

Article

Characterization of the Complete Mitochondrial Genomes of Two Species with Preliminary Investigation on Phylogenetic Status of Zyginellini (Hemiptera: Cicadellidae: Typhlocybae)

Xian Zhou ¹, Christopher H. Dietrich ² and Min Huang ^{1,*}

¹ Key Laboratory of Plant Protection Resources and Pest Management of Ministry of Education, Entomological Museum, Northwest A&F University, Yangling 712100, Shaanxi, China; zhouxian191@163.com

² Illinois Natural History Survey, Prairie Research Institute, University of Illinois, 1816 S. Oak St., Champaign, IL 61820, USA; chdietri@illinois.edu

* Correspondence: huangmin@nwsuaf.edu.cn

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Simple Summary: Typhlocybae currently is the second largest membracoid subfamily distributed worldwide and includes many important agricultural pests. The monophyly of Typhlocybae has been supported by several recent analyses, but the relationships of its included tribes remain largely unexplored, particularly that of the phylogenetic relationship of Zyginellini and Typhlocybini. This study presented the annotated complete mitochondrial genome sequences of two zyginelline species, *Limassolla* sp. and *Parazyginella tiani* Gao, Huang and Zhang, 2012, and a comparative analysis of mitochondrial genomes within the Typhlocybini and Zyginellini. Typhlocybae mitogenomes are highly conservative in overall organization, as have been found in some other Cicadellidae. The only unusual feature was found in the secondary structure of tRNAs, with the acceptor stem of *trnR* comprising only 5 or 6 bp in some species. This unusual feature was reported for the first time in Typhlocybae. Phylogenetic analyses showed that the monophyly of tribe Zyginellini was not supported and should be treated as a synonym of Typhlocybini. Nevertheless, a broader analysis with a much larger sample of taxa is needed to confirm the present results.

Abstract: To explore the characteristics of mitogenomes and reveal phylogenetic relationships of the tribes of Zyginellini and Typhlocybini in Typhlocybae, mitogenomes of two species of the Zyginellini, *Parazyginella tiani* and *Limassolla* sp., were sequenced. Mitogenomes of both species contain 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), two ribosomal RNA genes (rRNAs) and a large non-coding region (A + T-rich region). These characteristics are similar to other Membracoidea mitogenomes. All PCGs initiate with the standard start codon of ATN and terminate with the complete stop codon of TAA/G or with an incomplete T codon. All tRNAs have the typical clover-leaf structure, except *trnS1* which has a reduced DHU arm and the acceptor stem of *trnR* is 5 or 6 bp in some species, an unusual feature here reported for the first time in Typhlocybae. The A + T-rich region is highly variable in length and in numbers of tandem repeats present. Our analyses indicate that *nad6* and *atp6* exhibit higher evolutionary rates compared to other PCGs. Phylogenetic analyses by both maximum likelihood and Bayesian methods based on 13 protein-coding genes of 12 species of Typhlocybae suggest that Zyginellini are paraphyletic with respect to Typhlocybini.

Keywords: mitochondrial genome; Typhlocybae; Typhlocybini and Zyginellini; phylogenetic analysis

1. Introduction

Membracoidea is the largest hemipteroid superfamily, comprising nearly one-third of known hemipteran species, with approximately 24,000 valid species [1,2]. The leafhopper family Cicadellidae is among the 10 largest families of insects, and Typhlocybinae currently ranks as the second largest membracoid subfamily, with approximately 6000 described species [3–5]. The subfamily is distributed worldwide and includes many important agricultural pests such as the potato leafhopper (*Empoasca fabae* (Harris, 1841)), white apple leafhopper (*Zonocyba pomaria* (McAtee, 1926)), cotton leafhopper (*Amrasca biguttula* (Ishida, 1913)) and kaki leafhoppers (*Limassolla hebeiensis* Cai, Liang and Wang, 1992) [6–8].

The tribes of Typhlocybinae are most readily distinguished based on the wing venation. Although Oman et al. (1990) included ten valid tribes in Typhlocybinae [9], most recent eastern hemisphere workers recognize six tribes proposed by Dworakowska (1979): Alebrini, Dikraneurini, Empoascini, Erythroneurini, Typhlocybini and Zyginellini [10]. The monophyly of Typhlocybinae has been supported by several recent analyses [4,11–13], but the status and relationships of its included tribes remain largely unexplored, particularly that of the phylogenetic status of the tribe Zyginellini. Zyginellini and Typhlocybini were traditionally distinguished based on differences in hind wing venation [14] but more recently the former was considered to be a junior synonym of the latter [5]. The phylogenetic status and relationships of these two tribes have not yet been adequately tested by previous analyses of DNA sequence data and morphological characteristics [4,11,12]. Thus, additional data, such as complete mitogenomes, should be used to further investigate their phylogenetic relationships.

The insect mitogenome is typically a closed circular double-stranded DNA molecule, usually 15–18 kb in length and encoding 37 genes, including 13 protein-coding genes (PCG), 22 transfer RNA genes (tRNA), 2 ribosomal RNA genes (rRNA) and a control region (A + T-rich region) [15–17]. Because of maternal inheritance, absence of introns, high evolutionary rate, and rare recombination, the mitochondrial genome is considered ideal for phylogenetic and evolutionary analysis [18–23].

Currently, there are 14 complete or nearly complete mitogenomes of Typhlocybinae in GenBank [24–35]. To facilitate comparative studies and phylogenetic analyses, we sequenced the complete mitogenomes and provided functional annotations of two species in Zyginellini. Genomic structure, base composition, substitutional and evolutionary rates of six species of Typhlocybini and Zyginellini were comparatively analyzed. Combining mitogenome sequences of Typhlocybini and Zyginellini with previously available mitogenomes of other tribes of Typhlocybinae, i.e., Empoascini and Erythroneurini, we reconstructed phylogenetic relationships among major lineages of this subfamily to test the monophyly of Zyginellini and Typhlocybini.

2. Materials and Methods

2.1. Sample Preparation and DNA Extraction

Specimens of *Limassolla* sp. were collected at Hejiaping Village, Chongqing City, China and *Parazyginella tiani* Gao, Huang and Zhang, 2012 specimens were collected at Gutian Mountain, Quzhou City, Zhejiang Province, China. All specimens were preserved in 100% ethyl alcohol and stored at -20°C in the Entomological Museum of the Northwest A&F University, Yangling, Shaanxi Province, China. Identification of adult specimens was based on the venation of the hind wing and male genitalia characteristics. Total DNA was extracted from muscle tissues of the thorax using the EasyPureR Genomic DNA Kit following the manufacturer's instructions (TransGen, Beijing, China).

2.2. Sequence Analysis and Gene Annotation

Whole mitochondrial genome sequences of the 2 species, *Limassolla* sp. and *Parazyginella tiani*, were generated using an Illumina HiSeq platform with paired reads of 2×150 bp by the Biomarker Technologies Corporation (Beijing, China). First, the raw paired reads were quality-trimmed and assembled using Geneious 10.0.5 (Biomatters, Auckland, New Zealand) with default parameters [36]

using the previously published mitochondrial genomes of *L. lingchuanensis* Chou and Zhang, 1985 and *P. luodianensis* Yuan and Song, 2019 as bait sequences for *Limassolla* sp. and *P. tiani*, respectively [25,26]. The genomes were annotated using Geneious 8.1.3 and *L. lingchuanensis* and *P. luodianensis* as references, respectively [25,26]. A total of 13 PCGs were identified as open reading frames based on the invertebrate mitochondrial genetic code; rRNA genes and control regions were identified by alignment with homologous genes from other Typhlocybinae species; tRNA genes were identified using the MITOS Web Server (<http://mitos.bioinf.uni-leipzig.de/index.py>) [37] and secondary structures were plotted with Adobe Illustrator CS5. Finally, a circular mitogenome map was drawn using CGView (http://stothard.afns.ualberta.ca/cgview_server/) [38]. Nucleotide composition, codon usage, composition skewness and relative synonymous codon usage (RSCU) were analyzed using PhyloSuite v 1.1.15 [39]. Tandem repeats in the A + T-rich region were predicted using the Tandem Repeats Finder program (<http://tandem.bu.edu/trf/trf.html>) [40]. The nucleotide diversity (Pi) and sliding window analysis (a sliding window of 200 bp and a step size of 20 bp) based on 13 aligned protein-coding genes (PCGs) were performed using DnaSP v 5.0. [41]. Kimura-2-parameter genetic distances were calculated using MEGA 7.0 [42]. Non-synonymous (dN)/synonymous (dS) mutation rate ratios among the 13 PCGs were calculated with DnaSP v 5.0. [41].

2.3. Phylogenetic Analysis

Mitochondrial genomes of 12 species of Typhlocybinae were selected, including 2 species from Empoascini, 4 species from Erythroneurini, 2 species from Typhlocybini and 4 species from Zyginiellini. Two species belonging to different cicadellid subfamilies were chosen as outgroups: *Scaphoideus varius* Vilbaste, 1968 (Deltocephalinae: Scaphoideini) and *Taharana fasciana* Li, 1991 (Coelidiinae: Coelidiini) (Table 1) [43,44]. All previously available mitochondrial genomes in this study were acquired from GenBank. Statistical phylogenetic analysis of mitogenomes was conducted using PhyloSuite v 1.1.15 [39]. The nucleotide sequences of all 13 PCGs and 2 rRNA genes were used in our analyses. PCGs were aligned using the MAFFT v 7.313 plugin in PhyloSuite v 1.1.15 [39]. Nucleotide sequences were aligned using the G-INS-i (accurate) strategy and codon alignment mode; the two rRNAs were aligned with the Q-INS-i strategy [45]. Gaps and ambiguous sites were removed using Gblocks v 0.91b [46], then the results were concatenated in PhyloSuite v 1.1.15 [39]. PartitionFinder2 was used to select the optimal partition schemes and substitution models [47]; the results are shown in Supplementary Tables S2 and S3. We used the “greedy” algorithm with branch lengths estimated as “linked” and the Bayesian information criterion (BIC).

Alignments of individual genes were concatenated to generate 4 data sets: (1) the PCG12 matrix, including only the first and second codon positions of protein-coding genes; (2) the PCG12R matrix, including the first and second codon positions of protein-coding genes and the 2 rRNA genes; (3) the PCG123 matrix, including all 3 codon positions of protein-coding genes; (4) the PCG123R matrix, including all 3 codon positions of protein-coding genes and the 2 rRNA genes.

Phylogenetic trees were constructed using the IQ-TREE web server [48] and MrBayes 3.2.6 [49] based on maximum likelihood (ML) and Bayesian inference (BI), respectively. The ML tree was constructed with the ML + rapid bootstrap (BS) algorithm with 1000 replicates. For the Bayesian analysis, the default settings were used with 5×10^6 MCMC generations after reaching stationarity (average standard deviation of split frequencies <0.01), with estimated sample size >200 , and potential scale reduction factor ≈ 1 [50].

Table 1. Summary of mitogenomic sequence information used in the present study.

Subfamily	Tribe	Species	Accession Number	Reference	
Coelidiinae	Coelidiini	<i>Taharana fasciana</i>	NC_036015	[44]	
Deltocephalinae	Scaphoideini	<i>Scaphoideus varius</i>	KY817245	[43]	
		<i>Ghauriana sinensis</i>	MN699874	[28]	
Typhlocybinae	Empoascini	<i>Empoasca onukii</i>	NC_037210	[32]	
		<i>Mitjaevia protuberanta</i>	NC_047465	[30]	
		<i>Illinigina</i> sp.	KY039129	[27]	
	Erythroneurini	<i>Empoascanara dwalata</i>	MT350235	[33]	
		<i>Empoascanara sipra</i>	MN604278	[26]	
	Typhlocybini	<i>Typhlocyba</i> sp.		KY039138	[27]
			<i>Bolanusoides shaanxiensis</i>	MN661136	Unpublished
		<i>Limassolla lingchuanensis</i>	MN605256	[24]	
		Zyginellini	<i>Paraahimia luodianensis</i>	NC_047464	[25]
			<i>Limassolla</i> sp.	MT683892	This study
	<i>Parazyginella tiani</i>	MT683891	This study		

3. Results and Discussion

3.1. Genome Organization and Base Composition

The complete mitogenome sequences of *P. tiani* and *Limassolla* sp. were 17,562 bp and 17,053 bp in length, respectively (Figures 1 and 2). Known mitogenomes of Typhlocybinae range from 14,803 bp (*Illinigina* sp.) [27] to 16,497 bp (*P. luodianensis*) [25]. Length variation of Typhlocybinae mitogenomes is mainly due to variation in the size of the A + T-rich region. Each mitogenome includes the 37 standard mitochondrial genes; gene arrangement is the same as that of the hypothetical ancestral insect's mitogenome in order and direction [51] (Figures 1 and 2). Among the 37 mitochondrial genes, 23 genes (9 PCGs and 14 tRNAs) are transcribed from the majority strand (J-strand) and the remaining genes (four PCGs, eight tRNAs, and two rRNAs) are located on the minority strand (N-strand) (Table S1). The base composition of *P. tiani* is A = 42.1%, T = 34.3%, C = 13.2%, G = 10.4% and that of *Limassolla* sp. is A = 41.7%, T = 33.3%, C = 14.0%, G = 11.0%. The A/T nucleotide composition is 76.4% in *P. tiani* and 75.0% in *Limassolla* sp., thus exhibiting a strong A/T bias. The two species in this study have a positive AT skew and a negative GC as in the other four previously sequenced species of Typhlocybini and Zyginellini (Tables 2 and 3).

Table 2. Nucleotide composition and skewness of mitogenomes of *P. tiani* and *Limassolla* sp.

Regions	Size (bp)	T(U)	C	A	G	AT (%)	GC (%)	AT Skew	GC Skew
Full genome	17,562/17,053	34.3/33.3	13.2/14.0	42.1/41.7	10.4/11.0	76.4/75.0	23.6/25.0	0.103/0.112	-0.117/-0.119
PCGs	10,938/10,926	42.9/41.5	12.4/12.9	33.3/33.3	11.4/12.3	76.2/74.8	23.8/25.2	-0.126/-0.110	-0.041/-0.025
1st codon position	3646/3642	36.9/35.1	13.4/11.5	36.3/36.6	15.7/16.8	73.2/71.7	26.8/28.3	-0.008/0.022	0.172/0.187
2nd codon position	3646/3642	48.4/48.0	14.4/17.6	20.4/21.1	13.7/13.3	68.8/69.1	31.3/30.9	-0.407/-0.389	-0.123/-0.137
3rd codon position	3646/3642	43.4/41.4	15.4/9.7	43.3/42.1	4.8/6.7	86.7/83.5	13.3/16.4	-0.002/0.008	-0.278/-0.182
tRNAs	1422/1432	36.6/40.2	16.4/8.4	40.6/39.7	12.2/11.8	77.2/79.9	22.7/20.2	0.052/0.006	0.077/0.170
rRNAs	1892/1976	47.1/46.1	17.4/8.5	35.6/32.4	10.8/13.0	82.7/78.5	17.4/21.5	-0.139/-0.173	0.244/0.209
A + T rich-region	3230/2695	38.6/35.0	18.4/14.7	34.0/36.3	13.4/14.0	72.6/71.3	27.4/28.7	-0.063/0.019	-0.020/-0.026

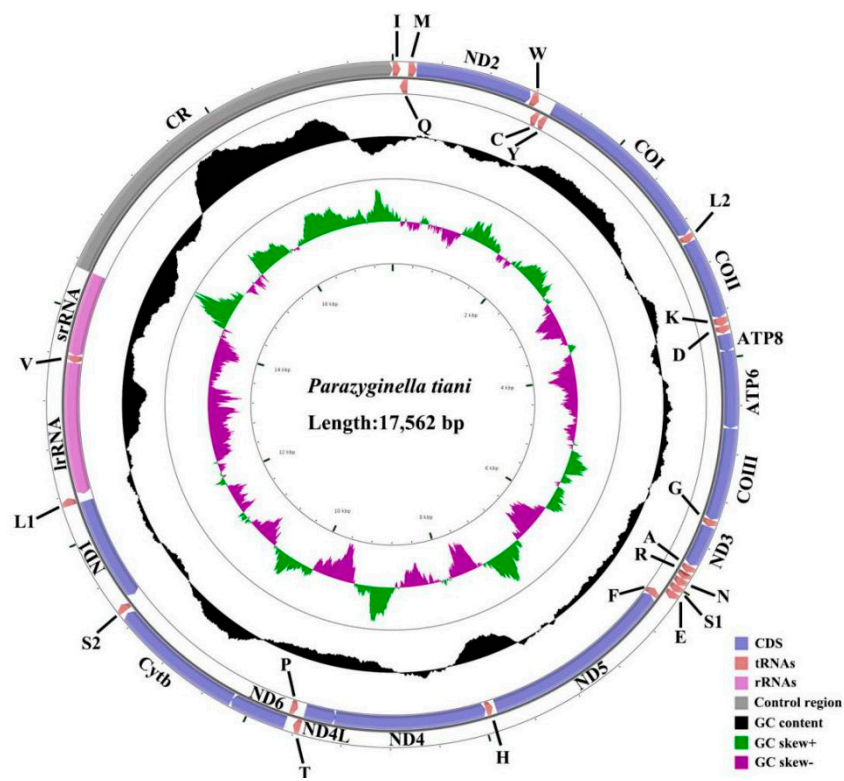


Figure 1. Organization of the complete mitogenome of *P. tiani*.

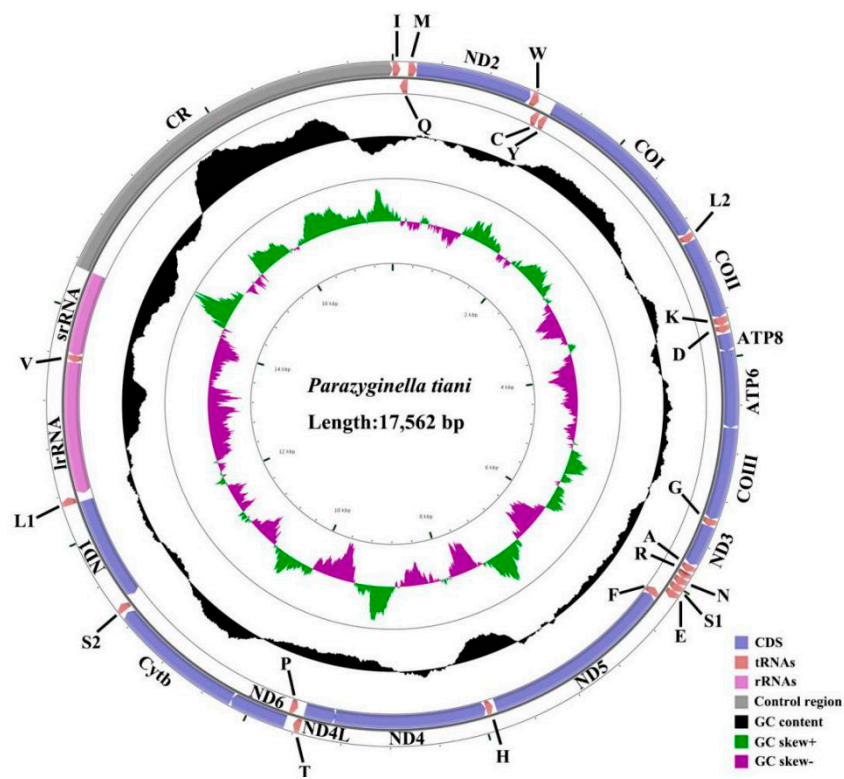


Figure 2. Organization of the complete mitogenome of *Limassolla* sp.

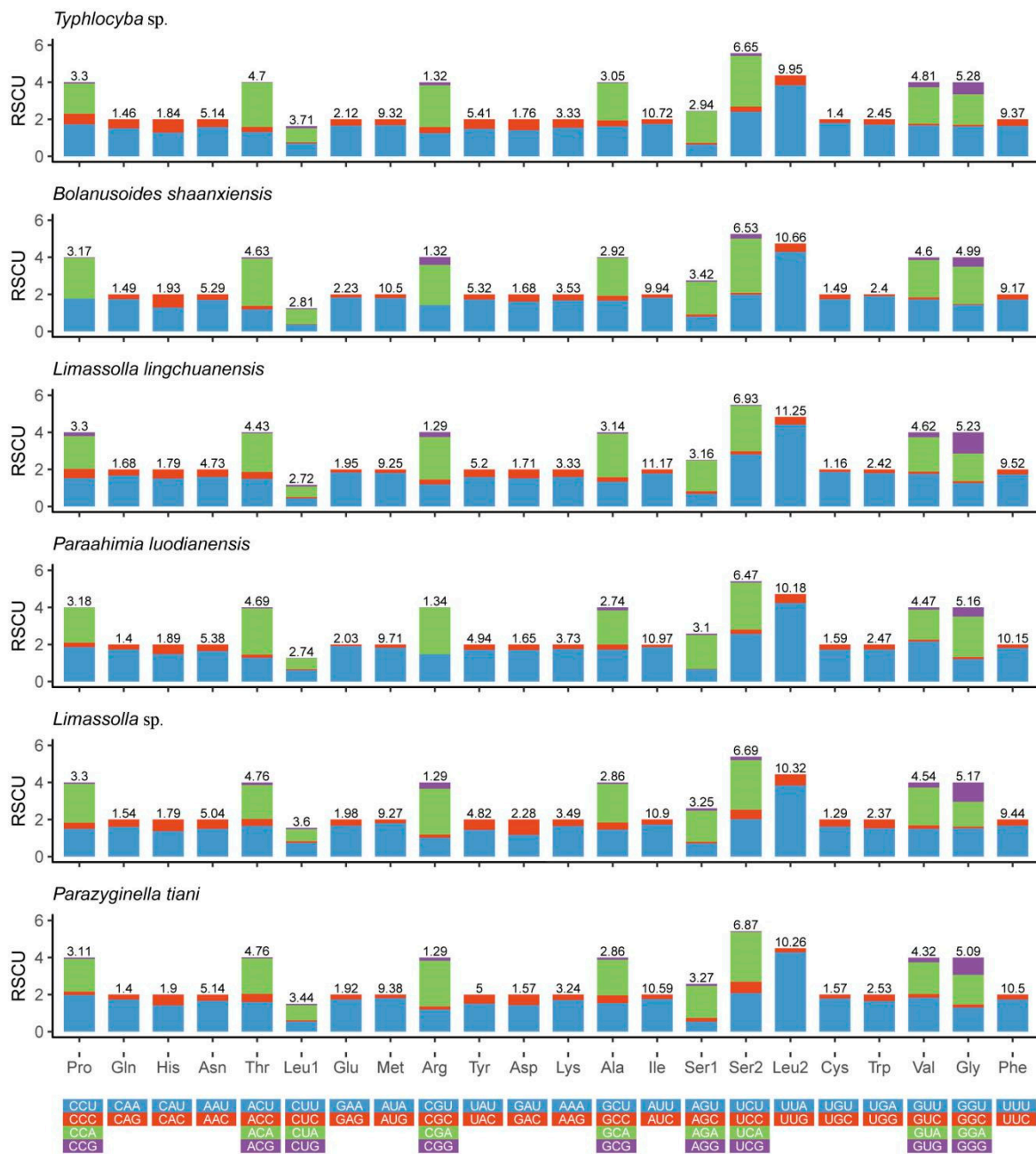


Figure 3. Relative synonymous codon usage (RSCU) in the mitogenomes of 6 species.

3.3. Transfer and Ribosomal RNA Genes

Each of the sequenced mitogenomes includes 22 tRNA genes; the total length of tRNA of *P. tiani* and *Limassolla sp.* is 1422 and 1432 bp, respectively; 14 tRNAs are encoded on the J-strand and the remainder are encoded on the N-strand; and the tRNAs of the two species have a positive AT and GC skew (Table 2). Secondary structures of all tRNAs are the typical cloverleaf secondary structure, except for that of *trnS1*, which lacks the dihydrouridine (DHU) arm forming a simple loop, as commonly found in other insect mitogenomes [16,53]. A cloverleaf secondary structure is conservative: 7 bp in the acceptor stem, 5 bp in the anticodon arm, with length of the DHU and TΨC arms variable. While the acceptor stem of *trnR* was 6 bp in *Limassolla sp.*, *L. lingchuanensis* and *Empoasca supra* Dworakowska, 1980 [24,26], this stem is 5 bp in *B. shaanxiensis*, *Ghauriana sinensis* Qin and Zhang, 2011, *Illinigina sp.* and *Mitjaevia protuberanta* Song, Li and Xiong, 2011 [27,28,30]. This result has not been previously reported in Typhlocybae but such characteristics may be due to mitochondrial genome variation among

different individuals [54]. Base pair mismatch is commonly found in the tRNA secondary structure of Cicadellidae [55]. In the arm structures of tRNAs of the two new mitogenomes, we recognized a total of six types of unmatched base pairs (G–U, U–U, A–A, G–A, U–C, A–C). The total number of unmatched base pairs found was 35 and 40 in *P. tiani* and *Limassolla* sp., respectively (Figures 4 and 5). The large and small ribosomal RNA genes are located between *trnL1* and *trnV*, and *trnV* and the A + T-rich region (Figures 1 and 2). The lengths of rRNAs of *P. tiani* and *Limassolla* sp. are 1892 and 1976 bp and the AT content is 82.7% and 78.5%, respectively, with negative AT-skew and positive GC-skew (Table 2).

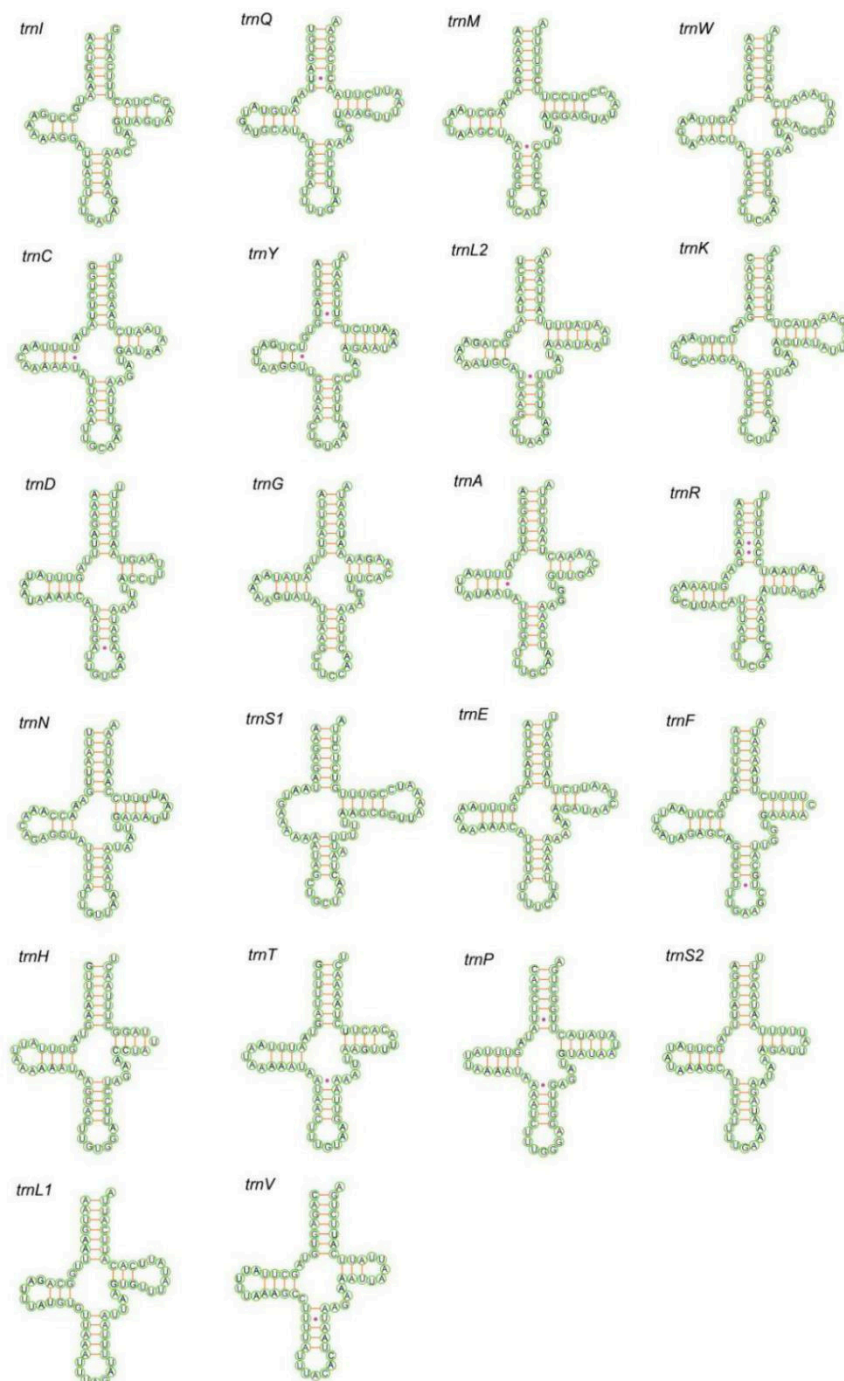


Figure 4. Predicted secondary cloverleaf structure for the tRNAs of *P. tiani*.

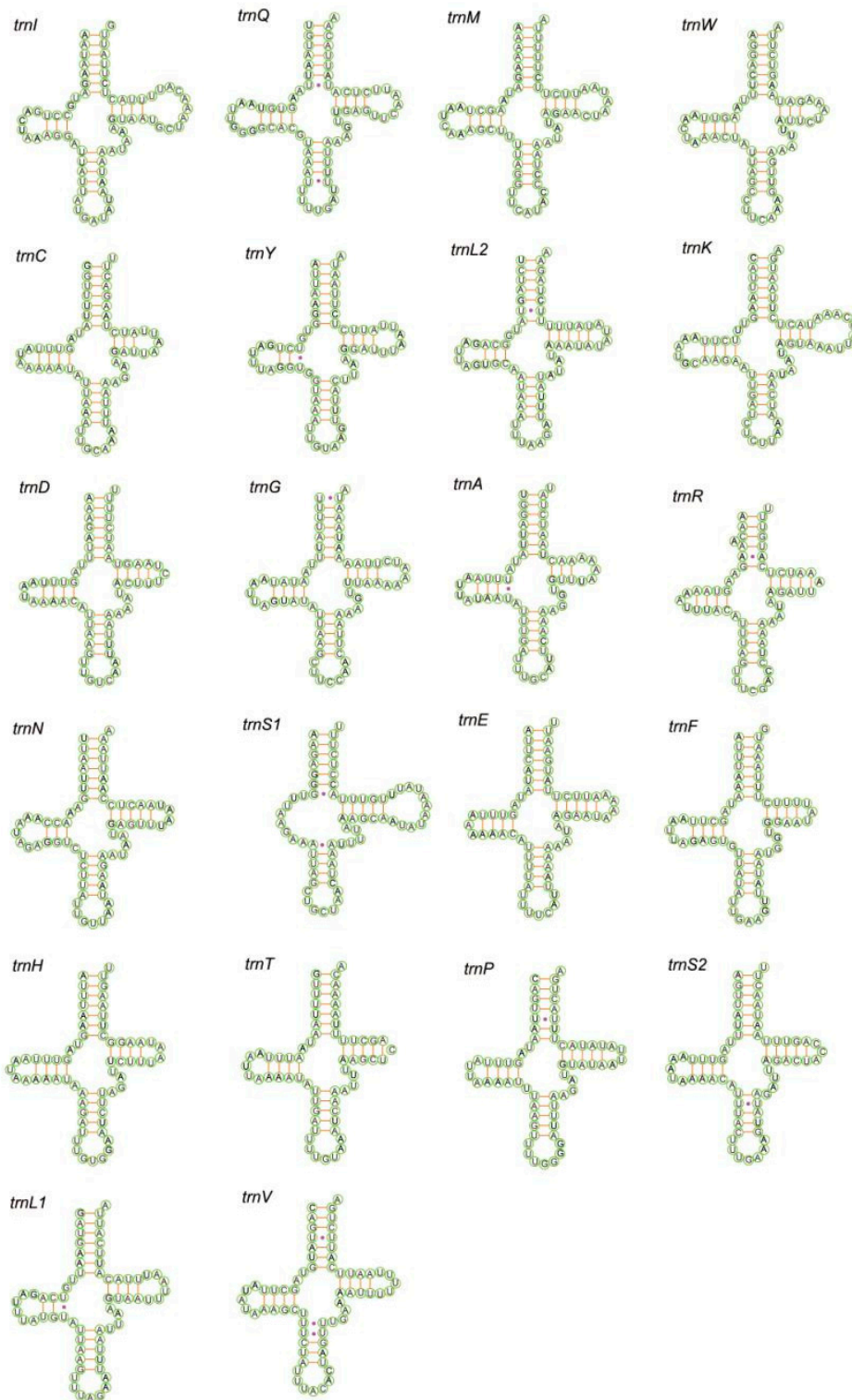


Figure 5. Predicted secondary cloverleaf structure for the tRNAs of *Limassolla* sp.

3.4. Gene Overlaps and Intergenic Spacers

There are 11 and 15 intergenic spacers in *P.tiani* and *Limassolla* sp. ranging from 1 to 28 bp in size. The longest (28 bp) intergenic spacer is found in *Limassolla* sp., between the *nad5* and *trnH* genes. Gene overlaps range from 1 to 8 bp in the two mitogenomes, with the longest (8 bp) found between *trnW* and *trnC* genes in both species (Table S1). tRNA genes have more gene overlaps, which may reflect fewer evolutionary constraints on such genes [56].

3.5. A + T-Rich Region

The A + T-rich region is the starting region of replication and the largest intergenic spacers are also believed to be involved in regulating transcription and replication of DNA in insects [57–59]. This region is located between *rrnS* and *trnM* in the two newly sequenced leafhoppers (Figures 1 and 2). The length of this region ranges from 912 to 945 bp in Typhlocybini, and from 1416 to 3230 bp in Zygynellini (Table 3). One tandem repeat region is found in the control region of *P. tiani*, four Poly (T) are located in non-repeat regions. Three tandem repeat regions in the control region of *Limassolla* sp. include a Poly (T) and Poly (A) between the first and second tandem repeat region. The other four species have a single tandem repeat region. The length, nucleotide sequences and copy numbers of repeat units in the control region are highly variable among known Typhlocybinae mitochondrial genomes (Figure 6).

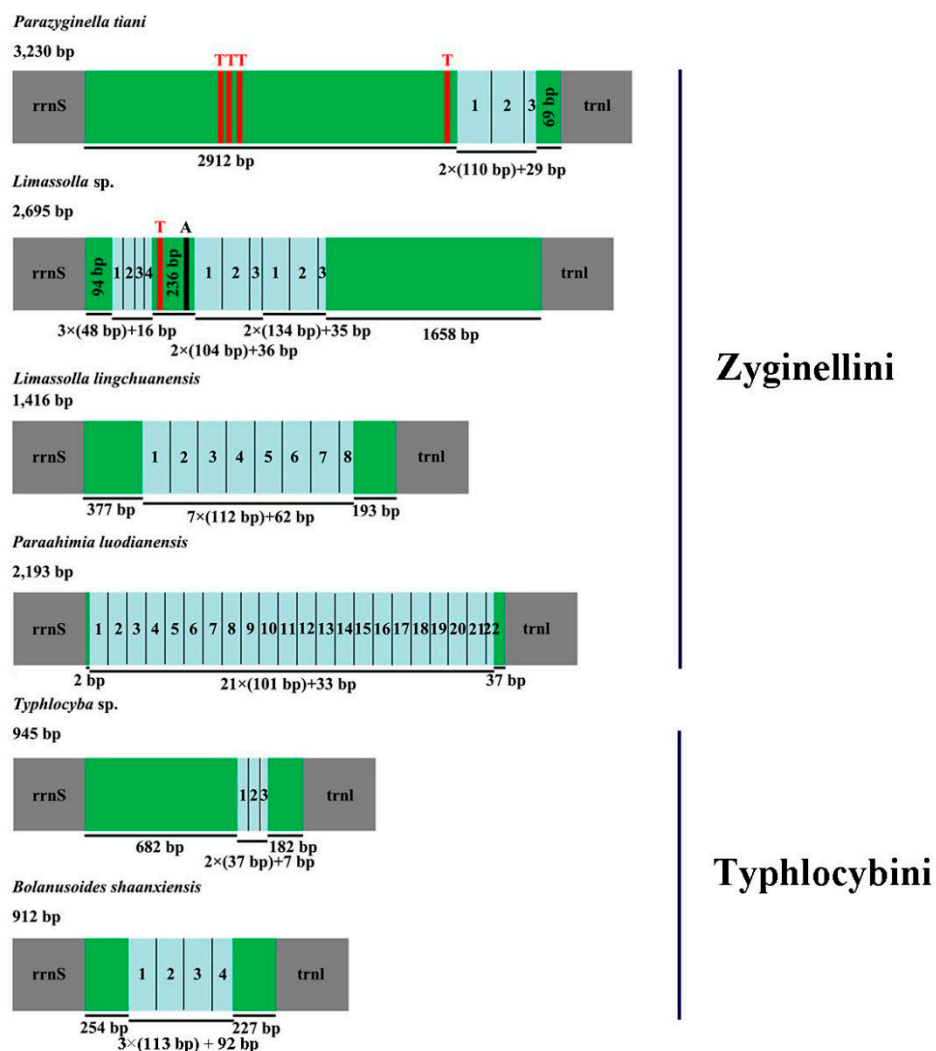


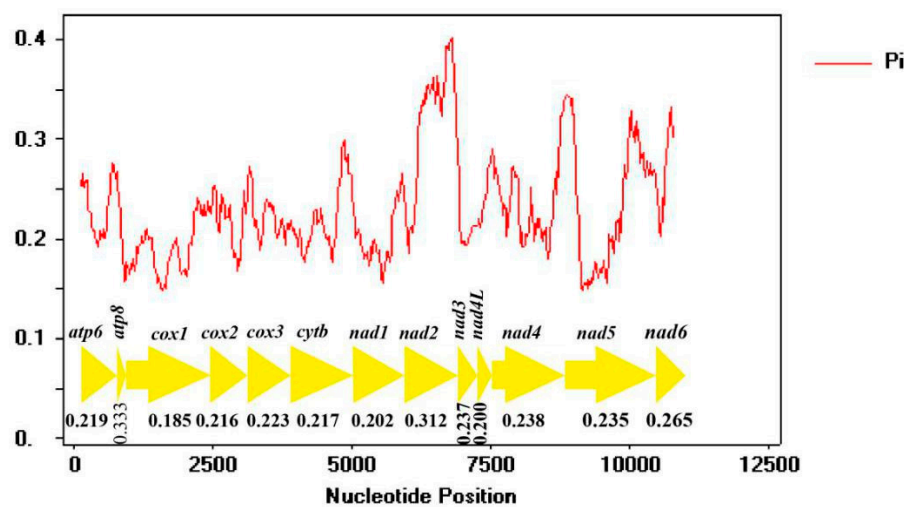
Figure 6. Organization of the control regions in the mitochondrial genomes of Typhlocybini and Zygynellini. The boxes colored blue indicate the tandem repeats. Non-repeat regions are shown by green boxes. The red and black blocks represent the structures of poly (T) and poly (A), respectively.

3.6. Nucleotide Diversity

Sliding window analysis was implemented to study the nucleotide diversity of 13 PCGs among six mitogenomes exhibited in Figure 7A. Nucleotide diversity values range from 0.185 (*cox1*) to 0.333 (*atp8*). Among the genes, *atp8* ($P_i = 0.333$) has the highest variability, followed by *nad2* ($P_i = 0.312$),

nad6 ($P_i = 0.265$) and *nad4* ($P_i = 0.238$). In contrast, *cox1* ($P_i = 0.185$) and *nad4L* ($P_i = 0.200$) have comparatively low values and are the most conserved of the 13 PCGs. The nucleotide diversity is highly variable among the 13 PCGs. Pairwise genetic distances among these six mitogenomes produced results in Figure 7B. *atp8* (0.444), *nad2* (0.406) and *nad6* (0.382) evolve comparatively faster, while *cox1* (0.213), *nad4L* (0.234) and *nad1* (0.236) evolve comparatively slowly (Figure 7B). Average non-synonymous (K_a) and synonymous (K_s) substitution rates can be used to estimate the evolutionary rate [60]. The pairwise K_a/K_s analyses showed that the average K_a/K_s ratios (ω) of 13 PCGs are from 0.169 to 0.615 ($0 < \omega < 1$) (Figure 7B), indicating that these genes are under purifying selection [61]. The genes *atp8* (0.615), *nad2* (0.499) and *nad5* (0.498) have comparatively high K_a/K_s ratios, while *cox1* (0.169), *cytb* (0.212) and *cox2* (0.281) have relatively low values (Figure 7B).

A



B

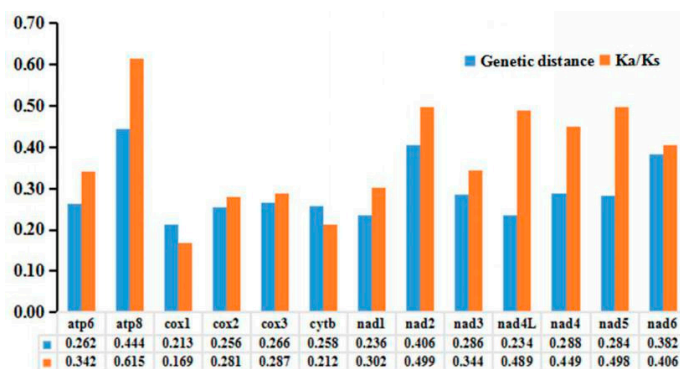


Figure 7. (A) Sliding window analyses of 13 protein coding genes among 6 species’ mitogenomes. The red line shows the value of nucleotide diversity (P_i) in a sliding window analysis (a sliding window of 200 bp with the step size of 20 bp); the P_i value of each gene is shown under the gene name. (B) Genetic distances and the ratio of non-synonymous (K_a) to synonymous (K_s) substitution rates of 13 protein-coding genes among 6 species of Typhlocybini. The average value for each PCG is shown under the gene name.

Nucleotide diversity analyses are crucial for designing species-specific markers useful for insects belonging to groups difficult to distinguish by morphology alone [62,63]. The mitochondrial gene *cox1* has long been used as a common barcode for identifying species and inferring the phylogenetic

relationships in insects [64,65]. In Typhlocybae, the *cox1* gene exhibits a relatively slow evolutionary rate compared to other PCGs, while *nad2* and *nad6* genes evolve more quickly. Thus, *nad2* and *nad6* may be more suitable as barcode genes for species identification in Typhlocybae.

3.7. Phylogenetic Analyses

The best partitioning scheme and models for the different datasets as selected by PartitionFinder are listed in Tables S2 and S3. The phylogenetic topologies were highly consistent based on the four analyzed datasets (PCG123, PCG123R, PCG12 and PCG12R) with high nodal support values in BI and ML trees. The results (Figure 8) recovered Typhlocybae as monophyletic. Empoascini, with *Empoasca* and *Ghauriana* sampled here, was resolved as monophyletic. Four included Erythroneurini, *Empoascanara sipra*, *Empoascanara dwalata* Dworakowska, 1971, *Illinigina* sp. and *Mitjaevia protuberanta*, forming a monophyletic clade sister to the clade comprising Typhlocybini and Zyginellini. All members of Typhlocybini and Zyginellini were grouped into a clade with high support (PP = 1, BS = 100), but the included Zyginellini formed a paraphyletic grade giving rise to Typhlocybini. Thus, the monophyly of tribe Zyginellini was not supported.

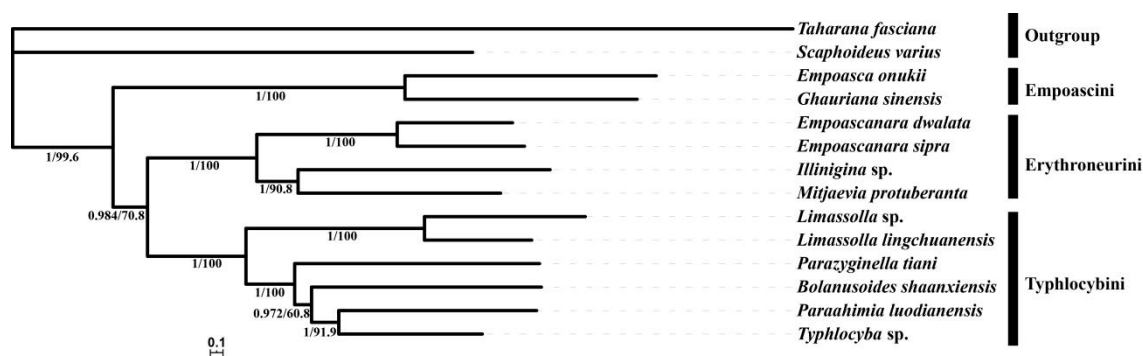


Figure 8. Phylogenetic tree produced from ML and BI analysis based on 13 protein-coding genes. Numbers on branches are Bayesian posterior probabilities (PP) and bootstrap values (BS) (The Typhlocybini including former Zyginellini).

The tribe Typhlocybini was proposed by Distant [14] and is currently divided into two informal groups according to the number of cross veins on the hind wing: the *Eupteryx* complex with three cross veins and another one with two cross veins, including the *Typhlocyba* complex, *Farynala* complex, and *Linnavuoriana* complex [66]. The tribe Zyginellini was proposed by Dworakowska based on the presence of one cross vein on the hind wing and with vein CuA apparently directly connected to MP [67], but its status as a separate tribe has been questioned [68]. Balme (2007) and Dietrich (2013) treated it as a synonym of Typhlocybini based on analysis of morphological characteristics and histone H3 and 16S rDNA sequences [4,5]. Recent phylogenetic analyses of Cicadellidae and Membracoidea [12,13] also grouped members of Zyginellini with Typhlocybini but these analyses did not include a large enough taxon sample to test the monophyly of the tribes. In our study, members of the *Typhlocyba* complex of Typhlocybini are derived from within Zyginellini, consistent with treatment of the two tribes as synonyms. Nevertheless, a broader analysis with a much larger sample of taxa is needed to confirm the present results.

4. Conclusions

This study presents the annotated complete mitochondrial genome sequences of *P. tinai* and *Limassolla* sp. and a comparative analysis of mitochondrial genomes within the Typhlocybini and Zyginellini. Typhlocybae mitogenomes are highly conservative in overall organization, exhibiting the hypothetical ancestral gene order for insects and lacking any gene rearrangements, as have been found in some other Cicadellidae. The only unusual feature was found in the secondary structure of

tRNAs, with the acceptor stem of *trnR* comprising only 5 or 6 bp in some species. This unusual feature is here reported for the first time in Typhlocybiinae. Phylogenetic analyses support treating Zyginellini as a synonym of Typhlocybini, as proposed by other recent authors. However, a much larger taxon with mitogenomes is still needed to reconstruct a better phylogeny tree to solve the tribal relationships within Typhlocybiinae.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2075-4450/11/10/684/s1>, Table S1: Mitogenomic organization of *P. tiani* and *Limassolla* sp. Table S2: The best partitioning schemes and models for the Maximum likelihood (ML) method based on four datasets selected by PartitionFinder. Table S3: The best partitioning schemes and models for the Bayesian inference (BI) method based on four datasets selected by PartitionFinder.

Author Contributions: Conceptualization, X.Z. and M.H.; Funding acquisition, M.H.; Methodology, X.Z. and M.H.; Validation, C.H.D. and M.H.; Writing—original draft, X.Z.; Writing—review and editing, X.Z., C.H.D. and M.H. All authors have read and agreed to the published version of the manuscript.

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References

1. McKamey, S.H. Taxonomic catalogue of the *Membracoidea* (exclusive of leafhoppers); second supplement to Fascicle 1-*Membracidae* of the General Catalogue of the *Hemiptera*. *Mem. Am. Entomol. Inst.* **1998**, *60*, 1–377.
2. McKamey, S.H. Leafhoppers of the World Database: Progress Report. In Proceedings of the 11th International Auchenorrhyncha Congress, Potsdam/Berlin, Germany, 5–9 August 2002; Museum für Naturkunde, Humboldt-Universität: Berlin, Germany, 2002; p. 85.
3. Hamilton, K.G.A. New world species of Chlorita, Notus, and Forcipata (Rhynchota: Homoptera: Cicadellidae: Typhlocybiinae) with a new tribe Forcipatini. *Can. Entomol.* **1998**, *130*, 491–507. [[CrossRef](#)]
4. Balme, G.R. Phylogeny and Systematics of the Leafhopper Subfamily Typhlocybiinae (Insecta: Hemiptera: Typhlocybiinae). Ph.D. Thesis, North Carolina State University, Raleigh, NC, USA, 2007; pp. 1–149.
5. Dietrich, C.H. South American leafhoppers of the tribe Typhlocybini (Hemiptera: Cicadellidae: Typhlocybiinae). *Zoologia* **2013**, *30*, 519–568. [[CrossRef](#)]
6. Cai, P.; Liang, L.J.; Wang, F. A new species of *Limassolla*, pest of Diospyros Kaki (Homoptera: Cicadelloidea: Typhlocybidae). *J. Anhui Agric. Univ.* **1992**, *19*, 324–326.
7. Zhang, Y.L. *A Taxonomic Study of Chinese Cicadellidae (Homiptera)*; Tianze Eldonejo: Yangling, China, 1990; pp. IV + 218.
8. Xu, Y.; Wang, Y.R.; Dietrich, C.H.; Fletcher, M.J.; Qin, D.Z. Review of Chinese species of the leafhopper genus *Ammasca Ghauri* (Hemiptera, Cicadellidae, Typhlocybinae), with description of a new species, species checklist and notes on the identity of the Indian cotton leafhopper. *Zootaxa* **2017**, *4353*, 360–370. [[CrossRef](#)]
9. Oman, P.W.; Knight, W.J.; Nielson, M.W. *Leafhoppers (Cicadellidae): A Bibliography, Generic Check-List and Index to the World Literature 1956–1985*; C.A.B. International Institute of Entomology: London, UK, 1990; pp. 1–368.
10. Dworakowska, I. The leafhopper tribe Zyginellini (Homoptera, Auchenorrhyncha, Cicadellidae, Typhlocybinae). *Rev. Zool. Afr.* **1979**, *93*, 299–331.
11. Dietrich, C.H. Phylogeny of the leafhopper subfamily *Evacanthinae* with a review of Neotropical species and notes on related groups (Hemiptera: Membracoidea: Cicadellidae). *Syst. Entomol.* **2004**, *29*, 455–487. [[CrossRef](#)]
12. Dietrich, C.H.; Allen, J.M.; Lemmon, A.R.; Lemmon, E.M.; Takiya, D.M.; Evangelista, O.; Walden, K.K.; Grady, P.G.; Johnson, K.P.; Wiegmann, B. Anchored Hybrid Enrichment-Based Phylogenomics of Leafhoppers and Treehoppers (Hemiptera: Cicadomorpha: Membracoidea). *Insect Syst. Divers.* **2017**, *1*, 57–72. [[CrossRef](#)]
13. Dietrich, C.H.; Rakitov, R.A.; Holmes, J.L.; Black, W.C. Phylogeny of the major lineages of *Membracoidea* (Insecta: Hemiptera: Cicadomorpha) based on 28S rDNA Sequences. *Mol. Phylogenet. Evol.* **2001**, *18*, 293–305. [[CrossRef](#)]

14. Distant, W.L. Rhynchota—Homoptera. In *The Fauna of British India including Ceylon and Burma*; Secretary of State for India in Council: London, UK, 1908; Volume 4, p. 501.
15. Boore, J.L. Animal mitochondrial genomes. *Nucleic Acids Res.* **1999**, *27*, 1767–1780. [[CrossRef](#)]
16. Cameron, S.L. Insect mitochondrial genomics: Implications for evolution and phylogeny. *Annu Rev. Entomol.* **2014**, *59*, 95–117. [[CrossRef](#)] [[PubMed](#)]
17. Wolstenholme, D.R. Genetic novelties in mitochondrial genomes of multicellular animals. *Curr. Opin. Genet. Dev.* **1992**, *2*, 918–925. [[CrossRef](#)]
18. Kim, M.J.; Kang, A.R.; Jeong, H.C.; Kim, K.G.; Kim, I. Reconstructing intraordinal relationships in *Lepidoptera* using mitochondrial genome data with the description of two newly sequenced *Lycaenids*, *Spindasis takanonis* and *Protantigius superans* (*Lepidoptera: Lycaenidae*). *Mol. Phylogenet. Evol.* **2011**, *61*, 436–445. [[CrossRef](#)]
19. Hebert, P.D.N.; Penton, E.H.; Burns, J.M.; Janzen, D.H.; Hallwachs, W. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc. Natl. Acad. Sci. India Sect. A* **2004**, *101*, 14812–14817. [[CrossRef](#)] [[PubMed](#)]
20. Li, Y.P.; Song, W.; Shi, S.L.; Liu, Y.Q.; Pan, M.H.; Dai, F.Y.; Lu, C.; Xiang, Z.H. Mitochondrial genome nucleotide substitution pattern between domesticated silkworm, *Bombyx mori*, and its wild ancestors, Chinese *Bombyx mandarina* and Japanese *Bombyx mandarina*. *Genet. Mol. Biol.* **2010**, *33*, 186–189. [[CrossRef](#)] [[PubMed](#)]
21. Simon, C.; Hadrys, H. A comparative analysis of complete mitochondrial genomes among *Hexapoda*. *Mol. Phylogenet. Evol.* **2013**, *69*, 393–403. [[CrossRef](#)]
22. Simon, C.; Buckley, T.R.; Frati, F.; Stewart, J.B.; Beckenbach, A.T. Incorporating molecular evolution into phylogenetic analysis, and a new compilation of conserved polymerase chain reaction primers for animal mitochondrial DNA. *Annu. Rev. Ecol. Evol. Syst.* **2006**, *37*, 545–579. [[CrossRef](#)]
23. Saccone, C.; Giorgi, C.D.; Gissi, C.; Pesole, G.; Reyes, A. Evolutionary genomics in *Metazoa*: The mitochondrial DNA as a model system. *Gene* **1999**, *238*, 195–209. [[CrossRef](#)]
24. Yuan, X.W.; Xiong, K.N.; Li, C.; Song, Y.H. The complete mitochondrial genome of *Limassolla lingchuanensis* (Hemiptera: Cicadellidae: Typhlocybinae). *Mitochondrial DNA Part B* **2020**, *5*, 229–230. [[CrossRef](#)]
25. Song, Y.H.; Yuan, X.W.; Li, C. The mitochondrial genome of *Paraahimia luodianensis* (Hemiptera: Cicadellidae: Typhlocybinae), a new genus and species from China. *Mitochondrial DNA Part B* **2020**, *5*, 1351–1352. [[CrossRef](#)]
26. Tan, C.; Chen, X.X.; Li, C.; Song, Y.H. The complete mitochondrial genome of *Empoasca supra* (Hemiptera: Cicadellidae: Typhlocybinae) with phylogenetic consideration. *Mitochondrial DNA Part B* **2020**, *5*, 260–261. [[CrossRef](#)]
27. Song, N.; Cai, W.; Li, H. Deep-level phylogeny of *Cicadomorpha* inferred from mitochondrial genomes sequenced by NGS. *Sci. Rep.* **2017**, *7*, 10429. [[CrossRef](#)] [[PubMed](#)]
28. Shi, R.; Yu, X.F.; Yang, M.F. Complete mitochondrial genome of *Ghauriana sinensis* (Hemiptera: Cicadellidae: Typhlocybinae). *Mitochondrial DNA Part B* **2020**, *5*, 1367–1368. [[CrossRef](#)]
29. Zhou, N.N.; Wang, M.X.; Cui, L.; Chen, X.X.; Han, B.Y. Complete mitochondrial genome of *Empoasca vitis* (Hemiptera: Cicadellidae). *Mitochondrial DNA Part A DNA Mapp. Seq. Anal.* **2016**, *27*, 1052–1053.
30. Yuan, X.; Li, C.; Song, Y.H. Characterization of the complete mitochondrial genome of *Mitjaevia protuberanta* (Hemiptera: Cicadellidae: Typhlocybinae). *Mitochondrial DNA Part B* **2020**, *5*, 601–602. [[CrossRef](#)]
31. Luo, X.; Chen, Y.; Chen, C.; Pu, D.Q.; Tang, X.B.; Zhang, H.; Lu, D.H.; Mao, J.H. Characterization of the complete mitochondrial genome of *Empoasca* sp. (Cicadellidae: Hemiptera). *Mitochondrial DNA Part B* **2019**, *4*, 1477–1478. [[CrossRef](#)]
32. Liu, J.H.; Sun, C.Y.; Long, J.; Guo, J.J. Complete mitogenome of tea green leafhopper, *Empoasca onukii* (Hemiptera: Cicadellidae) from Anshun, Guizhou Province in China. *Mitochondrial DNA Part B* **2017**, *2*, 808–809. [[CrossRef](#)]
33. Chen, X.X.; Yuan, Z.W.; Li, C.; Song, Y.H. Complete mitochondrial genome sequence of *Empoasca dwalata* (Hemiptera: Cicadellidae: Typhlocybinae). *Mitochondrial DNA Part B* **2020**, *5*, 2260–2261. [[CrossRef](#)]
34. Yang, X.; Yuan, Z.W.; Li, C.; Song, Y.H. Complete mitochondrial genome of *Eupteryx (stacla) minusula* (Hemiptera: Cicadellidae: Typhlocybinae) from China. *Mitochondrial DNA Part B* **2020**, *5*, 2375–2376. [[CrossRef](#)]
35. Jiang, J.; Yuan, X.; Yuan, Z.; Song, Y.H. The complete mitochondrial genome of *Parathailocyba orla* (Hemiptera: Cicadellidae: Typhlocybinae). *Mitochondrial DNA Part B* **2020**, *5*, 1981–1982. [[CrossRef](#)]

36. Kearse, M.; Moir, R.; Wilson, A.; Stones-Havas, S.; Cheung, M.; Sturrock, S.; Buxton, S.; Cooper, A.; Markowitz, S.; Duran, C. Geneious basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **2012**, *28*, 1647–1649. [[CrossRef](#)] [[PubMed](#)]
37. Bernt, M.; Donath, A.; Jühling, F.; Externbrink, F.; Florentz, C.; Fritzsch, G.; Pütz, J.; Middendorf, M.; Stadler, P.F. MITOS: Improved de novo metazoan mitochondrial genome annotation. *Mol. Phylogenet. Evol.* **2013**, *69*, 313–319. [[CrossRef](#)] [[PubMed](#)]
38. Grant, J.R.; Stothard, P. The CG View Server: A comparative genomics tool for circular genomes. *Nucleic Acids Res.* **2008**, *36*, 181–184. [[CrossRef](#)] [[PubMed](#)]
39. Zhang, D.; Gao, F.L.; Li, W.X.; Jakovlić, I.; Zou, H.; Zhang, J.; Wang, G.T. PhyloSuite: An integrated and scalable desktop platform for streamlined molecular sequence data management and evolutionary phylogenetics studies. *bioRxiv* **2018**, *20*, 489088. [[CrossRef](#)] [[PubMed](#)]
40. Benson, G. Tandem repeats finder: A program to analyze DNA sequences. *Nucleic Acids Res.* **1999**, *27*, 573–580. [[CrossRef](#)] [[PubMed](#)]
41. Librado, P.; Rozas, J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **2009**, *25*, 1451–1452. [[CrossRef](#)] [[PubMed](#)]
42. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870. [[CrossRef](#)]
43. Du, Y.M.; Dai, W.; Dietrich, C.H. Mitochondrial genomic variation and phylogenetic relationships of three groups in the genus *Scaphoideus* (Hemiptera: Cicadellidae: Deltocephalinae). *Sci. Rep.* **2017**, *7*, 16908. [[CrossRef](#)]
44. Wang, J.J.; Li, H.; Dai, R.H. Complete mitochondrial genome of *Taharana fasciana* (Insecta, Hemiptera: Cicadellidae) and comparison with other Cicadellidae insects. *Genetica* **2017**, *145*, 593–602. [[CrossRef](#)]
45. Katoh, K.; Standley, D.M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* **2013**, *30*, 772–780. [[CrossRef](#)]
46. Castresana, J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* **2000**, *17*, 540–552. [[CrossRef](#)] [[PubMed](#)]
47. Lanfear, R.; Frandsen, P.B.; Wright, A.M.; Senfeld, T.; Calcott, B. Partition Finder 2: New methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Mol. Biol. Evol.* **2016**, *34*, 772–773.
48. Nguyen, L.-T.; Schmidt, H.A.; von Haeseler, A.; Minh, B.Q. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* **2014**, *32*, 268–274. [[CrossRef](#)] [[PubMed](#)]
49. Ronquist, F.; Huelsenbeck, J.P. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **2003**, *19*, 1572–1574. [[CrossRef](#)]
50. Ronquist, F.; Teslenko, M.; Van Der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.A.; Huelsenbeck, J.P. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* **2012**, *61*, 539–542. [[CrossRef](#)]
51. Clary, D.O.; Wolstenholme, D.R. The mitochondrial DNA molecule of *Drosophila yakuba* nucleotide sequence, gene organization, and genetic code. *J. Mol. Evol.* **1985**, *22*, 252–271. [[CrossRef](#)]
52. Ojala, D.; Montoya, J.; Attardi, G. tRNA punctuation model of RNA processing in human mitochondria. *Nature* **1981**, *290*, 470. [[CrossRef](#)]
53. Li, H.; Leavengood, J.M.; Chapman, E.G.; Burkhardt, D.; Song, F.; Jiang, P.; Liu, J.P.; Zhou, X.G.; Cai, W.Z. Mitochondrial phylogenomics of Hemiptera reveals adaptive innovations driving the diversification of true bugs. *Proc. Biol. Sci.* **2017**, *284*, 20171223. [[CrossRef](#)]
54. Zhang, K.J.; Zhu, W.C.; Rong, X.; Ding, X.L.; Zhang, Y.K.; Liu, J.; Chen, D.S.; Du, Y.; Hong, X.Y. The complete mitochondrial genomes of two rice planthoppers, *Nilaparvata lugens* and *Laodelphax striatellus*: Conserved genome rearrangement in Delphacidae and discovery of new characteristics of *atp8* and *tRNA* genes. *BMC Genom.* **2013**, *14*, 417. [[CrossRef](#)]
55. Guo, Z.L.; Yuan, M.L. Research progress of mitochondrial genomes of Hemiptera insects. *Sci. Sin. Vitae* **2016**, *46*, 151–166.
56. Doublet, V.; Ubrig, E.; Alioua, A.; Bouchon, D.; Marcadé, I.; Maréchal-Drouard, L. Large gene overlaps and tRNA processing in the compact mitochondrial genome of the crustacean *Armadillidium vulgare*. *RNA Biol.* **2015**, *12*, 1159–1168. [[CrossRef](#)] [[PubMed](#)]
57. Zhang, D.X.; Szymura, J.M.; Hewitt, G.M. Evolution and structural conservation of the control region of insect mitochondrial DNA. *J. Mol. Evol.* **1995**, *40*, 382–391. [[CrossRef](#)] [[PubMed](#)]

58. Huang, W.; Zheng, J.; He, Y.; Luo, C. Tandem repeat modification during double-strand break repair induced by an engineered TAL effector nuclease in zebrafish genome. *PLoS ONE* **2013**, *8*, e84176. [[CrossRef](#)] [[PubMed](#)]
59. Levinson, G.; Gutman, G.A. Slipped-strand mispairing: A major mechanism for DNA sequence evolution. *Mol. Biol. Evol.* **1987**, *4*, 203–221.
60. Hurst, L.D. The Ka/Ks ratio: Diagnosing the form of sequence evolution. *Trends Genet.* **2002**, *18*, 486–487. [[CrossRef](#)]
61. Mori, S.; Matsunami, M.J.G. Signature of positive selection in mitochondrial DNA in *Cetartiodactyla*. *Genes Genet. Syst.* **2018**, *93*, 65–73. [[CrossRef](#)]
62. Jia, W.Z.; Yan, H.B.; Guo, A.J.; Zhu, X.Q.; Wang, Y.C.; Shi, W.G.; Chen, H.T.; Zhan, F.; Zhang, S.H.; Fu, B.Q. Complete mitochondrial genomes of *Taenia multiceps*, *T. hydatigena* and *T. pisiformis*: Additional molecular markers for a tapeworm genus of human and animal health significance. *BMC Genom.* **2010**, *11*, 447. [[CrossRef](#)]
63. Ma, L.Y.; Liu, F.F.; Chiba, H.; Yuan, X.Q. The mitochondrial genomes of three skippers: Insights into the evolution of the family *Hesperiidae* (Lepidoptera). *Genomics* **2019**, *112*, 422–441. [[CrossRef](#)]
64. Brabec, J.; Kostadinova, A.; Scholz, T.; Littlewood, D.T.J. Complete mitochondrial genomes and nuclear ribosomal RNA operons of two species of *Diplostomum* (Platyhelminthes: Trematoda): A molecular resource for taxonomy and molecular epidemiology of important fish pathogens. *Parasit. Vectors* **2015**, *8*, 336. [[CrossRef](#)]
65. Demari-Silva, B.; Foster, P.G.; Oliveira-de, T.M.P.; Bergo, E.S.; Sanabani, S.S.; Pessôa, R.; Sallum, M.A.M. Mitochondrial genomes and comparative analyses of *Culex camposi*, *Culex coronator*, *Culex usquatus* and *Culex usquatissimus* (Diptera: Culicidae), members of the Coronator group. *BMC Genom.* **2015**, *16*, 831. [[CrossRef](#)]
66. Young, D.A.J. A reclassification of western Hemisphere Typhlocybinae (Homoptera: Cicadellidae). *Univ. Kans. Sci. Bull.* **1952**, *35*, 3–217. [[CrossRef](#)]
67. Dworakowska, I. On some Typhlocybinae from Vietnam (Homoptera: Cicadellidae). *Folia Entomol. Hung.* **1977**, *30*, 9–47.
68. Hamilton, K.G.A. Classification, morphology and phylogeny of the family *Cicadellidae* (Rhynchota: Homoptera). In Proceedings of the 1st International Workshop on Biotaxonomy, Classification and Biology of Leafhoppers and Planthoppers (Auchenorrhyncha) of Economic Importance, London, UK, 4–7 October 1983; pp. 15–37.



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