

# Mitogenomic phylogeny of Typhlocybae (Hemiptera: Cicadellidae) reveals homoplasy in tribal diagnostic morphological traits

Bin Yan<sup>1</sup>, Christopher Dietrich<sup>2</sup>, Xiaofei Yu<sup>1</sup>, Meng Jiao<sup>3</sup>, Renhuai Dai<sup>1</sup>, and Maofa Yang<sup>1</sup>

<sup>1</sup>Guizhou University

<sup>2</sup>Illinois Natural History Survey

<sup>3</sup>Shandong Museum

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## Abstract

The subfamily Typhlocybae is a ubiquitous, highly diverse group of mostly tiny, delicate leafhoppers. The tribal classification has long been controversial and phylogenetic methods have only recently begun to test the phylogenetic status and relationships of tribes. To shed light on the evolution of Typhlocybae, we performed phylogenetic analyses based on 28 newly sequenced and 19 previously sequenced mitochondrial genomes representing all currently recognized tribes. The results support the monophyly of the subfamily and its sister group relationship to Mileewinae. The tribe Zyginellini is polyphyletic with some included genera derived independently within Typhlocybini. Ancestral character state reconstruction suggests that some morphological characters traditionally considered important for diagnosing tribes (presence/absence of ocelli, development of hind wing submarginal vein) are homoplastic. Divergence time estimates indicate that the subfamily arose during the Middle Cretaceous and that the extant tribes arose during the Late Cretaceous. Phylogenetic results support establishment of a new genus, *Subtilissimia* Yan & Yang **gen. nov.**, with two new species, *Subtilissimia fulva* Yan & Yang **sp. nov.** and *Subtilissimia pellicula* Yan & Yang **sp. nov.** (Typhlocybae: Typhlocybini); but indicate that two previously recognized species of *Farynala* distinguished only by the direction of curvature of the processes of the aedeagus are synonyms, i.e., *Farynala dextra* Yan & Yang, 2017 equals *Farynala sinistra* Yan & Yang, 2017 **syn. nov.** A key to tribes of Typhlocybae is provided.

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<sup>1</sup>Institute of Entomology, Guizhou University, Guiyang, Guizhou, 550025, China; <sup>2</sup>College of Tobacco Science, Guizhou University, Guiyang, Guizhou, 550025, China; <sup>3</sup>Shandong Museum, Jinan, Shandong, 250014, China; <sup>4</sup>Illinois Natural History Survey, Prairie Research Institute, University of Illinois, Champaign, IL, U.S.A.

**Running title:** Mitogenome-based phylogeny of Typhlocybae

**Correspondence:** Maofa Yang, Institute of Entomology, Guizhou University, Guiyang, Guizhou, 550025, China; College of Tobacco Science, Guizhou University, Guiyang, Guizhou, 550025, China. Tel: +86 0851–88292117; e-mail: mfyang@gzu.edu.cn.

## ABSTRACT

The subfamily Typhlocybae is a ubiquitous, highly diverse group of mostly tiny, delicate leafhoppers. The tribal classification has long been controversial and phylogenetic methods have only recently begun to test

the phylogenetic status and relationships of tribes. To shed light on the evolution of Typhlocybinae, we performed phylogenetic analyses based on 28 newly sequenced and 19 previously sequenced mitochondrial genomes representing all currently recognized tribes. The results support the monophyly of the subfamily and its sister group relationship to Mileewinae. The tribe Zyginellini is polyphyletic with some included genera derived independently within Typhlocybini. Ancestral character state reconstruction suggests that some morphological characters traditionally considered important for diagnosing tribes (presence/absence of ocelli, development of hind wing submarginal vein) are homoplastic. Divergence time estimates indicate that the subfamily arose during the Middle Cretaceous and that the extant tribes arose during the Late Cretaceous. Phylogenetic results support establishment of a new genus, *Subtilissimia* Yan & Yang **gen. nov.**, with two new species, *Subtilissimia fulva* Yan & Yang **sp. nov.** and *Subtilissimia pellicula* Yan & Yang **sp. nov.**; but indicate that two previously recognized species of *Farynala* distinguished only by the direction of curvature of the processes of the aedeagus are synonyms, i.e., *Farynala dextra* Yan & Yang, 2017 equals *Farynala sinistra* Yan & Yang, 2017 **syn. nov.** A key to tribes of Typhlocybinae is provided.

**KEYWORDS** classification; high-throughput sequencing; Membracoidea; microleafhopper; mitochondrial genes; phylogenetic analysis

## INTRODUCTION

The subfamily Typhlocybinae (Hemiptera, Membracoidea, Cicadellidae) is a large group of mostly tiny, delicate leafhoppers that feed primarily on leaf parenchymal cell contents of their host plants, thus differing from the phloem- or xylem-feeding (Cicadellinae) preferences exhibited by most other leafhoppers (Dietrich, 2013a; Bartlett et al., 2018). This group is distributed worldwide and comprises numerous agricultural pests (Nault & Ammar, 1989; Sun, 2004; Wearing et al., 2011; Shi et al., 2018). For example, the nymphs and adults of *Empoasca fabae* and *Matsumurasca onukii* attack crops of potato and tea, respectively (Backus et al., 2005; Chasen et al., 2014). However, the vast majority of species, which feed on a wide variety of plants, appear to be of little or no economic importance. The group remains poorly studied, partly because their delicate nature makes them difficult to preserve for morphological studies. Based on the large number of described species (approximately 5000), Typhlocybinae is the second largest cicadellid subfamily (after Deltocephalinae Bartlett et al., 2018) but the actual diversity of the group is probably much higher (Dietrich, 2013a). Typhlocybinae is readily distinguished from other leafhoppers by the following morphological characters: forewing fully developed without closed anteapical cells; hind tarsomere I acuminate, without transverse row of blunt setae (Dietrich, 2005).

Since Kirschbaum (1868) first recognized the subfamily, various authors employed different concepts of Typhlocybinae and its tribes (Table S1), with as few as four and as many as eleven tribes recognized (Metcalf, 1968; Mahmood & Ahmed, 1968; Dworakowska, 1979; Ahmed, 1983; Oman et al., 1990; Hamilton, 1998; Gebicki & Szewo, 2006; Dietrich, 2013a; Dietrich et al., 2017). Dworakowska's (1979) six-tribe typhlocybinae classification including Alebrini, Empoascini (synonym, Jorumini and Helionini), Erythroneurini (with synonym Bakerini), Dikraneurini (with synonym Forcipatini), Typhlocybini (with synonym Eupterygini) and Zyginellini has been followed by most recent authors. However, the status of Zyginellini has remained controversial, with recent authors regarding it either as a synonym of Typhlocybini (Ahmed 1984, Balme, 2007; Dietrich, 2013a; Zhou et al., 2020), or as an independent and valid tribe (Zhang, 1990; Song, 2010; Dietrich et al., 2017; Lu et al., 2021).

The current tribal classification of Typhlocybinae is based almost entirely on a few characters of the wing venation. Compared with other cicadellid tribes, typhlocybines have the venation of the fore- and hind wings relatively reduced. Thus, particular patterns of reduction and loss or consolidation of veins have traditionally been used to define the different tribes. Alebrini, the tribe traditionally considered to be the most "primitive", is the only tribe with an appendix on the forewing, a trait shared with non-typhlocybinae leafhoppers. Empoascini lack the section of the hind wing submarginal vein that extends along the costal margin. Erythroneurini and most Typhlocybini have the hind wing submarginal vein completely lacking at the wing apex. Erythroneurini and some Dikraneurini have hind wing vannal vein unbranched but Dikraneurini retain a complete submarginal vein.

Young (1965) transferred Mileewini (including genera *Amahuaka* and *Ujna* ) into Typhlocybinae based on intuitive morphological criteria but he later suggested that Mileewinae should be treated as a separate subfamily (Young, 1968), a classification that has been followed by most subsequent authors. Phylogenetic analyses of Membracoidea based on morphology and DNA sequence data yielded inconsistent results. The concatenated ML analysis of anchored-hybrid data by Dietrich et al. (2017) placed Eurymelinae (sensu lato) as sister to Typhlocybinae but with only 54% bootstrap support. An earlier morphology-based analysis of Cicadellidae (Dietrich 1999) placed Mileewinae (in part, Mileewini) as sister to Typhlocybinae but the first molecular phylogeny of this family (Dietrich et al., 2001) did not consistently recover Typhlocybinae as monophyletic and its relationships to other subfamilies were also inconsistently resolved. A subsequent combined analysis of morphological and 28S rDNA sequence data supported the monophyly of Typhlocybinae and its sister relationship to Mileewini (Dietrich et al., 2005). This sister-group relationship was also recovered in the recent multi-locus analysis of Lu et al. (2021) with moderate ML bootstrap support.

Most prior research on typhlocybine systematics has focused on species descriptions. New typhlocybine taxa continue to be described at a rapid pace, but the phylogeny of the group remains little explored. Previous analyses have supported the monophyly of Typhlocybinae but its sister-group has remained uncertain. Zhang (1990) proposed an intuitive morphology-based hypothesis of tribal relationships within Typhlocybinae, considering Alebrini to be the earliest diverging lineage based on the retention of an appendix (shared with other leafhoppers) in the forewing, with Dikraneurini sister to Empoascini based on the relatively well-developed submarginal vein (smv) of the hind wing. In an unpublished dissertation, Balme (2007) conducted the first explicit cladistics analysis of the group, using 73 discrete morphological characters and two molecular markers (16S rRNA and Histone 3) and proposed a classification including four tribes, Alebrini + (Typhlocybini + Empoascini) + Dikraneurini, with Erythroneurini treated as a synonym of the latter tribe. The anchored hybrid phylogenomic analysis of Membracoidea based on 388 genetic loci and more than 99,000 aligned nucleotides (Dietrich et al., 2017) included only 1-2 representatives of each tribe but recovered Alebrini as sister to Empoascini and this clade sister to a clade comprising Typhlocybini and Erythroneurini + Dikraneurini with strong support. This dataset did not include a taxon sample large enough to test the monophyly of individual tribes or examine relationships among genera within tribes. Most recently, Lu et al. (2021) analyzed a much larger sample of typhlocybine taxa using data from fragments of three nuclear and two mitochondrial genes, recovering the same tribal relationships found by Dietrich et al. (2017) and also recovering Zyginellini as sister to Typhlocybini but with low support.

Here we use sequence data from complete mitochondrial genomes in an attempt to improve resolution of phylogenetic relationships within Typhlocybinae and examine the evolution and stability of wing characters traditionally used for the classification of typhlocybine tribes. A total of 110 leafhopper mitochondrial genome assemblies have been previously deposited in GenBank. Among them, only 19 typhlocybine species are included representing the tribes Typhlocybini, Empoascini, Erythroneurini and Zyginellini. Data for species of Alebrini and Dikraneurini were not previously available. Therefore, prior to our study, mitogenome data for Typhlocybinae remained insufficient to facilitate a comprehensive phylogenetic analysis of the subfamily.

For this study, we assembled and annotated 28 new typhlocybine mitogenomes using next-generation sequencing (NGS) data, and conducted a comprehensive phylogenetic analysis to examine relationships among major lineages of the subfamily. In addition, we performed ancestral character state reconstruction (ACSR) to examine the evolution of key morphological characters. We also used molecular divergence time methods to estimate the times of origin of various typhlocybine clades.

## MATERIALS AND METHODS

### Taxon sampling and DNA extraction

In this study, single individuals of 28 typhlocybine species were newly sequenced, including one representative of Alebrini, three from Dikraneurini, three from Zyginellini and 21 from Typhlocybini. Vouchers are deposited at the Institute of Entomology, Guizhou University, Guiyang, China (GUGC). Nineteen additional mitogenome sequences were obtained from GenBank. The 47 mitogenomes cover all currently recognized

typhlocybinae tribes (*sensu* Dworakowska, 1979). Based on results of prior analyses of Membracoidea, we selected representatives of Mileewinae (three species), Cicadellinae (five species) and Evacanthinae (two species) as outgroups (Balme, 2007; Takiya, 2007; Dietrich et al., 2010; Dietrich, 2013b). Included taxa including voucher number, GenBank accession number, and collection locality are listed in Table S2. All specimens were identified to species before DNA extraction. Genomic DNA was extracted from the legs and from the thoracic muscle tissue using the DNeasy Blood and Tissue kit (Qiagen, Germany) following the animal tissue protocol.

#### Mitogenome sequencing, assembly and annotation

Illumina TruSeq libraries were prepared with an average insert size of 300 bp and sequenced on the Illumina NovaSeq 6000 platform (Beijing Berry Bioinformatics Technology Co., Ltd, China) generating 150 bp paired-end reads. The mitochondrial genome of typhlocybinae species was assembled with NOVOPlasty v2.7.0 (Dierckxsens et al., 2017), using COI sequences (MN661136, MN699874, MG397188, KY039138, MT488436 and NC\_046037) as seeds and the K-mer value set to 39. Annotation and visualization of the mitogenomes was accomplished with MitoZ v1.04 (Meng et al., 2019). Mitogenome sequences with low-quality assembly results were submitted to MITOS WebServer (Bernt et al., 2013), under default settings and the invertebrate genetic code. Gene boundaries were defined using the ARWEN v1.2 (Laslett & Canbäck, 2008), and Geneious Prime (Kearse et al., 2012). The 28 newly assembled mitogenome sequences were deposited in GenBank (Table S2).

#### Phylogenetic analyses

The nucleotide sequences of 13 protein-coding genes (PCGs) and two ribosome rRNAs (12S rRNA + 16S rRNA) and amino acids (13 PCGs) were aligned using MAFFT v7.394 (Katoh & Standley, 2013) with the highly accurate L-INS-I strategy, trimmed using trimAl v1.4.1 (Capella-Gutiérrez et al., 2009) with the heuristic method ‘automated1’ to remove gap-only and ambiguous-only positions, and concatenated using FASconCAT-G v1.04 (Kück & Longo, 2014). Finally, we generated three matrices for the tree inference: (1) amino acids sequence with the 13 protein-coding genes (PCGs\_faa); (2) nucleotide sequence of 13 protein code genes with the third codon excluded (PCG12\_fna); (3) nucleotide sequence of PCG12\_fna plus the two ribosomal RNAs (PCG12\_fna plus two rRNAs). Third codon positions were excluded from the nucleotide-based analyses to reduce the possibility of bias or long-branch attraction due to substitution saturation among species belonging to different genera. We applied both partitioned and non-partitioned approaches for phylogenetic inference. Partitioned maximum likelihood reconstructions were performed using IQ-TREE v1.6.3 (Nguyen et al., 2015) with 1,000 ultrafast bootstrap (UFBoot) (Hoang et al., 2018) and 1,000 SH-aLRT replicates (Guindon et al., 2010). The option ‘-m MFP+MERGE’ was performed in all three matrices. Non-partitioned reconstructions were made using site heterogeneous models in both maximum likelihood (ML) and Bayesian inference (BI). Posterior mean site frequency (PMSF) model (Wang et al., 2018) was used for the PCGs\_faa matrix by specifying a profile mixture model with the option ‘-mtInv+C60+FO+R’ in IQ-TREE. The corresponding partitioned tree (PCGs\_faa matrix) was treated as an initial guide tree. Bayesian inference using PhyloBayes MPI v1.8b (Lartillot et al., 2013) was performed for the PCGs\_faa matrix as well. Two separate chains were independently run for 10,000 generations under the CAT+GTR model (Lartillot & Philippe, 2004) using a starting tree derived from PMSF ML analyses. We used the program bpcomp (maxdiff value) and tracecomp (minimum effective size) to check for convergence, that is, when the maxdiff value is smaller than 0.3 and minimum effective sizes are larger than 50. In addition, pairwise distances (p-distance) using the nucleotide sequence of 13 PCGs are shown in Table S3.

#### Tree topology comparison

Three aspects of typhlocybinae phylogeny that have been previously inferred controversial were tested under a likelihood theory framework: a, the sister-group relationship (Cicadellinae, Typhlocybinae); b, the sister-group relationship (Typhlocybini, Zyginellini); c, ((Empoascini, Alebrini), (Typhlocybini, (Erythroneurini, Dikraneurini))). Approximately unbiased (AU) (Shimodaira, 2002), bootstrap proportion (BP) (Kishino et al., 1990), expected likelihood weight (ELW) (Strimmer, 2002), Kishino-Hasegawa (KH) test (Kishino, 1989), Shimodaira-Hasegawa (SH) (Shimodaira & Hasegawa, 1999), Weighted KH (WKH) and weighted SH

(WSH) tests were performed in IQ-TREE v.1.6.3 with the options: -au, -zb and -zw. The number of RELL replicates was specified as 10,000. Probability values ( $p$ -value) of the AU test smaller than 0.05 indicate that the hypothesis was rejected.

#### Ancestral character state reconstruction

Ancestral states were reconstructed for the following eight morphological characters that have been used previously to infer evolutionary trends within the group and to define tribes: a, ocelli (absent or present); b, forewing appendix (absent or present); c, forewing closed anteapical cells (absent or present); d, hind wing anal vein (branched or unbranched); e, hind wing with distal extension of CuA vein beyond submarginal vein (beyond submarginal vein or not); f, hind wing CuA vein (bifurcated near apex or not); g, hind wing RP and MA vein (connected by crossvein or confluent); h, hind wing submarginal vein apparently connected directly to CuA vein (absent or present). The eight characters were coded for terminal (tip) taxa as shown in Table S4.

The condition of character f is uncertain for species previously placed in Zyglinellini because the curved vein connected below the main stem of CuA could be interpreted either as a branch of CuA or as part of the submarginal vein, so this character was scored as uncertain (?) for the 6 included Zyglinellini species (Table S4). The description of each character and its states followed Dietrich (2013a) and Dworakowska (1993). Ancestral character state reconstruction (ACSR) was performed with a maximum likelihood approach using a single-rate Mk1 model in MESQUITE v3.20 (Maddison & Maddison, 2017). To account for phylogenetic uncertainty, we traced character history on 10,000 sampled posterior Bayesian trees and summarized results on the BI consensus tree. In addition, we also traced character history and summarized results on the ML tree based on the matrix of the PCG12.fna.

#### Divergence time estimates

The MCMCTREE program of PAML v4.9 package (Yang, 2007) was used to estimate divergence times with the approximate likelihood calculation method based on five fossil calibration points. These included an internal calibration for Dikraneurini (minimum age) based on a fossil from Dominican amber and three additional calibrations providing minimum age estimates for the outgroups Mileewini, Evacanthinae, and Cicadellinae (Table 1). The age of the root node was constrained based on the more comprehensive molecular timetree of Membracoidea provided by Dietrich et al. (2017). The input tree was retrieved from the PMSF topology. Two priors for the rgene gamma and sigma 2 gamma were set to (2, 20, 1) and (1, 10, 1), respectively. We used an independent rates model and alpha of 0.5. The first  $10^4$  iterations were discarded as burn-in, and 10,000 samples were sampled every 5 iterations.

#### Morphology

The length of the body reported in the descriptions includes the forewings at rest. Male genitalia were prepared using the techniques described by Oman (1949). Morphological terminology follows Dietrich (2005, 2011) and Dworakowska (1993). The genital segments of the specimens examined were macerated in 10% NaOH for 7–10 hours (or boiled for 1–3 minutes), then transferred to glycerin after rinsing in distilled water to remove traces of NaOH (10%) for further research. Male genitalia were drawn using a LEICA m125 microscope and drawings were processed using Adobe Illustrator CS6. Habitus photographs were taken with a KEYENCE VHX-6000 digital camera and optimized with Adobe Photoshop CS6. All specimens studied are housed in the Institute of Entomology, Guizhou University, Guiyang, China (GUGC).

## RESULTS

#### Mitogenome features

Newly sequenced mitochondrial genomes of 28 species representing 21 genera in the subfamily Typhlocybinae ranged from 14,396 bp to 16,931 bp in length, including 37 typical insect mitochondrial genes and a control region (Table S2). Mitochondrial gene arrangement is highly conservative and consistent with the hypothetical ancestral insect (Figure 1, a), except in the newly sequenced *Shaddai* sp. (GenBank, MW284820),

which has the gene *trnW* translocated behind *trnY*, with 691 bp and 460 bp intergenic spacers between *trnI* and *trnQ*, *trnY* and *trnW*, respectively (dashed line box in Figure 1, b). This is the first report of a mitochondrial gene rearrangement within this subfamily.

## Phylogeny

The aligned amino acid dataset (PCGs\_faa) included 3,467 sites. The aligned nucleotide sequence datasets included 6,934 sites (PCG12\_fna) and 9,218 sites (PCG12\_fna+2rRNAs), respectively. A total of five phylogenetic trees were generated, including one Bayesian inference tree (maxdiff value = 0.11, minimum effective sizes > 58) (Figure S1) and four maximum likelihood trees (Figure S2–S5). As expected, all phylogenetic analyses robustly supported the monophyly of Typhlocybinae with high nodal support values (PP=1, SH-aLRT=100, UFB=100) (Figure 2). Unlike the ML trees (Figure 2, c, e), which support the subfamilies Cicadellinae and Typhlocybinae as sister group, the other three phylogenetic trees consistently support that the latter as sister to Mileewinae (Mileewini) (Figure 2, a, b, d). However, these alternative phylogenetic arrangements received low to moderate nodal support. In tribal relationships of Typhlocybinae, the sister-group pairs Dikraneurini+Erythroneurini (Figure 3, b) and Alebrini+Empoascini (Figure 3, e) were recovered with moderate to strong nodal support. However, the results differed in the placement of Typhlocybini, with some analyses placing this tribe sister to Alebrini+Empoascini (Figure 2, a, b) and others placing Typhlocybini sister to Dikraneurini+Erythroneurini (Figure 2, c, d, e). The included representatives of Zyginellini (Figure 3, in bold) were never recovered as a monophyletic group but were derived from within Typhlocybini, consistent with the five tribe classification adopted by some previous authors (Ahmed, 1983; Dietrich, 2013a; Zhou et al., 2020). *Zyginella*, the type genus of Zyginellini, and *Limassolla* were consistently placed as sister to the rest of Typhlocybini with strong nodal support, but other included genera of Zyginellini (*Yangisunda* and *Paraahimia*) occupied more derived positions within Typhlocybini with low to moderate nodal support (Figure 3). In addition, the relationships of several deep internal nodes within Typhlocybini were unstable (Figure S1–S5).

Statistical tests of alternative tree topologies rejected hypotheses a (sister group relationship of Cicadellinae and Typhlocybinae) and b (sister group relationship of Typhlocybini and Zyginellini) but failed to reject either alternative hypothesis of the relationship of Typhlocybini to the other tribes (Table 2). Thus, the placement of Typhlocybini must be considered equivocal according to our results. The recent analysis of Lu et al. (2021) based on sequence data from 5 genes recovered Typhlocybini as sister to Dikraneurini+Erythroneurini but with only low to moderate nodal support. All analyses consistently supported the monophyly of the four tribes for which more than one exemplar was included: Dikraneurini, Empoascini, Erythroneurini, Typhlocybini (sensu lato, including Zyginellini). Zyginellini was consistently polyphyletic with the four included genera each representing an independent branch (Figure 3). Many branches pertaining to relationships within tribes were consistently well supported in all analyses but some were unresolved, particularly within Typhlocybini; for example, relationships among four major lineages of Typhlocybini were resolved inconsistently across analyses and form a polytomy in the Bayesian consensus tree (Figure 3).

The phylogenetic results, combined with evidence from morphological characters (see below) and genetic distances (Table S3) support the establishment of a new genus, *Subtilissimia* Yan & Yang **gen. nov.**, including two new species: *Subtilissimia fulva* Yan & Yang **sp. nov.** and *Subtilissimia pellicula* Yan & Yang **sp. nov.**, but suggest that the species *Farynala sinistra* Yan & Yang, 2017 and *Farynala dextra* Yan & Yang, 2017, which have the male aedeagus identical but with processes curved in opposite directions (Yan & Yang, 2017, Figs 13–32), should be treated as synonyms (p-distance < 0.7%, except COX3 gene), *Farynala sinistra* Yan & Yang, 2017 **syn. nov.** The previously sequenced species identified as “*Typhlocyba* sp.” (GenBank, KY039138) belongs to the genus *Aguriahana* Distant, 1918 (COX1 p-distance=1.2%; PP=1, SH-aLRT=100, UFB=100). Based on previous study (Qin et al., 2015; Yu et al., 2015) and analyses of phylogeny and genetic distance, we also show that the species previously identified as “*Empoasca vitis* (GenBank, NC-024838)” probably equals *Matsumurasca onukii* (COX1 p-distance = 0.3%). Other recent studies have shown that the latter species, a major pest of tea, has been widely misidentified in China (Qin et al., 2015). Unfortunately, we were unable to check voucher specimens from previous studies in order to confirm the

identifications suggested by our results.

### Ancestral character state reconstruction

Eight typhlocybinae morphological characters were traced over the BI consensus tree and ML tree (PCG12-fna). Overall, however, these reconstructions indicate that some characters used previously to diagnose tribes of Typhlocybinae exhibit considerable homoplasy (Figure 4; Figure S6). Ocelli were retained by Alebrini and Emposcini and lost in the common ancestor of Dikraneurini, Erythroneurini and Typhlocybini. However, a reversal to the ancestral state occurred in Typhlocybini corresponding to the genus *Hiratettix* (Figure 4, a). The forewing appendix is present in most non-typhlocybinae leafhoppers but absent in all Typhlocybinae except Alebrini; thus Alebrini has traditionally been viewed as the most “primitive” group of Typhlocybinae (Figure 4, b). However, the recovered sister-group relationship between Alebrini and Emposcini suggests that the appendix may either have been lost independently in Emposcini and the remaining tribes or lost in the common ancestor of Typhlocybinae and regained in Alebrini. The bifurcation of hind wing vein CuA vein, shared with the outgroup, was lost independently in Emposcini and Typhlocybini (Figure 4, f). The distal part of the hind wing submarginal vein (Figure 4, e) was lost independently in Erythroneurini and Typhlocybini and hind wing veins RP and MA (Figure 4, g) became confluent and separated multiple times independently within Typhlocybini. The unique condition of the submarginal vein connecting to CuA directly traditionally used to diagnose Zyginellini as a distinct tribe was acquired independently in three different lineages of Typhlocybini (sensu lato) (Figure 4, h). Except for the single derivation of the latter “Zyginellini” character state in the analysis of Lu et al. (2021) our results are consistent with this prior study.

### Divergence time estimates

A chronogram for Typhlocybinae divergence dates and topologies based on whole mitochondrial protein coding genes is shown in Figure 5. According to this result, Typhlocybinae arose ~118 million years ago (MYA) (94–142 MYA 95% HPD), during the middle Cretaceous period. Divergence of major lineages within Typhlocybinae was estimated to have begun ~99 MYA (78–119 MYA 95% HPD) with all tribes having first appeared during the Late Cretaceous. These date estimates are slightly younger than, but within the 95% confidence intervals, of those reported by Dietrich et al. (2017).

## DISCUSSION

### Mitogenome features

Most leafhopper mitochondrial genomes are highly conserved with an arrangement of the 37 genes consistent with that of the inferred ancestral insect, *Drosophila yakuba* (Clary & Wolstenholme, 1985). However, more than ten species in Cicadellinae, Deltocephalinae, Iassinae, Megophthalminae and Ledrinae have the phenomenon of gene rearrangements with the previous studies (Mao et al., 2017; Du et al., 2017, 2019; Song et al., 2019, Wang et al., 2020a) and our unpublished data. Only three types of gene rearrangements have been detected in Cicadellidae, which arise from three tRNA clusters: (1) in *Elymana* sp. (MK251130), *Osbornellus* sp. (MK251136), and *Stirellus bicolor* (MK251122), the tRNA cluster of trnA–R–N–S1–E–F is rearranged to trnR–E–F–A–N–S1; (2) in *Cofana unimaculata* (MK251095), the tRNA cluster of trnI–Q–M is rearranged to trnQ–I–M; (3) in *Cicadulina mbila* (MK251127), *Japananus hyalinus* (NC\_036298), *Macrosteles quadrilineatus* (NC\_034781) and *Macrosteles quadrimaculatus* (NC\_039560), the tRNA cluster of trnW–C–Y is rearranged to trnY–W–C or trnC–W–Y. In this study, *Shaddai* sp. (MW284820) has the gene trnW translocated behind trnY, trnC–Y–W, with an intergenic spacer of 460 bp (Figure 1, b). Gene rearrangements may represent an additional set of characters useful for phylogenetic reconstruction (Downton et al., 2002; Tyagi et al., 2020) but additional data on leafhopper species will be needed to determine whether any such rearrangements are shared by closely related taxa.

### Phylogeny, character evolution and tribal status

Our study indicates that mitogenome sequences are useful for resolving phylogenetic relationships of subfam-

ily Typhlocybae from the species to the tribal level. Analyses of both amino acid and nucleotide sequences yielded similar topologies with the main differences among analyses occurring among a few branches that received low bootstrap support in one or more analysis. Our results agree with the result of a recent analysis by Lu et al. (2021) that incorporated partial sequence data from three nuclear and two mitochondrial genes in recovering Typhlocybae as a well-supported monophyletic group sister to Mileewinae (Mileewini). A comprehensive analysis of Membracoidea based on anchored hybrid data (Dietrich et al., 2017) failed to recover this relationship but the relationship of Typhlocybae to other leafhopper subfamilies was poorly resolved by that study, so our results require further confirmation. Within Typhlocybae, our results consistently supported the monophyly of the tribes Dikraneurini, Emposcini, Erythroneurini and Typhlocybini (Alebrini was represented by a single species in our dataset).

Lu et al. (2021) recovered the three species of Zyginellini included in their dataset as a monophyletic sister group of Typhlocybini (*sensu stricto*) but our larger sample of Zyginellini genera indicates that this tribe is polyphyletic. This supports the treatment of some recent authors (e.g., Hamilton, 1983; Dietrich, 2013) of Zyginellini as a junior synonym of Typhlocybini. Based on our results, the hind wing character traditionally used to separate Typhlocybini from Zyginellini (absence of the distal segment of vein CuA; Figure 6, f6) is homoplasious and has been derived independently in at least three unrelated lineages. Another hind wing character (veins RP and MA separate versus confluent; Figure 4, character g) was previously used to distinguish Eupterygini from Typhlocybini but our results also indicate considerable homoplasy for this trait.

Details of relationships within individual tribes are difficult to compare between our results and those of Lu et al. (2021) given that the taxon samples of the two studies overlap only partially. Nevertheless, we note that several branches pertaining to relationships among genera of Typhlocybini, as well as a few branches within the other tribes, were extremely short and received low bootstrap support in both studies. This suggests that neither complete mitogenome data nor data from a few nuclear and mitochondrial genes will suffice to completely resolve typhlocybine phylogeny with high confidence, although increased taxon sampling may also help improve phylogenetic resolution in this group.

Our results are generally consistent with morphology-based classifications proposed recently. Young (1965) moved Mileewini (as Mileewanini) to Typhlocybae but this was rejected by Mahmood (1967). Young (1968) later suggested treating Mileewinae as a separate subfamily. Nevertheless, Mileewinae are similar to Typhlocybae in having relatively small, slender bodies, reduced forewing venation and slender hind tarsi (Dietrich, 2011). Previous phylogenetic studies have not reached a clear consensus on the sister-group relationship of Typhlocybae (Balme, 2007; Dietrich, 2013b; Wang et al., 2017; Dietrich et al., 2017). Our results are also equivocal in this regard. Two analyses (Figure 2, c, e) recovered a sister-group relationship between Typhlocybae and Cicadellinae (*sensu stricto*), but all other analyses (Figure 2, a, b, d) and the tree topology tests (Table 2) consistently support Typhlocybae as sister to Mileewinae. Considering the morphological similarities shared by Typhlocybae and Mileewinae, the latter hypothesis seems to be more plausible.

The tribal relationships in Typhlocybae have long been controversial (Wagner, 1951; Mahmood & Ahmed, 1968; Zhang, 1990; Balme, 2007; Dietrich et al., 2017). Our analyses consistently support a sister-group relationship between Alebrini and Emposcini, as well as between Erythroneurini and Dikraneurini, in agreement with other recent analyses (Dietrich et al., 2017; Lu et al., 2021). The former relationship is also consistent with Wagner's intuitive morphology-based hypothesis (1951), but not with those of Mahmood & Ahmed (1968) and Zhang (1990).

The main area of uncertainty appears to be the relationship of Typhlocybini to the other tribes and the relationships of deep internal nodes within Typhlocybini. Different analyses recovered Typhlocybini either as sister to Alebrini + Emposcini (Figure 2, a, b) or as sister to Erythroneurini + Dikraneurini (Figure 2, c, d, e), thus this relationship remains equivocal. Detailed morphology-based cladistics analyses of Typhlocybae have not been attempted but there appears to be some morphological support for the latter hypothesis (Typhlocybini+(Dikraneurini+Erythroneurini)). For example, all Erythroneurini and most Typhlocybini



(except a few Neotropical taxa) have the hind wing submarginal vein absent at the wing apex. The male subgenital plates of Typhlocybini, Dikraneurini and Erythroneurini also have relatively few macrosetae compared to those of Alebrini and Empoascini. These traits are potential synapomorphies supporting the sister relationship of Typhlocybini to Dikraneurini+Erythroneurini recovered in most of our analysis as well as the analyses of Lu et al. (2021) and Dietrich et al. (2017).

Ancestral state reconstructions of key morphological characters previously used to define and diagnose tribes within Typhlocybinae (Evans, 1963, 1971; Hamilton, 1983; Dworakowska, 1979, 1993; Zhang, 1990) (Table 3) indicate that some wing characters traditionally used to diagnose tribes are highly stable. Our analyses generally support the monophyly of tribes Empoascini, Dikraneurini, Erythroneurini and Typhlocybini as defined morphologically by most authors. Thus, our analyses suggest that the wing vein characters traditionally used to define these groups are reliable. Nevertheless, our results also indicate that some morphological characters have undergone homoplastic changes during the evolution of the group. Ocelli may be present or absent within Empoascini and Typhlocybini. In Empoascini, all genera have ocelli, except *Beamerana* and *Paulomanus*. The latter genera, which have hind wing venation identical to that of many Empoascini, nevertheless grouped with Typhlocybini in a recent morphology-based phylogeny and were tentatively placed in the latter tribe (Xu et al., 2021). Few genera in Typhlocybini have ocelli, e.g., *Hiratettix* and *Caknesia*, but we were able to include only the former in our analyses and it occupies a relatively derived position within the tribe, suggesting that ocelli were lost and regained at least once in this tribe (Figure 4, a). Loss of the hind wing submarginal vein is also apparently homoplastic with independent losses apparently occurring in Erythroneurini and Typhlocybini (Figure 4, e). Hind wing veins RP and MA have also apparently become confluent in the common ancestor of Erythroneurini and Dikraneurini and independently in Empoascini and Typhlocybini (Table 3). Loss of the branched hind wing anal vein occurs only in the Dikraneurini + Erythroneurini lineage but further analyses with a larger sample of taxa will be needed to determine the extent of homoplasy in this character.

The molecular divergence time estimates indicate that Typhlocybinae and extant tribes originated during the Middle and Late Cretaceous, respectively. Thus, diversification of major lineages in this group seems to have roughly coincided with the diversification of angiosperms during the Cretaceous (Hamilton, 1992, 1994; Ledyard, 1974; Foster et al., 2016). Our divergence time estimates for tribes of Typhlocybinae are considerably younger than those reported for the same branches by Dietrich et al. (2017), possibly due to the denser taxon sample of our study. Thus, in our study, the splits between Erythroneurini and Dikraneurini, and between Alebrini and Empoascini are both estimated at 76 MYA, compared to the 95 MYA and 112 MYA, respectively, reported by Dietrich et al. (2017). 95% confidence intervals of both studies are wide and broadly overlap, indicating that the available methods and data are only able to provide very approximate estimates of the times of origin of these major leafhopper lineages. Future analyses incorporating additional fossil evidence (unavailable at present), diversified clock models, dating methods, and rate priors may yield improved estimates.

### Species misidentifications

Song et al. (2017b) analyzed relationships within Cicadomorpha using mitochondrial genome data, including a species identified as “*Typhlocyba* sp.” (GenBank accession number, KY039138). In our phylogenetic results this sample did not group with *Typhlocyba choui*, but instead grouped with the genus *Aguriahana* with high support in all phylogenetic trees (PP=1, SH-aLRT=100, UFB=100) (Figure 1). In addition, the COX1 p-distance between “*Typhlocyba* sp.” (GenBank accession number, KY039138) and *Typhlocyba choui* was 18%, but the former was only 1.2% divergent from *Aguriahana juglandis* (Table S3). Based on the criteria of intraspecific genetic distance from 0% to 2% (Hebert et al., 2003), we suggest that “*Typhlocyba* sp.” (GenBank accession number, KY039138) should be defined a species of *Aguriahana*.

## CONCLUSION

This study sequenced 28 typhlocybinae mitogenomes, representing all currently recognized tribes. We report the first known mitochondrial gene rearrangement within Typhlocybinae (Alebrini). Despite some variability

among phylogenetic estimates based on different datasets, the results consistently support the monophyly of Typhlocybae and four tribes for which multiple representatives were included. In contrast to another recent analysis (Lu et al., 2021), Zyginellini was consistently polyphyletic in our results. Our results support the sister-group relationship of Typhlocybae to Mileewinae but this relationship received only moderate branch support. Ancestral character state reconstructions (ACSR) suggest that some morphological characters traditionally considered important for diagnosing tribes are homoplastic. A key to tribes of Typhlocybae is provided and new taxonomic changes are proposed based on the phylogenetic results, morphology and genetic distances. Although mitogenome sequence data appear to be broadly informative of relationships at various levels in the taxonomic hierarchy of Typhlocybae, further study incorporating a larger taxon sample and additional morphological and molecular evidence (e.g., nuclear genes, anchored hybrid loci) should help further clarify the phylogeny of this highly diverse group.

## TAXONOMY

### Key to tribes of Typhlocybae

1. Forewing appendix present (Figure 6, d1)..... **Alebrini**
- . Forewing appendix absent.....2
2. First forewing apical cell shorter than one third length of forewing (Figure 6, e1, e3, e5, f1, f3, f5).....3
- . First forewing apical cell longer than one third length of forewing (Figure 6, g1, g3, h1).....4
3. Hind wing submarginal vein extended beyond CuA vein but not surpassing MA vein or ‘MA + RP’ vein (Figure 6, e2, e4, e6)..... **Empoascini**
- . Hind wing submarginal vein not extended beyond CuA vein (Figure 6, f2, f4, f6)..... **Typhlocybini**
4. Hind wing submarginal vein complete, extending along costal margin and connected to apices of all longitudinal veins (Figure 6, g2, g4)..... **Dikraneurini**
- . Hind wing submarginal vein shorter than CuA vein (Figure 6, h2)..... **Erythroneurini**

### **Typhlocybini** Kirschbaum, 1868

*Subtilissimia* Yan & Yang **gen. nov.**

<http://zoobank.org:act:262A48A8-86C3-4B02-8EB6-5C4726AF13D2>

**Type species:** *Subtilissimia fulva* Yan & Yang

**sp. nov.**

**Diagnosis.** The new genus differs from other genera in this tribe by the following combined characters: small to medium sized, depressed and ovoid (Figure 7, a, b, i, j); head with a longitudinal reddish broad stripe from crown apex to posterior margin of pronotum; subgenital plate without macrosetae near base; style with numerous microsetae in middle part (outer margin); connective Y-shaped; aedeagus dorsal apodeme well developed, without process on base.

**Description.** Length of male 3.5 to 3.7 mm, female 3.5 to 3.6 mm. Dorsum brown-yellowish; head with longitudinal reddish broad stripe from crown apex to posterior margin of pronotum. Forewing semitransparent; veins pale white; apical cells infuscated; forewing clavus with reddish stripe, wave-shape; brochosoma area with two small dark spots; MP”+CuA’ vein with black spot on apex (Figure 7, a, b, i, j). Face yellow. Body flat in lateral view (Figure 7, b, j); crown produced; coronal suture distinct, not extended beyond the midline length of crown (Figure 7, a), or indistinct (Figure 7, b); Head subequal to pronotum width, length of crown at midline slightly longer than interocular width; scutellar suture distinct, nearly reach to sides; forewing apex rounded, the 2nd apical cell subequal to 4th apical cell; the 3rd apical cell smallest, triangular; hind wing R and MP veins directly connected at apex (Figure 7, d, l).

**Male genitalia.** Pygofer side with posterior margin rounded and with two clusters of setae on posterior and ventral parts; without macrosetae and process (Figure 7, c, k); subgenital plate gradually narrowed to apex, without big macrosetae at base; apex forked, or not forked (Figure 7, c, k); style slender, with a dense coverage of microsetae on outer margin; connective Y-shaped, with central lobe developed (Figure 7, f, n); aedeagus with dorsal apodeme well developed, preatrium weak; shaft slender, with pair of symmetrical processes; phallotreme apical (Figure 7, h, o).

**Remarks.** The new genus is similar to *Typhlocyba* in coloration and male genitalia but can be distinguished from the latter by the absence of basal macrosetae on the subgenital plate and presence of dense microsetae at the midlength of the paramere.

**Etymology.** This generic name is feminine, formed by the Latin word “*Subtilissimus*” which means “slender or fine”, referring to its aedeagal shaft in lateral view.

**Distribution.** China (Yunnan).

*Subtilissimia fulva* Yan & Yang **sp. nov.**

<http://zoobank.org:act:BFCDAE7D-C5E4-4896-A841-E3BA4A559DE3>

Figure 7, a–h

**Material examined.** Holotype, , Mengla County, Yunnan Province, 608 m, 12 May 2015, Coll. Bin Yan. Paratypes, 1, 3, same data as holotype; 1, Mengla County, Yunnan Province, 604 m, 16 Nov. 2018, Coll. Likun Zhong.

**Description.** Length of male 3.5 to 3.7 mm. Coronal suture indistinct (Figure 7, a). Forewing clavus with deeply reddish stripe, elongated to apex (Figure 7, a, b). Male pygofer side slightly convex on upper posterior margin (Figure 7, c); style slender, parallel-sided (Figure 7, f); aedeagal shaft tubular, with pair of lateral processes arising near mid length, extended distally and slightly divergent from shaft, almost reaching shaft apex (Figure 7, g, h).

**Etymology.** The species name was derived from the Latin adjective “fulvus” = reddish-yellow, tawny, amber-coloured, which refers to the coloration of the longitudinal patch on the crown.

**Remarks.** The new species is very similar to *Subtilissimiapellicula* **sp. nov.** in size and coloration, but differs in having the aedeagal processes arising near the shaft mid length, longer, and not forked near the apex in lateral view (Figure 7, g, h).

**Distribution.** China (Yunnan).

*Subtilissimiapellicula* Yan & Yang **sp. nov.**

<http://zoobank.org:act:2B04D9D2-665D-4A6D-B9C9-E75F3171D144>

Figure 7, i–p

**Material examined.** Holotype, , Simian Mountain, Chongqin, 1100 m, 12 Sep. 2017, Coll. Bin Yan. Paratypes, 3, same data as holotype.

**Description.** Body length 3.5 to 3.6 mm. Coronal suture distinct, short (Figure 7, i). Forewing with reddish stripe on hind margin, not reaching clavus apex (Figure 7, i, j). Male pygofer side rounded on posterior margin (Figure 7, m); style slender, apex sharp and slightly curved (Figure 7, n); Aedeagus tubular in ventral view, with membranous sides; shaft with pair of dorsal processes arising near apex, curved dorsally, forked near base (Figure 7, o, p).

**Etymology.** The species name was derived from the Latin noun “*pellicula*” = “small skin”, which refers to a membranous structure on the shaft. It should be treated as a noun in apposition

**Remarks.** The new species is very similar to *Subtilissimia fulva* **sp. nov.** in size and coloration, but differs in having the aedeagal processes arising near the shaft apex, curved dorsally, and forked near the apex in lateral view (Figure 7, o, p).

**Distribution.** China (Chongqin).

*Farynala Dworakowska*, 1970

*Farynaladextra* Yan & Yang, 2017

*Farynala dextra* Yan & Yang, 2017: 520.

*Farynala sinistra* Yan & Yang, 2017: 520 **syn. nov.**

**Note.** Yan & Yang (2017) described the species of *Farynala sinistra* and *Farynala dextra*, distinguishing them according to numbers of macrosetae on the pygofer side and the orientation of the aedeagal processes being opposite such that they appear to be mirror images of one another. Our current phylogenetic results (Figures S1-S5) and the small genetic distance between these two taxa based on COX1 p-distance=0.51% strongly indicates that these taxa are conspecific. Thus, the two species names are here treated as synonyms.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest to disclose.

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**TABLE 1** Fossil taxa used for node calibration.

Node no.	Fossil taxa	Current placement	Age estimate	Source
<b>a</b>	N/A	N/A	<1.741	N/A
<b>b</b>	<i>Jassoqualus hispaniolensis</i>	Evacanthinae: Nirvanini	>0.175<1.1	Oligo-Miocene Dominican amber
<b>c</b>	<i>Youngeawea bicolorata</i>	Mileewinae: Mileewini	>0.44	Eocene Baltic amber
<b>d</b>	Cicadellini gen? sp?	Cicadellinae: Cicadellini	>0.175<0.9	Oligo-Miocene Dominican amber
<b>e</b>	Dikraneurini gen. sp?	Typhlocybinae: Dikraneurini	>0.175<0.9	Oligo-Miocene Dominican amber

Note. The "N/A" indicates not applicable.

**TABLE 2** Statistical tests of alternative tree topology hypotheses conducted by IQ-TREE v.1.6.3. Monophyly constraints: **a** , sister-group relationship of Cicadellinae to Typhlocybinae; **b** , sister-group relationship (and reciprocal monophyly) of Typhlocybini and Zyginellini; **c** , alternative tribal topology recovered by some analyses ((Empoascini, Alebrini), (Typhlocybini, (Erythroneurini, Dikraneurini))).

Hypothesis	log L	deltaL	bp-RELL	p-KH	p-SH	p-WKH	p-WSH	c-ELW	p-AU
<b>a</b>	-127274.68	13898	0-	0-	0-	0-	0-	0-	2.2e-78-
<b>b</b>	-115531.50	2154.4	0-	0-	0-	0-	0-	0-	8.7e-06-
<b>c</b>	-113406.30	29.17	0.11+	0.11+	0.70+	0.11+	0.27+	0.11+	0.10+

**Note.** The value of deltaL indicates that logL differs from the maximal logL in the comparison. bp-RELL: bootstrap proportion using RELL method (Kishino et al., 1990; Kalyaanamoorthy et al., 2017). p-KH: p-value of one sided Kishino-Hasegawa test (Nguyen et al., 2015). p-SH: p-value of Shimodaira-Hasegawa test (Kishino et al., 1990). c-ELW: Expected Likelihood Weight (Hoang et al., 2018). p-AU: p-value of approximately unbiased (AU) test (Wang et al., 2018). Plus signs denote the 95% confidence sets. Minus signs denote significant exclusion. All test performed on 1000 replicates using the RELL method.

**TABLE 3** Comparison of tribal characters states in Typhlocybinae.

Feature	Tribe	Ocelli	Forewing appendix	Hind wing anal vein	Hind wing submarginal vein	Hind wing CuA vein	Hind wing RP and MP vein
Alebrini		+	+	+	+	+	+
Empoascini		+,-	-	+	+	+,-	+,-
Typhlocybini		+,-	-	+	-	-	+,-
Dikraneurini		-	-	+,-	+,-	+,-	-
Erythroneurini		-	-	-	-	+	-

**Note.** The symbols "+" and "-" indicated the following morphological characters. Ocelli and forewing

appendix: ‘+’, present; ‘-’, absent. Hind wing anal vein: ‘+’, bifurcated; ‘-’, not forked. Hind wing submarginal vein: ‘+’ extended beyond CuA vein; ‘-’ not extended beyond CuA vein. Hind wing CuA vein: ‘+’, bifurcated; ‘-’, not forked; Hind wing RP and MP vein: ‘+’, connected by crossvein; ‘-’, confluent. The symbol “+, -” denotes the presence of both character states.

**FIGURE 1** Schematic diagram of typhlocybinae mitochondrial genome. a, the usual type of mitogenome in Typhlocybinae; b, the type of *Shaddai* sp.

**FIGURE 2** Phylogenetic relationships of Typhlocybinae reconstructed with maximum likelihood (ML) and Bayesian inference (BI) from different mitochondrial datasets. A, BI tree (CAT + GTR model), using amino acid sequence of PCGs; B, ML tree (PMSF model), using amino acid sequence of PCGs; C, ML tree (Partitioning model), using amino acid sequence of PCGs; D, ML tree (Partitioning model), using nucleotide sequence of PCGs.12; E, ML tree (Partitioning model), using nucleotide sequence of PCGs.12 concatenated 2 rRNAs.

**FIGURE 3** Phylogenetic tree based on the PhyloBayes analysis of amino acid matrix with the site-heterogeneous CAT + GTR model. Node values for all analyses are plotted on or below respective clades as specified in the legend in the top-left corner, except those with consistently high support values (PP[?]0.99, SH-aLRT & UFB[?]98). Branches are colored matching the color chart on the left. For more information of trees, refer to figures S1-S5. Taxa previously included in Zyginellini are indicated in bold.

**FIGURE 4** Ancestral character states reconstructions analysis based on ML tree (PCGs12.fna) using maximum-likelihood method. The images at right illustrate seven morphological characters: a, ocelli (white ball, present; black ball, absent); b, forewing appendix (white ball, present; black ball, absent); c, forewing closed anteapical cells (white ball, present; black ball, absent); d, hind wing anal vein (white ball, bifurcated; black ball, not forked); e, hind wing with distal extension of CuA vein beyond submarginal vein (white ball, absent; black ball, present); f, hind wing CuA vein (white ball, bifurcated; black ball, not forked); g, hind wing RP and MP vein (white ball, connected by crossvein; black ball, confluent); h, hind wing submarginal vein apparently connected directly to CuA vein (white ball, absent; black ball, present). The gray balls indicate feature unknown. Taxa previously included in Zyginellini are indicated in bold.

**FIGURE 5** Chronogram showing divergence time estimates for major lineages of Typhlocybinae. Numbered nodes marked in red refer to fossil calibrations, a, root age, <174.1 MYA; b, 17.5–110 MYA; c, 44 MYA; d, 17.5–90 MYA; e, 17.5–90 MYA. Bars on nodes are the highest posterior density 95% confidence intervals (95% HPD). Scale: 1 = 100 MYA.

**FIGURE 6** The ocelli and wings are mapped to the BI tree, separately. a–f, ocelli on crown; a1–h2, wings, photographed with transmitted light to highlight venation. **Evacanthinae**, a (followed Zhang, 2011), a1–a2, *Convexana albicarinata* ; **Cicadellidae**, b, b1–b2, *Cicadella viridis* ; **Mileewinae**, c, c1–c2, *Mileewa albovittata* . **Typhlocybinae**, **Alebrini**, d, d1–d2, *Shaddai* sp.; **Empoascini**, e, e3–e4 *Ghauriana sinensis* , e1–e2, *Joruma* sp. and e5–e6, *Heliona* sp. (followed, Xu, 2019); **Typhlocybini**, f1–f2, *Aguriahana triangularis* , f3–f4, *Agnesiella aldera* , f5–f6, *Zyginella minuta* ; **Dikraneurini**, g1–g2, *Sweta bambusana* , g3–g4, *Dikraneura orientalis* ; **Erythroneurini**, h1–h2, *Tautoneura formosa* .

**FIGURE 7** a–h, *Subtilissimia fulva* Yan & Yang **sp. nov.** a, habitus, dorsal view; b, habitus, lateral view; c, male genitalia, lateral view; d, hind wing; e, subgenital plate, ventral view; f, style and connective, ventral view; g, aedeagus, lateral view; h, aedeagus, ventral view. i–p, *Subtilissimia pellicula* Yan & Yang **sp. nov.** i, habitus, dorsal view; j, habitus, lateral view; k, male genitalia, lateral view; l, hind wing; m, subgenital plate, ventral view; n, style and connective, ventral view; o, aedeagus, lateral view; p, aedeagus, ventral view.







