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Mitochondrial genome of the sweet potato hornworm, *Agrius convolvuli* (Lepidoptera: Sphingidae), and comparison with other Lepidoptera species

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Abstract

In the present study, we sequenced the complete mitochondrial genome (mitogenome) of *Agrius convolvuli* (Lepidoptera: Sphingidae) and compared it to previously sequenced lepidopteran species. The mitogenome was a circular molecule 15 349 base pairs (bp) long, containing 37 genes. The order and orientation of *A. convolvuli* genes were similar to the sequenced mitogenome of other lepidopterans. All protein-coding genes (PCGs) were initiated by ATN codons, except for the cytochrome c oxidase subunit 1 (*cox1*) gene that was seemingly started by the CGA codon as observed in other lepidopterans. Three of the thirteen PCGs had the incomplete termination codon T, while the remainder terminated with TAA. Additionally, the results of codon distribution of the 13 PCGs revealed that *Asn*, *Ile*, *Leu2*, *Lys, Phe*, and *Tyr* were the most frequently used amino acids. All transfer RNA (tRNA) genes were folded into the expected cloverleaf structure except for *trnS1* (AGN), which lacked a stable dihydrouridine arm. The length of the adenine (A) + thymine (T)-rich region was 331 bp, and included the motif ATAGA followed by a 19 bp poly-T stretch that is a microsatellite-like $(TA)_8$ element next to the ATTTA motif. Phylogenetic analyses (maximum likelihood and Bayesian methods) showed that *A. convolvuli* reside in the Sphingidae family.

Keywords: *Agrius convolvuli*; Lepidoptera; mitochondrial genome; Sphingidae

1. Introduction

The sweet potato hornworm, *Agrius convolvuli* (Lepidoptera: Sphingidae) is a well-known pest that is widely distributed in Asia, Africa, and Europe (Wen 2004). It is harmful to many economically important plants, such as *Dioscorea esculenta*, *Ipomoea pes-caprae*, *I. aquatica*, and so on (Li *et al*. 2005; Lin & Wu 2002). Several studies are available on the biological characteristics and chemical prevention of *A. convolvuli* (Lin & Wu 2002; Wen 2004), whereas, literature is scarce on the complete mitochondrial genome (mitogenome) of this species. This study aims to determine the mitochondrial genome of this species in the hope that it will further aid studies on the biological characteristics and chemical prevention of this pest species.

Animal mitochondrial DNA (mtDNA) generally constitutes 14–19 kb circular DNA molecules, and contains 13 protein-coding genes (PCGs: *atp6*, *atp8*, *cox1*, *cox2*, *cox3*, *cob*, *nad1*, *nad2*, *nad3*, *nad4*, *nad5*, *nad6*, and *nad4L*), 22 transfer RNA (tRNA) genes, and two ribosomal RNA (rRNA) genes (*rrnL* and *rrnS*) (Boore 1999; Dai *et al*. 2013). Additionally, mtDNA contains at least one variable sequence approximately 1 kb in size, known as the adenine (A) + thymine (T)-rich region (Kim *et al*. 2009). The mtDNA is maternally inherited and is subject to little, if any, sequence recombination, and is therefore useful for identifying species and characterizing population genetic structure and molecular evolution (Chen & Sun 2015; Sun *et al.* 2012).

The order Lepidoptera is one of the largest insect orders and includes greater than 160 000 described species that are classified into 45–48 superfamilies (Hao *et al.* 2012). Sphingidae is one of the most diverse superfamilies, and contains 203 genera and 1348 species distributed worldwide. Despite this enormous species diversity, only two complete mitogenomes are available in GenBank (Table 1) (Cameron & Whiting 2009). Newly accessible Lepidoptera mitogenomes will provide further insight into our understanding of evolutionary relationships between these species. In this study, polymerase chain reaction (PCR) amplification and DNA sequencing methods were used to determine the mitogenome of *A. convolvuli*. Moreover, phylogenetic analyses

were performed using the maximum likelihood (ML) and Bayesian inference (BI) methods based on the mitogenome sequences from various lepidopteran species.

2. Experimental Section

2.1. Sample Collection and DNA Extraction

The *A. convolvuli* larvae were collected from Hefei city, China. Our study generalized the species and did not account for variation within the species. The collected specimens were identified as *A. convolvuli*, preserved in 100% ethanol, and stored at −80 °C. Total genomic DNA was extracted from single specimens using the Takara Genomic DNA Extraction Kit, according to the manufacturer's instructions (Takara Co.; Dalian, China). The quality of DNA was examined by 1% agarose gel electrophoresis (w/v) and used to amplify the complete mitogenome of *A. convolvuli*.

2.2. PCR Amplification, Cloning, and Sequencing

To amplify the *A. convolvuli* mitogenome, nine pairs of primers were designed from full-length mitogenome sequences of several lepidopteran species and then synthesized (Beijing Sunbiotech Co., Ltd.; Beijing, China) (Table 2). All PCRs were performed in 50 μL reaction volumes, which contained 25 μL PCR Master mix (2 ×; Aidlab Co.; Beijing, China), 1.5 μL extracted DNA as a template, 2 μL of each primer (10 μM), and 19.5 μL sterilized distilled water. The PCR was performed under the following conditions: an initial denaturation for 4 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 40 s at 46–58 °C (depending on the primer combination), 1–3 min (depending on the putative length of the fragments) at 72 °C, and a 10 min final extension at 72 °C.

The PCR products were separated by agarose gel electrophoresis (1% w/v) and were purified using a DNA gel extraction kit (TransGen Co.; Beijing, China). The obtained fragments were cloned using T-vector (Takara Co.; Dalian, China) in XL-1 blue competent cells (TransGen Co.; Beijing, China). The positive recombinant clone with an insert was sequenced at least three times (Invitrogen Co., Ltd.; Shanghai,

China).

2.3. Sequence Assembly and Gene Annotation

The mtDNA final consensus sequence was assembled using the SeqMan II program from the Lasergene software package (DNASTAR Inc.; Madison, USA). Sequence annotation was performed using online blast tools from NCBI (http://blast.ncbi.nlm.nih.gov/Blast) and compared with other lepidopteran sequences available in GenBank. The overlapping regions and intergenic spacers between genes were counted manually. The composition skewness was determined by AT skew = [A − T]/[A + T] and GC skew = [G − C]/[G + C]) formulae to describe the base composition of nucleotide sequences (Junqueira *et al.* 2004). The PCGs were initially identified by sequence similarity with *Manduca sexta* (Cameron & Whiting 2009) and aligned with other lepidopteran mitogenome sequences using Clustal X version 2.0 (Larkin *et al.* 2007). The PCGs nucleotide sequences were translated into putative proteins based on invertebrate mtDNA genetic codons. The relative synonymous codon usage values were calculated using MEGA 5.0 (Tamura *et al.* 2011). The tRNA genes were identified using the tRNAscan-SE program software available online at http://lowelab.ucsc.edu/tRNAscan-SE/ or edited by eye as sequences having the appropriate anticodon (Lowe & Eddy 1997). The secondary structures of tRNA genes were drawn by the RNAstructure program. The tandem repeats in A + T-rich regions were predicted by the tandem repeats finder available online (http://tandem.bu.edu/trf/trf.html) (Benson 1999).

2.4. Phylogenetic Analysis

To reconstruct the phylogenetic relationship among lepidopterans, the PCG nucleotide sequences of the 13 lepidopteran mitogenome were initially aligned (1 from this study and 12 downloaded from GenBank) using Clustal X with default gap penalties and then concatenated. In addition, the mitogenomes of *Drosophila*

incompta (NC_025936) and *Anopheles gambiae* (NC_002084) were selected as outgroups (Beard *et al.* 1993; Re *et al.* 2014). Two analytical approaches, ML and BI, were used to infer phylogenetic trees. The ML analyses were used to infer phylogenetic trees with 1000 bootstrap replicates. Substitution model selection was also conducted based on the lowest Bayesian information criterion scores using MEGA 5.0. The mtREV24 + G + F model was the appropriate model for the amino acid sequence dataset.

3. Results

3.1. Genome Organization and Base Composition

The length of the *A. convolvuli* complete mitogenome was 15 349 base pairs (bp), containing an entire set of 13 PCGs, 22 tRNA genes, 2 rRNA genes, and a major non-coding region known as the A + T-rich region (Figure 1 and Table 3). The heavy strand (H-strand) encoded more genes (9 PCGs and 14 tRNAs) than the light strand (L-strand) that encoded 4 PCGs, 8 tRNAs, and 2 rRNAs. The order of the genes and the orientation of the *A. convolvuli* mitogenome were identical to completely sequenced lepidopteran mitogenomes of other species, in the order of *trnM-trnI-trnQ*, which was different from the ancestral order of *trnI-trnQ-trnM* (Boore *et al.* 1998). The entire mitogenome of *A. convolvuli* was biased towards using A and T, with an A + T content of 81.49%. The AT skew for the majority strand was –0.001, while the GC skew was –0.169, referring to the occurrence of more Ts than As and more Cs than Gs.

3.2. Overlapping and Intergenic Spacer Regions

The *A. convolvuli* mitogenome contained 26 bp gene overlaps in total at 9 locations. Additionally, the longest overlap of eight bp was located between *trnW* and *trnC* (Table 3).

Agrius convolvuli contained 212 bp intergenic spacer sequences in mtDNA in total. The intergenic spacers were spread over 18 regions, ranging in size from 1 to 53

bp (Table 3). The longest spacer was located between *trnQ* and *nad2*. In addition, the spacer region between *trnS2 (UCN)* and *nad1* contained an 'ATACTAA' motif.

3.3. Protein-Coding Genes

A total of 13 PCGs were identified in *A. convolvuli*, with a length of 11 095 bp. All PCGs started with the canonical putative initiation codon ATN, except for the *cox1* gene, which had CGA as the start codon. Ten of the thirteen PCGs had TAA termination codons; while *cox1*, *cox2*, and *nad4* terminated with a single T. The complete nucleotide sequences of six lepidopteran species were downloaded from GenBank to illustrate codon usage among lepidopterans. These species (four species belonging to Bombycoidea and one each from Pyraloidea and Papilionoidea) were examined and the results revealed that *Asn*, *Ile*, *Leu2*, *Lys, Phe*, and *Tyr* were the most frequently used amino acids (Figure 2). There were at least 7 codon families with no less than 60 codons per thousand codons (*Asn*, *Ile*, *Leu2*, *Lys, Met*, *Phe*, and *Tyr*), and 2 families with at least 90 codons per thousand codons (*Ile* and *Phe*) that were observed in the 6 insect species (Table 4). The relative synonymous codon usage values for Lepidoptera were shown in Figure 3. The codons with high content of G and C, such as CGG, CGC, GCG, and CGC were abandoned in some species (Figure 4).

3.4. Ribosomal RNA Genes and Transfer RNA Genes

There were two rRNAs in *A. convolvuli* with a total length of 2137 bp. The large ribosomal gene (*rrnL*) was 1392 bp long, whereas the small ribosomal gene (*rrnS*) was only 777 bp long. The *rrnL* and *rrnS* genes were located between *trnL1* (CUN) and *trnV*, and *trnV* and the A + T-rich region, respectively. The AT skew was positive (0.017), while the GC skew was negative (–0.327), indicating the presence of more Ts than As and more Cs than Gs. Similar to other known lepidopteran rRNAs, the A + T content of the *rrnL* and *rrnS* was 85.27% and 85.46%, respectively.

Agrius convolvuli mitochondrial tRNA genes were scattered throughout the

molecule, with 14 encoded by the H-strand, while the rest were encoded by the L-strand. All the tRNAs could be folded into the expected secondary cloverleaf structure except for the *trnS1 (*AGN*)* gene that lacked a stable dihydrouridine arm (Figure 4 & Figure S1).

3.5. The A + T-rich Region

The A + T-rich region of *A. convolvuli* extended over 331 bp (15 019–15 349 nt) and was located between the *rrnS* and *trnM* genes. This region contained the highest A + T content (93.35%) in the mitogenome. Sequence analysis of *A. convolvuli* A + T-rich region revealed that there was a conserved structure including the motif 'ATAGA' and a 19 bp poly-T stretch downstream of the *rrnS* gene. In addition, we identified a microsatellite-like $(TA)_8$ element next to the 'ATTTA' motif and a poly-A element upstream of the *trnM* gene (Figure 5).

3.6. Phylogenetic Analyses

To reconstruct the phylogenetic relationship among lepidopteran insects, the nucleotide sequences of the 13 PCGs were firstly aligned and then concatenated. The phylogenetic analyses showed that *A. convolvuli* have a close relationship to *M. sexta* and *Sphinx morio* that was well supported by both the ML and BI analyses (Figures 6A & 6B). Within the Pyraloidea, *P. nepenthes* and *C. nephalonica* form a separate but lower-supported lineage from both ML and BI analyses. *Agrius convolvuli* was within the family Sphingidae (Bombycoidea) and clustered with other superfamilies, including the Noctuoidea, Pyraloidea, Papilionoidea, and Tortricoidea. Additionally, Bombycoidea was closer to Noctuoidea than any other superfamily.

Discussion

In the present study, we sequenced and determined the complete mitochondrial genome of *A. convolvuli*, based on the primers designed from previously known mitogenome of lepidopterans. The mitochondrial genome structure of *A. convolvuli*

was typical of other known insect mitogenomes, with a similar arrangement and order of genes. This was particularly true in the arrangement of *trnM-trnI-trnQ*, which is different from the ancestral order of *trnI-trnQ-trnM* (Boore *et al.* 1998). Lavrov et al. (1999) found that the placement of *trnM* might therefore represent a molecular feature exclusive to lepidopteran mtDNAs. The H-strand was found biased to the distribution of genes, and rRNA only resided on the L-strand (Figure 1 and Table 3). The mitogenome of six lepidopteran species representing four lepidopteran superfamilies (Bombycoidea, Noctuoidea, Pyraloidea, and Tortricoidea) were downloaded and used for comparison with the *A. convolvuli* mitogenome (Table 5). The length of the *A. convolvuli* mitogenome (15 349 bp) was more closely related to *Actias selene* (15 236 bp) and *Acleris fimbriana* (15 933 bp) within the superfamily Bombycoidea, when considering the sequenced lepidopteran mitogenomes to date (Liu *et al.* 2012). The *A. convolvuli* mtDNA was highly biased to A + T content (81.49%), which is higher than *A. selene* (78.91%), *Ochrogaster lunifer* (77.84%), *Tyspanodes hypsalis* (81.41%), and *Cydia pomonella* (80.13%), and lower than that of *M. sexta* (81.78%) (Table 4) (Cameron & Whiting 2009; Liu *et al.* 2012; Salvato *et al.* 2008; Shi *et al.* 2013; Wang *et al.* 2014). The AT skew for the majority strand was –0.001, while the GC skew was –0.169, indicating the occurrence of more Ts than As and more Cs than Gs.

The *A. convolvuli* mitogenome contained 26 bp gene overlaps in total at 9 locations. Additionally, the largest eight bp was located between *trnW* and *trnC* (Table 3). This was similar to *A. yamamai* and *Bombyx mori* strain H9, while *A. selene* had a 16 bp long overlap (Dai *et al.* 2013; Kim *et al.* 2009; Liu *et al.* 2012).

The length of the intergenic spacer region in *A. convolvuli* (212 bp in 18 regions) was longer than that of other lepidopteran species including *Artogeia melete* (118 bp over 10 regions), *Camponotus raphaelis* (178 bp over 17 regions), and *Caligula boisduvalii* (194 bp over 16 regions), although shorter than *O. lunifer* (371 bp over 20 regions) (Hong *et al.* 2008; Hong *et al.* 2009; Salvato *et al.* 2008). Furthermore, the 'ATACTAA' motif, between *trnS2* (*UCN*) and *nad1*, was a conserved region and widely documented in most lepidopteran mtDNAs (Figure S2) (Liu *et al.* 2008; Timmermans *et al.* 2014; Zhu *et al.* 2013). This seven bp motif was possibly

fundamental to site recognition by the transcription termination peptide (Taanman 1999).

The *A. convolvuli* PCGs had a typical start codon (ATN) except for the *cox1* (CGA) gene, which is quite similar to other lepidopterans (Dai *et al.* 2014; Liu *et al.* 2013a). Several authors have maintained the problematic translational start at the *cox1* locus in many insect species, with TTAG, ACG, and TTG proposed as start codons for *cox1* (Lee *et al.* 2008; Ogoh & Ohmiya 2004; Sabine *et al.* 2004). TAA was the most frequent stop codon in *A. convolvuli* and the single T nucleotide was the incomplete stop codon. This has been well documented in other invertebrate mitogenome and is a common evolutionary feature shared by mtDNA. The single T stop codon was typically located close to *trnK*, recognized by endonucleases processing the polycistronic pre-mRNA transcription, and produced functional stop codons by polyadenylation from its contiguous PCGs (Lu *et al.* 2002).

All the tRNAs could be folded into the expected secondary cloverleaf structure except for the *trnS1* (AGN) gene that lacked a stable dihydrouridine arm (Figure 6), which was similar to several published lepidopteran mitogenomes (Chai & Du 2012; Liu *et al.* 2014). In addition, there were several mismatched bps in the *A. convolvuli* tRNAs. In *Amata emma*, 24 mismatched bp were observed, while in *Cerura menciana* 10 mismatched bps were obtained (Dai *et al.* 2015; Lu *et al.* 2013). These mismatched bps were corrected via RNA editing mechanisms (Lavrov *et al.* 1999).

Sequence analysis of the A + T-rich region of *A. convolvuli* revealed there was a conserved structure that included the motif 'ATAGA' and a 19 bp poly-T stretch downstream of the *rrnS* gene. In addition, we identified a microsatellite-like $(TA)_8$ element next to the 'ATTTA' motif and also a poly-A element, which may be involved in controlling transcription and/or replication initiation or may have some other unknown function. These structures were quite similar to the mitogenome of previously known insects and sequenced lepidopterans (Dai *et al.* 2013; Liao *et al.* 2010; Zhu *et al.* 2013; Liu *et al.* 2016). The presence of multiple tandem repeat elements has been reported to be a characteristic feature of the insect A + T-rich region (Jiang *et al.* 2009). In *A. convolvuli*, the A + T-rich region harbored a repeat

element of 62 bp. This characteristic feature has been documented in a small number of lepidopterans, for example in *M. separate*, which contains 51 bp repeat elements that reside twice in the A + T region (Hao *et al.* 2012). Whereas, in several other insect species (e.g., *C. menciana* and *Helicoverpa armigera*) sequenced to date, no conspicuous multiple tandem repeat element was observed (Dai *et al.* 2013; Yin *et al.* 2010).

The phylogenetic analyses showed that Bombycoidea was phylogenetically closer to Noctuoidea. These phylogenetic relationships were consistent with previously documented studies of lepidopterans (Jiang *et al.* 2009; Kawahara *et al.* 2009; Liao *et al.* 2010). We concluded from the present study that more research on the diverse Lepidoptera species is needed, to be able to understand better the relationships among them.

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Conflicts of Interest

The authors declare no conflict of interest.

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Figure and table captions

Figure 1. Map of the mitogenome of *A. convolvuli*. Protein-coding genes coded on the majority strand are blue colored, while the remainder plus two rRNA genes coded on the minority strand are pink colored. The tRNA genes with a single letter above the central axis are coded on the majority strand. Underscores under the axis with F1–F9 indicate positions of nine overlapping PCR amplified fragments.

Figure 2. Comparison of the codon usage of mitochondrial genome in Lepidoptera

Figure 3. The relative synonymous codon usage of mitochondrial genome across five superfamilies in Lepidoptera.

Figure 4. Predicted secondary cloverleaf structures for two tRNA genes of *A. convolvuli*

Figure 5. Features present in the A + T-rich region of *A. convolvuli*

Figure 6. Inferred phylogenetic relationship among Lepidoptera based on amino acid sequence of mitochondrional 13 PCGs using maximum likelihood (ML) (A) and Bayesian inference (BI) (B). The number at each node shows bootstrap percentages (A) and posterior probabilities (B). *Drosophila incompta* and *Anopheles gambiae* are used as outgroups. The star next to *A. convolvuli* is a mark.

Table 1. Lepidopteran mitogenomes used in the present study

Table 2. The primers used in the present study

Table 3. Annotation and gene organization of the *Agrius convolvuli* mitogenome

Table 4. Codon distribution in Lepidoptera

Table 5. Composition and skewness in the lepidopteran mitogenomes

Table 1. Lepidopteran mitogenomes used in this study

Subfamily	Family	Species	Acc.number	Refference
Bombycoidea	**Sphingidae**	***Agrius convolvuli***		**This study**
		Sphinx morio	KF163965.1	Kim *et al.* 2008
		Manduca sexta	GU188273.1	Cameron & Whiting 2008
	Bombycidae	*Bombyx mori*	NC_002355.1	Lee *et al.* 2008
		Bombyx mandarina	AY301620.2	Pan *et al.* 2008
	Saturniidae	*Actias selene*	NC_018133.1	Liu *et al.* 2012
		Antheraea pernyi	AY242996.2	Hong *et al.* 2009
		Eriogyna pyretorum	FJ685653.1	Jiang *et al.* 2009
Gelechioidea	Oecophoridae	*Endrosis sarcitrella*	KJ508037.1	Zhu *et al.* 2013
Noctuoidea	Noctuidae	*Spodoptera litura*	NC_021756.1	Liu *et al.* 2014
		Helicoverpa armigera	GU188273.1	Yin *et al.* 2010
	Notodontidae	*Ochrogaster lunifer*	AM946601.1	Salvato *et al.* 2008
		Phalera flavescens	JF440342.1	Sun *et al.* 2012
Pyraloidea	Crambidae	*Tyspanodes hypsalis*	NC_023978.1	Wang *et al.* 2014
Papilionoidea	Papilionidae	*Papilio syfanius*	NC_023978.1	Dong *et al.* 2014
	Nymphalidae	*Polyura nepenthes*	NC_026073.1	Shi & Hao 2015
Tortricoidea	Tortricidae	*Acleris fimbriana*	NC_018754.1	Zhao *et al.* 2014
		Cydia pomonella	JX407107.2	Shi *et al.* 2013

Table 2. The primers used in this study

Primer pair	Primer sequence (5' →3')
F1	GCTTTTGGGCTCATACCTCA
R1	GATGAAATACCTGCAAGATGAAG
F2	TGGAGCAGGAACAGGATGAAC
R2	GAGACCADTACTTGCTTTCAG
F3	ATTCTATATTTCTTGAAATATTAT
R3	CATAAATTATAAATCTTAATCATA
F4	TCGACCTGGAACTTTAGC
R4	GCAGCTATAGCCGCTCCTACT
F5	TAAAGCAGAAACAGGAGTAG
R5	ATTGCGATATTATTTCTTTTG
F6	GGAGCTTCTACATGAGCTTTTGG
R6	GTTTGCGACCTCGATGTTG
F7	CGGTTTGAACTCAGATCATGTAAG
R7	TATTGTATCTTGTGTATCAGAGTTTA
F8	GGTCCCTTACGAATTTGAATATATCCT
R8	AAACTAGGATTAGATACCCTATTAT
F9	TCTAGAAACACTTTCCAGTACCTC
R9	ACTTAATTTATCCTATCAGAATAA

Table 3. Annotation and gene organization of the *Agrius convolvuli* mitogenome

Gene	Direction	Location	Size (bp)	Anticodon	Start codon	Stop codon	Intergenic Nucleotides
trnM	F	1−67	67	CAT	—	—	6
trnI	F	74−138	65	GAT	—	—	-3
trnQ	R	136−204	69	TTG	—	—	53
nad2	F	258−1271	1014	—	ATT	TAA	-1
trnW	F	1271−1337	67	TCA	—	—	-8
trnC	R	1330−1394	65	GCA	—	—	9
trnY	R	1404−1468	65	GTA	—	—	3
cox1	F	1472−3002	1531	—	CGA	T	4
trnL2(UUR)	F	3007−3072	66	TAA	—	—	0
cox2	F	3073−3754	682	—	ATG	T	0
trnK	F	3755−3825	71	CTT	—	—	3
trnD	F	3829−3894	66	GTC	—	—	0
atp8	F	3895−4059	165	—	ATC	TAA	-7
atp6	F	4053−4731	679	—	ATG	TAA	17
cox3	F	4749−5540	792	—	ATG	TAA	6
trnG	F	5547−5613	67	TCC	—	—	0
nad3	F	5614−5967	354	—	ATT	TAA	32
trnA	F	6000−6063	64	TGC	—	—	-1
trnR	F	6063−6127	65	TCG	—	—	2
trnN	F	6130−6195	66	GTT	—	—	3
trnS1(AGN)	F	6199−6264	65	GCT	—	—	-1
trnE	F	6264−6331	68	TTC	—	—	0
trnF	R	6332−6399	68	GAA	—	—	-1
nad5	R	6399−8140	1742	—	ATA	TAA	-3
trnH	R	8138−8204	67	GTG	—	—	6

nad4	R	8211−9541	1331	—	ATA	T	3
nad4L	R	9545−9835	291	—	ATT	TAA	14
trnT	F	9850−9914	65	TGT	—	—	-1
trnP	R	9914−9980	67	TGG	—	—	1
nad6	F	9982−10,512	531	—	ATG	TAA	13
cob	F	10,526−11,679	1154	—	ATG	TAA	20
trnS2(UCN)	F	11,700−11,764	65	TGA	—	—	17
nad1	R	11,782−12,717	936	—	ATG	TAA	0
trnL1(CUN)	R	12,718−12,784	67	TAG	—	—	0
rrnL	R	12,785−14,176	1392	—	—	—	0
trnV	R	14,177−14,241	65	TAC	—	—	0
rrnS	R	14,242−15,018	777	—	—	—	0
A+T-rich regi		15,019−15,349	331	—	—	—	—

Table 4. Codon distribution in Lepidoptera

Agrius convolvuli		*Sphinx morio*		*Manduca sexta*		*Actias selene*		*Tyspanodes hypsalis*		*Polyura nepenthes*	
AA	**CDpT**	**AA**	**CDpT**	**AA**	**CDpT**	**AA**	**CDpT**	**AA**	**CDpT**	**AA**	**CDpT**
Ala	14.04	Ala	8.27	Ala	7.69	Ala	22.91	Ala	19.74	Ala	23.09
Arg	6.73	Arg	5.02	Arg	3.55	Arg	8.20	Arg	8.30	Arg	9.06
Asn	81.90	Asn	110.20	Asn	108.80	Asn	90.50	Asn	103.50	Asn	93.22
Asp	25.15	Asp	13.88	Asp	14.19	Asp	13.86	Asp	14.59	Asp	14.61
Cys	4.68	Cys	9.16	Cys	11.82	Cys	8.20	Cys	10.58	Cys	10.52
Glu	32.17	Glu	18.02	Glu	16.85	Glu	15.84	Glu	22.60	Glu	19.58
Gln	34.81	Gln	17.13	Gln	19.51	Gln	24.04	Gln	21.45	Gln	17.83
Gly	20.77	Gly	13.29	Gly	14.78	Gly	30.83	Gly	25.17	Gly	30.68
His	26.91	His	16.24	His	16.85	His	25.45	His	18.02	His	16.95
Ile	97.10	Ile	104.80	Ile	106.70	Ile	95.02	Ile	115.30	Ile	118.10
Leu1	66.39	Leu1	32.78	Leu1	34.88	Leu1	58.82	Leu1	40.90	Leu1	40.91
Leu2	111.70	Leu2	77.38	Leu2	78.92	Leu2	95.31	Leu2	89.53	Leu2	76.27
Lys	85.41	Lys	69.99	Lys	74.19	Lys	81.17	Lys	77.52	Lys	75.10
Met	83.36	Met	74.42	Met	78.33	Met	66.18	Met	64.07	Met	72.18
Phe	99.74	Phe	99.53	Phe	94.00	Phe	96.15	Phe	94.68	Phe	102.00
Pro	25.15	Pro	22.15	Pro	19.80	Pro	33.37	Pro	28.89	Pro	37.11
Ser1	13.75	Ser1	64.09	Ser1	58.53	Ser1	30.54	Ser1	37.76	Ser1	28.05
Ser2	37.15	Ser2	63.79	Ser2	66.21	Ser2	52.60	Ser2	56.92	Ser2	61.95
Thr	29.25	Thr	47.84	Thr	43.45	Thr	41.86	Thr	38.33	Thr	40.91
Trp	15.50	Trp	24.22	Trp	26.01	Trp	23.19	Trp	20.59	Trp	29.51
Tyr	66.98	Tyr	95.39	Tyr	94.00	Tyr	64.20	Tyr	72.65	Tyr	59.61
Val	21.35	Val	12.40	Val	10.94	Val	21.78	Val	18.88	Val	22.79

Table 5. Composition and skewness in the Lepidopteran mitogenomes

Species	Size (bp)	A%	G%	T%	C%	A+T %	ATskewness	GCskewness
Whole genome								
A. convolvuli	**15,349**	**40.71**	**7.69**	**40.78**	**10.82**	**81.49**	**-0.001**	**-0.169**
M. sexta	15,516	40.67	7.46	41.11	11.76	81.78	-0.005	-0.195
B. mandarina	15,682	43.11	7.40	38.48	11.01	81.59	0.057	-0.196
A. selene	15,236	38.54	8.05	40.37	13.03	78.91	-0.023	-0.236
O. lunifer	15,593	40.09	7.56	37.75	14.60	77.84	0.030	-0.318
T. hypsalis	15,329	40.00	7.67	41.42	10.92	81.41	-0.017	-0.175
C. pomonella	15,253	39.92	7.88	40.21	11.99	80.13	-0.004	-0.207
PCG								
A. convolvuli	**11,095**	**39.91**	**8.40**	**40.16**	**11.53**	**80.07**	**-0.003**	**-0.157**
M. sexta	11,178	40.40	8.22	39.89	11.49	80.29	0.006	-0.166
B. mandarina	11,196	42.83	8.26	37.04	11.87	79.87	0.072	-0.179
A. selene	11,231	37.93	8.74	39.44	13.89	77.37	-0.020	-0.228
O. lunifer	11,266	32.47	12.08	43.26	12.19	75.73	-0.142	-0.004
T. hypsalis	11,188	39.31	8.46	40.66	11.57	79.97	-0.017	-0.155
C. pomonella	11,199	39.55	8.69	39.00	12.76	78.55	0.007	-0.190
tRNA								
A. convolvuli	**1447**	**40.91**	**8. 85**	**40.22**	**10.02**	**81.13**	**0.009**	**-0.062**
M. sexta	1470	41.09	8.16	40.68	10.07	81.77	0.005	-0.105
B. mandarina	1472	41.78	7.81	39.95	10.46	81.73	0.022	-0.145
A. selene	1459	40.37	8.16	40.23	11.24	80.60	0.002	-0.159
O. lunifer	1666	41.78	7.32	39.86	11.04	81.63	0.023	-0.202
T. hypsalis	1456	40.73	7.90	41.35	10.03	82.07	-0.008	-0.119
C. pomonella	1451	41.14	7.93	40.32	10.61	81.46	0.010	-0.145
rRNA								
A. convolvuli	**2137**	**43.38**	**4.93**	**41.95**	**9.73**	**85.34**	**0.017**	**-0.327**
M. sexta	2168	41.37	4.84	44.05	9.73	85.42	-0.031	-0.335
B. mandarina	2134	43.86	4.78	41.05	10.31	84.91	0.028	-0.366
A. selene	2126	39.93	4.99	43.79	11.29	83.73	-0.046	-0.387
O. lunifer	2157	41.96	4.82	40.19	13.03	82.15	0.022	-0.460
T. hypsalis	2156	42.02	4.92	43.09	9.97	85.11	-0.013	-0.339
C. pomonella	2147	40.48	5.03	43.92	10.57	84.40	-0.041	-0.355

A+T-rich region								
A. convolvuli	**372**	**41.99**	**2.11**	**51.36**	**4.53**	**93.35**	**-0.100**	**-0.364**
M. sexta	324	45.06	1.54	50.31	3.09	95.37	-0.055	-0.335
B. mandarina	484	46.49	2.69	47.93	2.89	94.42	-0.015	-0.036
A. selene	339	43.07	5.90	44.84	6.19	87.91	-0.020	-0.024
O. lunifer	319	44.5	1.6	48.9	5.0	93.4	-0.047	-0.524
T. hypsalis	350	43.43	1.14	52.00	3.43	95.43	-0.090	-0.501
C. pomonella	351	43.30	1.14	52.42	3.13	95.73	-0.095	-0.466

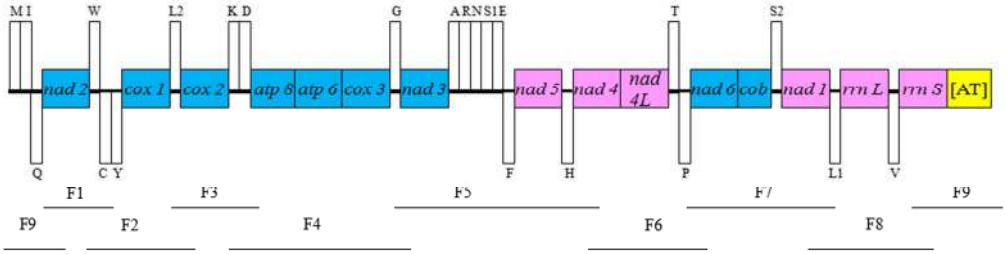


Figure 1. Map of the mitogenome of A. convolvuli. Protein-coding genes coded on the majority strand are blue colored, while the remainder plus two rRNA genes coded on the minority strand are pink colored. The tRNA genes with a single letter above the central axis are coded on the majority strand. Underscores under the axis with F1–F9 indicate positions of nine overlapping PCR amplified fragments.

253x75mm (300 x 300 DPI)

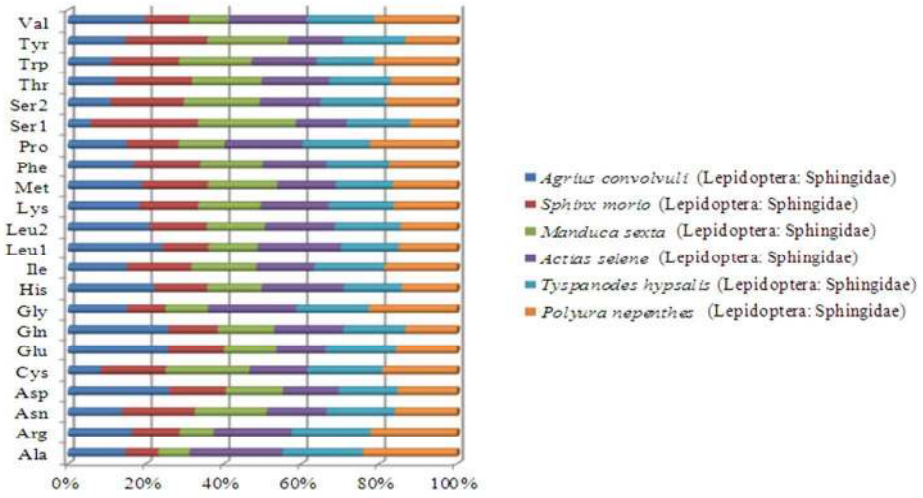


Figure 2. Comparison of the codon usage of mitochondrial genome in Lepidoptera

71x38mm (300 x 300 DPI)

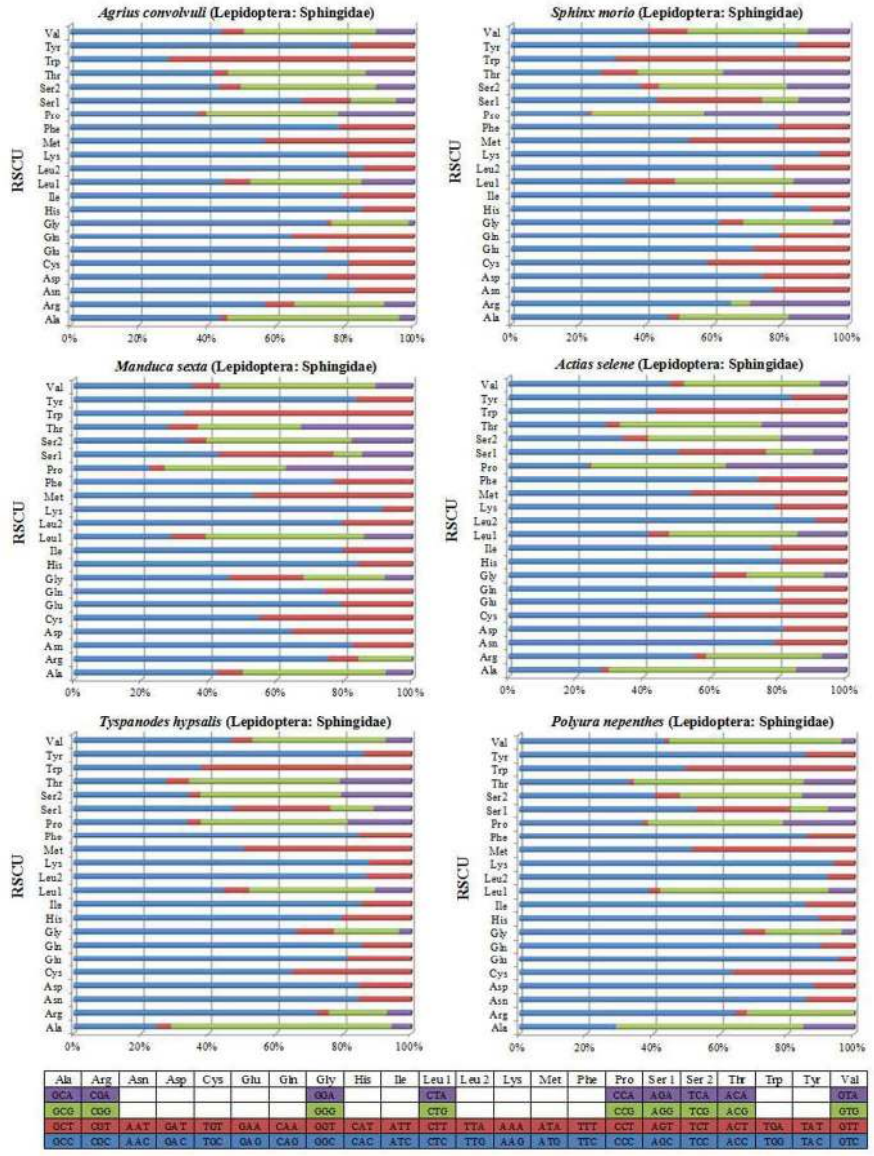


Figure 3. The relative synonymous codon usage of mitochondrial genome across five superfamilies in Lepidoptera.

195x263mm (300 x 300 DPI)

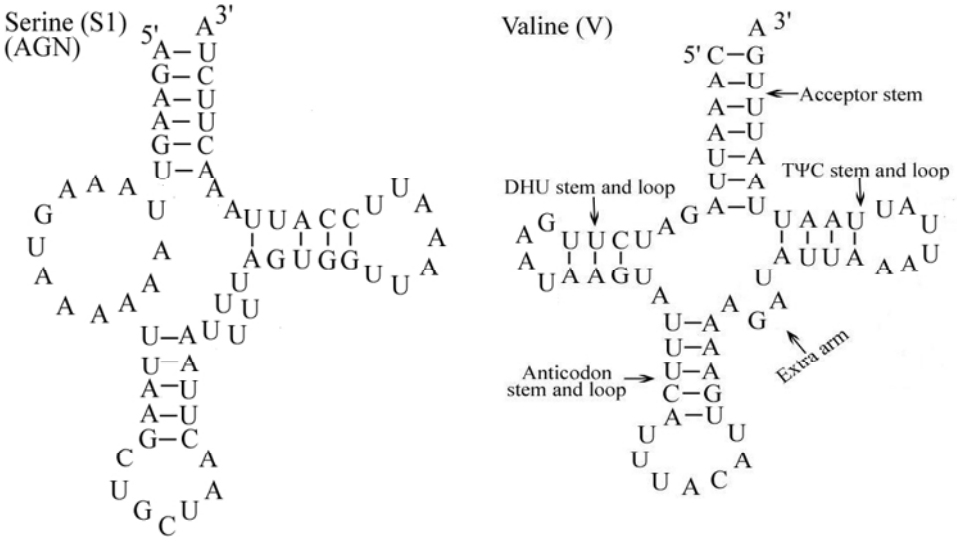


Figure 4. Predicted secondary cloverleaf structures for two tRNA genes of A. convolvuli

84x46mm (300 x 300 DPI)

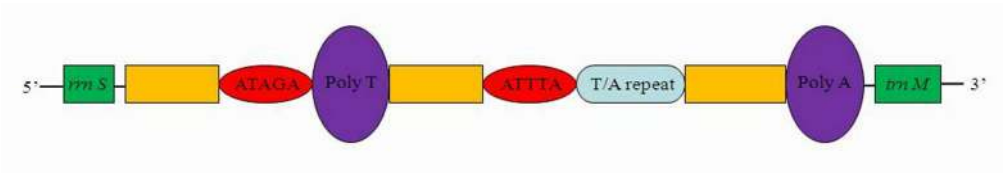
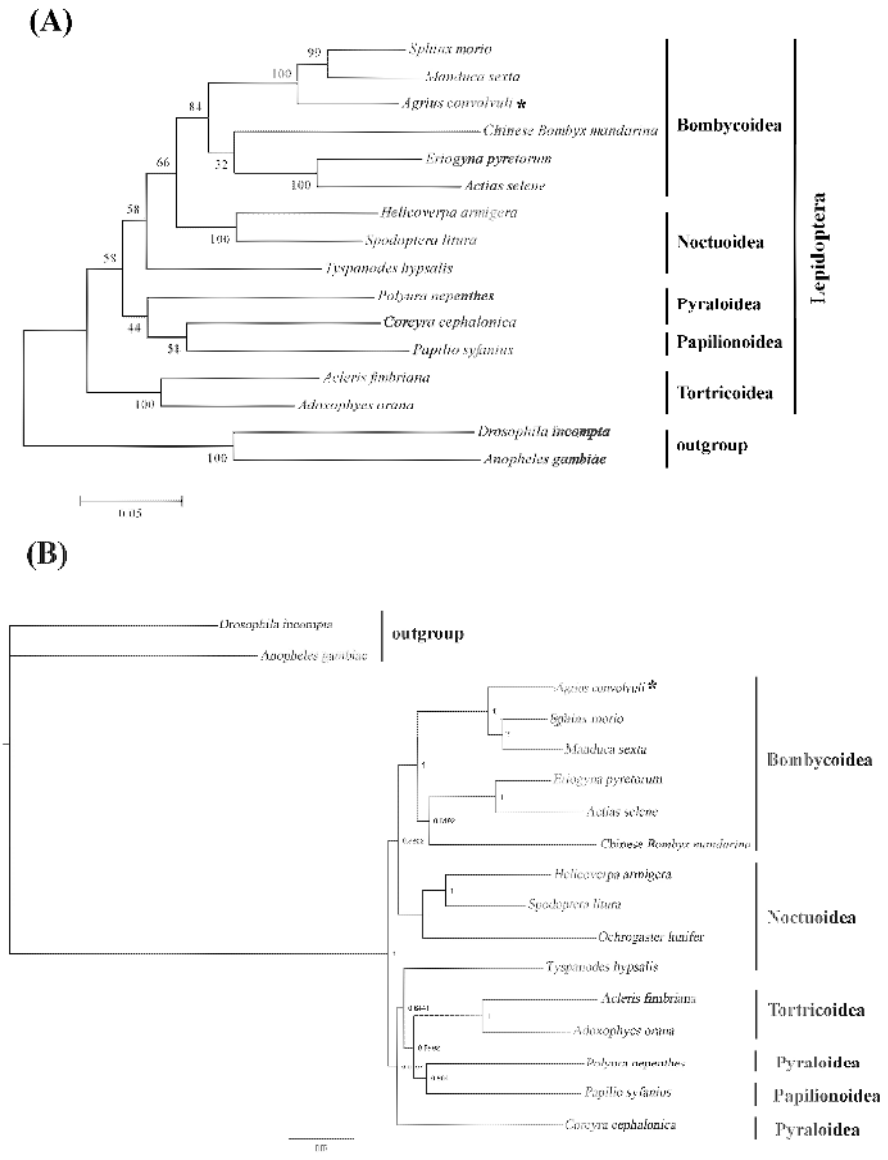


Figure 5. Features present in the A + T-rich region of A. convolvuli

82x13mm (300 x 300 DPI)



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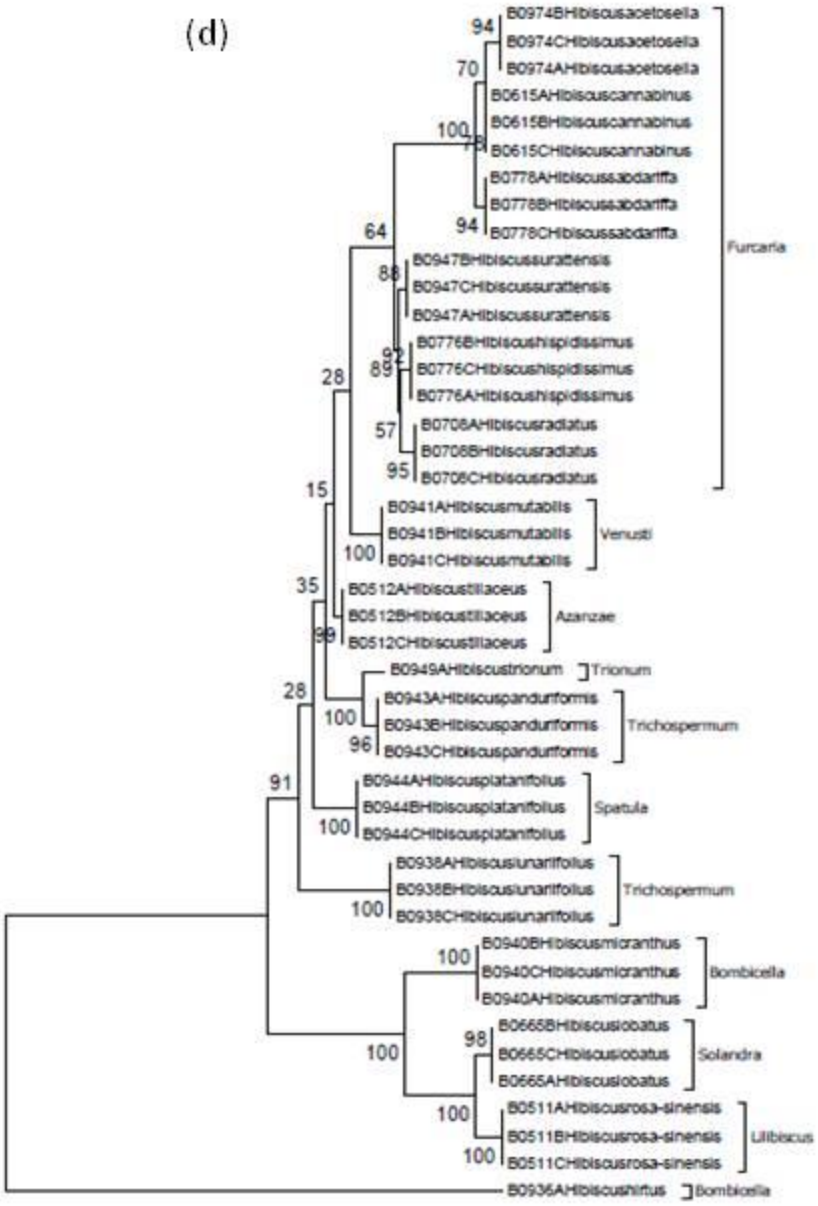
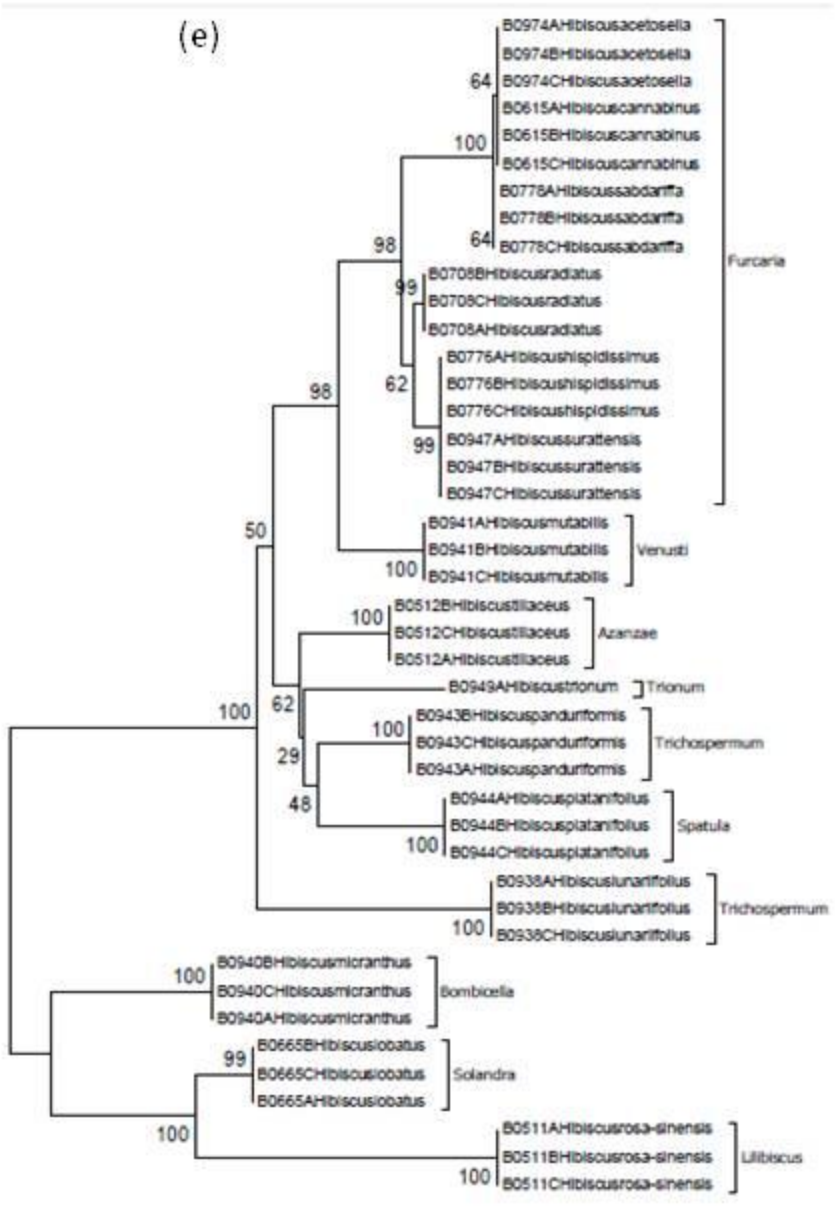
240x318mm (300 x 300 DPI)


(d)
B0974BHibiscusacetosella
B0974CHibiscusacetosella
B0974AHibiscusacetosella
B0615AHibiscuscannabinus
B0615BHibiscuscannabinus
B0615CHibiscuscannabinus
B0778AHibiscussabdariffa
B0778BHibiscussabdariffa
B0778CHibiscussabdariffa
B0947BHibiscussurattensis
B0947CHibiscussurattensis
B0947AHibiscussurattensis
B0776BHibiscushispidissimus
B0776CHibiscushispidissimus
B0776AHibiscushispidissimus
B0706AHibiscusradiatus
B0706BHibiscusradiatus
B0706CHibiscusradiatus
Furcaria
B0941AHibiscusmutabilis
B0941BHibiscusmutabilis
B0941CHibiscusmutabilis
Venusti
B0512AHibiscustiliaceus
B0512BHibiscustiliaceus
B0512CHibiscustiliaceus
Azanzae
B0949AHibiscustrionum
Trionum
B0943AHibiscuspandurtiformis
B0943BHibiscuspandurtiformis
B0943CHibiscuspandurtiformis
Trichospermum
B0944AHibiscusplatanifolius
B0944BHibiscusplatanifolius
B0944CHibiscusplatanifolius
Spatula
B0938AHibiscuslunariifolius
B0938BHibiscuslunariifolius
B0938CHibiscuslunariifolius
Trichospermum
B0940BHibiscusmicranthus
B0940CHibiscusmicranthus
B0940AHibiscusmicranthus
Bombicella
B0665BHibiscusiobatus
B0665CHibiscusiobatus
B0665AHibiscusiobatus
Solandra
B0511AHibiscusroga-sinensis
B0511BHibiscusroga-sinensis
B0511CHibiscusroga-sinensis
Lilibiscus
B0936AHibiscushirtus
Bombicella


(e)
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B0974CHibiscusacetosella
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B0615CHibiscuscannabinus
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B0706CHibiscusradiatus
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Furcaria
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B0941BHibiscusmutabilis
B0941CHibiscusmutabilis
Venusti
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B0512CHibiscustiliaceus
B0512AHibiscustiliaceus
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Trionum
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Trichospermum
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Trichospermum
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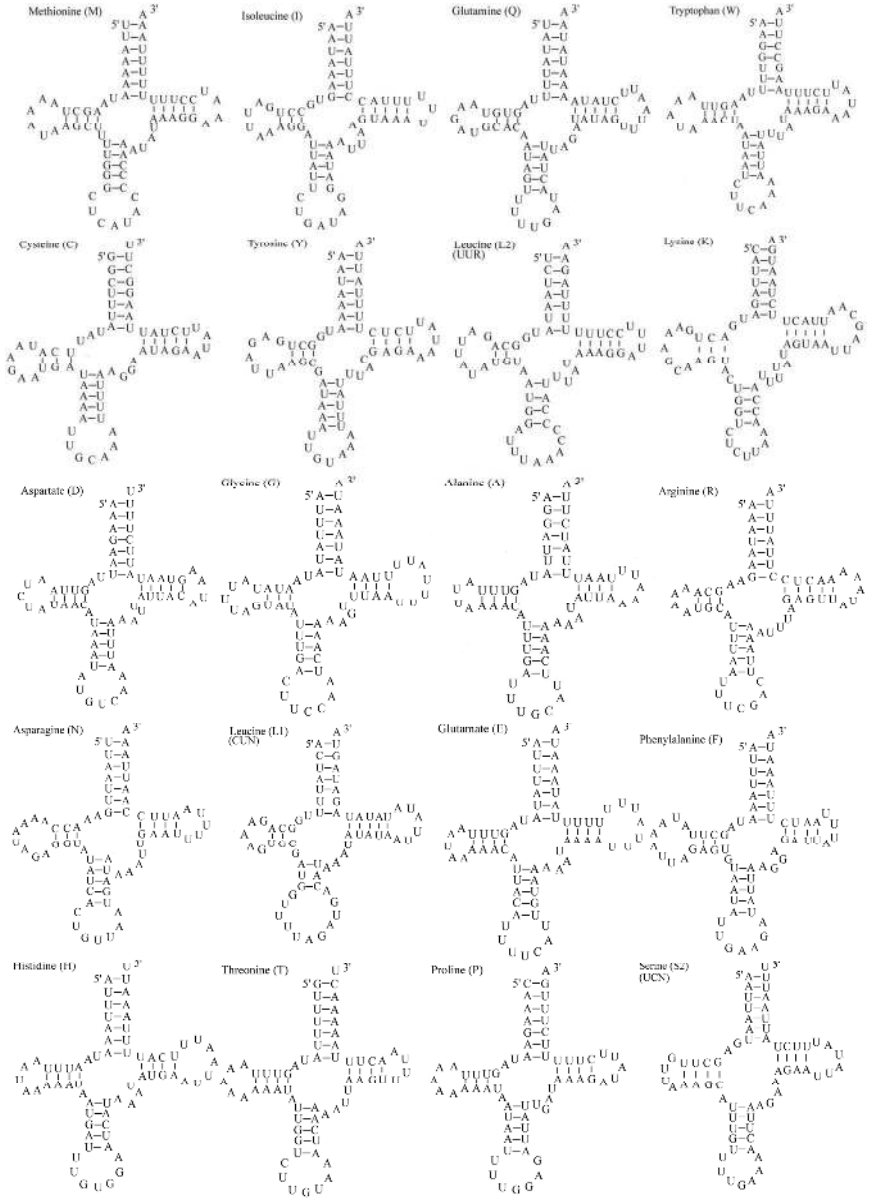
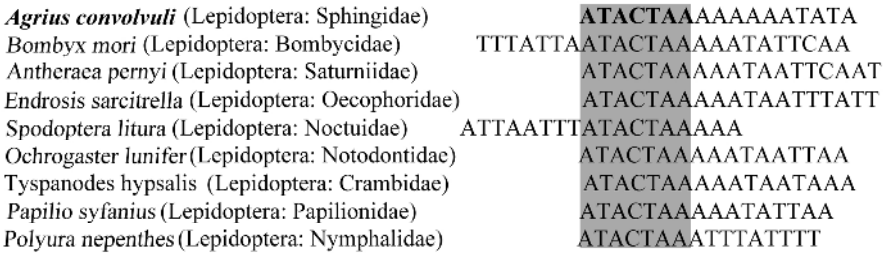


Predicted secondary cloverleaf structures for the other tRNA genes of A. convolvuli.

163x227mm (300 x 300 DPI)



Alignment of the intergenic spacer region between trnS2 (UCN) and ND1 of lepidopteran insects. The shaded 'ATACTAA' motif was conserved across the Lepidoptera order.

167x55mm (300 x 300 DPI)