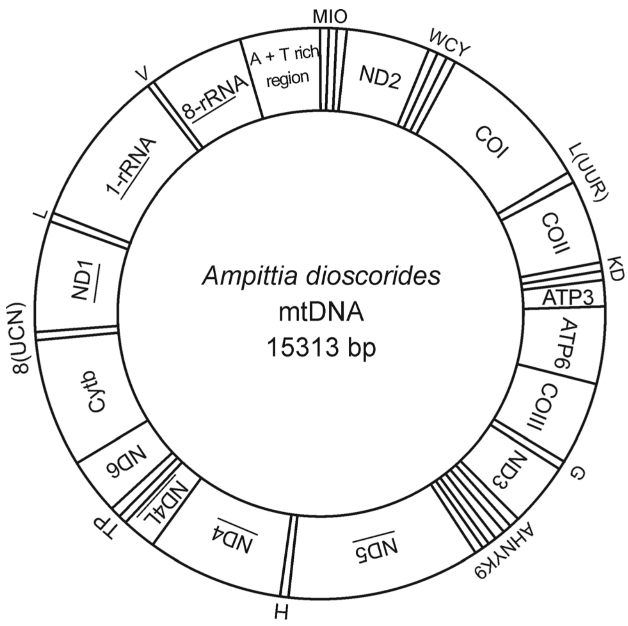


Figure 1 Circular map of the mitochondrial genome of *Ampittia dioscorides.*

ATPase8, ATPase6, COIII, ND5, ND4L, ND6, and *Cytb*); TAG (*ND1* and *ND3*); and the incomplete stop codon T (*COI, COII,* and *ND4*). Incomplete termination codons are frequently observed in most insect mitogenomes (Cha et al., 2007; Hong et al., 2008; Kim et al., 2010), and are often activated through post-transcriptional polyadenylation, in which two A residues are added to create a TAA terminator (Anderson et al., 1981; Ojala et al., 1981).

tRNA and rRNA genes

The 22 tRNAs vary in size from 57 bp [tRNA^{Ser}(AGN)] to 71 bp (tRNA^{Lys}) and have a typical clover-leaf structure, with the unique exception of tRNA^{Ser} (AGN), which lacks the dihydrouridine (DHU) stem (Fig. 2). The tRNAs contain a total of 34 pair mismatches in their stems, including 10 pairs in the DHU stems, nine pairs in the amino acid acceptor stems, four pairs in the TΨC stems, and 11 pairs in the anticodon stems. The number of mismatches in the *A. dioscorides* tRNAs detected in the present study is well within the range reported in previous studies on other lepidopteran insect tRNAs (Liu et al., 2008; Jiang et al., 2009; Kim et al., 2010). These tRNA mismatches can be corrected through RNA editing mechanisms, which are well known in arthropod mtDNA (Lavrov et al., 2000).

As in all other insect mitogenome sequences, two rRNA genes (*rrnL* and *rrnS*) were detected in *A. dioscorides*. The lrRNA and srRNA genes of the *A. dioscorides* mitogenome are 1,316 and 721 bp in length, respectively. They are located between tRNA^{Leu} (CUN) and tRNA^{Val} and between tRNA^{Val} and the A + T-rich region, respectively (Fig. 1).

A + T-rich region

The A + T-rich region of *A. dioscorides* is located between the 12S rRNA and tRNA^{Met} genes. The 389-bp-long A + T-rich region exhibits a higher A + T content (93.32%) than any other region of the *A. dioscorides* mitogenome. A conserved sequence (5′-AGATTTTTTTTTTTTTTTT-3′) was identified at the 5′-end of the A + T-rich region. A poly-A sequence (AAAAAAAAAAAAA) was found at the 3′-end of the A + T-rich region. Additionally, other short microsatellite-like repeat regions were observed throughout the A + T-rich region, without noticeable macro-repeats.

Phylogenetic analysis

The newly sequenced mitogenome and *wingless* gene of *A. dioscorides* were used for phylogenetic analyses, together with the mitogenomes of 21 other butterflies, representing six families (Nymphalidae, Danaidae, Lycaenidae, Pieridae, Papilionidae, and Hesperiidae). The phylogenetic analyses were carried out using Bayesian Inference (BI) and Maximum parsimony (MP) algorithms for the concatenated amino acid sequences of 13 protein-coding genes and nuclear *wingless* gene. Both BI and MP trees had the same topological structure: ((((Nymphalidae + Danaidae) + Lycaenidae) + Pieridae) + Papilionidae) + Hesperiidae). In the two phylogenetic trees, species of the six families were divided into two major branches (Figs. 3 and 4). The first clade included species in the Papilionoidea, whereas the second clade included only Hesperiidae. Within Papilionoidea, five families (Nymphalidae, Danaidae, Lycaenidae, Pieridae, Papilionidae) of butterflies are presented as monophyletic in the phylogenetic trees.

Conclusion

We sequenced the complete mitochondrial genome and *wingless* gene of *Ampittia dioscorides* and analyzed the phylogenetic relationships between Papilionoidea and Hesperioidea. The results based on the concatenated amino acid sequences of 13 protein-coding genes and the nuclear *wingless* gene are congruent with traditional classification based on morphology and support the view that butterflies are divided into two superfamilies, Papilionoidea and Hesperioidea.

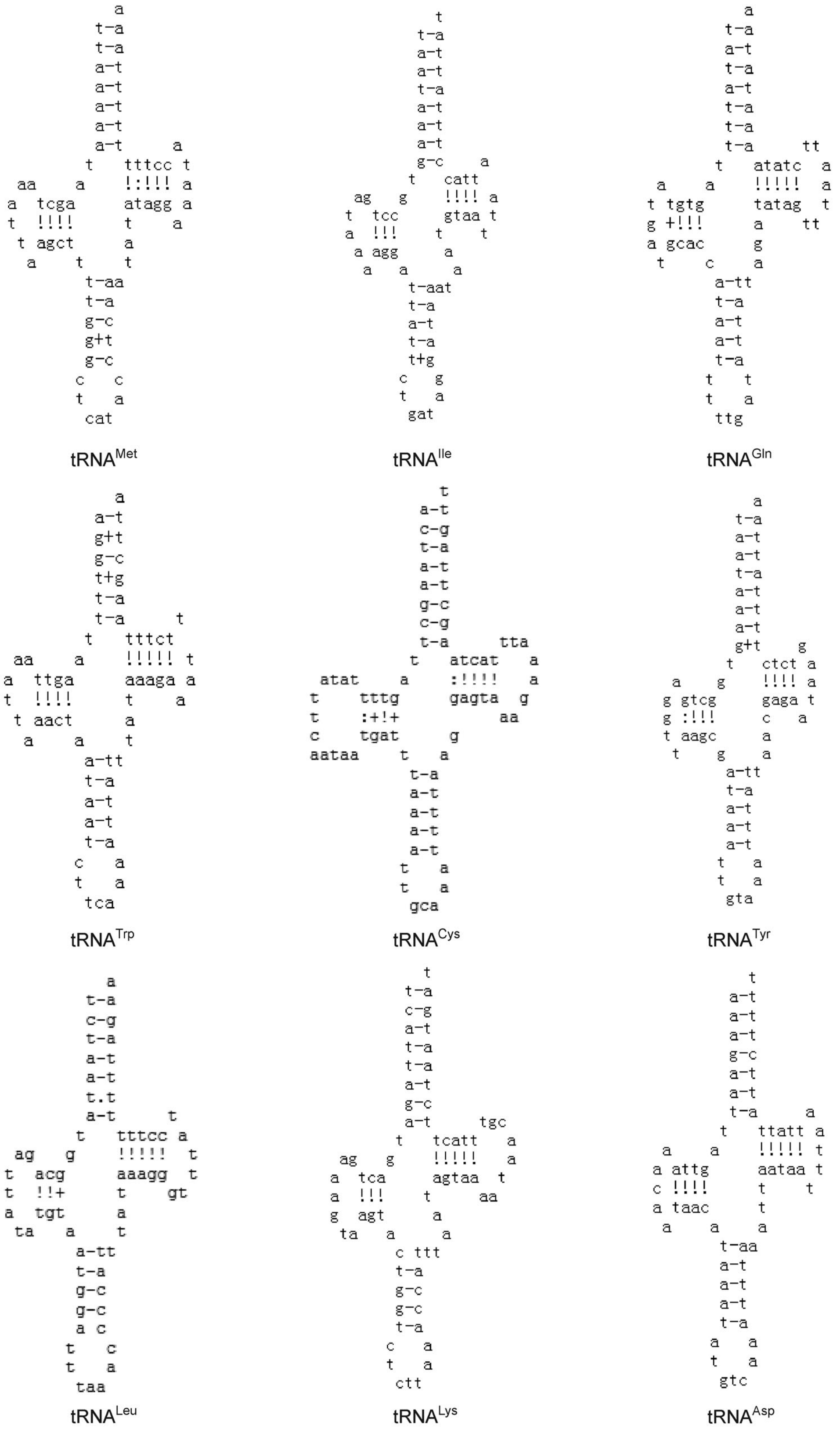
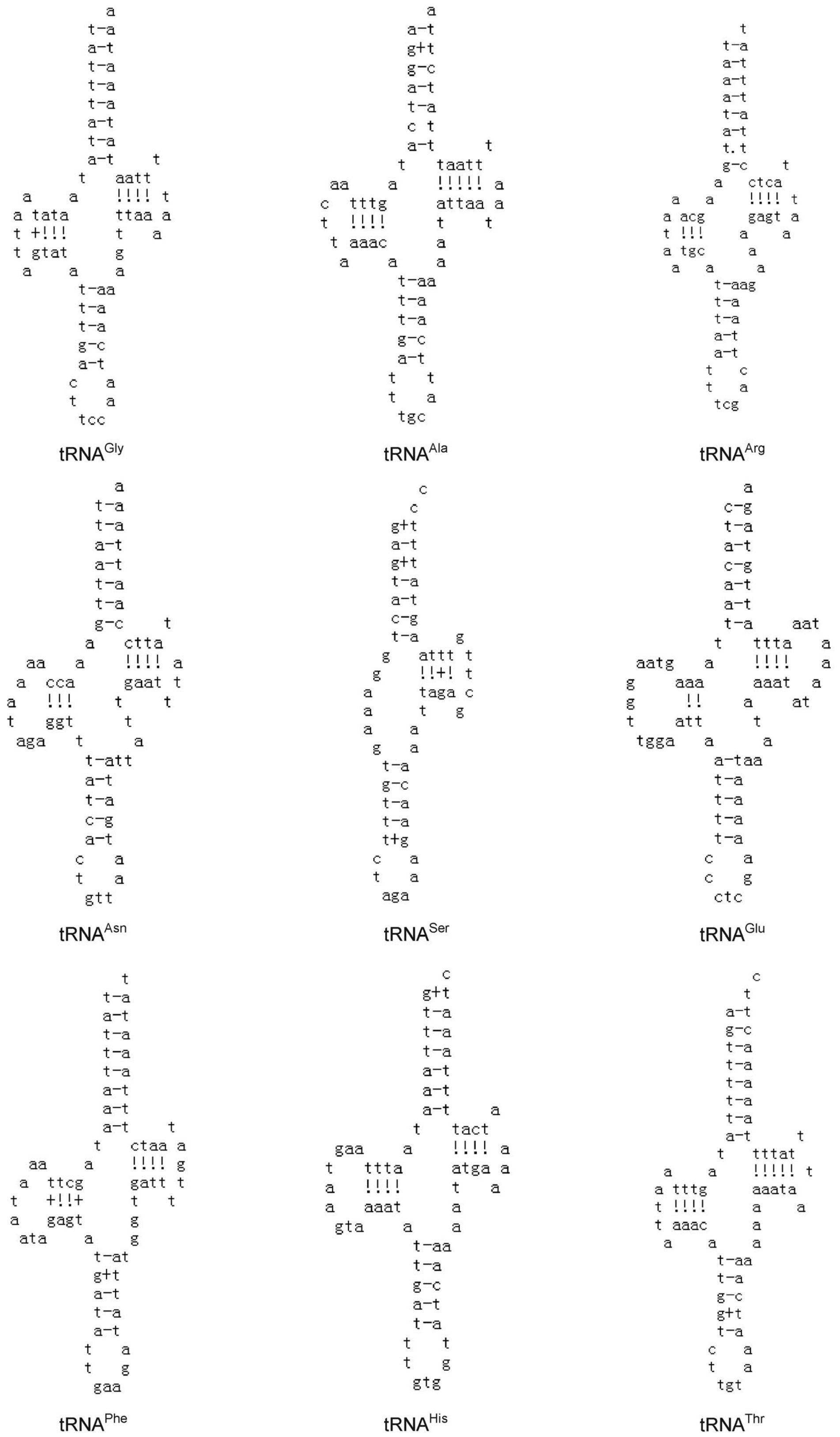


Figure 2 Predicted secondary clover-leaf structure for the tRNA genes of *Ampittia dioscorides*

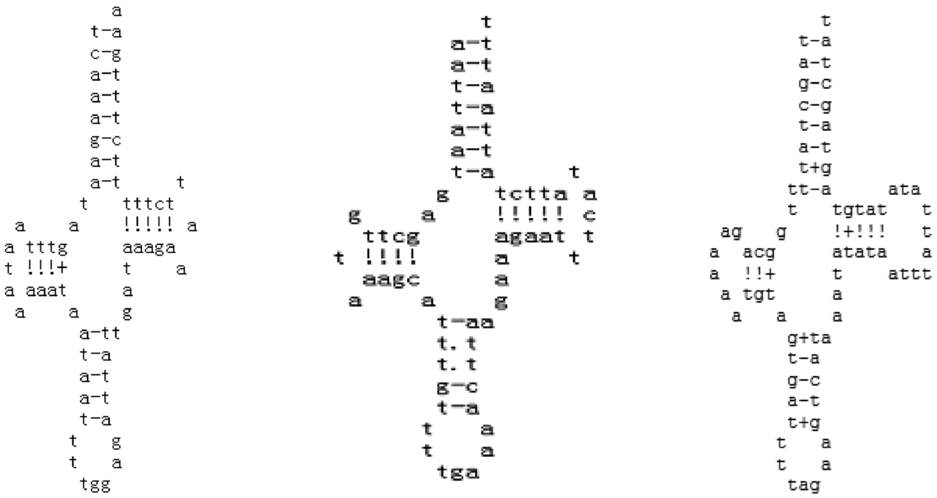


tRNA^{Gly} tRNA^{Ala} tRNA^{Arg}

tRNA^{Asn} tRNA^{Ser} tRNA^{Glu}

tRNA^{Phe} tRNA^{His} tRNA^{Thr}

Figure 2 (*Continued*)



```
          a
        t-a
        c-g
        a-t
        a-t
        a-t
        g-c
        a-t
        a-t     t
       t   tttct
 a    a    !!!!! a
a tttg     aaaga
t !!!+     t    a
a aaat     a
 a    a    g
       a-tt
       t-a
       a-t
       a-t
       t-a
      t   g
      t   a
       tgg
```

tRNA^{Pro}

```
          t
        a-t
        a-t
        t-a
        t-a
        a-t
        a-t
        t-a     t
       g   tctta a
 g    a    !!!!! c
  ttcg     agaat t
t !!!!     a    t
  aagc     a
 a    a    g
       t-aa
       t.t
       t.t
       g-c
       t-a
      t   a
      t   a
       tga
```

tRNA^{Ser}

```
          t
        t-a
        a-t
        g-c
        c-g
        t-a
        a-t
        t+g
       tt-a     ata
       t   tgtat   t
 ag   g    !+!!!   t
a  acg     atata   a
a  !!+     t    attt
 a tgt     a
  a   a    a
       g+ta
       t-a
       g-c
       a-t
       t+g
      t   a
      t   a
       tag
```

tRNA^{Leu}

```
          a
        c-g
        a-t
        a-t
        a-t
        t-a
        t-a
        a-t    t
       g   taat t
 g    a    !!!!  a
t ttcg     atta  t
t !!!+     a   tt
  aagt     a
 t    a    g
       t-aa
       t-a
       t-a
       c-g
       c t
      t   t
      t   a
       tac
```

tRNA^{Val}

Figure 2 (*Continued*)

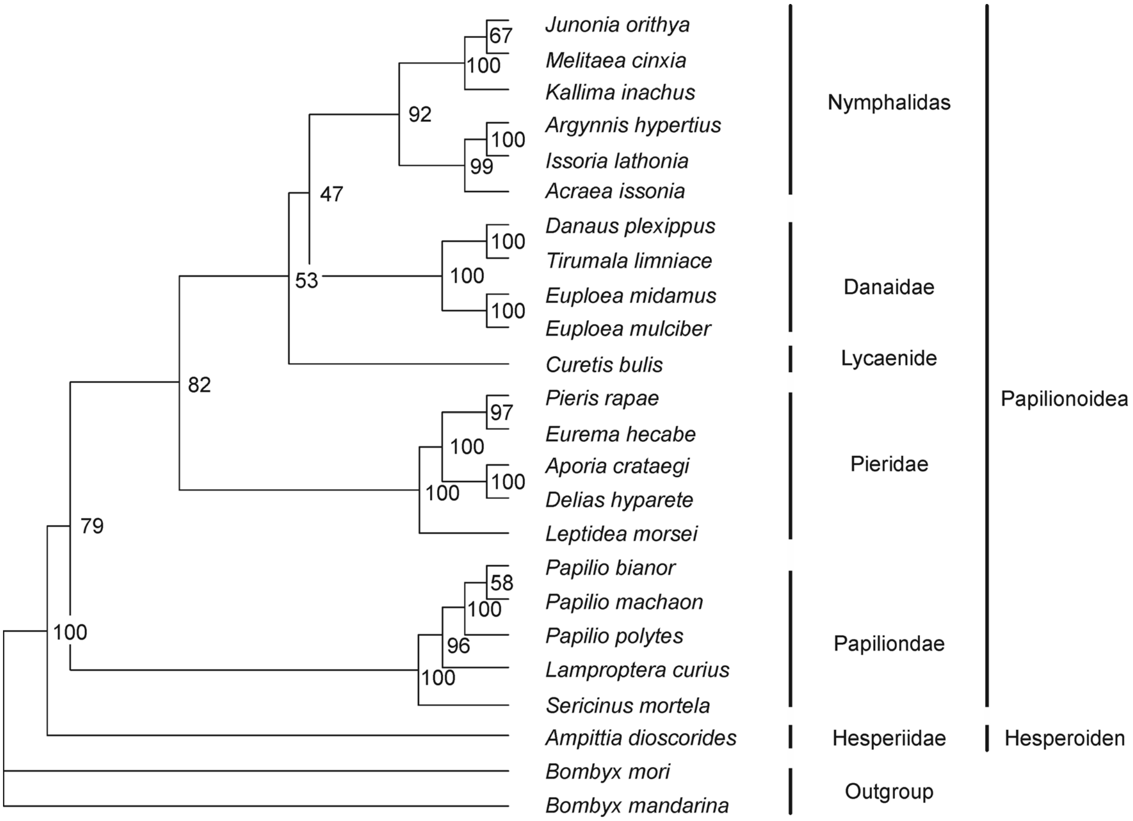


Figure 3 Maximum parsimony phylogenetic tree based on the concatenated amino acid sequences of 13 protein coding genes + *wingless* genes.

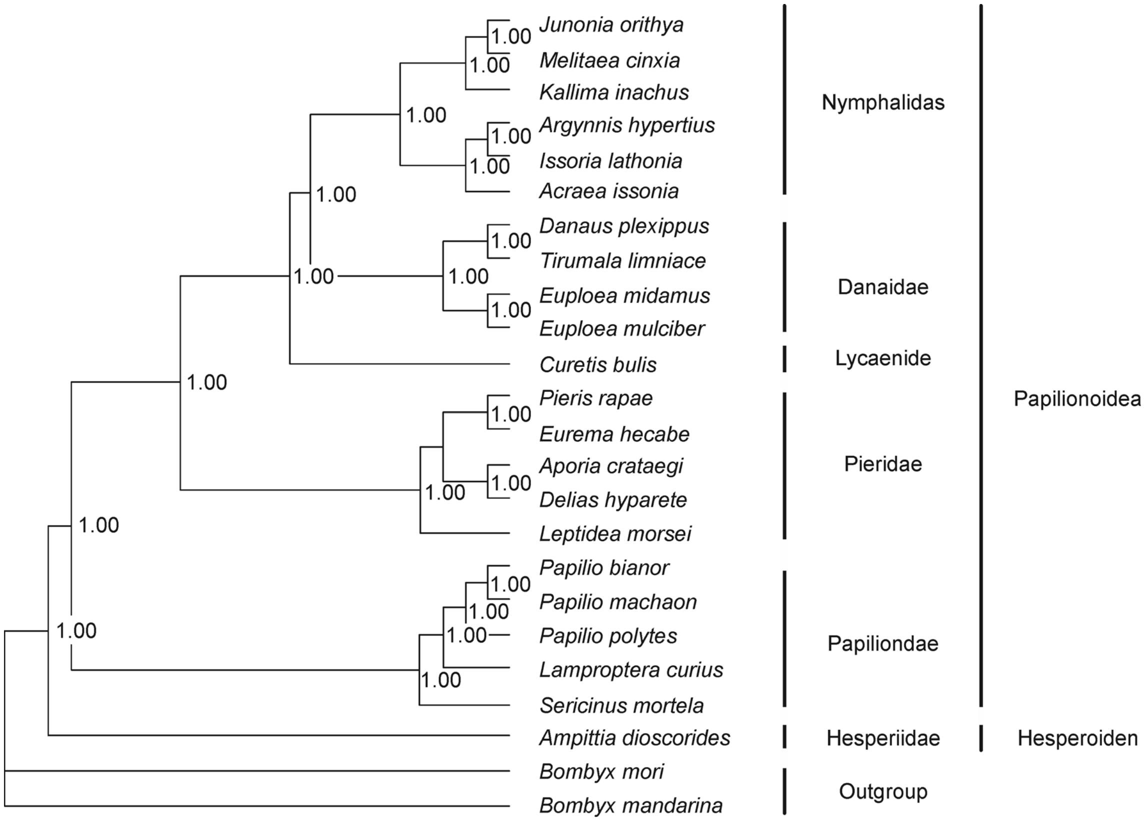


Figure 4 Bayesian inference phylogenetic tree based on the concatenated amino acid sequences of 13 protein coding genes + *wingless* genes.

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Compliance with ethics guidelines

Xin-Min Qin, Xiao-Wen Yang, Li-Xia Hou, and Hui-Min Li declare that they have no conflict of interests. All institutional and national guidelines for the care and use of laboratory animals were followed.

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