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A multi-gene phylogenetic analysis of the leafhopper subfamily Typhlocybinae (Hemiptera: Cicadellidae) challenges the traditional view of the evolution of wing venation

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ABSTRACT

Genera of the diverse leafhopper subfamily Typhlocybinae have traditionally been classified into tribes primarily based on characters of the wing venation and an intuitive phylogeny of this group was previously proposed based on the hypothetical pattern of wing vein evolution. Some recent authors suggested that wing vein characters are not always reliable but few attempts have been made to examine the status and relationships of typhlocybine tribes using rigorous phylogenetic analyses. Phylogenetic analysis of a dataset comprising DNA sequences from five gene regions (*H3*, *H2A*, *28S* rDNA *D2*, *16S* rDNA, and *COI* with a total length of 2413 bps) and 61 species representing six previously recognized tribes of Typhlocybinae provides strong support for the monophyly of the subfamily and five of the previously recognized tribes. Most branches received moderate to strong maximum likelihood bootstrap support. The following intertribal relationships were recovered: (Alebrini + Empoascini) + ((Dikraneurini + Erythroneurini) + Typhlocybini). Maximum likelihood analysis recovered Zyginellini (treated as a separate tribe by some authors) as sister to Typhlocybini with low branch support, but the former tribe was derived from within the latter in Bayesian analysis of the same dataset and relationships within Typhlocybini (sensu lato) were generally poorly resolved in both analyses. The relationship of Typhlocybini to other tribes is also unstable, suggesting that more data are needed to resolve the position of this tribe with confidence. Parts of the phylogeny receiving strong support in both analyses contradict the traditional view that Alebrini, the only tribe retaining an appendix in the forewing, is the earliest diverging lineage and possibly gave rise to the other tribes. Ancestral state reconstructions indicate that characters of the wing venation traditionally used for diagnosing typhlocybine tribes are generally conservative but exhibit some homoplasy and may not, by themselves, be reliable for recognizing monophyletic groups within this subfamily.

1. Introduction

1.1. Taxonomy and classification

The subfamily Typhlocybinae (Cicadomorpha: Cicadellidae) includes mostly small, delicate leafhoppers that feed primarily on leaf parenchyma cell contents, thus differing from other leafhoppers that feed preferentially on plant vascular fluids. Typhlocybinae are distributed worldwide and, based on known species, are the second largest cicadellid subfamily, comprising ~5000 valid species, after Deltocephalinae (~7080 valid species) (Dmitriev, 2003). However, large numbers of typhlocybine species continue to be described and tropical faunas remain poorly documented (Dietrich, 2013). Many typhlocybine species are economically damaging pests of crops and forestry, mainly injuring plants through direct feeding, although a few have been implicated in the spread of plant pathogens (Kuoh, 1966; Backus, 1988; Weintraub and Beanland, 2006; Wilson and Weintraub, 2007).

Typhlocybinae are distinguished from other cicadellids by their small size and delicate appearance, lack of closed anteapical cells in the forewing, and acuminate first hind tarsomere. The composition and tribal classification of Typhlocybinae has been confusing and controversial with various authors recognizing as few as five to as many as eleven tribes (Melichar, 1903; Distant, 1908, 1918; Matsumura, 1931; McAtee, 1926, 1934; Oman, 1949; Oman et al., 1990; Young, 1952,

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1965; Dlabola, 1958; Mahmood, 1967; Metcalf, 1968; Mahmood and Ahmed, 1968; Ahmed, 1983; Dworakowska, 1970, 1977, 1979; Knight, 1976; Ruppel, 1987; Zhang, 1990; Dietrich, 2005, 2013). Most taxonomic research on the group has been confined to regional studies, with authors from different regions adopting different classifications. Melichar (1903) divided the group into two taxonomic divisions, Empoascaria and Typhlocybaria, the former with the hindwing distal cells closed and the latter with these cells open. Distant (1908, 1918) and Matsumura (1931) followed this classification. McAtee (1934) divided Typhlocybinae into four tribes–Alebrini, Dikraneurini, Jorumini and Eupterygini, also based on wing venation. Young (1952) also presented a four-tribe system for the New World fauna (Alebrini, Dikraneurini, Erythroneurini, and Typhlocybini) that was followed by Mahmood (1967), who added a new tribe, Bakerini, based on study of the Oriental fauna. Dworakowska (1970, 1977, 1979 and numerous other papers) the principal worker on the Old World fauna for several decades, recognized six tribes–Alebrini, Dikraneurini, Empoascini, Erythroneurini, Typhlocybini, Zyginellini. This system has been followed by Zhang (1990) and most other taxonomists studying the Old World fauna. However, Ahmed (1983) recognized five tribes, considering Zyginellini to be an artificial group and treating it as a junior synonym of Typhlocybini. Ruppel (1987) presented a key to 10 tribes based on the venation of the fore- and hind wing but stated that Mileewini (as "Mileewanini") is more properly placed in Cicadellinae. In their world catalogue and checklist of Cicadellidae, Oman et al. (1990) provided a "provisional" higher classification that listed eight typhlocybine tribes as valid. Dietrich (2013) adopted Ahmed's five-tribe classification in his revision of South American Typhocybini leafhoppers. Zhang (1990) presented an intuitive phylogeny of the tribes based on wing venation (Fig. 1). The most comprehensive phylogenetic analysis of the group, incorporating molecular and morphological data, was presented in the unpublished dissertation of Balme (2007) who recognized four tribes (Alebrini, Dikraneurini, Empoascini, Typhlocybini).

1.2. Phylogenetic relationship

Previous phylogenetic studies of Membracoidea as a whole have included few representatives of Typhlocybinae and have been equivocal with regard to the monophyly of the subfamily. Analysis of partial 28S rDNA sequences failed to recover the four included exemplars as a monophyletic group (Dietrich et al., 2001). A subsequent analysis combining 28S sequence data with morphological characters recovered the three included exemplars as monophyletic with strong support. Based on a more taxon-rich phylogenetic analysis of Typhlocybinae using DNA sequences (*16S* rDNA and histone *H3*) and morphological characters, (Balme, 2007) proposed treating Jorumini McAtee and Helioninae Haupt as junior synonyms of Empoascini, and also treated Erythroneurini and Forcipatini as synonyms of Dikraneurini but this doctoral dissertation remains unpublished. A recent phylogenomic analysis of Membracoidea (Dietrich et al., 2017) based on data for 388 loci obtained using anchored hybrid enrichment included representatives of 10 genera representing 7 previously recognized tribes (Alebrini, Dikraneurini, Empoascini, Erythroneurini, Forcipatini, Typhlocybini, Zyginellini). This analysis supported the monophyly of Typhlocybinae and resolved relationships among tribes mostly with strong branch support (Fig. 2) but did not include a large enough taxon sample to test the monophyly of individual tribes. A recent morphology-based phylogenetic analysis focused primarily on relationships within Empoascini recovered this tribe as sister to Dikraneurini with Typhlocybini and

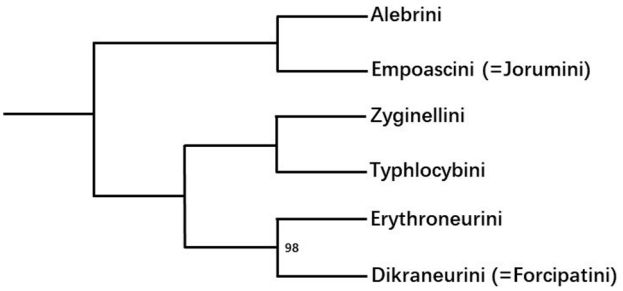

Fig. 2. Relationships among Typhlocybinae tribes recovered in anchored-hybrid-based phylogenomic analysis of Dietrich et al. (2017). ML bootstrap score is indicated only for one branch that received <100% support.

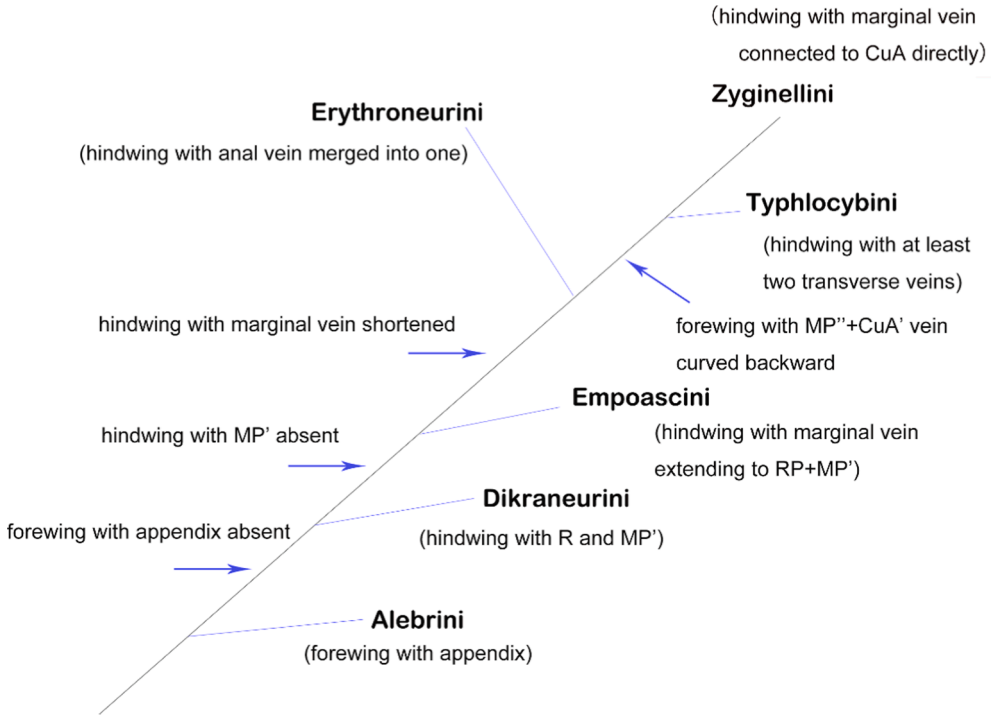

Fig. 1. Hypothesized evolution of Typhlocybinae based on wing venation (Zhang, 1990). The vein terminology shown here is followed Dworakowska (1993).

Erythroneurini forming a paraphyletic grade subtending the former (Xu et al., 2021). Recent phylogenetic studies based on mitochondrial genome sequences yielded results that were generally consistent with those of Dietrich et al. (2017) but did not include Alebrini and suggested that Zyginellini is derived from Typhlocybini (Zhou et al., 2020; Jiang et al., 2021).

In this study, we analyze a dataset comprising partial sequences from five gene regions (*H2A*, *H3*, *28S* rDNA *D2*, *16S* rDNA, *COI*) for a much larger sample of typhlocybine taxa to further elucidate the status and relationships of the tribes of Typhlocybinae.

2. Materials and methods

2.1. Taxon sampling and identification

Wild collected typhlocybine leafhoppers were preserved in vials of 95% ethanol and were stored in ultracold (−80 °C) freezers at Northwest A&F University (NWAFU, China). Following Dworakowska's classification, all tribes of Typhlocybinae were sampled. The dataset contains 61 representative taxa of 33 genera (including undescribed groups) of 6 tribes within Typhlocybinae, and 13 representatives of five other subfamilies, Deltocephalinae, Cicadellinae, Evacanthinae, Mileewinae and Signoretiinae as outgroups. Molecular data were newly obtained for 49 representatives of Typhlocybinae and data for the remaining 25 taxa were obtained from GenBank (NCBI). Voucher numbers, collection localities and GenBank accession numbers are summarized in Table S1. Specimens were identified based on male genitalia and other characters using the taxonomic literature. Vouchers are deposited in the Entomological Museum, Northwest A&F University.

2.2. DNA extraction, amplification and sequencing

To positively identify each species, the genital capsule was removed from one male specimen of each morphospecies and cleared in 10% NAOH solution to allow examination of the diagnostic parts of the genitalia for positive species identification. DNA was then extracted from the remainder of the body of the same specimen utilizing an Insect Genomic DNA Extraction Kit (Bioer Technology Co., China) following manufacturer protocols. The body was allowed to soak in the extraction buffer for 6–8 h, after which it was removed from the buffer and saved along with the genital capsule as a voucher specimen, along with remaining intact specimens of the same species (when available). Five genes (*16S* rDNA, *COI*, *28S* rDNA *D2*, *H2A*, *H3*, total length 2413bps) were amplified by PCR (C1000, Bio-Rad) and sequenced using primers listed in Table S2 (Fang et al., 1993; Folmer et al., 1994; Dietrich et al., 1997; Cryan et al., 2000; Dietrich et al., 2001; Ogden and Whiting, 2003; Hebert et al., 2003; Cryan, 2005; Zahniser and Dietrich, 2010; Dai et al., 2008; Le Roux and Rubinoff, 2009; Cryan and Urban, 2011). 25ul reaction volumes of PCR amplification consisted of 1ul DNA template, Taq polymerase (TransFast, TransGene Biotech), 0.5ul each primer and the remaining volumes of mix in Table S3. The thermal cycling protocols were as follows: 94 °C for 3 min, 30 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min, and a final extension of 72 °C for 7 min of *28S* rDNA *D2*. Except for the annealing temperature 50 °C for 1 min for *16S* rDNA, *H2A* and *H3*, the protocols were same. 94 °C for 3 min, then 5 cycles of 94 °C for 1 min, 45 °C for 1 min, 72 °C for 1.5 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 5 min for COI. After target fragments were amplified and checked by electrophoresis, PCR products were sequenced by Sunny Ltd. (Shanghai).

2.3. Outgroups

Recent phylogenetic analyses (e.g., Dietrich et al., 2017) provided strong support for the monophyly of Typhlocybinae but have not consistently resolved the relationship of this group to other leafhoppers. Therefore, we selected outgroups representing several other cicadellid subfamilies, including Mileewinae. Earlier analyses based on morphology (Dietrich, 1999), morphology and *28S* rDNA sequences combined (Dietrich, 2005), and mitochondrial genomes recovered Mileewinae as sister to Typhlocybinae (Chen et al., 2021).

2.4. Phylogenetic analysis

Sequences were assembled and adjusted using MEGA X v.10.1.8 (Kumar et al., 2018). Multiple alignments were performed using MAFFT v.7.313 (Katoh and Standley, 2013) with the '-auto' strategy. Regions of unclear homology were removed using TRimAl v1.4.1 (Capella-Gutierrez et al., 2009) with the '-automated1′ method, and resulting alignments were concatenated with FASConCAT-g v1.04 (Kück and Longo, 2014). Partitioning schemes and substitution models were estimated by the best-fit partition model (Edge-unlinked) using the BIC criterion by ModelFinder (Kalyaanamoorthy et al., 2017) or PartitionFinder 2 (Lanfear et al., 2017) as implemented in IQ-TREE v.1.6.8 (Nguyen et al., 2015) and included in PhyloSuite v.1.2.2 (Zhang et al., 2020). According to the BIC, the best-fit models were: GTR + F + R4 for partition 28S + H2A + H3 and GTR + F + I + G4 for partition 16S + COI. Maximum likelihood reconstructions were performed using IQ-TREE with node support calculated based on 1,000 SH-aLRT replicates (Guindon et al., 2010) and 1,000 ultrafast bootstraps (UFBoot2, Hoang et al., 2018). In reporting results of phylogenetic analyses, we consider ML boostrap and Bayesian PP scores of 95% or higher to be "high" support and values between 70 and 94% to be "moderate".

Bayesian analyses (BI) was performed with Mrbayes v3.2.6 (Ronquist et al., 2012) and conducted in the CIPRES Science Gateway (Miller et al., 2010). The best model choice was GTR + I + G for each gene in PartitionFinder 2.1.1 (Lanfear et al., 2017). Four independent chains were run for a maximum of 20 million generations. Trees were sampled every 1000 generations with the first 25% discarded as burn-in. With runs converged, the average standard deviation of split frequencies was 0.008136 (<0.01). Convergence between runs was examined with Tracer v.1.7 (Rambaut et al., 2018).

2.5. Character evolution

To evaluate the evolution of wing venation of the typhlocybine leafhoppers, we selected ten morphological characters of the wing based on previous taxonomic studies (summarized in Table S4) and reconstructed their evolution on the molecular phylogenetic tree: (1) absence or presence of anteapical cells of forewing; (2) absence or presence of appendix of forewing; (3) straight or curved vein MP''+CuA' of forewing; (4) veins R and MP of hindwing confluent or not; (5) absence or presence of submarginal vein of hindwing at wing apex; (6) submarginal vein of hindwing, extent (Xu et al., 2020); (7) vein CuA of hindwing branched or not; (8) absence or presence of distal segment of CuA of hindwing; (9) number of hind wing crossveins; (10) anal vein of hindwing branched or unbranched. Combinations of these wing characters were traditionally used to distinguish tribes of Typhlocybinae (Zhang, 1990; Dietrich, 2005; Xu et al., 2021). Terminology for labeling veins follows Dworakowska (1993). Character changes were reconstructed on the ML bootstrap consensus tree using the Bayesian Binary MCMC (BBM) method in RASP v4 (Yu et al., 2020).

3. Results

3.1. Phylogenetic analysis

ML analysis based on data from five genes for 74 taxa with 2 partitions and 2413 total sites yielded a well-resolved phylogeny with most branches receiving moderate to strong bootstrap support (Fig. 3). The result strongly supports the monophyly of Typhlocybinae placing Mileewinae in part (Mileewini) as sister group. Currently recognized

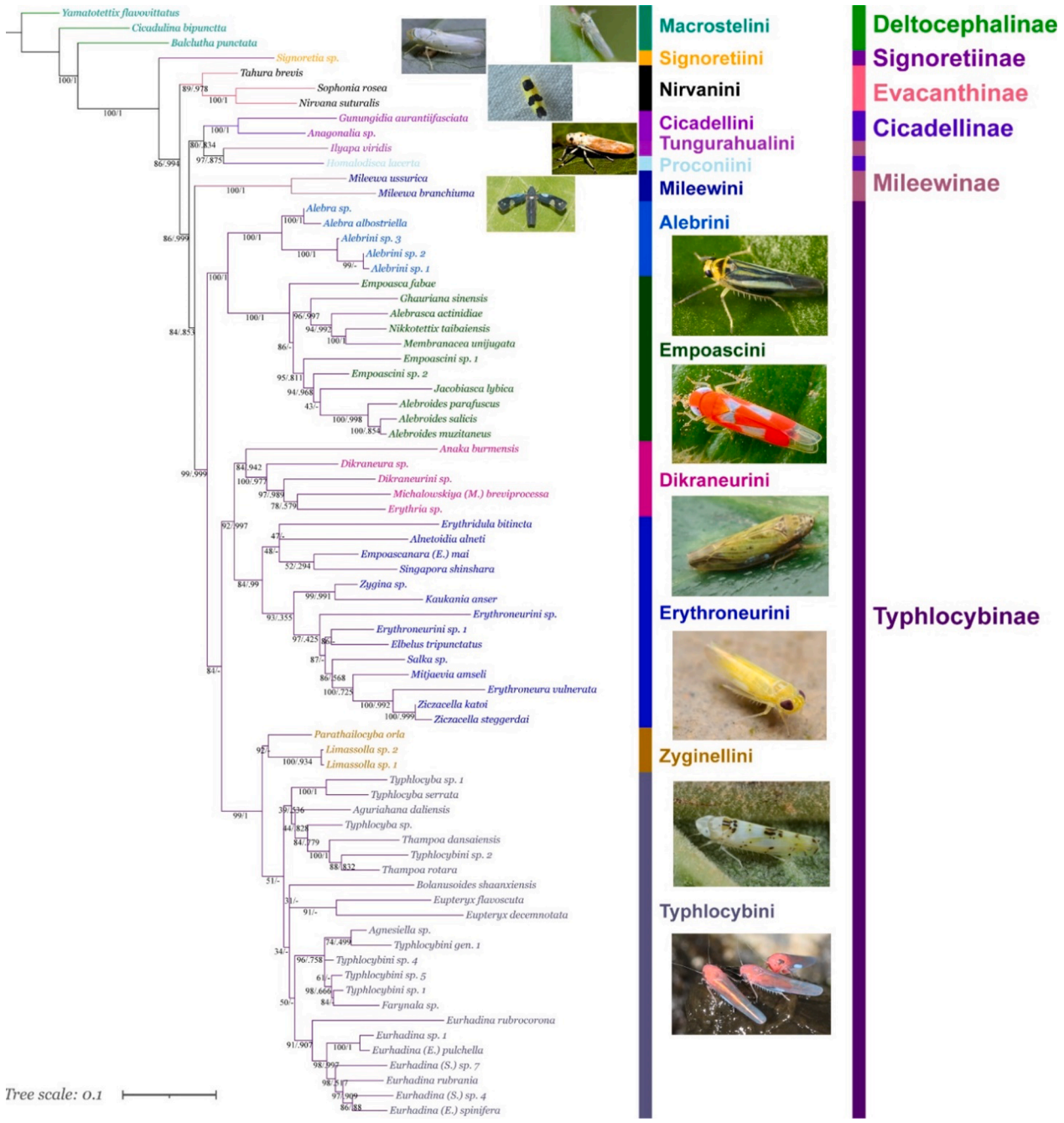

Fig. 3. Maximum likelihood bootstrap consensus of higher-level relationships of the subfamily Typhlocybinae inferred from multi-gene data. The numbers below the branches are maximum likelihood bootstrap scores and Bayesian posterior probabilities ("-" indicates branch not recovered in Bayesian analysis). Bayesian consensus is shown in Fig. S1.

tribes Alebrini, Empoascini, Dikraneurini, Erythroneurini and Typhlocybini (sensu lato) are also supported as monophyletic with moderate to high bootstrap support. Relationships among tribes are consistent with the anchored hybrid phylogenomic results of Dietrich et al. (2017) although bootstrap support for the clade comprising Dikraneurini, Erythroneurini and Typhlocybini is only moderate (84%) in the present results. The three included representatives of Zyginellini form a monophyletic group sister to Typhlocybini (sensu stricto). Branches pertaining to relationships within tribes received mostly moderate to strong support except for a few deep internal branches within Typhlocybini and one internal branch in Erythroneurini that received <50% support. Genera for which multiple representatives were included are recovered as monophyletic except the paraphyletic typhlocybine genus *Typhlocyba*. Within Typhlocybini, representatives of Eupterygini (with hind wing veins RP and MA separate and connected by a crossvein) were not recovered as monophyletic, consistent with treatment of the latter taxon as a synonym of the former by most recent authors.

Bayesian analysis (BI) of the same dataset recovered Typhlocybinae as monophyletic with strong support (PP = 0.999) and also recovered the sister pairs Dikraneurini + Erythroneurini and Alebrini + Empoascini, consistent with the ML results, but placed the latter lineage as sister to Typhlocybini (Fig. 3; Fig. S1). BI analysis also failed to recover Zyginellini and Typhlocybini as sister groups, suggesting instead that the former is derived from the latter. Areas of incongruence between the Bayesian and ML results received moderate to low branch support in one or both analyses, suggesting that some relationships among and within

tribes are unstable. In both ML and BI analysis, Typhlocybinae is sister to Mileewini consistent with Chen et al. (2021) but this relationship received only moderate branch support. Within Typhlocybinae, many relationships were consistent and well supported in both analyses. The main areas of instability are in the relationship of Typhlocybini to the other tribes and relationships among genera within Typhlocybini (sensu lato).

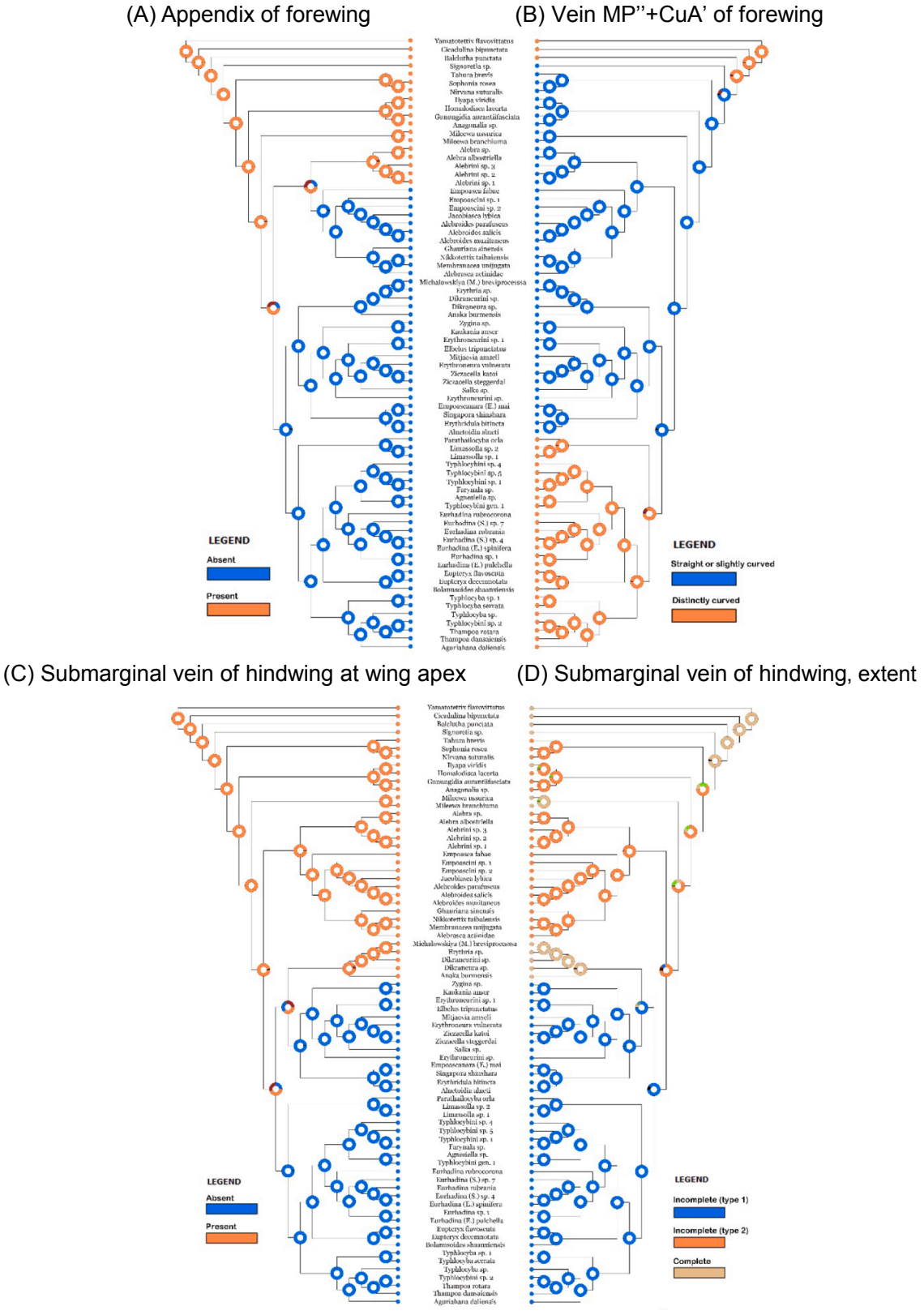

Fig. 4. Hypothesized evolution of four wing characters traditionally used to diagnose tribes of Typhlocybinae, reconstructed using the Bayesian Binary MCMC (BBM) method in RASP. Pie charts on each node indicate the likelihood of the estimated ancestral states. See also Supplemental Fig. S2.

3.2. Ancestral character state reconstruction (ACSR)

Reconstruction of wing characters on the phylogeny resulting from ML analysis of molecular data (Fig. 4) indicates that characters traditionally used to classify typhlocybine leafhoppers into tribes are generally conservative but show some homoplasy. Loss of the r-m and m-cu crossveins that form closed anteapical cells in the forewings of most leafhoppers occurred in the common ancestor of Typhlocybinae and was retained through the evolution of this subfamily but parallel loss of closed anteapical cells also occurred in some members of the outgroup Evacanthinae (Nirvanini, Fig. S2). Presence of an appendix in the forewing is also characteristic of most leafhoppers but absent in all Typhlocybinae except Alebrini. Thus, previous authors (e.g., Zhang, 1990) inferred that Alebrini are the most plesiomorphic members of the subfamily. As reconstructed here, the appendix was retained by Alebrini and lost independently in Empoascini and the common ancestor of the other typhlocybine tribes (Fig. 4). Within Typhlocybinae, a straight forewing vein MP''+CuA' of forewing is an ancestral feature with the strongly curved MP''+CuA' derived in the common ancestor of Typhlocybini + Zyginellini (Fig. 4). Hind wing veins R and MP are not confluent in the outgroups and this trait is retained by Alebrini but these two veins became confluent independently in Empoascini and in the ancestor of the other three tribes mostly. A reversal to the ancestral state occurred in three clades of Typhlocybini corresponding to *Eurhadina*, *Eupteryx* and *Aguriahana* (Fig. S2). Loss of the submarginal vein at the apex of the hind wing was reconstructed as having occurred independently in the ancestors of Erythroneurini and Typhlocybini (Fig. 4) but treating different patterns of loss of the submarginal vein as distinct states results in two different partially reduced states having arisen separately in Alebrini + Empoascini and the ancestor of Dikraneurini, Erythroneurini and Typhlocybini, with a reversal to the ancestral (complete submarginal vein) condition in Dikraneurini (Fig. 4). Acquisition of an unbranched vein CuA of hindwing (Fig. S2) and loss of the distal segment of CuA (Fig. S2) are both limited to Zyginellini. Loss of hind wing crossveins occurred independently in Zyginellini, Empoascini and Dikraneurini (except for *Michalowskiya*; Fig. S2). The unique unbranched anal vein was acquired in the ancestor of Erythroneurini (Fig. S2).

4. Discussion

The most taxonomically comprehensive phylogenetic analyses of Typhlocybinae attempted to date provide strong support for the monophyly of Typhlocybinae as defined by most recent authors. Although Mileewini, which Young (1965) included as a tribe of Typhlocybinae, is recovered as sister to Typhlocybinae sensu stricto, bootstrap and posterior probability support for this relationship is moderate (BS = 84% and PP = 0.853) in contrast to the strong support for Typhlocybinae (sensu stricto; BS = 99% and PP = 0.999). Therefore, we follow Dietrich (2011) and other recent authors (Chen et al., 2021) who treated Mileewinae as a separate subfamily. Within Typhlocybinae sensu stricto, our results are generally consistent with the recognition of five tribes that have been widely accepted by recent authors: Alebrini, Dikraneurini, Empoascini, Erythroneurini and Typhlocybini. This suggests that the characters of the fore- and hind wing traditionally used to diagnose these groups are generally stable and phylogenetically informative. Zyginellini, treated as a separate tribe by some authors (e.g., Dworakowska, 1970, 1977, 1979; Zhang, 1990), is a monophyletic group sister to Typhlocybini (sensu stricto) in the ML results but the branch separating Zyginellini from Typhlocybini received low bootstrap support (51%) and BI analysis not only failed to recover Zyginellini as monophyletic but also suggested that this group is derived from within Typhlocybini. This is consistent with the proposals of Ahmed (1983) and Dietrich (2013) to treat these two taxa as synonyms. Dietrich (2013) noted that specimens of individual species of South American Typhlocybini (not included in our dataset) may vary in the single hind wing character traditionally used to separate Zyginellini from Typhlocybini. Similarly, our analysis indicates that the *Eupteryx*-group (represented in our dataset by *Eupteryx, Agnesiella* and *Eurhadina*), treated by some previous authors (e.g., Ruppel 1987) as a separate tribe (Eupterygini) also based on one hind wing character, is polyphyletic and derived from within Typhlocybini sensu stricto. Reconstruction of the evolution of the hind wing character traditionally used to separate Eupterygini from Typhlocybini (RP and MA confluent or not) indicates that the non-confluent condition may have been derived independently in different genera. Further analyses incorporating larger numbers of taxa from all of these previously recognized groups are needed to elucidate their status and relationships with more confidence.

Recovery of Alebrini and Empoascini as monophyletic sister groups in our analysis contradicts the suggestion of some previous authors (e.g., Zhang 1990) that Alebrini by themselves are the earliest diverging lineage of Typhlocybinae or that Alebrini gave rise to the other tribes. Alebrini have been traditionally distinguished from other Typhlocybinae based on the retention of an appendix in the forewing, a trait shared with non-typhlocybine leafhoppers but absent in other tribes of Typhlocybinae. Presence of an appendix has been considered a plesiomorphic trait supporting the "basal" position of Alebrini within the subfamily. Our reconstruction of the evolution of this character suggests that, although the appendix was retained by Alebrini it was lost independently in Empoascini and the common ancestor of the other typhlocybine tribes. Morphological synapomorphies supporting the monophyly of Alebrini have not yet been identified so their recovery as a monophyletic group by our analysis is interesting. Further analyses of a larger sample of Alebrini genera, including representatives of endemic New World genera, will be needed to confirm the monophyly of this group. The endemic Neotropical genus *Protalebrella*, the only alebrine included in the phylogenomic analysis of Dietrich et al. (2017), was sister to the two included representatives of Empoascini, consistent with our results.

Our results indicate that, although the wing characters traditionally used to diagnose tribes of Typhlocybinae are generally conservative, they show some homoplasy and, by themselves, may not be reliable for diagnosing tribes within this subfamily. Thus, other morphological and molecular characters need to be considered in revising the higher classification of this large and diverse group.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.

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