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Dense sampling of taxa and characters improves phylogenetic resolution among deltocephaline leafhoppers (Hemiptera: Cicadellidae: Deltocephalinae)

Yanghui Cao[1] | **Christopher H. Dietrich**[1] | **James N. Zahniser**[2] | **Dmitry A. Dmitriev**[1]

[1]Illinois Natural History Survey, Prairie Research Institute, University of Illinois, Champaign, Illinois, USA

[2]United States Department of Agriculture, APHIS-PPQ-NIS, MRC-168, National Museum of Natural History, Smithsonian Institution, Washington, District of Columbia, USA

Correspondence
Christopher H. Dietrich, Illinois Natural History Survey, Prairie Research Institute, University of Illinois, 1816 S. Oak Street, Champaign, IL 61820, USA.
Email: chdietri@illinois.edu

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Abstract

We analysed a phylogenomic dataset comprising 730 terminal taxa and >160,000 nucleotide positions obtained using anchored hybrid enrichment of genomic DNA for a sample of deltocephaline leafhoppers and outgroups. Maximum likelihood analyses of concatenated nucleotide and amino acid sequences as well as coalescent gene tree analysis, yielded well-resolved phylogenetic estimates that were highly congruent with most branches receiving maximum support. Some topological incongruence occurs among the trees resulting from different analyses, mainly distributed among very short branches at intermediate levels in the phylogeny, pertaining to relationships among some tribes and multi-tribe lineages restricted to particular continents. Coalescent gene tree analysis revealed extensive gene tree conflict at these nodes, suggesting that certain relationships may remain difficult to resolve consistently even with genome-scale datasets and dense taxon sampling. Ancestral character state reconstruction of feeding preference indicates that grass specialization has been acquired three or fewer times in some highly diverse but relatively derived lineages. Molecular divergence time estimation suggests that the earliest divergences in the subfamily occurred during the Cretaceous but that most modern tribes did not appear until after the Cretaceous–Palaeogene boundary and that grass specialist lineages generally diversified and spread at the same time as grasslands were becoming widespread globally. These analyses also show a high level of global biogeographic structure, with several large lineages of primarily arboreal deltocephalines restricted to particular regions or continents and long-distance dispersal among continents occurring primarily among grass-specialist lineages. The results represent a major improvement over previous analyses of this diverse subfamily, which were based on 152 or fewer taxa and data from morphology and partial sequences of two genes.

KEYWORDS
anchored-hybrid enrichment, Auchenorrhyncha, biogeography, divergence time estimation, host specialization, Insecta, phylogenomics

INTRODUCTION

The leafhopper subfamily Deltocephalinae sensu lato (Zahniser & Dietrich, 2013) is a well-supported monophyletic group comprising >7200 valid species (>50% more than in Mammalia) placed in 970 genera and 39 tribes, making it the largest subfamily of Cicadellidae, itself one of the ten largest families of insects with >23,000 described species. The only undoubted fossil deltocephaline is known from Oligo-Miocene Dominican amber (Zahniser, unpublished), but molecular divergence time analysis suggests that the subfamily originated in the lower Cretaceous (Catanach, 2013; Dietrich et al., 2017); thus, their evolutionary diversification coincided with that of their primarily angiosperm host plants. Like other leafhoppers, deltocephalines are all sap-sucking herbivores and are ubiquitous in terrestrial ecosystems wherever their host plants are found. They feed preferentially on the phloem sap of a wide variety of vascular plants, with available records spanning >50 plant families including both woody and herbaceous dicots and monocots, and show varying degrees of host fidelity (Nickel & Remane, 2002). In grasslands and savannas, deltocephalines are among the most numerically dominant and diverse groups of herbivores (K. G. A. Hamilton, 1995; Whitcomb, Hicks, et al., 1987; Whitcomb, Kramer, et al., 1987) with many species specializing on grasses and others alternating between woody and herbaceous host plants. This prevalence of grass specialization has attracted attention in evolutionary studies (Catanach, 2013; Dietrich, 1999; Dietrich et al., 1997; Whitcomb et al., 1994; Whitcomb & Hicks, 1988; Zahniser & Dietrich, 2013), which indicate that specialization on particular grasses, shifts among grass species and geographic partitioning of host ranges have promoted speciation (Whitcomb, Hicks, et al., 1987; Whitcomb, Kramer, et al., 1987). Because of their abundance, host-plant specialization and sensitivity to disturbance, conservationists have recognized deltocephalines as good indicators of habitat quality (Biedermann et al., 2005; K. G. A. Hamilton, 1995; Wallner et al., 2012). Association of many deltocephalines with grasslands, the ecosystems most heavily impacted by human activities, has led to some species being considered for listing as threatened or endangered (K. G. A. Hamilton, 1999; Pinedo-Escatel et al., 2021), although the vast majority of species are too poorly known for their conservation status to be assessed.

The classification and composition of Deltocephalinae have long been controversial. Oman et al. (1990) proposed a provisional classification comprising 23 tribes, embodying a traditional definition of the subfamily based primarily on the structure of the head and leg chaetotaxy. However, other authors (e.g., K. G. A. Hamilton, 1975; Zhang & Webb, 1996) pointed out shortcomings of this definition and the first explicit phylogenetic analyses of Cicadellidae based on morphological (Dietrich, 1999; K. G. A. Hamilton, 1983) and molecular data (Dietrich et al., 2001) indicated that Deltocephalinae, sensu Oman et al. (1990) is paraphyletic. These results supported treatment of several subfamilies recognized by Oman et al. (1990) as tribes of Deltocephalinae (Anufriev & Emeljanov, 1988; Dietrich & Rakitov, 2002; Emeljanov, 1999). Subsequent, more detailed analyses of Deltocephalinae and related groups based on additional morphological characters (Zahniser & Dietrich, 2008, 2010) and molecular data supported synonymizing several additional groups treated as subfamilies by Oman et al. (1990) with Deltocephalinae. The most comprehensive phylogenetic analysis of Deltocephalinae to date (Zahniser & Dietrich, 2013) combined morphology with DNA sequences from two gene regions (~2800 bp of *28S* and ~350 bp of *histone H3*) for 152 exemplar species representing all the 38 recognized tribes at that time and 7 unplaced genera. The phylogeny resulting from this analysis was reasonably well-resolved and supported near the base and toward the tips of the tree, and largely congruent with a previous morphology-based phylogeny (Zahniser & Dietrich, 2008), but many intermediate branches received only low to moderate support based on maximum likelihood (ML) bootstrap scores and Bayesian posterior probabilities. Although results of this analysis were used to revise the tribal classification, the largest tribe, Athysanini, remains polyphyletic as presently defined and the analysis included too few representatives of this and other tribes to provide a robust test of the status of recognized tribes or to resolve relationships among genera within tribes.

Several taxonomic studies (e.g., Dietrich & Dmitriev, 2003; Dietrich & Rakitov, 2002; Emeljanov, 1999; Knight & Webb, 1993; Linnavuori & Al-Ne'amy, 1983; Viraktamath & Anantha Murthy, 1999; Webb & Godoy, 1993; Zahniser, 2008; Zhang & Webb, 1996) have improved knowledge of the diversity of Deltocephalinae, but few have included explicit phylogenetic analyses (Bennett & O'Grady, 2012; Dietrich et al., 1997, 1998; Fang et al., 1993; Kamitani, 1999; Knight & Webb, 1993; Zahniser, 2021). Together, these analyses included only 17% of known deltocephaline genera; thus, relationships among the vast majority of genera and species remain unknown. Study of recently obtained samples from various biodiversity hotspots also indicates that many genera and species remain to be described, named and placed phylogenetically. A robust cyberinfrastructure platform already developed for deltocephaline leafhoppers (Zahniser, 2014) includes a complete nomenclatural database, country-level distribution data for all genera and species, an illustrated interactive key to tribes and standard images for ~450 genera. This platform was created using 3I software (Dmitriev, 2006a). The database was recently merged with the World Auchenorrhyncha Database and migrated into TaxonWorks (M. J. Yoder & Dmitriev, 2017).

A recent phylogenomic analysis of Membracoidea (sensu lato, including Cicadellidae; Dietrich et al., 2017) confirmed the monophyly of Deltocephalinae (sensu Zahniser & Dietrich, 2013) and resolved relationships among the included taxa with strong support but included only 22 representatives of the subfamily. To provide a more robust and detailed phylogeny of Deltocephalinae, we modified the anchored hybrid probe kit used by Dietrich et al. (2017) to obtain sequence data for a large number of additional taxa and used these data to provide improved estimates of the relationships among major deltocephaline lineages.

MATERIALS AND METHODS

Taxon sampling

To provide the most comprehensive taxon sample possible, we attempted to obtain representatives of as many of the known genera of Deltocephalinae as possible. Most of the specimens were obtained through collaboration with various terrestrial insect bioinventory projects and through our own collecting over the past 18 years. Specimens were collected using a variety of methods including sweeping, vacuuming, Malaise traps and at lights and placed directly into 95% ethanol in the field. Bulk samples were returned to the lab and sorted to morphospecies with each species placed in a separate cryo storage vial and kept in a −20°C freezer. Numerous specimens apparently representing undescribed genera and species were also included. Representatives of Neocoelidiinae (three samples) and Aphrodinae (Portanini, one sample) were included as outgroups based on placement of these taxa as sister to Deltocephalinae with strong support in a previous anchored-hybrid-based study (Dietrich et al., 2017). Totally, 726 deltocephaline samples were included in the dataset, representing 39 tribes, 479 genera and 725 species of Deltocephalinae (Table 1). The included taxa are listed in Table S1. Vouchers are deposited in the Illinois Natural History Survey Insect Collection, Champaign (USA).

Anchored hybrid probe design

The anchored hybrid probe kit used by Dietrich et al. (2017) was modified to increase the targeting specificity and efficiency for orthologues shared across Membracoidea. Initial target regions included the 514 loci selected by Dietrich et al. (2017) and the 2395 single-copy orthologues across arthropod species identified by Skinner et al. (2020). Individual alignments of the 388 loci used for phylogenetic analysis in Dietrich et al. (2017) were directly employed for probe design after removing the alignments with <120 consecutive base pairs (bp) of nucleotide sequence. For the remaining loci, we scanned a sharpshooter genome (*Homalodisca vitripennis* (Germar)) and 45 transcriptomes of membracoids (Table S2) to identify the best matching sequences for each species x locus combination (Ayala-Ortiz, 2019; Ettinger et al., 2021; Galetto et al., 2018; Johnson et al., 2018; Skinner et al., 2020). MAFFT (Katoh & Standley, 2013) with automatic selection of alignment algorithms was used to generate the alignment for each locus. We first selected the loci containing ≥30 taxa and at least one consecutive 120 bp region with >50% pairwise sequence identity. Identification of exon boundaries and further requirements for loci selection followed C. A. Hamilton et al. (2016) and Young et al. (2016). Specifically, selected loci should: (1) be at least 150 bp in length, (2) contain no exon boundaries and (3) contain no indels. Pairwise sequence identity was calculated using a sliding window of 120 bp to assess the conservation of sequences. Probes of 120 bp were designed from the most conserved regions with a spacing of 30–50 bp. The final probe kit includes ~50,000 probes targeting 685 loci of Membracoidea, 425 of which were included in Dietrich et al. (2017) with the remaining loci newly selected. Our probe kit also targets some bacterial genes (endosymbionts and plant pathogens), which are not analysed in this study.

TABLE 1 Representation of currently recognized deltocephaline tribes (with number of valid known genera/species in parentheses) and number of genera and species included in this study

Tribe	Number of genera	Number of species
Acinopterini (2/30)	1	3
Acostemmini (12/27)	6	9
Arrugadini (1/4)	1	1
Athysanini (227/1181)	106	114
Bahitini (25/187)	11	19
Bonaspeiini (26/150)	19	21
Chiasmini (21/329)	18	66
Cicadulini (14/118)	7	14
Cochlorhinini (11/105)	6	10
Deltocephalini (72/601)	31	38
Dorycephalini (1/2)	1	1
Drabescini (46/238)	30	44
Drakensbergenini (1/18)	1	1
Eupelicini (7/62)	4	7
Faltalini (13/63)	7	11
Fieberiellini (10/49)	3	3
Goniagnathini (4/62)	1	4
Hecalini (25/196)	15	42
Hypacostemmini (1/4)	1	1
Koebeliini (6/16)	3	3
Limotettigini (4/88)	3	7
Luheriini (1/1)	1	1
Macrostelini (37/376)	10	21
Magnentiini (2/2)	1	1
Mukariini (16/79)	11	18
Occinirvanini (1/1)	1	1
Opsiini (42/343)	25	47
Paralimnini (141/937)	62	63
Pendarini (8/255)	6	26 (27 samples)
Penthimiini (46/215)	20	24
Phlepsiini (5/83)	4	7
Punctulini (5/7)	6	7
Scaphoideini (64/748)	29	29
Scaphytopiini (5/176)	1	10
Selenocephalini (21/213)	8	15
Stegelytrini (30/90)	11	11
Stenometopiini (7/106)	4	18
Tetartostylini (1/11)	1	1
Vartini (8/29)	3	6
Total	479	725 (726 samples)

DNA extraction, sequencing and assembling

DNA was extracted from the abdomens of specimens (mostly males) using Qiagen DNeasy kits following manufacturer protocols except incubation time was increased to 48 hours to maximize yield. Library preparation was performed at Rapid Genomics LLC (Gainesville, FL), followed by Illumina paired-end 150 bp sequencing. Raw reads were processed using TrimmomaticPE (Bolger et al., 2014) to remove adaptors and poor-quality data with a minimum length of 50, leading and trailing settings of 5, sliding window setting of 4:15 and an Illuminaclip setting of 2:30:10. Cleaned reads were assessed for quality using FASTQC (Andrews, 2010) and then assembled using ABySS v2.1.0 (Simpson et al., 2009) with minimum mean k-mer coverage of 3. For each sample, three assemblies were generated using a k-mer length setting of 29, 50 and 90 bp, respectively.

Orthologue identification, alignment and filtering

To identify the orthologues, protein sequences of the sequences used for probe design were employed as queries. TBLASTN searches were performed against the assemblies generated in the last step with a cut-off E-value of 10^{-5}, and the results were sorted by bit score first, then E value. Then, the sequences of the best hit of each sample were extracted and used as queries for BLASTX search against the protein database comprising the original query sequences. Sequences eligible for reciprocal best BLAST hits were screened as candidate orthologues. To further filter paralogue sequences and bacterial genes, the candidate orthologues were also confirmed through TBLASTN searches against the full protein files of three insects, including *H. vitripennis* (Ettinger et al., 2021), *Nilaparvata lugens* (Stål) (Ma et al., 2021) and *Drosophila melanogaster* Meigen (Drysdale et al., 2005), as well as six bacteria that may either be associated with leafhoppers or cause contamination, including '*Candidatus* Sulcia muelleri' Moran (Bennett et al., 2016), '*Candidatus* Nasuia deltocephalinicola' Noda (Bennett et al., 2016), *Wolbachia* Hertig (Neupane et al., 2020), '*Candidatus* Phytoplasma' Firrao (Oshima et al., 2004), *Escherichia coli* (Migula) (Welch et al., 2002) and *Cutibacterium acnes* (Gilchrist) (Scholz et al., 2016). Orthologous relationships among the original protein query sequences and the full protein files of the three insects were determined by OrthoFinder v2.5.1 (Emms & Kelly, 2019). Sequences with the best hit to a bacterial gene, or an insect gene but not the orthologue of the original reference sequences, were excluded. In addition to the region matching the reference sequences, 20 bp flanking areas, which may provide additional informative characters for phylogenetic analysis, were also retrieved by a custom script. Nucleotide sequences were translated to amino acid sequences after introns were removed.

Sequences of each individual locus were aligned using MAFFT (Katoh & Standley, 2013) with automatic selection of alignment algorithms. The relatively short nucleotide sequences of many loci may contain limited numbers of informative characters useful for gene tree construction; therefore, loci from the same gene were concatenated, which resulted in 429 final alignments for gene tree construction. These alignments were further concatenated by a custom script to generate the complete datasets then trimmed by trimAl v1.4 (Capella-Gutiérrez et al., 2009) with a gapthreshold setting of 0.3. The 429 individual nucleotide alignments were also processed using the same trimming method and manually checked for misalignments and to exclude outliers before being used for gene tree construction. Percentage of missing data was calculated for each taxon using the complete nucleotide and amino acid alignments (Table S1). Taxa with >90% missing data were excluded from further analysis (these taxa were not included in the total reported under Taxon Sampling above).

Phylogenetic analysis

Three separate analyses were performed: (1) a ML analysis of the complete concatenated nucleotide sequence data; (2) a ML analysis of the same dataset (introns removed) translated into amino acids and (3) a coalescent gene-tree analysis performed using ASTRAL (Mirarab & Warnow, 2015). For the latter analysis, because the nucleotide sequences obtained for many loci were relatively short, trees were constructed based on the nucleotide sequences (rather than amino acids) of each gene to provide the largest number of potentially informative characters for constructing each gene tree using ML. All the alignments and partition schemes in this study were deposited in the University of Illinois at Urbana-Champaign Illinois Data Bank (UIUC IDB) (https://doi.org/10.13012/B2IDB-8842653_V1).

IQ-TREE v1.6.12 (Nguyen et al., 2015) was employed for model selection and ML analysis. To reduce the computational requirements for such large concatenated datasets, the -m TEST option and a fast relaxed clustering of 10% of partition pairs (−rclusterf 10) were chosen to select the best-fit substitution model for each gene and find the best partition schemes. One thousand ultrafast bootstrap replicates were performed with a -bnni option to reduce the risk of overestimating branch support with UFBoot due to severe model violations. To construct individual gene trees, the -m TEST option was used for model selection, and 1000 ultrafast bootstrap replicates were performed with the -bnni option. Then, the consensus trees for individual genes were used as input for multispecies coalescent analysis by ASTRAL v 4.10.5 (Mirarab & Warnow, 2015) under default settings. The -t 2 option was chosen to enable full annotation, including quartet support, quartet frequency and local posterior probability (LPP).

Divergence time estimation

Divergence times were estimated for branches on the concatenated nucleotide ML tree using the RelTime-OLS algorithm in MEGA v11.0.8 (Tamura et al., 2021), which relies on the estimation of branch lengths by the ordinary least-squares approach and is suitable for large datasets (Mello, 2018). Two calibrations were applied: (1) a minimum age of 17.5 million years ago (Ma) for *Frequenamia* DeLong based on an undescribed fossil belonging to this genus from Dominican amber (Zahniser unpublished) and (2) a mean age of normal distribution as 128 Ma with standard deviation of 10 Ma for the root node calibration of Deltocephalinae based on results of the comprehensive timetree for Membracoidea provided by Dietrich et al. (2017). Default settings were applied except the gamma distributed rates among sites with a gamma parameter of 1 was chosen.

To check the stability of divergence time estimates across methods, we also analysed our data using an alternative Bayesian dating method. The Bayesian method is very computationally intensive and, unfortunately, could not be used to analyse the complete dataset in our study. To generate a reduced dataset that was more computationally tractable, we performed both gene and sample selection. Eighty-six genes were chosen from the 429 individual gene alignments (~1/5 of the total genes) filtering first to obtain tree-like loci, then filtering for the most clock-like loci and finally filtering for tree length using SortaDate (Chen et al., 2021; Smith et al., 2018). We then selected 107 samples (~1/7 of the total samples) to represent all the tribes and major lineages with a preference of those with less missing data (missing nucleotides <20% in the 86-gene dataset). ML analysis based on the reduced concatenated nucleotide dataset was conducted using IQ-TREE v1.6.12 with the same settings used for the complete concatenated datasets. To estimate the divergence times, we first estimated the branch lengths and substitution rates by the approximate likelihood calculation with a GTR model in BASEML of PAML v4.9 (Yang, 2007). Then, MCMCTree in PAML v4.9 was implemented for Bayesian dating analysis with the following settings: clock = 2 (independent rates), model = 7 (REV, equal to GTR), rgene_gamma = 1 5.1, sigma2_gamma = 1 10, burnin = 2,000,000, sampfreq = 100 and nsample = 100,000. Calibrations were set at the root with a gamma distribution of (163.84, 128) and at the *Frequenamia* clade with a minimum age of 17.5 Ma. MCMCTree was run twice with different seeds to assess convergence. Parameters were checked in Tracer v1.7.1 to ensure the effective sample sizes were over 200 (Rambaut et al., 2018).

Ancestral character state reconstruction

Ancestral host preference and biogeographic areas were reconstructed based on the timetree generated in the last step using MrBayes Ancestral States with R (MBASR; Heritage, 2021) with a sampling setting of 500. Host preference was scored as a binary character with states: nongrass/sedge feeder and grass/sedge feeder. Biogeographic areas are scored as a multi-state character, divided into Oriental, Palaearctic, Afrotropical, Australian and the New World. We combined Neotropical and Nearctic distributions into a single category because many of the included genera belonging to various tribes have distributions that span these two biogeographic realms. Due to the large size of our dataset and the presence of polytomies in the input timetree, we were not able to use reconstruction programs (e.g., RASP, BioGeoBEARS) that incorporate more complex evolutionary models.

RESULTS

We obtained sequences for all 685 loci, 675 of which include sequences for >70% of samples and 649 of which include sequences for >90% of samples. For 711 samples, the percentage of missing nucleotides is less than 20% (Table S1). The final analysed dataset comprised aligned nucleotide sequences for 675 loci representing 429 genes comprising 163,365 nucleotide positions, 79,921 of which are phylogenetically informative. The concatenated amino acid alignment contains 53,969 positions, 15,615 of which are parsimony-informative. Trees resulting from the three analyses were generally very well resolved with nearly all branches receiving maximum branch support (Figures S1–S3): ML bootstrap support in the ML analysis and local posterior probability in the coalescent gene tree analysis. Most branches were also stable across analyses suggesting that the anchored hybrid sequence data are decisive with regard to most deltocephaline relationships. Nevertheless, several areas of the phylogeny appear to be unstable, as indicated by topological differences among trees produced by the three separate analyses. Many of these unstable branches received less than maximum support in one or more analyses, but there are also some cases where conflicting topologies received maximum branch support in all analyses based on ML bootstrap scores and local posterior probabilities. This accentuates the need to explore possible sources of conflict in large phylogenomic datasets.

In our results, topological conflicts may be attributable, at least in part, to conflicting phylogenetic signal among individual genetic loci. However, many of the included loci are short (275 of the 429 genes are represented by 300 or fewer nucleotide positions), and only 148 of the individual gene trees have average bootstrap scores >70%. Thus, many of the individual anchored hybrid loci do not include enough informative characters to yield a fully resolved phylogeny with strong branch support. The pie charts provided on Figure S3 illustrate the extent of apparent gene-tree conflict by showing the proportion of loci supporting the three alternative arrangements at each node. In all cases where different analyses yielded different topologies, support for the branch shown on the ASTRAL tree is less than 50% and, in many cases, gene tree support is nearly evenly distributed among the three alternative quartets. Most branches toward the tip and near the base of the tree are consistently resolved, but branches pertaining to relationships among several major, relatively derived, lineages are very short with high levels of apparent gene tree conflict. Nevertheless, given that many included loci do not include enough informative characters to completely resolve the phylogeny, much of the apparent gene tree conflict suggested by the ASTRAL results may be due to lack of signal, rather than conflicting signal. Therefore, the ASTRAL results should be interpreted with caution, and we suggest that the results of the concatenated ML analyses may be more reliable for our dataset.

Aside from a few genera that are probably misplaced to tribe in the present classification, the following tribes, as presently defined based on morphological criteria (Zahniser & Dietrich, 2013), are monophyletic (Figures 1 and S1–S3): Stegelytrini (except *Sychentia* Wei & Webb), Acostemmini, Goniagnathini, Acinopterini, Fieberiellini, Selenocephalini, Phlepsiini, Drabescini (except *Nirvanguina* Zhang & Webb), Bahitini (except *Bergolix* Linnavuori, *Brincadorus* Oman and *Concepciona* Linnavuori & DeLong), Scaphytopiini, Cicadulini (except *Knullana* DeLong), Hecalini (except *Hecalusina* He, Zhang & Webb), Macrostelini (except *Evinus* Dlabola), Limotettigini, Cochlorhinini, Koebeliini, Mukariini (except *Pseudobalbillus* Jacobi), Vartini, Punctulini, Stenometopiini, Chiasmini, Faltalini, Deltocephalini (except *Loeia* Duan and *Yuanamia* Zhang & Duan) and Paralimnini. Penthimiini is consistently paraphyletic with respect to Magnentiini, Bonaspeiini is consistently paraphyletic with respect to Selenocephalini, and Athysanini, Opsiini and Scaphoideini are consistently polyphyletic. Among the latter three tribes, Athysanini and Scaphoideini are poorly defined morphologically (Zahniser & Dietrich, 2013).

Several genera currently classified in the largest tribe, Athysanini, which previous studies have also shown to be polyphyletic, are confidently placed phylogenetically in other, better defined tribes by our results (e.g., *Phlepsanus* Oman in Phlepsiini, *Hardya* Edwards and *Watanabella* Vilbaste in Cicadulini and *Arawa* Knight in Limotettigini). There are also some diverse lineages comprising large groups of genera currently placed in Athysanini. The largest such group is a clade including most of the endemic New World athysanine genera that is sister to the clade comprising the endemic New World tribe Scaphytopiini. Most remaining genera of Athysanini, including the type genus, belong to an unrelated lineage of genera mostly endemic to Eurasia but also including the endemic western North American tribe Cochlorhinini and the Holarctic and Oriental pine-specialist tribe Koebeliini.

Genera of Scaphoideini are mostly divided among two independent lineages: one comprises the large and widespread genera *Scaphoideus* Uhler and *Osbornellus* Ball and all of the Old World endemic members of the tribe and is paraphyletic with respect to the Old World tribe Drabescini. Most endemic New World genera currently placed in Scaphoideini are included in an almost exclusively New World clade that also includes endemic New World tribes Bahitini, Pendarini and Scaphytopiini, as well as most endemic New World genera of Athysanini.

Opsiini is defined based on the divided shaft of the male aedeagus, with each branch bearing a gonopore. However, this trait also occurs in some Mukariini, and one genus currently placed in the latter tribe, *Pseudobalbillus*, consistently groups with the clade

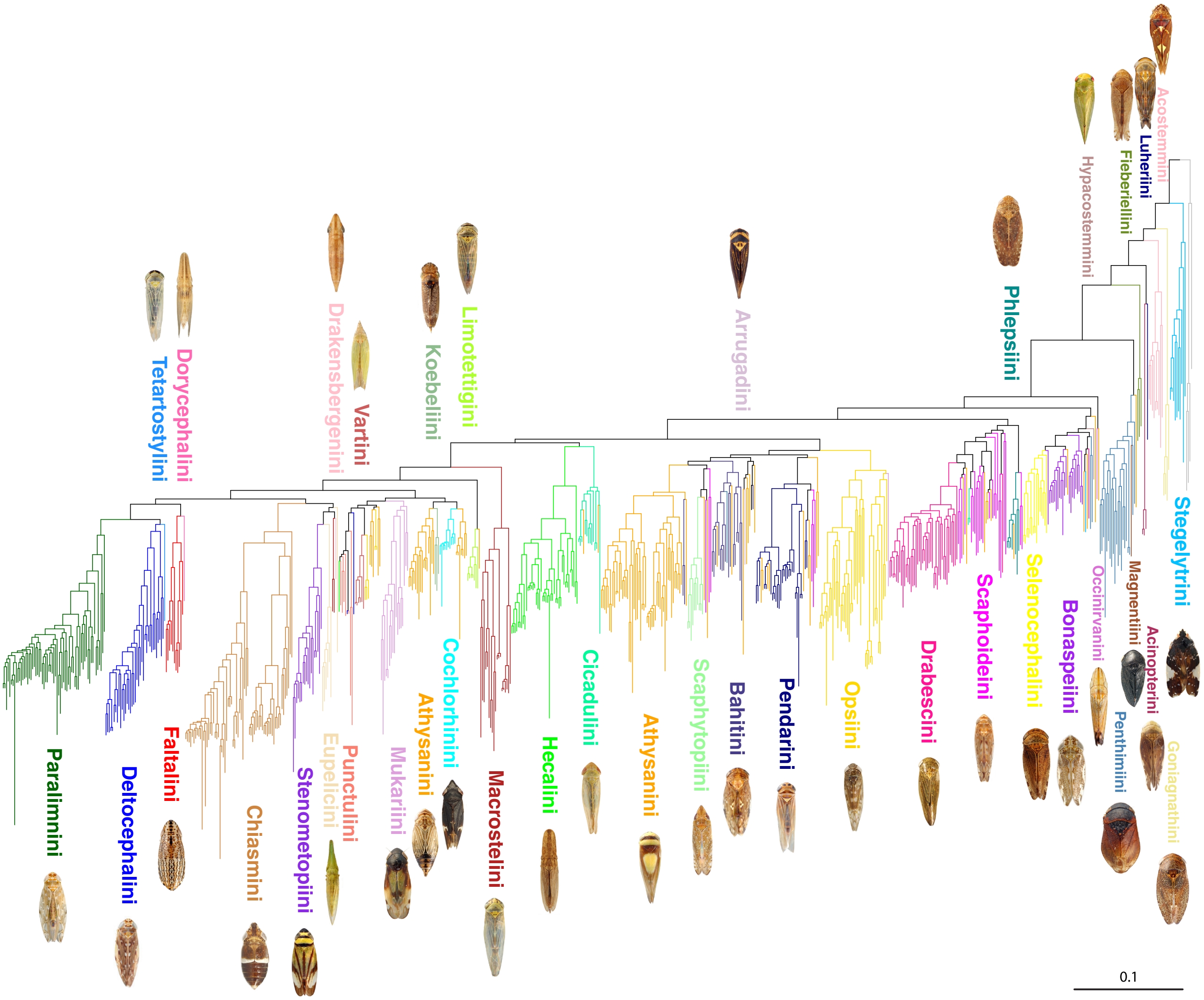

FIGURE 1 Consensus maximum likelihood tree of Deltocephalinae based on a concatenated nucleotide dataset comprising 163,365 positions. Branches are coloured according to tribes. Nearly all (705 of the 729) nodes received maximum bootstrap support, thus bootstrap scores are not shown here. The corresponding phylogram with details of taxon names and branch support is shown in Figure S1

comprising a majority of included Opsiini although its position within this group is unstable (Figures S1–S3).

Some larger lineages comprising multiple tribes are also stable in composition across results. One diverse lineage of grass-specialist leafhoppers comprising Deltocephalini, Paralimnini, Tetartostylini, Faltalini, Dorycephalini, Chiasmini, Stenometopiini, Eupelicini and Drakensbergenini was consistently recovered in all analyses (Figures 1, 2, S1–S4), although there is some conflict among results among the deepest splits in this lineage, for example, with the monobasic tribe Tetartostylini placed either as sister to Deltocephalini + Paralimnini (Figures S2 and S3) or as sister to Deltocephalini alone (Figures 1 and S1), and the group comprising Eupelicini, Drakensbergenini and the macrosteline genus *Evinus* either forming a monophyletic group sister to the remainder of the lineage (Figure S3) or a paraphyletic grade subtending Stenometopiini (Figures 1, S1, S2). Another diverse lineage that was consistently recovered includes a monophyletic clade comprising most Opsiini, all included members of the endemic New World tribes Arrugadini, Bahitini, Pendarini and Scaphytopiini, and all endemic New World genera currently placed in Athysanini (except *Extrusanus* Oman) and Scaphoideini (except *Omanana* DeLong and *Spathanus* DeLong) (Figures 1, 3, S5). Relationships within this clade are also somewhat unstable, with Opsiini (comprising mostly Old World taxa) sister to the remaining members of the clade in the concatenated nucleotide ML tree (Figures 1 and S1) but derived from among the endemic New World lineages in the other two analyses (Figures S2 and S3).

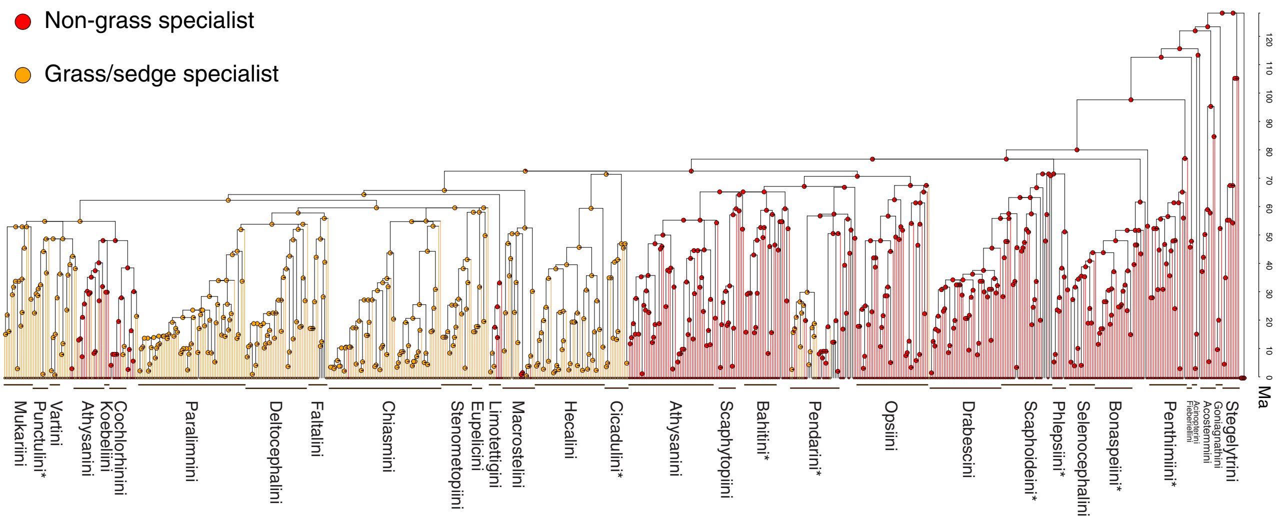

FIGURE 2 Ancestral state reconstruction of host plant preference (grass/sedge versus nongrass feeding) based on the RelTime timetree of Deltocephalinae. Clade marked with '*' indicates that the clade mainly consists of members of a particular tribe, but representatives of one or more other tribes are present. Details including tip labels are provided on the fully resolved tree in Figure S4

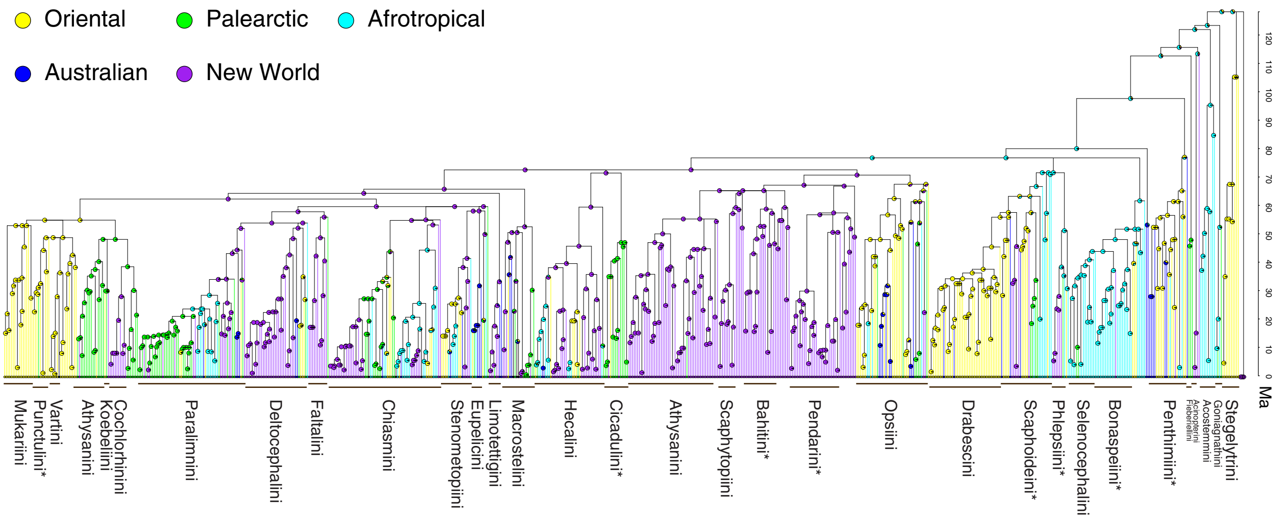

FIGURE 3 Ancestral state reconstruction of biogeography based on the RelTime timetree of Deltocephalinae. Clade marked with '*' indicates that the clade mainly consists of members of a particular tribe, but representatives of one or more other tribes are present. Details including tip labels are provided on the fully resolved tree in Figure S5

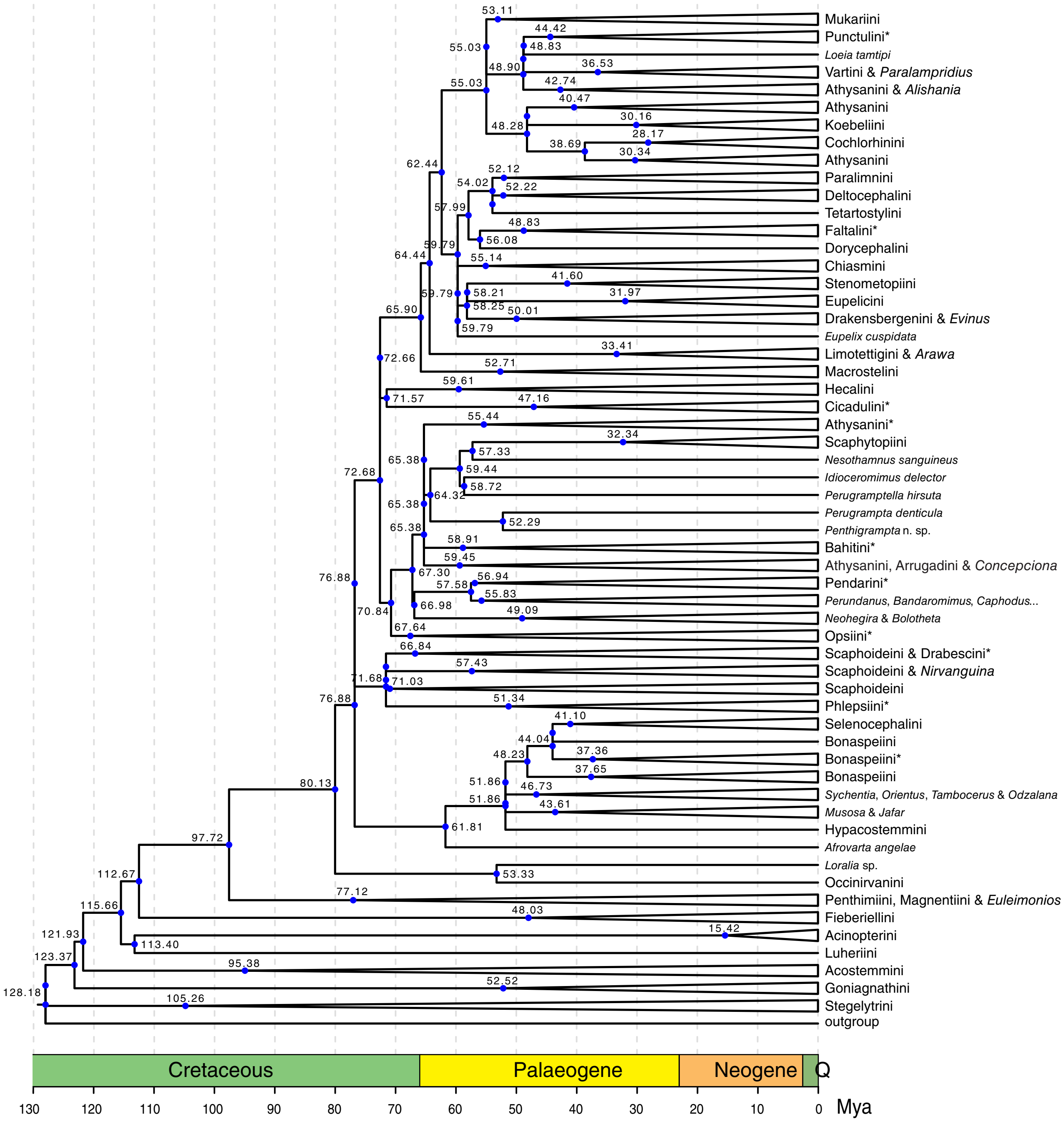

FIGURE 4 Chronogram of Deltocephalinae showing divergence times for major lineages resulted from the RelTime-OLS method. Clade marked with '*' indicates that the clade mainly consists of members of a particular tribe, but representatives of one or more other tribes are present. Details including tip labels are provided on the fully resolved tree in Figure S6

A third large clade consistently recovered in all analyses comprises Mukariini (excluding *Pseudobalbillus*), Punctulini, Vartini, Cochlorhinini, Koebeliini and nearly all the included Old World endemic Athysanini (= the '*Athysanus* group' sensu Zahniser & Dietrich, 2013) although, again, relationships among major lineages within this clade were somewhat unstable. The first three mentioned tribes are associated with bamboo, and they usually grouped with genera currently placed in Athysanini and Opsiini that are also associated with bamboo. Within the same large clade, Cochlorhinini, a tribe endemic to the southwestern USA, was consistently sister to a group of Palearctic Athysanini, including the type genus of that tribe (Figures S1–S3, S5). This group is consistently sister to a clade comprising the northern hemisphere pine-specialist tribe Koebeliini and another clade of mostly Palaearctic Athysanini.

Toward the base of the tree, two large clades, one comprising a paraphyletic grade of mostly Old World Scaphoideini subtending the widely distributed tribe Phlepsiini and Old World endemic tribe Drabescini, and another comprising a lineage of largely Afrotropical groups including Bonaspeiini, Selenocephalini and a few genera currently placed in other tribes, are largely stable, except for a few genera that switch positions among these major lineages in different analyses (Figures 1, 3, S1–S3, S5).

In all three analyses, the six deepest splits within Deltocephalinae (branches giving rise to Stegelytrini, Acostemmini, Goniagnathini, Luheriini, Acinopterini, Fieberiellini and including the split of Penthimiini and the remaining deltocephalines) are resolved consistently with the exception of the relative positions of Acostemmini and Goniagnathini, which are switched in the ML tree based on concatenated nucleotides compared to the other two results (Figures S1–S3).

Divergence times

Divergence time analysis based on our phylogenetic results, using the RelTime algorithm and incorporating the same internal fossil calibration point for Deltocephalinae used by Dietrich et al. (2017) with the age of the root node of Deltocephalinae calibrated based on the results of the previous analysis of Membracoidea as a whole, yielded slightly younger divergence times for most nodes (Figures 4 and S6). This is probably because the present analysis included much denser taxon sampling and was able to place the internal calibration point (based on an undescribed fossil from Oligo-Miocene Dominican amber attributable to the modern genus *Frequenamia*) more precisely. Analysis of a reduced set of taxa and characters with the Bayesian method implemented in MCMCTree (Figure S7) yielded divergence times that were consistently within the 95% confidence intervals for comparable branches in the RelTime analysis but were, on average, somewhat older than those obtained from RelTime. This may also reflect the much smaller taxon sample of the latter analysis. Both divergence time analyses indicate that a majority of the currently recognized modern tribes of Deltocephalinae originated either shortly before or shortly after the K-P boundary, 66 Ma with the root node of the subfamily occurring in the lower Cretaceous (~128 Ma) and some groups that mostly inhabit forests and feed on woody plants (e.g., Stegelytrini, Acostemmini, Penthimiini) originating in the middle Cretaceous.

Ancestral state reconstructions

Reconstruction of the binary character ‘nongrass/sedge specialist’ versus ‘grass/sedge specialist’ on the phylogeny from RelTime divergence time analysis (Figures 2 and S4) suggests that there may have been as few as two shifts to specialization on grasses or sedges, one in the New World endemic tribe Pendarini and another in the large lineage comprising 11 tribes thought to consist almost exclusively of grass/sedge feeders (Chiasmini, Cicadulini, Deltocephalini, Eupelicini, Faltalini, Hecalini, Mukariini, Paralimnini, Punctulini, Stenometopiini and Vartini) and a few additional tribes including both grass/sedge and nongrass/sedge feeders (e.g., Cochlorhinini, Limotettigini, Macrostelini). Within the latter lineage, a few shifts to nongrass/sedge hosts, as well as reversals back to grass/sedge feeding apparently occurred, e.g., in the lineage including Cochlorhinini, Koebeliini and Athysanini (in part).

Biogeographic reconstructions, also based on the timetree from RelTime analysis (Figures 3 and S5), reveal substantial large-scale biogeographic structure within Deltocephalinae, with several major lineages largely or entirely restricted to a particular biogeographic realm or region. The earliest diverging groups are predominantly Old World, including the predominantly Oriental Stegelytrini, predominantly Malagasy Acostemmini and the widespread Palaeotropical Penthimiini, as well as a large predominantly Afrotropical clade comprising Bonaspeiini and Selenocephalini and a more widespread Old World clade comprising Drabescini and Old World representatives of Scaphoideini. A highly diverse New World lineage comprises endemic New World tribes Bahitini, Pendarini, Scaphytopiini and nearly all New World endemic representatives of Athysanini. More derived, grass/sedge specialist tribes also show strong continental-scale biogeographic structure with major lineages mostly restricted to particular regions and relatively few apparent transcontinental dispersal events.

DISCUSSION

Phylogenomic analysis of the most taxon- and character-rich dataset of Deltocephalinae ever compiled provides a well-resolved phylogeny with most branches consistently resolved among analyses and receiving maximum ML bootstrap and LPP support. The most comprehensive prior phylogenetic analysis of Deltocephalinae (Zahniser & Dietrich, 2013) was based on morphology combined with sequence data from only two genes and a taxon sample nearly five times smaller. Although these prior results are consistent with the present results in many respects, the present results yielded trees with greater stability and stronger branch support overall. This, in addition to the much larger taxon sample of the present study, with nearly five times as many terminals as the most comprehensive previous analysis of Deltocephalinae, it provides a more robust basis for assessing the status and relationships of deltocephaline tribes and tracing the evolution of traits of potential importance to the diversification of the subfamily.

Divergence times

The molecular timetree of Deltocephalinae based on the RelTime analysis places divergence of the root node in the lower Cretaceous, 128 Ma. Crown diversification of Stegelytrini, the earliest diverging lineage, a group presently restricted to Eurasia, is dated at ~105 Ma. Acostemmini, a group now largely restricted to Madagascar, diverged from the remaining Deltocephalinae ~122 Ma. The earliest split in this tribe occurred shortly thereafter, ~95 Ma, but diversification of the largest lineage did not begin until ~59 Ma, after initiation of orographic precipitation in Madagascar during the late Palaeocene/early Eocene (A. D. Yoder & Nowak, 2006). Despite the lack of direct fossil evidence, several other deltocephaline tribes also appear to have originated during the Cretaceous. These include Goniagnathini (~123 Ma), Fieberiellini (~113 Ma), Luheriini and Acinopterini (~113 Ma) and Penthimiini (~98 Ma). Our analyses date the origin of most remaining tribes during the Palaeogene with many originating 0–15 million years after the Cretaceous–Palaeogene boundary (K-P boundary) of 66 Ma. The large lineage comprising nearly all grass-specialist deltocephaline tribes is inferred to have originated very close to the K-P boundary marking the global extinction crisis attributed to the Chicxulub asteroid impact. The first undoubted grass macrofossils are from North America and have been dated to 55 Ma (Crepet & Feldman, 1991), and slightly younger fossils are known from Eurasia and Australia, but fossil pollen attributed to grasses occurred in South America as early as 70 Ma (Strömberg, 2011), and molecular divergence time analyses place the origins of grasses between 107 and 129 Ma (Prasad et al., 2011). Although these dates agree with our estimates of the time of origin of Deltocephalinae as a whole, our results suggest that most grass-specialist deltocephaline lineages may not have arisen until shortly before grass-dominated landscapes first appeared ~40 Ma and that diversification and spread of these lineages occurred as grasslands became widespread globally over the past 20 million years. Most deltocephaline lineages that primarily inhabit forests also show a pattern of crown diversification during the past 50 million years suggesting that most modern genera arose during the Palaeogene or Neogene. The very short branches separating many major lineages that appeared shortly after the K-P boundary suggest that deltocephaline leafhoppers underwent an explosive rapid radiation during the Palaeogene, filling a wide variety of niches distributed among geographic regions, biomes and host plants. Nevertheless, although numerous leafhoppers have been described from the relatively well-studied Eocene Baltic amber fauna, no Deltocephalinae have been reported from fossil ambers of this age or older. This suggests that Deltocephalinae did not become a numerically dominant group until relatively recently.

Diet evolution

Several previous authors (e.g., Whitcomb, Kramer, et al., 1987; Whitcomb & Hicks, 1988; Zahniser & Dietrich, 2010, 2013) have suggested that grass-specialist groups of Deltocephalinae are more highly derived than those that feed on woody or herbaceous dicots. The most comprehensive previous phylogeny of the subfamily consistently grouped several grass-specialist tribes into a single clade but also suggested that grass specialization arose more than once within Deltocephalinae. Our results also suggest that grass specialization is a conservative trait. Although the number of possible origins of this trait varies somewhat depending on which phylogenetic result is used, our ancestral state reconstruction for the binary character nongrass/sedge feeder versus grass/sedge feeder on the timetree produced by analysis of concatenated nucleotide data supports only two or three main independent origins of grass-sedge specialization (Figures 2 and S4), one in a clade of the New World tribe Pendarini, and one or two in the major lineage comprising Cicadulini, Hecalini and their diverse sister group which includes the remaining grass/sedge-specialist Deltocephalinae.

The latter very large clade comprising most grass/sedge specialist leafhoppers includes the tribes Deltocephalini and Paralimnini, which share a linear connective in the male genitalia and were previously suggested to represent the most derived group of Deltocephalinae (Whitcomb, Kramer, et al., 1987). This clade also includes Chiasmini, Dorycephalini, Drakensbergenini, Eupelicini, Faltalini and Stenometopiini, which, with the exception of one composite-feeding genus (*Driotura* Osborn & Ball), specialize on grasses.

Our results also include in this larger clade a diverse group of Old World bamboo specialists, including Mukariini, Punctulini and Vartini as well as several bamboo-feeding genera now included in Athysanini and Opsiini. This indicates that, despite apparently sharing some morphological traits with some nongrass specialist groups, grass-specialist genera placed in the latter two tribes are closely related to other grass-specialist deltocephalines.

Some lineages of this largest grass-specialist clade include clades that apparently underwent reversals from grass-specialization to feeding on nongrass hosts (as well as some subsequent shifts back to grass feeding in the same lineages). These include Macrostelini, most of which are apparently grass- or sedge-specialists but include *Davisonia* Dorst and *Sonronius* Dorst, as well as some *Macrosteles* Fieber species, which feed on nongrass hosts. Ancestral Limotettigini apparently specialized on woody hosts, but one lineage of this group shifted to sedge- or grass-specialization, as previously suggested by K. G. A. Hamilton (1994). The largely Palaearctic clade that includes the type genus of Athysanini and the endemic western North American tribe Cochlorhinini both also apparently underwent reversals from grass specialization to specialization on nongrass hosts, mostly in dry grassland habitats. Derivation of another clade comprising non-grass-specialist deltocephalines that inhabit open habitats includes the Holarctic tribe Koebeliini, which specialize on pines, and a clade of mostly Palaearctic Athysanini.

The remaining Deltocephalinae that feed on either woody or herbaceous hosts and those inhabiting forests form a large paraphyletic grade towards the base of the tree. Several large clades of forest-dwelling deltocephalines are largely restricted to particular continents (Figures 2, 3, S4, S5), suggesting that long-distance dispersal has been less frequent among arboreal lineages than among lineages that inhabit grasslands.

Biogeography

Due to the large size of our dataset, we were not able to use methods that incorporate sophisticated biogeographic models. Additionally, the conclusions drawn regarding biogeographic history can be influenced by taxon sampling, which was necessarily limited within tribes and genera in this study. Thus, the results of our ancestral area reconstructions using the relatively simple Bayesian model available in MBASR should be interpreted with caution. Nevertheless, the overall patterns of biogeographic association among and within major deltocephaline lineages are relatively simple and straightforward, so we think the present results provide a reasonable approximation of the main distributional patterns across the subfamily. Our reconstruction of large-scale biogeographic patterns reveals considerable biogeographic structure within Deltocephalinae, with many major lineages largely or entirely restricted to particular regions (Figures 3 and S5), even more so than may have been predicted by the current tribal classification. The Afrotropical region is slightly favoured as the ancestral area of the subfamily, although this is somewhat equivocal because one of the earliest diverging deltocephaline lineages, Stegelytrini, is almost exclusively Oriental and the sister group comprising Neocoelidiinae and Portanini is restricted to the New World. Aside from the clade comprising endemic New World tribes Luheriini + Acinopterini, most early diverging lineages originated in the Old World, either Oriental or Afrotropical regions and subsequently colonized other regions. Our divergence time estimates suggest that Deltocephalinae may not have arisen until after the modern continents had already begun to separate and no obvious continental-scale vicariant patterns are evident in the distributions of regional faunas among clades. This suggests that rare long-distance dispersal events followed by diversification have been the dominant force shaping modern biogeographic patterns of the subfamily overall. Penthimiini apparently originated in the Oriental region, separately colonized the Afrotropical and Australian regions, and most recently colonized North America. A large lineage originating in the Afrotropical region comprising members of various tribes at its base, the African-endemic tribes Bonaspeiini and Hypacostemmini, and Selenocephalini colonized the Palaearctic and Oriental regions. Most of the major grass-specialist lineages appear to have arisen in the New World and subsequently colonized different parts of the Old World through several separate dispersal events, with some subsequently re-colonizing the New World. For example, Stenometopiini is reconstructed as having originated in the New World but subsequently colonized Asia and, from there, Africa. Similar New World to Asia to Africa colonizations apparently also occurred in Chiasmini and Paralimnini although some dispersal in the opposite direction also apparently occurred, for example, the genus *Exitianus* Ball, the *Icaia* Linnavuori + *Athysanella* Baker clade, and several independent lineages of Paralimnini apparently dispersed from the Palaearctic into the Nearctic region. Colonizations of Australia have occurred independently multiple times with dispersal from the New World and Asia being most common but with one Afrotropical lineage of Hecalini also colonizing Australia. One grass-specialist lineage comprising Mukariini, Punctulini, Vartini and related genera currently placed in Athysanini originated in the Oriental region. In the RelTime timetree that was used for the biogeographic reconstruction, this group forms a polytomy with another clade comprising grass- and nongrass-specialist Palaearctic members of Athysanini, the Nearctic tribe Cochlorhinini, which includes both grass- and nongrass specialists and the pine-specialist tribe Koebeliini.

Implications for higher classification

Overall, our results generally support the monophyly of most previously recognized tribes (sensu Zahniser & Dietrich, 2013). However, a few small tribes are derived from within larger, paraphyletic tribes (e.g., Magnentiini derived from Penthimiini) and two of the largest tribes, Scaphoideini and Athysanini, are highly polyphyletic. Several genera belonging to these and other tribes appear to be misplaced to tribe based on our results. This may reflect morphological convergence among unrelated regional faunas based on adaptation to similar niches. For example, one of the characters diagnostic for Scaphoideini is the presence of reflexed, darkly pigmented veins on the forewing costal margin. Often these veins are accompanied by a dark preapical spot on the forewing and enlarged tufts of setae at the tip of the abdomen which, in combination, give the appearance of a false head at the posterior end of the body. Selection for such disruptive coloration, which may confuse visual predators such as birds and lizards, may have occurred independently in different regional faunas of arboreal deltocephalines, giving rise to convergent morphological syndromes that led to these unrelated leafhoppers being included in the same tribe. Most of the Old World or cosmopolitan genera placed in Scaphoideini form a large paraphyletic grade that gave rise to Drabescini. Endemic New World genera currently assigned to Scaphoideini group with a large, almost exclusively New World clade that includes Bahitini, Pendarini, Scaphytopiini and Athysanini in part. Pendarini could be expanded to include several genera currently placed in Athysanini or Scaphoideini. Scaphytopiini could be expanded to include *Perugrampta* Kramer, *Nesothamnus* Linnavuori and *Idioceromimus* Dietrich & Rakitov. A large clade comprising many athysanine genera endemic to Neotropical and Nearctic deciduous forests gave rise to *Platymetopius* Burmeister, the only member of this clade occurring in the Old World (Palaearctic). Thus, Platymetopiini, recently treated as a junior synonym of Athysanini (or as subtribe Platemetopiina; Dmitriev, 2006b), could be reinstated and expanded to include members of this forest-dwelling clade.

Among the grass-specialist tribes, most of those previously recognized based on morphological traits are monophyletic. Chiasmini is consistently monophyletic, in contrast to previous analyses in which the two main lineages of this group (one comprising the macropterous genera *Nephotettix* Matsumura and *Exitianus*, the other comprising the remaining, mostly brachypterous genera) did not consistently group together (Zahniser & Dietrich, 2013, 2015). Stenometopiini, which in some previous analyses was derived from within Chiasmini, is here consistently recovered as monophyletic and within a clade sister to Chiasmini. Eupelicini is paraphyletic, having given rise to the monobasic African tribe Drakensbergenini.

Most other tribes were consistently recovered as monophyletic, as presently defined. But a few genera appear to be misplaced to tribe. These include the Afrotropical genera *Jafar* Kirkaldy and *Musosa* Linnavuori, which are currently included in Penthimiini but grouped with the largely Afrotropical clade comprising Bonaspeiini, Selenocephalini and several other genera of questionable tribal placement (*Afrovarta* Zahniser, *Hypacostemma* Linnavuori, *Odzalana*

Linnavuori, *Tambocerus* Zhang & Webb, *Sychentia and Orientus* DeLong). The endemic New World tribes Bahitini and Pendarini could be rendered monophyletic by including several New World genera currently included in the polyphyletic tribe Athysanini. Several additional genera currently placed in Athysanini apparently also belong instead to other, smaller tribes, including *Dagama* Distant, which consistently groups with Bonaspeiini, *Phlepsanus*, which consistently groups with Phlepsiini, and *Hardya* and *Watanabella*, which consistently group with Cicadulini. More detailed analyses of individual lineages consistently recovered as monophyletic in our results are needed to further elucidate relationships among genera and to inform further revisions to the classification.

CONCLUSIONS AND FUTURE DIRECTIONS

Despite a massively increased sample of taxa and genes in the present dataset, a few areas of deltocephaline phylogeny, mostly pertaining to relationships among tribes and other major lineages at intermediate levels of divergence, are unstable. Given that most branches at both deeper and shallower levels within the phylogeny are consistently resolved by our data with strong branch support, our results suggest that these very short, intermediate-level areas may remain difficult to resolve even with larger numbers of characters and taxa. These short, moderately deep internal branches suggest that a rapid radiation gave rise to several major deltocephaline lineages almost simultaneously during the early Palaeogene. Such ancient rapid radiations have been reported in many other studies and continue to present major difficulties for phylogenetic inference, even in the genomic era (Whitfield & Lockhart, 2007).

Overall, our results provide a robust framework for future, more detailed analyses of individual deltocephaline lineages. Such analyses are needed because, although our present dataset includes >700 terminal taxa, these still represent only a small fraction (~10%) of the known diversity of Deltocephalinae. Future analyses incorporating more complex evolutionary models may provide additional insight on the phylogeny of Deltocephalinae, but computational constraints currently limit the application of many such methods to very large datasets. Given the recent reductions in cost of generating very large phylogenomic datasets, improved strategies are needed for using such taxon- and character-rich datasets to analyse the evolution of traits, such as host plant associations, that may have played important roles in generating the high levels of taxonomic and phenotypic diversity exhibited by highly diverse groups of insects such as deltocephaline leafhoppers.

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DATA AVAILABILITY STATEMENT

Raw sequence data have been uploaded to the Sequence Read Archive on GenBank (BioProject number: PRJNA780295; SRA accession numbers: SRR17212755-SRR17213484). Alignments and partition schemes that support the findings of this study are available in the University of Illinois at Urbana-Champaign Illinois Data Bank (UIUC IDB) at https://doi.org/10.13012/B2IDB-8842653_V1.

ORCID

Yanghui Cao https://orcid.org/0000-0002-0515-0767
Christopher H. Dietrich https://orcid.org/0000-0003-4005-4305

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Figure S1. Consensus maximum likelihood tree of Deltocephalinae based on a concatenated nucleotide dataset comprising 163,365 positions. Bootstrap values are 100 unless indicated. Branches are coloured according to tribes.

Figure S2. Consensus maximum likelihood tree of Deltocephalinae based on a concatenated amino acid dataset comprising 53,969 positions. Bootstrap values are 100 unless indicated. Branches are coloured according to tribes.

Figure S3. Cladogram of Deltocephalinae resulting from the multispecies coalescent analysis in ASTRAL based on 429 gene trees. Pie charts show the quartet supports of the main topology (navy), the first alternative quartet topology (yellow) and the second alternative quartet topology (grey). Effective number of genes, that is, number of gene trees that contain quartets around a specific branch, are indicated above each branch. Local posterior probability support values are one unless shown below each branch. Branches are coloured according to tribes.

Figure S4. Ancestral state reconstruction of host plant preferences based on the RelTime timetree of Deltocephalinae.

Figure S5. Ancestral state reconstruction of biogeography based on the RelTime timetree of Deltocephalinae.

Figure S6. Divergence times of Deltocephalinae resulting from the RelTime-OLS method in MEGA based on the concatenated nucleotide ML tree. Calibration nodes are marked in red. Bars on nodes are 95% confidence intervals.

Figure S7. Divergence times of Deltocephalinae resulting from MCMCTree based on a reduced concatenated nucleotide dataset (107 taxa × 86 genes). Calibration nodes are marked in red. Bars on nodes are 95% confidence intervals.

Table S1. Information of taxon sampling in this study.

Table S2. Genome and transcriptomes used for probe design.

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