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Mitogenomics of Three *Ziczacella* Leafhoppers (Hemiptera: Cicadellidae: Typhlocybinae) from Karst Area, Southwest China, and Their Phylogenetic Implications

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Abstract: Leafhoppers (Hemiptera, Auchenorrhyncha, Cicadellidae) are distributed worldwide and include around 2550 genera, more than 21,000 species, including almost 2000 species in China. Typhlocybinae is the second largest subfamily in Cicadellidae after Deltocephalinae. Previously, morphological characteristics were the diagnostic basis of taxonomy, but they were not combined with molecular biology. The genus *Ziczacella* Anufryev, 1970 has only six known species worldwide. The mitogenomes of *Ziczacella steggerdai* Ross, 1965, *Ziczacella dworakowskae* Anufriev, 1969 and *Ziczacella heptapotamica* Kusnezov, 1928 were sequenced and identified here for the first time. They all contained 13 PCGs, 22 tRNA genes, 2 rRNA genes, and a control region, and the complete mitochondrial genomes were 15,231 bp, 15,137 bp, and 15,334 bp, respectively. The results show heavy AT nucleotide bias. Phylogenetic analysis yielded the following topology: (Empoascini + Alebrini) + ((Erythroneurini + Dikraneurini) + (Zyginellini + Typhlocybini)). In this study, three newly sequenced species were closely related to *Mitjaevia dworakowskae* and *M. shibingensis*. We confirmed the monophyly of the four tribes within Typhlocybinae again, and Zyginellini should be combined with Typhlocybini, which supports Chris's points.

Keywords: mitochondrial genome; phylogenetic analysis; *Ziczacella*; karst

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1. Introduction

Erythroneurini, belonging to Typhlocybinae of Cicadelladae, is the largest tribe of Typhlocybinae, with about 2000 species worldwide [1]. The leafhoppers feed on the sap of host plants and are abundant in forests and grasslands [2]. They are not only important agricultural and forestry pests, but also carriers of plant pathogens [3,4]. *Ziczacella* Anufryev, 1970, as one genus of this tribe, is widely distributed in the Palaearctic and Oriental regions, and only six known species have been reported until now [5,6].

The traditional classification of leafhoppers has attracted great attention, including the classification of Typhlocybinae. At present, Typhlocybinae mainly contains six tribes, but this taxonomy remains controversial [7–9]. Previously, people have tried to use morphological data or sequence data of a few genes to estimate the phylogenetic relationships between leafhopper groups, but little research has been performed on Typhlocybinae [10–12]. Nowadays, the emergence of a new generation of sequencing technology has brought a breakthrough to solve this problem so that the mitochondrial genome data can verify the existing classification of Typhlocybinae [13–15].

The insect mitochondrial genomic DNA has a molecular weight of 14–20 KB and is a closed double-stranded DNA molecule. Usually, it contains 37 genes, including 22 transfer RNA (tRNAs) genes,13 protein-coding genes (PCGs), including cytochrome c oxidase subunits 1–3 (*cox1–3*), NADH dehydrogenase 1–6 and 4L (*nad1–6* and *nad4L*), ATPase

subunit 6 and 8 (*atp6* and *atp8*), cytochrome b (*cytb*), two ribosomal RNAs genes (*16S* and *12S*), and a control region, which is the region rich in A + T. The mitochondrial genome is very suitable for genomics research [16,17]. Compared with nuclear genes, the mitochondrial genome has many advantages, such as easy detection, low molecular weight, simple structure, conservative arrangement, and so on. Therefore, the mitochondrial genome is widely used to identify phylogenetic relationships and population genetic structure at different taxonomic levels [18,19].

Leafhoppers are important agricultural and forestry pests, feeding on the sap of various economic crops, and they are also vectors of plant pathogens [2–4]. These characteristics make it very important to study the genetic information and biological evolution of leafhoppers. In order to further enrich the mitochondrial genome data of leafhoppers and provide comparative data for related species, the complete mitochondrial genome sequences of *Ziczacella steggerdai* Ross, 1965, *Ziczacella dworakowskae* Anufriev, 1969, and *Z. heptapotamica* Kusnezov, 1928 from Karst areas in southwest China were sequenced and analyzed [5,20,21], and their phylogenetic relationship with other leafhoppers was analyzed (Figure 1). At present, there are no gene data about the erythroneurine genus *Ziczacella* in the GenBank, and this study is the first time the complete mitochondrial gene and phylogenetic relationship of *Ziczacella* has been analyzed. These new molecular data will contribute to the identification of leafhopper species, the comparison of genetic relationships, and the study of population genetics and evolution in the future. Thirty mitogenomic sequences were downloaded from the National Center for Biotechnology Information (NCBI) to estimate phylogenetic relationships (Table 1).

Figure 1. External morphology of *Z. steggerdai* (ZS), *Z. dworakowskae* (ZD), and *Z. heptapotamica* (ZH).

Table 1. List of the mitochondrial genomes analyzed in the present study.

Tribe	Species	Length (bp)	GenBank No.	Reference
Alebrini	*Shaddai* sp.	17,575 bp	MZ014457.1	[22]
	Sobrala sp.	16,732 bp	MZ014458.1	[22]
Dikraneurini	*Dikraneura zlata*	15,330 bp	MZ014450.1	[22]
	Dikraneurini sp.	15,306 bp	MZ014451.1	[22]
	Michalowskiya breviprocessa	15,591 bp	MW264489.1	Unpublished

Table 1. *Cont.*

Tribe	Species	Length (bp)	GenBank No.	Reference
	Uniformus sp.	14,440 bp	MW272457.1	Unpublished
	Uzeldikra longiprocessa	14,752 bp	NC_069160.1	Unpublished
Empoascini	*Empoasca onukii*	15,167 bp	NC_037210.1	[23]
	Ghauriana sinensis	15,491 bp	MN699874.1	[24]
	Empoasca serrata	15,131 bp	MZ014453.1	[22]
	Alebroides salicis	15,890 bp	MZ014449.1	[22]
Erythroneurini	*Empoascanara dwalata*	15,271 bp	MT350235.1	[25]
	Empoascanara sipra	14,827 bp	NC_048516.1	[26]
	Empoascanara wengangensis	14,830 bp	MT445764.1	[25]
	Mitjaevia dworakowskae	16,399 bp	MT981880.1	[27]
	Mitjaevia shibingensis	15,788 bp	MT981879.1	[27]
	Ziczacella steggerdai	15,231 bp	OQ657302	This study
	Ziczacella dworakowskae	15,137 bp	OQ657303	This study
	Ziczacella heptapotamica	15,334 bp	NC064506.1	This study
Typhlocybini	*Eupteryx gracilirama*	17,173 bp	MT594485.1	[28]
	Eupteryx minuscula	16,944 bp	MN910279.1	[28]
	Eurhadina acapitata	15,419 bp	MZ457331.1	Unpublished
	Eurhadina dongwolensis	15,708 bp	MZ457332.1	Unpublished
	Eurhadina jarrayi	15,332 bp	MZ014455.1	[22]
Zyginellini	*Limassolla emmrichi*	14,677 bp	MW272458.1	Unpublished
	Limassolla lingchuanensis	15,716 bp	NC_046037.1	[29]
	Parazyginella tiani	17,562 bp	NC_053918.1	[30]
	Zyginella minuta	15,544 bp	NC_052876.1	[31]
outgroup	*Atkinsoniella thalia*	15,034 bp	NC_062847.1	Unpublished
	Scaphoideus maculatus	15,486 bp	NC_060770.1	Unpublished

2. Materials and Methods

2.1. Sampling and DNA Extraction

Samples of *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica* were collected from roadside weeds in Jiulongpo District, Chongqing City, China (29°5′51″ N, 107°19′57″ E), Shizhong District, Leshan City, Sichuan Province, China (30°01′39″ N, 103°46′37″ E), and Qixingguan District, Bijie City, Guizhou Province, China (27°09′42″ N, 105°05′47″ E), respectively. Samples were placed in a freezer at −20 °C and then stored in the Cicadellidae specimen room, School of Karst Science, Guizhou Normal University, under the voucher numbers GZNU-ELS-20220321, GZNU-ELS-20220322, and GZNU-ELS-20190716. Total genomic DNAs were extracted from three leafhopper species with abdomens and wings removed using a DNeasy Blood & Tissue kit (QIAGEN, Beijing, China) in turn, according to the manufacturer's instructions. Due to the small size of the leafhopper body, in order to successfully extract DNA, each species used six leafhoppers.

2.2. DNA Sequencing and Assembly

Sequencing libraries were generated using the Truseq Nano DNA HT Sample Preparation Kit (Illumina, Alameda, CA, USA). Whole mitochondrial genome sequencing (150 bp paired-end reads) was performed using the Illumina Novaseq 6000 platform (Illumina, Alameda, CA, USA) at Berry Genomics, Beijing, China. The raw data were filtered using SOAPnuke v.1.3.0, and approximately 6.2 GB (*Z. heptapotamica*), 5.7 GB (*Z. steggerdai*), and 5.4 GB (*Z. dworakowskae*) of clean data were obtained and saved in fastq format. The average depth was 703.0 X, 529.8 X, and 424.1 X, respectively. After quality-proofing of the obtained fragments, the complete mt genome sequence was assembled manually using Geneious Prime V 2021.1.1 and then manually proofread based on sequencing peak figures. A homology search was performed by the Blast function in NCBI to verify the amplified sequence as the correct sequence [32,33].

2.3. Sequence Annotation and Analyses

The assembled mitogenome sequence was subsequently annotated using Geneious Prime V 2021 and the mitogenome of *Mitjaevia protuberanta* (GeneBank accession number: NC_047465.1) as the reference. All tRNA genes were identified with MITOS Web Server

(http://mitos.bioinf.uni-leipzig.de/index.py, accessed on 19 December 2021) [34]. The nucleotide base composition, codon usage, A + T content values, and relative synonymous codon usage (RSCU) were calculated using MEGA 7.0 [35]. The typical second structures for tRNAs were manually drawn with Adobe Illustrator 2021 in accordance with the MITOS predictions. Circular mitogenome maps were drawn using the CG View server [36] (http://cgview.ca/, accessed on 6 January 2023) and Photoshop CS 6. The skewing of the nucleotide composition was calculated with the formulas AT skew = (A − T)/(A + T) and GC skew = (G − C)/(G + C) [37]. The complete mitochondrial genome sequences of *Z. steggerdai* (Genbank: OQ657302), *Z. dworakowskae* (Genbank: OQ657303), and *Z. heptapotamica* (NC064506.1) were submitted to NCBI.

2.4. Phylogenetic Analyses

The phylogenetic analysis used the mitochondrial genomes of three newly sequenced species and other Typhlocybinae species downloaded from GenBank, containing four species from Empoascini, eight species from Erthroneurini, two species from Alebrini, five species from Typhlocybini, five species from Dikraneurini, and four species from Zyginellini. *Atkinsoniella thalia* and *Scaphoideus maculatus* were regarded as outgroups (Table 1). For phylogenetic analyses, 13 PCG and 2 rRNA genes were selected to construct phylogenetic trees. Thirty mitogenomic sequences were aligned and corrected using MAFFT v7; gaps and ambiguous sites in the alignments were then removed using Gblocks 0.91b [38,39]. The trimmed datasets were used to estimate the phylogeny by Bayesian inference (BI) using MrBayes 3.2.7 and maximum likelihood (ML) using IQ-TREE [40–42]. The best model was inferred by PartitionFinder (v2.1.1) [43]. BI selected GTR + I + G as the optimal model, running 10 million generations twice, sampling once every 1000 generations after the average standard deviation of the segmentation frequency drops below 0.01, with the first 25% of the samples being discarded burn-in, and the posterior probability (PP) of each branch was calculated. ML constructed with the IQ-TREE used an ultrafast bootstrap approximation approach with 10,000 replicates and calculated bootstrap scores for each node (BP).

3. Results and Discussion

3.1. Organization and Composition of the Genome

The genome organization and nucleotide composition of three new mitogenomes sequenced in this study are similar to other Typhlocybine species previously reported [26,29,44,45]. The complete mitogenomes of *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica* are double-stranded plasmids with 15,231 bp, 15,137 bp and 15,334 bp, respectively (Table 2). Usually, it contains 13 PCGs, 22 tRNA genes, 2 rRNA genes, and a control region. Twenty-four genes encode in the majority strand (L-strand), while the other fourteen genes encode in the minority strand (H-strand) (Figure 2). The mitogenome of *Z. steggerdai* has a total of 36 bp space in thirteen gene overlaps, ranging in length from 1 to 8 bp; the longest overlap region fell between tRNA-*Trp* and tRNA-*Cys* genes. In addition, there were ten 1–6 bp coding gene spacer regions, with a total length of 29 bp; the longest 6 bp intergenic spacer sequences were located between *cox3* and tRNA-*Gly*. In the *Z. dworakowskae* mitogenome, gene overlaps were found at 13 gene junctions and involved a total of 51 bp, the longest 16 bp overlapping located between *nad4* and *nad4L*. Intergenic spacer sequences were found at 12 gene junctions and involved a total of 29 bp; the longest 6 bp intergenic spacer sequences were located between *cox3* and tRNA-*Gly*. The mitogenome of *Z. heptapotamica* harbors a total of 32 bp in 12 overlapping genes (1–8 bp-long), and 11 coding gene spacer regions (1–6 bp-long) are present. The longest 8 bp overlap region fell between tRNA-*Cys* and tRNA-*Tyr* genes, and the longest 6 bp intergenic spacer sequences were located between *cox3* and tRNA-*Gly*.

The nucleotide composition of the whole mitogenome of *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica* was as follows: (A) 42.2%, 42.4%, and 42.4%; (T) 37.2%, 36.9%, and 36.9%; (G) 8.9%, 8.9%, and 9.0%; and (C) 11.7%, 11.8%, and 11.8%. The mitochondrial genomes of the three mitogenomes show heavy AT nucleotide bias, with an A + T% content

for the whole sequence of 79.4%, 79.3%, and 79.2%, respectively (Figure 3). The PCGs show the lowest A + T% among whole genes, while the control region (CR) has the strongest A + T% bias. Analysis of 37 individual genes of the three mitogenomes shows that AT skews are mostly positive, while for GC skews, all the GC skews are negative. Positive AT skews indicate that the content of base A is higher than that of base T, while a negative value indicates the opposite. Only the AT skews of CR are negative (−0.255, −0.196, and −0.148). In conclusion, the genetic composition of the three species is mostly biased towards A and C (Table 3, Figure 3).

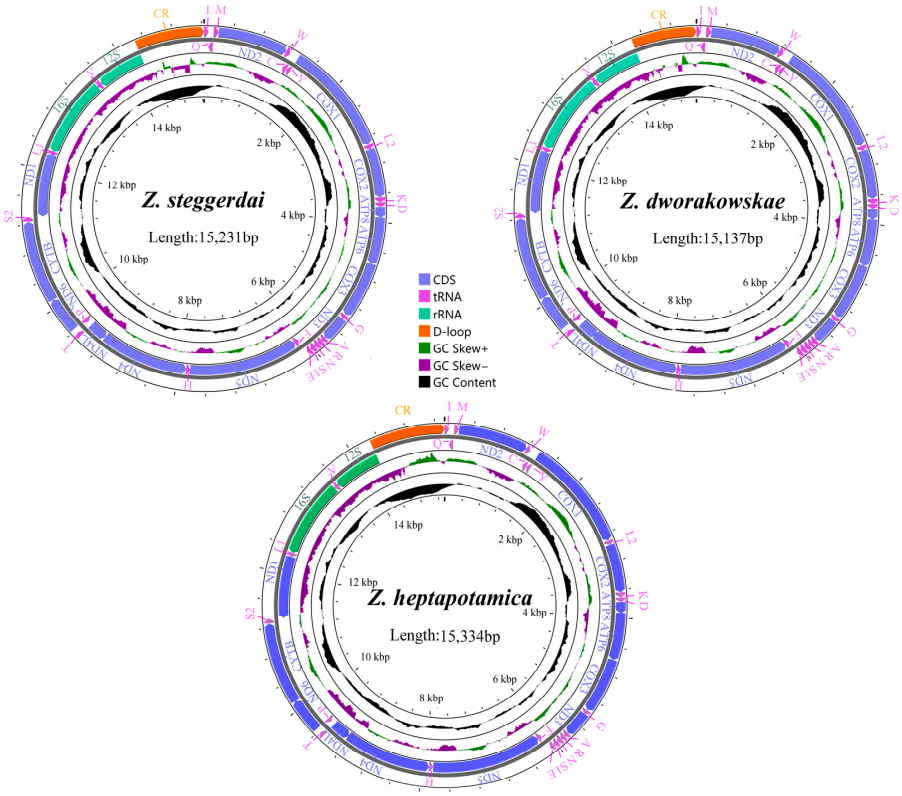

Figure 2. Circular maps of the mitochondrial genome of *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica*.

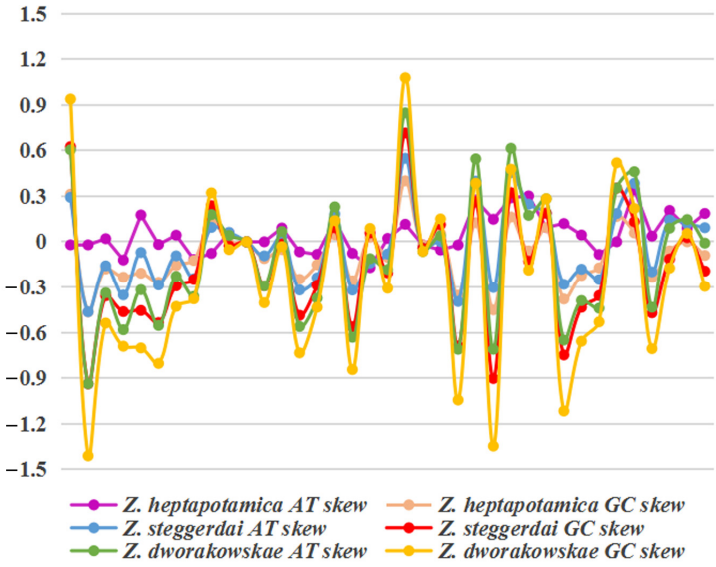

Figure 3. AT and GC skews were calculated for 37 mitochondrial genes of *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica*.

Table 2. Annotations of the mitogenomes of *Z. steggerdai* (ZS), *Z. dworakowskae* (ZD), and *Z. heptapotamica* (ZH).

Gene	Position			Size (bp)			Intergenic			Start Codon			Stop Codon			Strand
	ZS	ZD	ZH	ZS	ZD	ZH	ZS	ZD	ZH	ZS	ZD	ZH	ZS	ZD	ZH	
tRNA-*Ile*	1–64	1–64	1–64	64	64	64	0	0	0							H
tRNA-*Gln*	62–130	62–130	63–129	69	69	67	−3	−3	−2							L
tRNA-*Met*	134–202	134–202	134–202	69	69	69	3	3	4							H
nad2	203–1174	203–1174	203–1174	972	972	972	0	0	0	ATA	ATA	ATA	TAA	TAA	TAA	H
tRNA-*Trp*	1174–1237	1174–1237	1174–1237	64	64	64	−1	−1	−1							H
tRNA-*Cys*	1230–1292	1230–1292	1230–1292	63	63	63	−8	−8	−8							L
tRNA-*Tyr*	1296–1357	1296–1357	1296–1356	62	62	61	3	3	3							L
cox1	1360–2901	1360–2901	1359–2900	1542	1542	1542	2	2	2	ATA	ATA	ATA	TAA	TAA	TAA	H
tRNA-*Leu2*	2907–2971	2907–2971	2906–2970	65	65	65	5	5	5							H
cox2	2972–3650	2972–3650	2971–3649	679	679	679	0	0	0	ATT	ATT	ATT	T	T	T	H
tRNA-*Lys*	3651–3720	3651–3720	3650–3719	70	70	70	0	0	0							H
tRNA-*Asp*	3721–3785	3721–3785	3720–3784	65	65	65	0	0	0							H
atp8	3784–3936	3784–3936	3783–3935	153	153	153	−2	−2	−2	TTG	TTG	TTG	TAA	TAA	TAA	H
atp6	3933–4583	3933–4583	3932–4582	651	651	651	−4	−4	−4	ATA	ATA	ATA	TAA	TAA	TAA	H
cox3	4584–5363	4584–5363	4583–5362	780	780	780	0	0	0	ATG	ATG	ATG	TAA	TAA	TAA	H
tRNA-*Gly*	5370–5431	5370–5431	5369–5430	62	62	62	6	6	6							H
nad3	5432–5785	5432–5785	5431–5784	354	354	354	0	0	0	ATT	ATT	ATT	TAA	TAA	TAA	H
tRNA-*Ala*	5787–5847	5787–5847	5786–5846	61	61	61	1	1	1							H
tRNA-*Arg*	5847–5907	5847–5907	5846–5906	61	61	61	−1	−1	−1							H
tRNA-*Asn*	5907–5972	5907–5972	5906–5971	66	66	66	−1	−1	−1							H
tRNA-*Ser1*	5972–6038	5972–6038	5971–6037	67	67	67	−1	−1	−1							H
tRNA-*Glu*	6040–6103	6040–6102	6039–6102	64	63	64	1	1	1							H
tRNA-*Phe*	6105–6169	6104–6168	6104–6168	65	65	65	1	1	1							L
nad5	6170–7826	6169–7804	6169–7843	1657	1636	1675	0	0	0	ATT	ATT	ATT	T	T	T	L
tRNA-*His*	7824–7887	7802–7865	7841–7904	64	64	64	−3	−3	−3							L
nad4	7887–9209	7865–9202	7904–9226	1323	1338	1323	−1	−1	−1	ATA	ATT	ATA	TAA	TAA	TAA	L
nad4L	9209–9487	9187–9465	9226–9504	279	279	279	−1	−16	−1	ATG	ATG	ATG	TAA	TAA	TAA	L
tRNA-*Thr*	9490–9552	9468–9530	9507–9569	63	63	63	2	2	2							H
tRNA-*Pro*	9553–9618	9531–9596	9570–9635	66	66	66	0	0	0							L
nad6	9621–10,106	9599–10,084	9638–10,123	486	486	486	2	2	2	ATT	ATT	ATT	TAA	TAA	TAA	H
cytb	10,107–11,243	10,085–11,221	10,124–11,260	1137	1137	1137	0	0	0	ATG	ATG	ATG	TAA	TAA	TAA	H
tRNA-*Ser2*	11,247–11,310	11,225–11,288	11,264–11,327	64	64	64	3	3	3							H
Nad1	11,304–12,245	11,282–12,223	11,321–12,259	942	942	939	−7	−7	−7	ATA	ATA	ATT	TAA	TAA	TAA	L
tRNA-*Leu1*	12,243–12,307	12,221–12,285	12,260–12,324	65	65	65	−3	−3	0							L
16S	12,308–13,483	12,286–13,461	12,325–13,503	1176	1176	1179	0	0	0							L
tRNA-*Val*	13,484–13,553	13,462–13,531	13,504–13,569	70	70	66	0	0	0							L
12S	13,554–14,278	13,532–14,254	13,570–14,294	725	723	725	0	0	0							L
D-loop	14,279–15,231	14,255–15,137	14,295–15,334	953	883	1040	0	0	0							H

Table 3. Nucleotide compositions, AT skew, and GC skew in different regions of *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica* mitochondrial genomes.

Region	C%			A%			G%			T%			A + T%			AT Skew			GC Skew		
	ZS	ZD	ZH	ZS	ZD	ZH	ZS	ZD	ZH	ZS	ZD	ZH	ZS	ZD	ZH	ZS	ZD	ZH	ZS	ZD	ZH
whole	11.7	11.8	11.8	42.2	42.4	42.4	8.9	8.9	9.0	37.2	36.9	36.8	79.4	79.3	79.2	0.064	0.070	0.071	−0.134	−0.136	−0.135
PCGs	12.7	12.8	12.8	41.7	41.6	41.6	10.0	10.0	10.1	35.6	35.6	35.5	77.3	77.2	77.1	0.077	0.078	0.079	−0.119	−0.123	−0.118
tRNA	12.0	12.0	12.0	40.5	40.4	40.3	9.6	9.7	9.8	37.9	37.9	37.9	78.4	78.3	78.2	0.033	0.032	0.031	−0.110	−0.110	−0.101
rRNA	11.0	11.1	11.0	49.3	49.4	49.6	6.4	6.3	6.3	33.3	33.2	33.1	82.6	82.6	82.7	0.195	0.197	0.199	−0.269	−0.273	−0.272
CR	0.8	0.5	2.1	36.8	39.9	41.0	0.5	0.3	1.6	61.9	59.3	55.3	98.6	99.2	96.3	−0.255	−0.196	−0.148	−0.231	−0.143	−0.135

3.2. Protein-Coding Genes and Codon Usage

The three *Ziczacella* mitogenomes contained 13 PCGs; the total length is 10,955 bp, 10,949 bp, and 10,970 bp, respectively. Among the 13 protein-coding genes of *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica*, nine PCGs (*nad2*, *nad3*, *nad6*, *cox1*, *cox2*, *cox3*, *atp8*, *atp6*, and *cytb*) encode on the majority strand (H-strand), while the other four PCGs (*nad1*, *nad4*, *nad5*, and *nad4L*) encode on the minority strand (L-strand) (Table 2). The longest gene was the *nad5* gene (1657, 1636, and 1675), and the shortest was the *atp8* gene (153, 153, and 153) in *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica*, respectively. The proportion of A, T, G, and C is 41.7%, 35.6%, 10.0%, and 12.7% in *Z. steggerdai* mitogenome; 41.6%, 35.6%, 10.0%, and 12.8% in *Z. dworakowskae* mitogenome; and 41.6%, 35.5%, 10.1%, and 12.8% in *Z. heptapotamica*. The A + T content of the PCGs in *Z. steggerdai* mitogenome (77.3%) is higher than in *Z. dworakowskae* mitogenome (77.2%) and *Z. heptapotamica* mitogenome (77.1%) (Table 3).

Among the 13 protein-coding genes, the *atp8* genes started with TTG, while all other PCGs contained the usual ATN (ATA, ATT, and ATG) start codon. In *Z. steggerdai* mitogenome and *Z. heptapotamica* mitogenome, three PCGs utilize ATG (*cox3*, *nad4L*, and *cytb*), four PCGs utilize ATT (*cox2*, *nad3*, *nad5*, and *nad6*), one PCG utilizes TTG (*atp8*), and five PCGs utilize ATA (*nad1*, *nad2*, *nad4*, *atp6*, and *cox1*) as the start codon. Eleven PCGs (*nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad6*, *cox1*, *atp6*, *atp8*, *cox3*, and *cytb*) have TAA as a stop codon, whereas the *cox2* and *nad5* genes use a single T. However, in the *Z. dworakowskae* mitogenome, unlike the other two mitochondrial genomes, *nad4* is started by ATT.

The 13 PCGs in the three mitogenomes comprised 5076 codons, 5045 codons, and 5111 codons, respectively. Statistics on the available codon count and the relative synonymous codon usage (RSCU) of *Z. steggerdai* found that the most abundant codons were AAA (Lys), AAU (Asn), AUU (Ile), and AUA (Met). *Z. dworakowskae* mitogenome, where the five most frequently used codons are AAU (Asn), AAA (Lys), UAU (Tyr), UUA (Leu2), and AUA (Met). The five most frequently found codons in the mitochondrial genome of *Z. heptapotamica* were AAU (Asn), AAA (Lys), UAU (Tyr), UUA (Leu2), and AUU (Ile). Relative synonymous codon usage (RSCU) showed that the most frequently utilized amino acids are Asn, Lys, Phe, Ile, Tyr, and Met (Table 4). The majority of codons all end with A or U, which leads to the high A + T bias of the entire mitogenome (Figures 4 and 5).

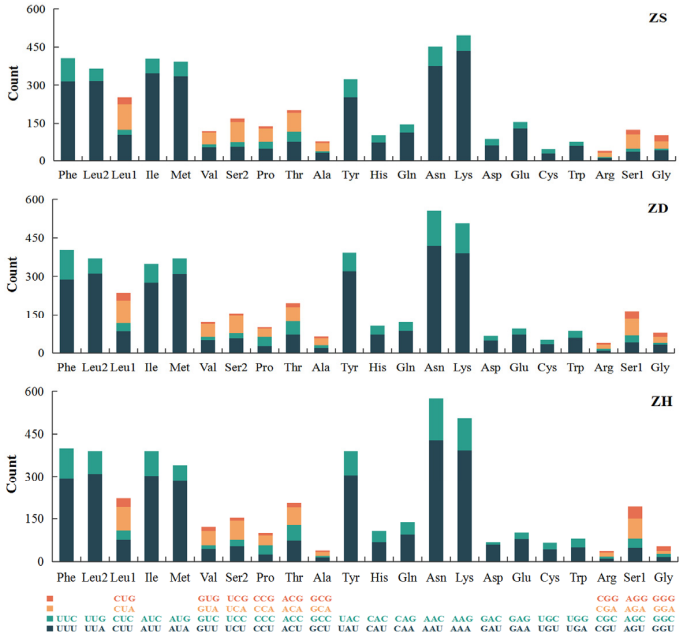

Figure 4. Count in the mitogenomes of *Z. steggerdai* (ZS), *Z. dworakowskae* (ZD), and *Z. heptapotamica* (ZH).

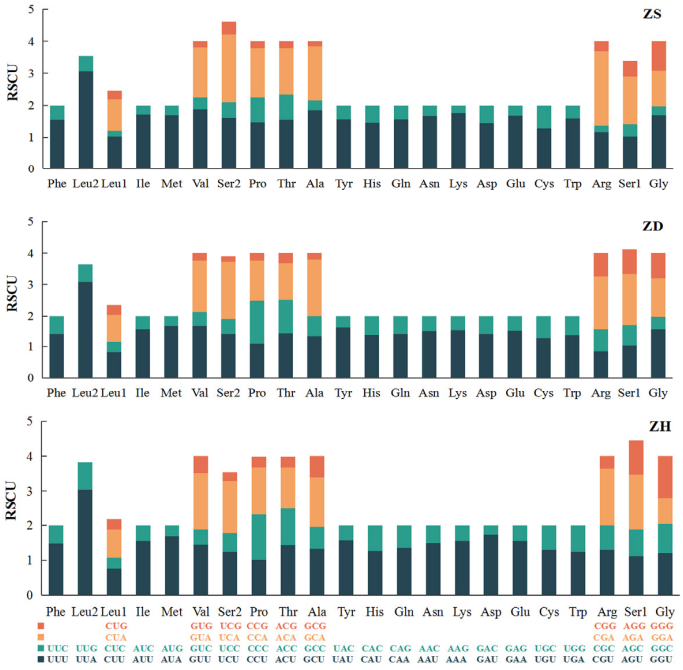

Figure 5. Relative synonymous codon usage (RSCU) in the mitogenomes of *Z. steggerdai* (ZS), *Z. dworakowskae* (ZD), and *Z. heptapotamica* (ZH).

Table 4. Codon and relative synonymous codon usage (RSCU) in the mitogenomes of three mitogenomes (* stands for stop codon).

Amino Acid	Codon	*Z. steggerdai*		*Z. dworakowskae*		*Z. heptapotamica*		Amino Acid	Codon	*Z. steggerdai*		*Z. dworakowskae*		*Z. heptapotamica*	
		Count	RSCU	Count	RSCU	Count	RSCU			Count	RSCU	Count	RSCU	Count	RSCU
Phe	UUU	315	1.55	289	1.43	294	1.47	Tyr	UAU	253	1.56	321	1.63	305	1.56
	UUC	91	0.45	114	0.57	106	0.53		UAC	72	0.44	72	0.37	85	0.44
Leu2	UUA	317	3.07	312	3.08	309	3.03	His	CAU	75	1.46	72	1.37	68	1.27
	UUG	49	0.47	58	0.57	81	0.79		CAC	28	0.54	33	0.63	39	0.73
Leu1	CUU	106	1.03	83	0.82	76	0.75	Gln	CAA	114	1.56	85	1.43	94	1.36
	CUC	19	0.18	33	0.33	33	0.32		CAG	32	0.44	34	0.57	44	0.64
	CUA	101	0.98	90	0.89	82	0.8	Asn	AAU	377	1.67	420	1.51	428	1.49
	CUG	27	0.26	31	0.31	31	0.3		AAC	75	0.33	135	0.49	147	0.51
Ile	AUU	347	1.71	276	1.58	302	1.55	Lys	AAA	436	1.75	390	1.54	392	1.55
	AUC	58	0.29	74	0.42	88	0.45		AAG	61	0.25	118	0.46	114	0.45
Met	AUA	334	1.7	310	1.68	286	1.68	Asp	GAU	64	1.44	47	1.42	58	1.73
	AUG	59	0.3	60	0.32	54	0.32		GAC	25	0.56	19	0.58	9	0.27
Val	GUU	56	1.87	50	1.68	44	1.44	Glu	GAA	130	1.68	72	1.53	79	1.55
	GUC	11	0.37	13	0.44	13	0.43		GAG	25	0.32	22	0.47	23	0.45
	GUA	47	1.57	49	1.65	50	1.64	Cys	UGU	32	1.28	33	1.27	42	1.29
	GUG	6	0.2	7	0.24	15	0.49		UGC	18	0.72	19	0.73	23	0.71
Ser2	UCU	59	1.6	57	1.43	54	1.24	Trp	UGA	63	1.59	58	1.36	50	1.23
	UCC	18	0.49	19	0.48	23	0.53		UGG	16	0.41	27	0.64	31	0.77
	UCA	78	2.12	72	1.81	66	1.52	Arg	CGU	11	1.16	8	0.84	11	1.29
	UCG	15	0.41	7	0.18	11	0.25		CGC	2	0.21	7	0.74	6	0.71
Pro	CCU	51	1.47	27	1.08	25	1.01		CGA	22	2.32	16	1.68	14	1.65
	CCC	27	0.78	35	1.4	32	1.29		CGG	3	0.32	7	0.74	3	0.35
	CCA	53	1.53	32	1.28	34	1.37	Ser1	AGU	38	1.03	41	1.03	48	1.11
	CCG	8	0.23	6	0.24	8	0.32		AGC	14	0.38	27	0.68	33	0.76
Thr	ACU	78	1.54	71	1.44	73	1.42		AGA	55	1.49	65	1.63	69	1.59
	ACC	40	0.79	53	1.08	55	1.07		AGG	18	0.49	31	0.78	43	0.99
	ACA	73	1.45	57	1.16	61	1.19	Gly	GGU	44	1.69	31	1.57	16	1.21
	ACG	11	0.22	16	0.32	16	0.31		GGC	7	0.27	8	0.41	11	0.83
Ala	GCU	35	1.84	20	1.33	13	1.33		GGA	29	1.12	24	1.22	10	0.75
	GCC	6	0.32	10	0.67	6	0.62		GGG	24	0.92	16	0.81	16	1.21
	GCA	32	1.68	27	1.8	14	1.44	*	UAA	324	1.69	351	1.65	365	1.66
	GCG	3	0.16	3	0.2	6	0.62		UAG	59	0.31	75	0.35	74	0.34

3.3. Transfer and Ribosomal RNA Genes

The mitogenomes of *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica* included 22 transfer RNA genes, as in most invertebrates [46,47], of which 14 are encoded in the major strand (H-strand), while the other eight are encoded in the minor strand (L-strand) (Table 2). Their nucleotide lengths ranged from 61 (Ala, Arg) to 70 bp (Lys, Val), and the total lengths of tRNA genes were 1429 bp, 1428 bp, and 1422 bp, respectively. The tRNA of the three species has a positive AT and negative GC skew; the AT skew of 22 tRNA is positive, and the GC skew is positive. Compared to the conventional insect mitochondrial gene order, no tRNA gene rearrangements are found. All of the tRNA genes can be folded into typical cloverleaf secondary structures except for tRNA-*Ser1* in three newly sequenced mitochondrial genomes, which lack the dihydrouridine (DHU) stem and form a simple loop [48,49]. It can be clearly seen from the secondary structure of *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica* that, in addition to the typical Watson–Crick pairings (A-U and G-C), there are also some typical pairings such as U-G, and a total of 14, 16, and 16 G-U weak base pairs are found, respectively (Figures 6–8).

The two ribosomal RNAs (*12S* and *16S* ribosomal RNA) are separated by tRNA-*Val*. The three *Ziczacella* mitogenomes and the 16s rRNA gene (*Z. steggerdai*: 1176 bp, *Z. dworakowskae*: 1176 bp, and *Z. heptapotamica*: 1179 bp) are located between tRNA-*Leu1* and tRNA-*Val*; the *12S* rRNA gene (*Z. steggerdai*: 725 bp, *Z. dworakowskae*: 723 bp, *Z. heptapotamica*: 725 bp) is between tRNA-*Val* and the A + T rich region. Both rRNA genes are encoded on the L-strand. The rRNA genes displayed a positive AT skew and a negative GC skew, with an A + T content of 82.6% in *Z. steggerdai*, 82.6% in *Z. dworakowskae*, and 82.7% in *Z. heptapotamica*.

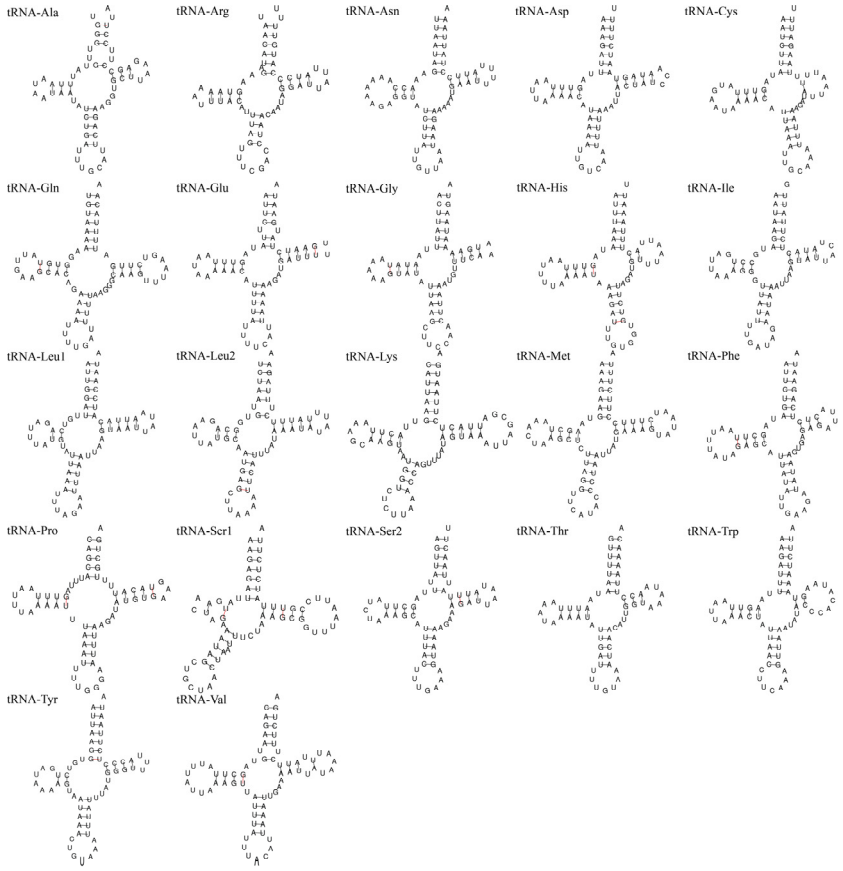

Figure 6. Predicted secondary cloverleaf structure for the tRNAs of *Z. steggerdai*.

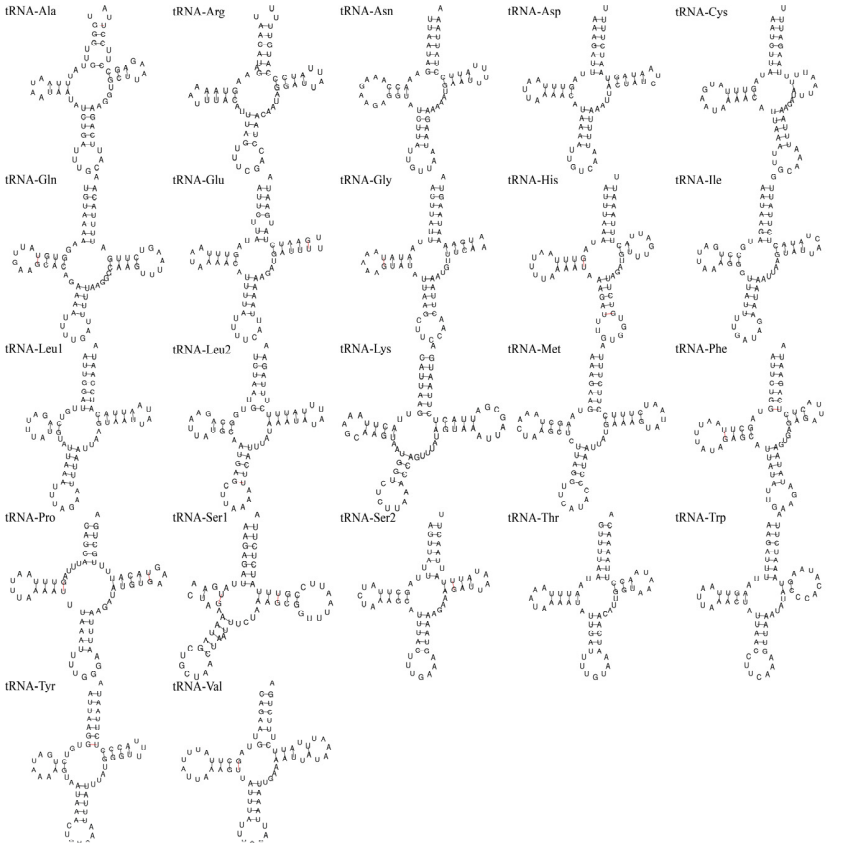

Figure 7. Predicted secondary cloverleaf structure for the tRNAs of *Z. dworakowskae*.

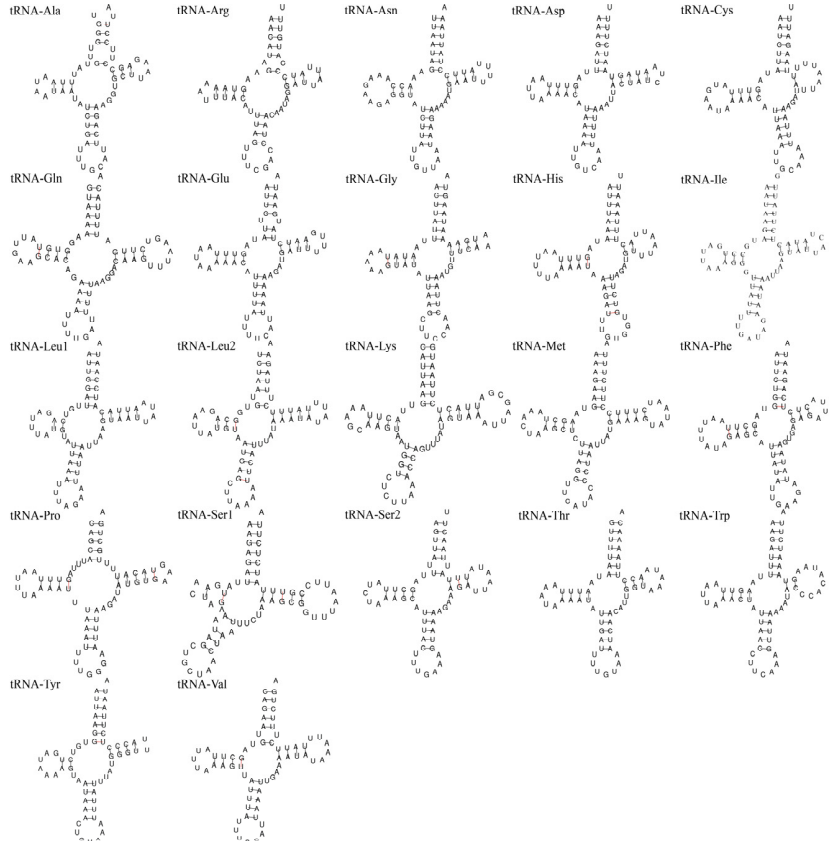

Figure 8. Predicted secondary cloverleaf structure for the tRNAs of *Z. heptapotamica*.

3.4. Control Region

Like the typical insect mitochondrial genome, *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica* have a control region, which is located between *12S rRNA* and tRNA-*Ile*, which is the longest in the mitogenomes. The nucleotide composition of the A + T rich region of the three mitogenomes is as follows: *Z. steggerdai* 98.6%; *Z. dworakowskae*: 99.2%; and *Z. heptapotamica*: 96.3%, respectively (Table 3). The AT content in this region was the highest in the whole mitochondrial genome.

3.5. Phylogenetic Relationships

In this study, the phylogenetic tree was constructed based on 13 PCGs and 2 rRNAs from 28 species of Typhlocybinae (Alebrini, Dikraneurini, Empoascini, Erythroneurini, Typhlocybini, and Zyginellini) and two outgroups. Phylogenetic analysis showed that *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica* are sister groups with *Mitjaevia dworakowskae* and *Mitjaevia shibingensis*. The result yielded the following topology: (Empoascini + Alebrini) + ((Erythroneurini + Dikraneurini) + (Zyginellini + Typhlocybini)) (Figure 9). Empoascini, Dikraneurini, Alebrini, and Erythroneurini are identified as monophyletic, with the sister group of Alebrini and Empoascini as the base branch, followed by the sister group of Erythroneurini and Dikraneurini, and finally, the species of Zyginellini and Typhlocybini belonging to the same branch.

In previous studies, Typhlocybinae was often divided into six monophyletic tribes: Alebrini, Dikraneurini, Empoascini, Erythroneurini, Typhlocybini, and Zyginellini [50]. Zyginellini was proposed by Dworakowska in 1979, and its morphological difference from Typhlocybini is the hind wing vein CuA connected to vein MP. Maximum likelihood (ML) and Bayesian inference (BI) are highly supported and have consistent topologies in most phylogenetic analyses. Both methods support that Typhlocybini and Zyginellini merge into a single branch, and the two tribes should be treated as synonyms, because their species are so intertwined that they are hard to separate from each other. The results are

slightly different from the traditional classification system, but similar to the results of most phylogenetic relationships of Typhlocybinae based on molecular data. We support the view proposed by Dietrich (2013); that is, Zyginellini may not be a monophyletic group and should be merged with Typhlocybini [9]. The Typhlocybine species are very rich, so like most phylogenetic studies based on molecular data, this study is only based on a small number of species to explore the phylogenetic status of newly sequenced species. However, more sequencing data of leafhoppers are needed to construct a more complete phylogenetic tree in order to clarify the relationship between the tribes of Typhlocybinae.

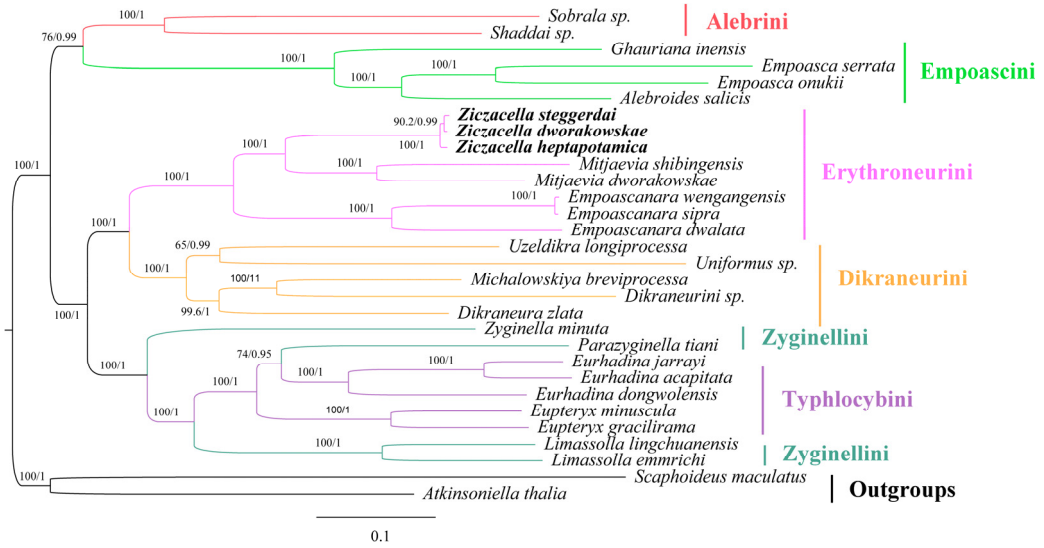

Figure 9. Maximum likelihood (ML) and Bayesian inference (BI) phylogenetic tree for three newly sequenced species based on 13 PCGs and 2 rRNAs of Typhlocybinae. The first number is a bootstrap proportion (BP) of maximum likelihood (ML) analyses, and the second number at each node is Bayesian posterior probabilities (PP).

4. Conclusions

In summary, the complete mitochondrial genome sequences of *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica* were sequenced for the first time. We analyzed the basic composition, location, and other characteristics of PCGs, tRNA genes, rRNA genes, and control regions and further elucidated the relationship between them and other species in Typhlocybinae. They are close to most other sequenced leafhoppers in structures and compositions. In addition, based on the mitochondrial gene sequences of 30 leafhopper species, a phylogenetic tree was established by the maximum likelihood method and Bayesian method. The result showed that this collection of *Z. steggerdai*, *Z. dworakowskae*, and *Z. heptapotamica* is a sister group to the collection of *Mitjaevia dworakowskae* and *Mitjaevia shibingensis*. Meanwhile, Alebrini, Dikraneurini, Empoascini, and Erythroneurini are proven again as monophyletic, while Zyginellini and Typhlocybini should be gathered into a single branch. The results of this study confirm that Zyginellini is a junior synonym of Typhlocybini; that is, the two tribes should be combined and placed into the same taxon as a monophyletic group.

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