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Mitogenome and phylogenetic analysis of typhlocybinae leafhoppers (Hemiptera: Cicadellidae)

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Abstract

Mitogenomes play an active role in determining the relationship between insect phylogeny and evolution and solve some problems encountered in traditional morphological classification. In order to increase the mitogenome data of leafhoppers the mitogenomes of *Cassianeura cassiae* and *C. bimaculata* were sequenced in this study and found to be 15,423 bp and 14,597 bp in length respectively. The gene structure was found to be similar to other published leafhopper mitogenomes, but we found the length of the control region of *C. bimaculata* to be the shortest in existing studies. The phylogenetic analysis of 13 PCGs resulted in a well-supported tree topology but species in Typhlocybini and Zyginellini were mixed. Meanwhile, this study also provided the phylogenetic analysis based on the body external morphology, female genitalia morphology and male genitalia morphology of 9 species of Typhlocybinae. The results showed that the female sternite VII of different species is quite different, but the female valvulae of different species in the same genus shows a certain consistency. The morphological phylogenetic tree is basically the same structure as the molecular phylogenetic tree. In the two different kinds of phylogenetic trees, Typhlocybini and Zyginellini clustered into one clade, showing a more closed relationship.

Typhlocybinae (Hemiptera, Auchenorrhyncha, Cicadellidae) is the second-largest leafhopper subfamily after Deltocephalinae, and comprises six tribes, 446 genera, and >4,400 species, widely distributed in the six major zoogeographic regions of the world¹. Unlike most other Cicadellidae members of the group feed directly from plant cells and have a strong host specificity^{2,3}; many are also important agricultural pests.

Insect mitogenome is a covalently closed circular double-stranded DNA molecule, usually ~16 kb in size, containing 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, and two ribosomal RNA (rRNA) genes and a non-coding region (A+T-rich region)⁴. Owing to its simple structure, small size, multiple copies, maternal inheritance, lack of recombination, and fast evolution speed^{5,6}, it is considered to provide phylogenetic relationships at various taxonomic levels from which to determine morphological homologies⁷.

In recent years, with the maturity of sequencing technology and the application of universal primers for mitochondrial genes^{8,9}, insect mitochondrial genomes have begun to be widely measured. So far, the National Center for Biotechnology Information (NCBI) has only included the complete mitogenome data of 21 species of Typhlocybinae, including eight species of Erythroneurini, one species of Typhlocybini, seven species of Zyginellini, and five species of

Empoascini. This data is therefore far from sufficient to conduct systematic research on the subfamily Typhlocybinae. In order to enrich the complete mitogenomes data of Typhlocybinae, two species of the genus *Cassianeura* were sequenced and annotated in this study (Table 1). Meanwhile, the structure and composition of mitochondrial genomes of two of the three species of *Cassianeura* were analyzed and compared. Specimens of the two species were recently collected by us from the *Cassia* tree in Pattaya, Chonburi Province, Thailand¹⁰.

Materials and methods

Sample collection and DNA extraction. The leafhopper specimen information collected in this study is shown in Table 1, and the data downloaded from GenBank is shown in Table 2. The specimens were immersed in absolute ethanol and stored in a refrigerator at -20°C. After morphological identification, the prepared voucher specimens with male genitalia and female genitalia were deposited in the insect specimen room of Guizhou Normal University.

Genome Sequencing, Assembly, and Annotation. Mitogenomes were obtained by PCR amplification and sequencing. The PCR reaction was performed with LA-Taq polymerase. The cycling conditions comprised a pre-denaturation step for 2 min at 94°C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 1 min/kb, and then the final extension for 10 min at 72°C. PCR products are directly sequenced. After quality-proofing of the obtained DNA fragments, DNASTar¹¹ was used to manually assemble the complete mt genome sequence, and homology search was performed through the Blast function in NCBI to verify whether the amplified sequence is the target Sequence^{12,13}.

The positions and secondary structures of 22 tRNA genes were determined using tRNAscan SE version 1.21¹⁴ and ARWEN version 1.2¹⁵. The base composition of each genes, relative synonymous codon usage (RSCU), and A+T content values were analyzed with MEGA 6.06¹⁶. The tandem repeat sequence of the control area was determined by the online search tool Tandem Repeats Finder¹⁷. Strand asymmetry was calculated through the formula: AT-skew = $[A - T] / [A + T]$ and GC-skew = $[G - C] / [G + C]$ ¹⁸. Using DnaSP 5.0 software¹⁹ to estimate the ratio of the non-synonymous (Ka) to the synonymous substitution rate (Ks) of 13 PCGs and calculate the nucleotide diversity (Pi) of 13 PCGs which from 23 species of Typhlocybinae. The sliding window analysis was set to sliding window: 200 bp, step size: 20 bp.

Phylogenetic analysis. Phylogenetic analysis was performed based on 13 protein-coding genes of 21 species in Typhlocybinae, and one species from Ricaniidae were selected as outgroups (Table 1 and 2). Using the Gblocks Server online platform to eliminate the poorly aligned positions and divergent areas of the DNA protein alignment and check and correct all the alignments in MEGA 6.06³⁶. The phylogenetic tree is constructed using the Bayesian Inference (BI) method and the Maximum Likelihood (ML) method. The BI analysis was performed using MrBayes 3.2.7³⁷. BI selected GTR+I+G as the optimal model, running 10 million generations twice, sampling once every 1000 generations, with a burn-in of 25%, and the remaining trees used to generate a consensus tree and calculate the probability of each branch of the posterior probability (PP). The ML analysis was conducted using IQ-TREE³⁸ under an ML + rapid bootstrap (BS) algorithm with 10,000 replicates, and calculate bootstrap scores for each node (BP).

Photographing and illustration of female genitalia, and establishment of phylogenetic tree in morphology.

An Olympus SZX16 dissecting microscope was used for specimen study and an Olympus BX53 stereoscopic microscopes for drawing of the dissected female genitalia. Morphological terminology used in this study follow Dietrich & Rakitov³⁹. All specimens examined were deposited in the collection of the School of Karst Science, Guizhou Normal University, China (GZNU). Photographs were taken by using a KEYENCE VHX-5000 digital microscope. The classification and selection of morphological traits refer to Dietrich & Dmitriev⁴⁰, using the traditional search method in TNT to establish a phylogenetic tree.

Results and discussion

Mitochondrial genomic structure and composition. In GenBank, known mitogenomes of Typhlocybinae range from 14,803 bp to 16,945 bp^{23,24} (Table 2). The complete mitogenomes of *C. cassiae* and *C. bimaculata* are 15,423 bp and 14,597 bp in size, respectively (Fig. 1, Table 1). In this study, it is found that the length of the complete mitogenome of *C. bimaculata* was the smallest, which is mainly due to the difference in the length of the A+T region. Like most insects, the mitogenomes of *C. cassiae* and *C. bimaculata* contain 13PCGs, 22 tRNA genes, two rRNA genes, non-coding regions, and with no gene rearrangement phenomenon. Twenty-three genes encode in the minority strand (H-strand) while the others encode in the majority strand (J-strand) (Table S1).

The mitogenome of *C. cassiae* has 12 intergenic spacers, 47 bp in total, ranging in length from 1 bp to 10 bp. The longest intergenic spacer is between *trnA* and *trnR*; 14 gene overlapping regions, ranging in length from 1 bp to 8 bp, the longest gene overlapping region is between *trnW* and *trnC* (Table S1). The mitogenome of *C. bimaculata* has 10 intergenic spacers of 41 bp in total, and the length also varies from 1 bp to 10 bp. The longest intergenic spacer is between *trnQ* and *trnM*; 14 gene overlapping regions, ranging in length from 1bp to 8 bp, the longest gene overlapping region is the same position as *C. cassiae* (Table S1). Such gene structure is common among leafhoppers^{23,41}.

The nucleotide composition of *C. cassiae* and *C. bimaculata* are as follows (Table 3): The content of A+T are 78.4~93.6% and 73.3~87.8%, respectively. The highest value of A+T content of both appears in the control region (CR), the lowest value of *C. bimaculata* appears in the 1st codon position while the *C. cassiae* appears in the N-strand. Each genes component of the two species mostly showed positive AT-skew and negative GC-skew. The nucleotide composition of J-strand of *C. cassiae* and *C. bimaculata* show relatively high A+T content, 86.2% and 79.3%, respectively. Most metazoan species exhibit clear strand asymmetry⁴². *C. cassiae* and *C. bimaculata* are no exception, showing positive AT-skew and negative GC-skew on the J-strand. Such mitogenome structure is common in Arthropods^{43,44}. However, the N-strand of *C. cassiae* and *C. bimaculata* show negative AT-skew and negative GC-skew.

Protein-coding genes and codon usage. Like most leafhoppers, only four genes (*nd5*, *nd4*, *nd4L*, and *nd1*) of the 13 PCGs of *C. cassiae* and *C. bimaculata* are encoded on the N-strand, while other genes are encoded on the J-strand (Table S1). Except for *atp8* and *nad5* that use TTG as the start codon, the remaining 13 PCGs start codons all follow the ATN rule. Among the 13 PCGs, standard stop codons (TAA and TAG) and an incomplete stop codon (T) are used. The *cox2* of both species are terminated by T, the *nad5* gene of *C. bimaculata* is also terminated by T, but

the *nad5* gene of *C. cassiae* is terminated by TAA. Most of the start and stop codons of the two species are the same; only the four genes *nad2*, *atp8*, *nad3*, and *nad5* are different. Such subtle differences in Cicadellidae insects are ubiquitous^{41,45}.

The relative synonymous codon usage (RSCU) for the 13 PCGs is summarized in Table S2 and Fig. 2. Both *C. cassiae* and *C. bimaculata* mitotic genes encode 3,655 amino acids. In the two mitochondrial genomes, it is observed that the frequency of the codon UUA (Leu2) is much higher than other codons, and the RSCU is 3.93 and 3.12, respectively. The codon GCG (Ala) has not been found in *C. cassiae*, and this codon deletion has been observed in existing studies. For example, the *Ricania speculum* of the Ricaniidae family lacks Thr (ACG), and *Aphaena discolors nigrotibiata* of the Fulgoridae family lacks CCG (Pro), GCG (Ala), ACG (Thr) three codons^{46,47}. In addition, both species showed greater codon preference.

Transfer and ribosomal RNA genes. The tRNA lengths of *C. cassiae* and *C. bimaculata* are 1,453 bp and 1,433 bp, respectively, of which 14 tRNAs are on the N-strand, and the rest are on the J-strand. 22 tRNAs are between 62~72 bp in size. The *trnK* are longest of two species, and the shortest tRNA of *C. cassiae* is *trnC* while *C. bimaculata* has many, including *trnC*, *trnY*, *trnD*, *trnG*, *trnA*, and *trnR* (Tables S1 and 4). The secondary structure of 22 tRNAs in the mitotic genomes of two species are shown in Fig. S1.

The secondary structure of tRNA is shown in Fig. S1. *C. cassiae* and *C. bimaculata* are the same as other leafhoppers, except that the dihydrouridine (DHU) arm of *tRNA-S1* forms a simple loop, the remaining 21 tRNAs exhibit a typical clover-leaf secondary structure^{41,48,49}. 17 and 16 weak G-U base pairs are found in the tRNAs of *C. cassiae* and *C. bimaculata*, respectively. In addition, there are differences in the number of base pairs on the Anticodon arm of Arg (R), Asp (D), and Met (M) of the two species. This difference is not common in two species of the same genus^{41,45}.

The two highly conserved rRNA genes of *C. cassiae* and *C. bimaculata* are encoded on the short-chain (J-strand). *16S* are located between *trnL2* and *trnV*, with lengths of 1184 bp and 1168pb, respectively, and *12S* are located after *trnV*, with lengths of 733 bp and 731 bp, respectively (Table S1). Both *16S* and *12S* have positive AT-skew and negative GC-skew (Table 3).

Control region. The control region, also known as the A+T region, is the initiation region of mitochondrial DNA replication and the largest non-coding region in the metazoan mitotic genome; due to the change in the length of tandem repeat units (TRs) and the difference in copy number, the control region has a larger change in size than other regions of the mitotic genome^{44,50,51}. The lengths of the control regions in the mitotic genomes of *C. cassiae* and *C. bimaculata* are 1078 bp and 302 bp, respectively, and the A+T% are 93.6% and 87.8%, respectively (Table 3). *C. cassiae* in the control region has a TRs, a length of 746bp, 289bp repeating units (Fig. 3). The length of the control region of *C. bimaculata* is the smallest that has been found in the Typhlocybinae, without TRs. So far, no obvious regularity has been found for the structural changes in the control region of different species.

Phylogenetic analysis. The phylogenetic analysis of Typhlocybinae was estimated using 13 PCG sequences of 19 species (17 Typhlocybinae and two outgroups). The BI and ML analysis

produced a consistent tree topology comprising ((Zyginellini+Typhlocybini)+Erythroneurini)+Empoascini (Fig. 4), with the two *Cassianeura* species forming a sister group with *Mitjaevia protuberanta*. However, some species of Zyginellini were included in Typhlocybini and some of its members with low branch support (PP=1, BS=44), findings which add to the controversy of the tribes monophyly⁵². Zyginellini was erected by Dworakowska, 1977 based on the hind wing vein CuA connected to vein MP compared to their separation in Typhlocybini⁵³. This distinction has been accepted for many years, e.g., Zhang⁵⁴ but more systematic analyses are clearly needed before conclusions can be made on the validity of the tribe.

Female genitalia. Studies have shown that female sternite VII of different kinds of leafhoppers has large differences in morphology^{39,55-57}. Female sternite VII of nine species in subfamily Typhlocybinae had been studied in this paper (Fig. 5). All sternite VII of the leafhoppers has a convex and narrower posterior edge than the base; except for *E. sipra*, the base connecting the abdomen relatively smooth, without protrusions, sternite VII of different species has different shades of color. *M. protuberanta* with a longer female sternite VII, which covers about half of the ovipositor, and the posterior edge extremely convex, much narrower than the base; the female sternite VII of *E. sipra* resembles the connective of male genitalia, with pair of darker lateral arms; the female sternite VII of *E. dwalata* with posterior edge slightly concave medially; the shape of the female sternite VII of *C. cassiae* is similar to that of *M. protuberanta*, but the degree of protrusion of the posterior edge is relatively small; the posterior edges of *C. bimaculata*, *E. minuscula* and *E. gracilivramus* are less protruding than other leafhoppers; the female sternite VII of *L. lingchuanensis* is extremely transparent; the female sternite VII of *Z. minuta* covers most of the ovipositor, with two longitudinal dark oval areas in the middle.

The female valvula I of the nine leafhoppers are shown in Fig. 6, and the paired valvula I is connected basally by membranes. One single valvula I widen at base and tapering towards apex, and with many imbricate sculptures dorsally and ventroapically. In addition to the valvula I of *Z. minuta* relatively wide, others elongated compared. In this study, the majority species of Typhlocybinae leafhoppers with valvula I long and slim^{55,56}, broadened valvula I is not common among leafhoppers. Most Deltocephalinae and Cicadellinae leafhoppers with wider female valvula I^{39,57,58}. Here, the end of female valvula I of *E. dwalata* is slightly blunt, and the rest species are sharper; the end of *Z. minuta* is produced at apex distinctly.

Usually, the leafhopper valvulae II are divided into two branches; the two branches are slightly different in shape³⁹. The female valvulae II of 9 species of leafhoppers in this paper are shown in Fig. 7. In lateral view, valvulae II narrow at base, distinctly expanded in middle part and then gradually narrowed towards apex, which blunt, not very sharp. Valvulae II of *M. protuberanta* and *L. lingchuanensis* have coarsened serrations on dorsal margin apically, the area between each larger tooth is composed of many small serrations, and the ventral preapical tooth distinctly. *Z. minuta* also with some stout serrations on dorsal margin and the part between each larger tooth also with numerous small serrations. However, compared with *M. protuberanta* and *L. lingchuanensis*, *Z. minuta* with more blunt serrations on dorsal side, and the ventral apical teeth absent. The valvulae II of *E. sipra* and *E. dwalata* with serrations of varying sizes on the dorsal side, the ventral apical tooth of *E. sipra* and *E. dwalata* present. *C. cassiae* and *C. bimaculata* with almost uniform serrations in size on dorsal margin apically; and ventral margin smooth, without

tooth at apex. The valvulae II of *E. minuscula* and *E. gracilivramus* with slanted large serrations at apex dorsally, but *E. gracilivramus* with sharper serrations on dorsal side. Therefore, the valvulae II of most leafhoppers with an obvious saw-like structure, which can cut the leaf epidermis during the spawning process and form a fallopian guide tube⁵⁹.

The female valvula III of 9 species of leafhoppers is shown in Fig. 8. Valvula III is located on both sides of valvula I and valvulae II; its base connected with valvula I, shaped like a small boat, with some irregularly arranged small setae on ventral surface. Thereinto, *M. protuberanta*, *E. sipra*, *E. dwalata*, *E. gracilivramus*, and *Z. minuta* with many slender and dense setae on surface of valvula III, while the other leafhoppers relatively few setae. The color of valvula III of different leafhopper species is quite different, mostly similar to the color of sternite VII^{39,57}.

In the research on the female genitalia of 9 existing species, we found that the female valvula I, II, and III of the same group have a certain consistency. Combining the characteristics of body morphology and male genitalia, a morphological phylogenetic tree was obtained (Table S3, S4, Fig. 9). The internal relationship of the morphological phylogenetic tree is: (Zyginellini+Typhlocybini)+Erythroneurini. Comparing with the molecular phylogenetic tree, the structures of the two are basically the same, all showing the two tribes of Typhlocybini and Zyginellini clustered into one blade, have a closed relationship. However, in Erythroneurini, the position of *M. protuberanta* has changed, which may be caused by the difference in genome composition brought about by geographical isolation. Does this mean that the combination of morphology and molecules can mutually verify and adjust the classification of species? Should the leafhopper tribe Zyginellini be merged into Typhlocybini? A deeper systematic study with more leafhopper sample data is needed to solve these scientific questions in the future.

Conclusions

The Oriental leafhopper genus *Cassianeura* (Cicadellidae: Typhlocybinae: Erythroneurini) contains three species which feed and reproduce on Cassia trees. In this study, we sequenced and annotated the complete mitogenomes of two *Cassianeura* species (*C. cassiae* and *C. bimaculata*) and compared them with other Typhlocybinae leafhoppers. Comparative mitogenomes analysis showed that the gene content and gene arrangement of the two mitotic genomes are both conservative. Phylogenetic analysis of the nucleotide sequences of 13 PCGs resulted in a well-supported topology, most branches received the greatest support, and most of the relationships were consistent with the results of other recent phylogenetic studies. For example, Zhou (2020) studied the phylogenetic relationship between Zyginellini and Typhlocybini insects in his article and found that there is an inseparable close relationship between them.

In addition, we have also studied the morphological characteristics of female genitalia of 9 Typhlocybinae leafhoppers. The research results have shown that species in the same genus have a certain consistency in female valvulae, but an obvious morphological differences among different genera. However, the morphology of female sternite VII of different species is quite different. Combining the morphologies of the body external surface, the female and male genitalia, a phylogenetic tree is very similar to the molecular phylogenetic tree. Both support that Typhlocybini and Zyginellini have a close relationship. The results of this study will help to improve the understanding of the mitogenomes of Typhlocybinae in the future, provide more information on the research of the female reproductive system of Typhlocybinae, and partly verified the classification of Typhlocybinae by combining morphological and molecular methods.

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Author Contributions

J.J., X.X.C. and Y.H.S. conceived and designed the experiments. C.L. collected insect specimens. J.J. and X.X.C. performed the experiments. J.J. analyzed the data and wrote the paper. All authors discussed results and implications. All authors have read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Table 1. Information on samples

subFamily	Species	Length(bp)	GenBank No.	Sampling time	Collecting sites	Collector
Typhlocybinae	<i>Cassianeura cassiae</i>	15,423	MT985380	26 I 2019	Thailand	Can Li
	<i>Cassianeura bimaculata</i>	14,597	MT985381	26 I 2019	Thailand	Can Li

Table 2. Sequence information of 17 species of Typhlocybinae and one outgroup downloaded from GenBank

tribe	Species	Length(bp)	GenBank No.	Reference
Erythroneurini	<i>Mitjaevia protuberanta</i>	15472	NC_047465.1	20
	<i>Empoascanara dwalata</i>	15271	MT350235.1	21
	<i>Empoascanara sipra</i>	14827	NC_048516.1	22
	<i>Illinigina sp.</i>	14803	KY039129.1	23
Typhlocybini	<i>Eupteryx minuscula</i>	16945	MN910279.1	24
	<i>Bolanusoides shaanxiensis</i>	15274	MN661136.1	25
	<i>Typhlocyba sp.</i>	15233	KY039138.1	23
Zyginellini	<i>Limassolla lingchuanensis</i>	15716	NC_046037.1	26
	<i>Zyginella minuta</i>	15544	MT488436.1	27
	<i>Paraahimia luodianensis</i>	16497	NC_047464.1	28
	<i>Parathailocyba orla</i>	15382	MN894531.1	29
Empoascini	<i>Empoasca onukii</i>	15167	NC_037210.1	30
	<i>Empoasca vitis</i>	15154	NC_024838.1	31
	<i>Empoasca flavescens</i>	15152	MK211224.1	32
	<i>Ghauriana sinensis</i>	15491	MN699874.1	33
Outgroup (Cercopidae)	<i>Phymatostetha huangshanensis</i>	17785	NC_039157.1	34
	<i>Callitettix braconoides</i>	15637	NC_025497.1	35

Figure 1. The organization of the mitogenomes of *C. cassiae* and *C. bimaculata*

Table 3. Nucleotide composition of the *C. cassiae* (C) and *C. bimaculata* (B) mitogenomes

Feature	A%		C%		G%		T%		A+T%		AT-skew		GC-skew		Length(bp)	
	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B
Whole	42.8	39.9	10.4	11.8	8.5	10.6	38.3	38.7	81.1	78.6	0.055	0.015	-0.101	-0.054	15423	14597
PCGs	42.2	38.5	10.9	12.3	9.4	11.4	37.5	37.8	79.7	76.3	0.058	0.009	-0.074	-0.038	10966	10967
1st codon position	44.5	40.5	9.6	13.3	8.7	13.5	37.1	32.8	81.6	73.3	0.091	0.105	-0.049	0.007	3656	3656
2nd codon position	41.4	35.6	10.5	14.3	9.8	11.3	38.2	38.8	79.6	74.4	0.040	-0.043	-0.034	-0.117	3655	3656
3rd codon position	40.6	39.5	12.6	9.2	9.8	9.3	37.9	42.9	78.5	82.4	0.034	-0.041	-0.125	0.005	3655	3655
tRNA	41.0	40.0	11.4	11.0	9.6	10.5	38.1	38.5	79.1	78.5	0.037	0.019	-0.086	-0.023	1453	1433
16S	47.9	47.7	10.0	10.4	6.2	6.1	36.0	35.8	83.9	83.5	0.142	0.143	-0.235	-0.261	1184	1168
12S	48.7	46.2	10.2	11.8	6.0	6.8	35.1	35.2	83.8	81.4	0.162	0.153	-0.259	-0.269	733	731
CR	41.7	42.4	4.1	5.0	2.3	7.3	51.9	45.4	93.6	87.8	-0.109	-0.034	-0.281	0.187	1078	302
N	36.6	33.3	11.0	12.3	10.7	12.0	41.8	42.4	78.4	75.7	-0.066	-0.120	-0.02	-0.01	7680	7664
J	50.1	47.3	10.7	11.6	7.1	9.0	36.1	32.0	86.2	79.3	0.162	0.193	-0.202	-0.126	6656	6635

Figure 2. Relative Synonymous Codon Usage (RSCU) of mitogenomes for *C. cassiae* (top) and *C. bimaculata* (bottom).

Figure 3. Structural organization of the Control region of *C. cassiae* and *C. bimaculata*. R: repeat unit.

Figure 4. Phylogenetic tree from Typhlocybae based on nucleotide sequence of 13 PCGs. Numbers above the nodes refer to the bootstrap proportion (left) of maximum likelihood (ML) analyses and posterior probability (right) of Bayesian (BI) analyses.

Figure 5. Female sternite VII: A. *Mitjaevia protuberanta*. B. *Empoascanara sipra*. C. *Empoascanara dwalata*. D. *Cassianeura cassiae*. E. *Cassianeura bimaculata*. F. *Eupteryx minuscula*. G. *Eupteryx gracilivramus*. H. *Limassolla lingchuanensis*. I. *Zyginella minuta*.

Figure 6. Female valvula I: A-I: A. *Mitjaevia protuberanta*. B. *Empoascanara sipra*. C. *Empoascanara dwalata*. D. *Cassianeura cassiae*. E. *Cassianeura bimaculata*. F. *Eupteryx minuscula*. G. *Eupteryx gracilivramus*. H. *Limassolla lingchuanensis*. I. *Zyginella minuta*. A₁-I₁: Enlarged end. A₂-I₂: Local enlargement.

Figure 7. Female valvula II: A-I: A. *Mitjaevia protuberanta*. B. *Empoascanara sipra*. C. *Empoascanara dwalata*. D. *Cassianeura cassiae*. E. *Cassianeura bimaculata*. F. *Eupteryx minuscula*. G. *Eupteryx gracilivramus*. H. *Limassolla lingchuanensis*. I. *Zyginella minuta*. A₁-I₁: Enlarged end.

Figure 8. Female valvula III: A-I: A. *Mitjaevia protuberanta*. B. *Empoascanara sipra*. C. *Empoascanara dwalata*. D. *Cassianeura cassiae*. E. *Cassianeura bimaculata*. F. *Eupteryx minuscula*. G. *Eupteryx gracilivramus*. H. *Limassolla lingchuanensis*. I. *Zyginella minuta*.

Figure 9. Phylogenetic tree established by traditional search methods based on 63 morphological traits. (Outgroup: *Dikraneura* sp.)

Figures

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Figure 7

Female valvula II: A-I: A. *Mitjaevia protuberanta*. B. *Empoascanara sipra*. C. *Empoascanara dwalata*. D. *Cassianeura cassiae*. E. *Cassianeura bimaculata*. F. *Eupteryx minuscula*. G. *Eupteryx gracilivramus*. H. *Limassolla lingchuanensis*. I. *Zyginella minuta*. A1-I1: Enlarged end.

Figure 8

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Phylogenetic tree established by traditional search methods based on 63 morphological traits. (Outgroup: *Dikraneura sp.*)

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