Phylogenomics of palynophagous pine cone weevils (Coleoptera: Cimberididae) recovers the monophyly of Cimberidini and Doydirhynchini and reveals the paraphyly of *Cimberis*

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Abstract. The beetle family Cimberididae (pine cone weevils) consists of one extant subfamily, Cimberidinae, and two tribes, Cimberidini and Doydirhynchini, both limited in distribution to the Northern Hemisphere. Cimberidini comprise the genera *Acromacer* Kuschel, *Cimberis* Gozis and *Pityomacer* Kuschel and Doydirhynchini the genera *Doydirhynchus* Dejean and *Lecontellus* Kuschel. The larvae and adults of all known extant Cimberididae are palynophagous (pollen-feeding) in the male strobili of conifers of the genus *Pinus* (L.) (Pinaceae). We reconstructed the phylogeny of Cimberididae using data from 420 nuclear genes obtained via anchored hybrid enrichment. Our taxon sample included six species representing all described extant genera of Cimberididae except *Pityomacer*, which was unavailable for study. Most relationships in the resulting maximum-likelihood trees based on nucleotide and amino-acid sequence data were recovered with maximal bootstrap support. Both trees showed a monophyletic family Cimberididae, containing two clades corresponding to the tribes Cimberidini and Doydirhynchini. In Cimberidini, *Cimberis* was rendered paraphyletic by *Acromacer bombifrons* (LeConte). Notably, the internal and terminal branches of Cimberidini were very short compared to other parts of the tree. Generic relationships are discussed according to the phylogenetic results. To make the genus *Cimberis* monophyletic, we synonymize the names *Cimberis* Gozis, 1881 and *Acromacer* Kuschel, 1989 (syn. n.) and re-establish the combination *Cimberis bombifrons* (LeConte, 1876), proposed by Kuschel (1959). This study provides the first molecular phylogenetic hypothesis for the palynophagous weevil family Cimberididae.

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INTRODUCTION

The orthocerous (i.e., possessing straight antennae) weevil family Cimberididae Gozis, 1882 has been thought to be the sister group of all other extant weevils (Shin et al., 2018). Cimberididae contains one extant subfamily, Cimberidinae Gozis, 1882, and the tribes Doydirhynchini Pierce, 1916 and Cimberidini Gozis, 1882 (Alonso-Zarazaga et al., 2023). The Cimberidini comprise the genera *Acromacer* Kuschel, 1989, with one Nearctic species, *Cimberis* Gozis, 1881 (Fig. 1), with at least seven Nearctic

species and one or more species in the Palaearctic (Friedman, 2009), and *Pityomacer* Kuschel, 1989, with three Nearctic species (Kuschel, 1989; Anderson et al., 2014). The Doydirhynchini contain the genera *Doydirhynchus* Dejean, 1821, with two Palaearctic species, and *Lecontellus* Kuschel, 1989, with three Nearctic species (Kuschel, 1989; Anderson et al., 2014).

All Cimberididae are associated with conifers in the genus *Pinus* (L.) (Pinaceae) (Kuschel, 1989, 1993). Adults and larvae are palynophagous (pollen-feeding) (Hamilton,

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Fig. 1. *Cimberis attelaboides* (Cimberididae: Cimberidini) photographed by Olli Pihlajamaa at Lökbergen, Siuntio, Finland, on May 28, 2016. Used with permission.

1994) and larvae develop in and on the male strobili (Thomas & Herdy, 1961; May, 1993). Whereas the degree of host specificity is unclear for some species, most Cimberididae appear to feed on one or a few locally abundant species of *Pinus* (Hamilton, 1994). The extant distribution of Cimberididae roughly mirrors the native distribution of *Pinus*; however, the family is not (yet?) known from the Oriental Region (Anderson et al., 2014). Cimberididae likely have Jurassic origins (e.g., Shin et al., 2018; McKenna et al., 2019) and are represented in the Mesozoic fossil record, but the relationships of most Mesozoic weevil fossils to extant families remain obscure (e.g., Oberprieler et al., 2007; Anderson et al., 2014).

To provide phylogenetic context for taxonomic, systematic and evolutionary research on Cimberididae, we used anchored hybrid enrichment (AHE) (Lemmon et al., 2012; Lemmon & Lemmon, 2013; Haddad et al., 2018; Shin et al., 2018) to generate DNA sequence data from more than 500 nuclear genes for six species representing all described extant genera of Cimberididae except *Pityomacer*, which was not available for our study. We used the resulting data to reconstruct the phylogeny of Cimberididae and investigate tribe- and genus-level relationships.

MATERIAL AND METHODS

Taxon sampling

In total, 14 taxa were included in this study, eight of which were outgroups; the outgroup genomic data have previously been published. Outgroups represented the families Anthribidae (one species), Belidae (three species), Chrysomelidae (one species), Nemonychidae (two species) and Orsodacnidae (one species). Of these, the weevil families Anthribidae, Belidae and Nemonychidae are the closest living relatives of Cimberididae (Oberprieler et al., 2007; McKenna et al., 2009, 2018, 2019; McKenna, 2011; Shin et al., 2018). We sampled only a subset of the available potential outgroup species, e.g., from Shin et al. (2018), to maximize the number of loci available for analysis. Including more outgroups reduces the number of loci because most potential outgroup taxa have some missing data and some loci were not 1 : 1 orthologs in all possible outgroup species. Chrysomelidae + Orsodacnidae were used to root the resulting phylogenetic trees. For ingroup taxon sampling, six species of Cimberididae were included — with sequences newly produced here for five of them — representing four of the five extant genera and both subfamilies (Table 1). Newly generated raw data used in the present study are available from Zenodo (https://doi.org/10.5281/zenodo.14271180).

DNA extraction, library preparation and Illumina sequencing

Total DNA was extracted from legs or thoracic muscle, depending on the size of the specimen, using the G-Biosciences OmniPrep kit (G-Biosciences, Catalog #786-136, St. Louis, MO, U.S.A.), following the manufacturer's protocol, but with one modification: samples were incubated for 15 h instead of 15 min. Final DNA extractions were eluted with 60 µL of nuclease-free water and treated with RNaseA. The remaining body parts were preserved in 95% ethanol as vouchers and are stored in the McKenna Lab. Genomic DNA QC statistics were generated for

Table 1. Taxa included in molecular phylogenetic analyses, their sample ID and the total number of loci used for phylogenetic analyses.

Family	Subfamily	Species	ID number	Loci	Source
Chrysomelidae	Galerucinae	*Diabrotica undecimpunctata*	DDM2511	346	Shin et al. (2018); Haddad et al. (2018)
Orsodacnidae	Orsodacninae	*Orsodacne cerasi*	DDM2125	342	Shin et al. (2018); Haddad et al. (2018)
Anthribidae	Urodontinae	*Urodontus mesemoides*	DDM2579	288	Shin et al. (2018)
Nemonychidae	Rhinorhynchinae	*Bunyaeus monteithi*	DDM2640	352	Shin et al. (2018)
Nemonychidae	Rhinorhynchinae	*Rhynchitomacerinus kuscheli*	DDM2861	372	Shin et al. (2018); Haddad et al. (2018)
Belidae	Belinae	*Apagobelus brevirostris*	DDM0114	354	Li et al. (2024)
Belidae	Oxycoryninae	*Hydnorobius hydnorae*	DDM2555	215	Shin et al. (2018)
Belidae	Oxycoryninae	*Notorhopalotria montgomeryensis*	DDM0540	357	Shin et al. (2018)
Cimberididae	Cimberidinae	*Acromacer bombifrons*	DDM0386	391	Present study
Cimberididae	Cimberidinae	*Cimberis pilosa*	DDM2538	396	Shin et al. (2018)
Cimberididae	Cimberidinae	*Cimberis turbans*	DDM4252	326	Present study
Cimberididae	Cimberidinae	*Cimberis elongata*	DDM3045	7	Present study
Cimberididae	Cimberidinae	*Doydirhynchus austriacus*	DDM0518	341	Present study
Cimberididae	Cimberidinae	*Lecontellus byturoides*	DDM0385	356	Present study

each extracted specimen using a Qubit fluorometer, and DNA quality (fragmentation/degradation and/or contamination with RNA) was further assessed via gel electrophoresis.

The DNA was fragmented by sonication with a Q800R2 Sonicator (Illumina TruSeq), using 50 µL of the DNA extractions in 0.2 mL strip tubes, targeting a modal fragment size of 350 base pairs. Libraries were constructed using the NEBNext Ultra II DNA Library Prep Kit (NEB #E7645L) with NEBNext Multiplex Oligos for Illumina (Dual Index Primers Sets 1 and 2) (NEB #E7600S and #E7780S), with two-sided size selection around a mode of 480 base pairs. Target enrichment through hybridization followed the myBaits Hybridization Capture for Targeted NGS (Version 5), with a 65°C hybridization temperature. We used our published Coleoptera AHE Probe set (Shin et al., 2018; Haddad et al., 2018) to target 599 loci.

Enriched libraries were amplified using KAPA HiFi HotStart ReadyMix. PCR cycling consisted of an initial denaturing step at 98°C for 2 min, followed by eight cycles of denaturing at 98°C for 20 s, annealing at 60°C for 30 s, elongation at 72°C for 45 s and a final elongation step at 72°C for 5 min. The enriched and multiplexed libraries were sequenced using 150-bp paired-end reads on an Illumina HiSeq Lane at Novogene Corporation Inc. (Sacramento, CA, U.S.A.).

Dataset preparation

We followed Breinholt et al. (2018) for dataset preparation and Haran et al. (2023) for the reference set used for assembly. Raw reads were assembled using an iterative baited assembly (IBA) after filtering with Trim Galore! V.0.4.0 (bioinformatics. babraham.ac.uk). Orthology was determined with the genome of *Tribolium castaneum* Herbst, 1797 as a reference, and single-hit and genome mapping location criteria were used with NCBI Blastn (Camacho et al., 2009). Cross-contamination checks were conducted with USEARCH (Edgar, 2010), and sequences with > 99% identity across different subfamilies were identified and removed. Cleaned sequences were aligned in MAFFT v.7.245 (Katoh & Standley, 2013), and isoform consensuses were generated using FASconCAT-G 1.02 (Kück & Longo, 2014).

AliView v1.18 (Larsson, 2014) was used to manually check each nucleotide alignment and ensure the probe region was in the correct open reading frame (ORF). We used a long-branch detection protocol to investigate the possibility of external contamination, paralogous sequences and/or major sequencing/assembly errors following Li et al. (2022) (longbranchpruner.pl available on Osiris, http://galaxy-dev.cnsi.ucsb.edu/osiris/). We produced maximum-likelihood (ML) gene trees from nucleotide (NT) multiple sequence alignments (MSAs) in IQ-TREE v.2.1.3

(Nguyen et al., 2015), conducting a full model test for each gene. We pruned tip sequences that exceeded eight standard deviations from the mean tip length of the gene tree from NT MSAs. Loci with < 50% taxon coverage were excluded. We used Phyx v.1.1 (Brown et al., 2017) to translate corresponding amino acid alignments. We also used Degen1 v1.4 (Regier et al., 2010; Zwick et al., 2012) to eliminate synonymous changes in NT MSAs. We used this approach because including synonymous changes can result in compositional bias, leading to the inference of incorrect phylogenetic relationships, especially for deep divergences (Regier et al., 2010; Zwick et al., 2012). Three datasets, NT, NTDegen and AA, were generated by concatenating MSAs using Phyx v.1.1. Five hundred seventy loci were assembled across 14 taxa, and 420 loci were ultimately selected for phylogeny inference after removing those with low coverage and other issues noted above.

Phylogenetic analyses and tests of node support

We conducted ML phylogenetic analyses using IQ-TREE v.2.1.3 (Nguyen et al., 2015). The NT dataset was partitioned by locus and codon position (1260 partitions), the NTDegen and AA datasets were partitioned by locus (420 partitions), and all datasets were model-tested in ModelFinder (Kalyaanamoorthy et al., 2017) as implemented in IQ-TREE. We found the best partition scheme after merging possible partitions ('-MFP+MERGE' command), determining the best scheme under the Bayesian information criterion (BIC). An initial number of 1000 parsimony trees generated in IQ-TREE with the command '-ninit 1000', and 100 trees with the fewest steps were used to initialize the candidate set (-ntop 100), considering all possible nearest-neighbor interchanges (-allnni). These 100 trees were maintained in the candidate set during the ML tree search (-nbest 100), and unsuccessful runs were terminated after 1000 iterations (-nstop 1000). Perturbation strength was set to 0.2 (-pers 0.2), as recommended for datasets with many short sequences. We applied nearest-neighbor interchange (NNI) branch swapping to improve the tree search and limit overestimating branch supports due to severe model violations ('-bnni' command). Node supports were computed with 1000 UFBoot ('-B' command) replicates (Hoang et al., 2018; Minh et al., 2020) and SH-aLRT ('-alrt' command) (Guindon et al., 2010). Nodes were classified as "maximally statistically supported" if they were recovered with support values of UFBoot = 100 AND SH-aLRT = 100, "robustly supported" if they were recovered with support values of UFBoot ≥ 95 AND SH-aLRT ≥ 80, "moderately supported" if UFBoot ≥ 95 OR SH-aLRT ≥ 80 and "weakly supported" if UFBoot < 95 AND SH-aLRT < 80 (Hoang et al., 2018; Minh et al., 2020).
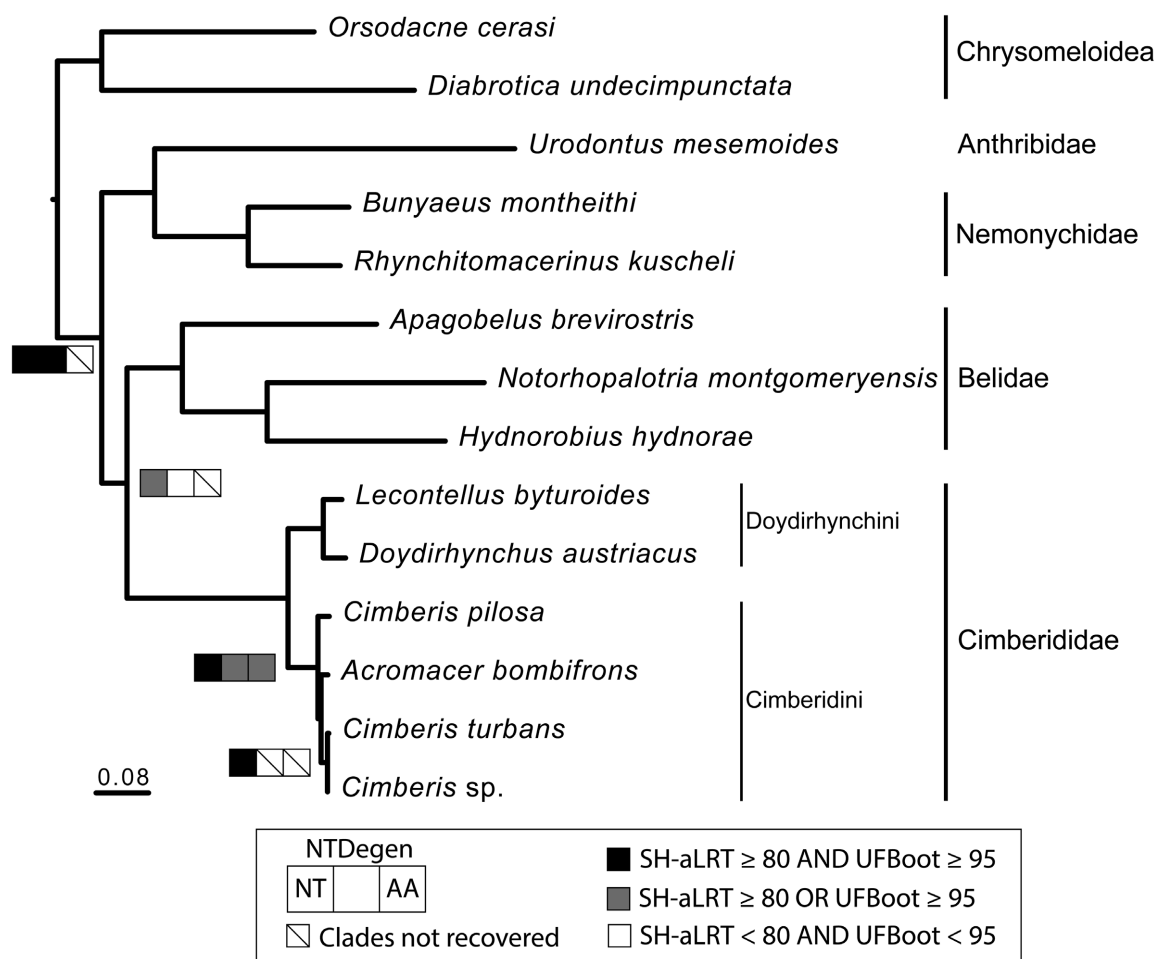
Fig. 2. Maximum-likelihood tree (phylogram) of Cimberididae with branch lengths proportional to the number of substitutions, resulting from a maximum-likelihood (ML) analysis of nucleotide sequences (NT) from 420 nuclear genes partitioned by gene and codon position. Squares at the branches are ML SH-aLRT and UFBoot values recovered from analyses of the NT, NTDegen, and AA datasets and are shaded according to the legend. Nodes without squares are robustly (maximally) supported in analyses of all three datasets.

RESULTS AND DISCUSSION

Phylogenomic data coverage and partitioning

A total of 599 orthologs were extracted across the 14 taxa studied. Loci with less than 50% taxon coverage (fewer than seven taxa with data) were excluded to reduce the amount of missing data in the resulting data matrix, leaving 420 loci for inclusion in phylogenetic analyses. For the final datasets, the length of each locus ranged from 144 to 576 bp (average of 231 bp), and the number of loci ranged from 215 to 396 (average of 341 loci) among all taxa, except for *C. elongata* (LeConte, 1876), which only yielded seven loci. The NT and NTDegen datasets comprised 97200 nucleotides, with 28880 and 3017 parsimony-informative sites, respectively. The AA dataset contained 32400 amino acids with 2968 parsimony-informative sites. The datasets have 24.0% missing data. In analyses of partitioned datasets, 1260, 420 and 420 partitions were pre-defined for the NT, NTDegen and AA datasets and were merged into 27, 10 and 14 partitions, respectively.

Phylogenetic relationships

All nodes had maximal statistical support in the resulting phylogenetic trees, except for the node suggesting the sister-group relationship between Belidae (three species sampled) and Cimberididae, the monophyly of Cimberidini and the sister-group relationship between *C. elongata* and *C. turbans* Kuschel, 1989, the latter of which is likely due to the low number of loci (7) recovered for *C. elongata*. Nemonychidae (two genera sampled, both Rhinorhynchinae Mecomacerini) were recovered as the sister-group of Anthribidae (one species sampled, in Urodontinae) (Fig. 2), as in most other recent molecular phylogenetic studies (e.g., McKenna et al., 2009; McKenna, 2011; Shin et al., 2018), and with maximal statistical support. The clade Nemonychidae plus Anthribidae was recovered as sister-group of the clade Belidae plus Cimberididae with maximal statistical support by NT and NTDegen datasets but was not recovered by the AA dataset (Fig. 2). The AA dataset, instead, weakly supported Nemonychidae plus Anthribidae as sister-group of Belidae (SH-aLRT/UFBoot = 33.3/49) (Fig. S3). A sister-group relationship between Belidae and Cimberididae, which conflicts with the results of previous studies (e.g., Shin et al., 2018), was moderately supported (SH-aLRT/UFBoot = 96/93) by the NT dataset (Fig. S1), weakly supported (23.4/48) by the NTDegen dataset (Fig. S2) and not recovered by the AA dataset (Fig. S3).

The phylogenetic placement of Cimberididae as a family separate from Nemonychidae, in which Cimberididae had previously been classified as a subfamily (Cimberidinae), was supported, as found by Shin et al. (2018). The monophylies of Cimberididae, Doydirhynchini and Nemonychidae were recovered with maximal statistical support. The monophyly of Cimberidini was maximally supported in analyses of the NT dataset and moderately supported by the NTDegen (85.4/77) and AA (92.3/88) datasets (Fig. S2 and S3). Doydirhynchini and Cimberidini were sister groups to each other. The species of the monotypic genus *Acromacer*, *A. bombifrons* (LeConte, 1876), was recovered inside *Cimberis* with maximal statistical support, rendering the latter genus paraphyletic. *Cimberis pilosa* (LeConte, 1876) was consistently recovered as sister-taxon of the remaining Cimberidini, but the relationships between *A. bombifrons*, *C. turbans* and *C. elongata* were not consistently resolved. Analyses of the NT dataset recovered maximal statistical support for the relationship *C. turbans* + *C. elongata*, whereas the relationship *A. bombifrons* + *C. elongata* was recovered by the NTDegen (0/57) and AA (0/57) datasets with weak support (Figs S2 and S3). Nonetheless, *C. turbans* never clustered together with *C. pilosa*, thus rendering the *C. pilosa* group (Kuschel, 1989), which Legalov (2009) formalized as a subgenus *Amerocimberis*, also as paraphyletic and the latter name superfluous. Based on the results of this phylogenetic study, we synonymize the names *Cimberis* Gozis, 1881 and *Acromacer* Kuschel, 1989 (syn. n.) and consequently resurrect the combination *Cimberis bombifrons* (LeConte), as previously established (Kuschel, 1959).

The phylogenetic position of *Acromacer* inside *Cimberis* revealed by our analysis makes it likely that *Pityomacer*, which was not included in our study but is very similar in phenotypic characters to *Acromacer* (Kuschel, 1989), falls inside *Cimberis* as well. *Acromacer* and *Pityomacer* were separated from *Cimberis* based on the shape of their mandibles (angled on the outside and without an inner tooth in the former two, rounded on the outside and with a large inner tooth in *Cimberis*). However, it is unclear how significant these differences are and whether other character sets, e.g., the presence of setiferous sex patches on the ventrites of the females (present in *Cimberis* and *Pityomacer* but absent in *Acromacer*) or the shape and setation of the labrum, may reveal a different and phylogenetically more pertinent pattern of relationships in Cimberidini. Judging from the very short branches of the four species included in our analysis, Cimberidini may comprise only a single genus (*Cimberis*).

The genus *Pityomacer* was unavailable for this study and should be included in future research on the phylogeny of Cimberididae. Additionally, the relationship of the genus *Nemonyx* Redtenbacher, 1845 (subfamily Nemonychinae), currently combined with Rhinorhynchinae into the family Nemonychidae, needs to be investigated to clarify and further solidify our understanding of the interrelationships of Anthribidae, Belidae, Cimberididae and Nemonychidae (McKenna et al., 2009, 2015; McKenna, 2011; Shin et al.,

2018; Li et al., 2024; McKenna et al., in prep.). The Palaearctic *Cimberis attelaboides* (Fabricius, 1787) (Fig. 1), the type species of *Cimberis*, should also be included in future studies to elucidate its relationship to the Nearctic species of Cimberididae. *Cimberis attelaboides* differs from the Nearctic species in that it has longer third antennal segment, but it has the same unusual ciliate endophallic sclerite as the American *C. pilosa* (Kuschel, 1993), which suggests that some Nearctic species are very closely related to *C. attelaboides*.

Sampling the remaining known species of Cimberididae and temporal calibration of the cimberidid phylogeny would help clarify patterns of morphological evolution, particularly in *Cimberis*, and timing and geographic patterns of taxonomic diversification. Moreover, temporal calibration of the phylogeny will help elucidate the timing of the divergences of sister taxa that have Palaearctic versus Nearctic distributions, such as *Doydirhynchus* and *Lecontellus*, which may result from ancient vicariance, e.g., as a consequence of the opening of the North Atlantic, or from relatively recent trans-oceanic dispersal. Any studies involving temporal calibration of the Cimberididae phylogeny should include a thorough evaluation of potentially relevant Mesozoic fossil records of orthocerous weevils, such as that undertaken by Li et al. (2024).

Given their palynophagous habits on Pinaceae, it would be illuminating to explore the genes encoded in the genomes of Cimberididae species that comprise the defensome (Marrone et al., 2012; McKenna et al., 2016). These include genes with known or putative involvement in the evolution of specialized phytophagy (McKenna et al., 2019; McKenna, 2020). However, outside of target enrichment data, there are no genomic resources available to facilitate exploration of the genes underlying cimberidid biology, notably including their pollen-feeding habits. It would be interesting, for example, to see if the genomes of Cimberididae exhibit similar suites of metabolic genes as those of other palynophagous Cucujiformia, such as the nitidulid beetle *Aethina tumida* Murray, 1867 (Evans et al., 2018), which includes pollen in its diet but is not strictly palynophagous. Moreover, there is nothing known about the bacterial or fungal symbionts of Cimberididae, which play important roles in the metabolism of plant material in other groups of Phytophaga beetles (e.g., McKenna, 2020; Salem & Kaltenpoth, 2021).

CONCLUSIONS

Our findings represent the first molecular phylogenetic hypothesis for the family Cimberididae and provide a basis for better understanding the evolution of palynophagy and Pinaceae feeding in orthocerous weevils. The previously recognized genus *Acromacer* is recovered as nested in *Cimberis* and is therefore subsumed into *Cimberis*. The genus *Pityomacer* and several other potentially notable ingroup and outgroup taxa should be included in future research to further resolve the phylogenetic position of Cimberididae, its internal relationships and timing and patterns of taxonomic diversification. However, any such studies

involving temporal calibration should include a thorough evaluation of the Mesozoic fossil record of orthocerous weevils. Finally, genomic resources are lacking for Cimberididae and would provide new opportunities for exploring the evolution of weevil trophic habits and beyond.

AUTHOR CONTRIBUTIONS. D.D.M. and X.L. performed the research; D.D.M. designed the study; X.L. and D.D.M. analyzed the data; D.D.M. and X.L. wrote the paper, and all authors provided feedback on a manuscript draft.

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SUPPLEMENTARY MATERIAL. Supplementary Figs S1–S3 are on the following page. Final sequence alignments and Newick tree files for Cimberididae are available from Zenodo (https://doi.org/10.5281/zenodo.14271180).
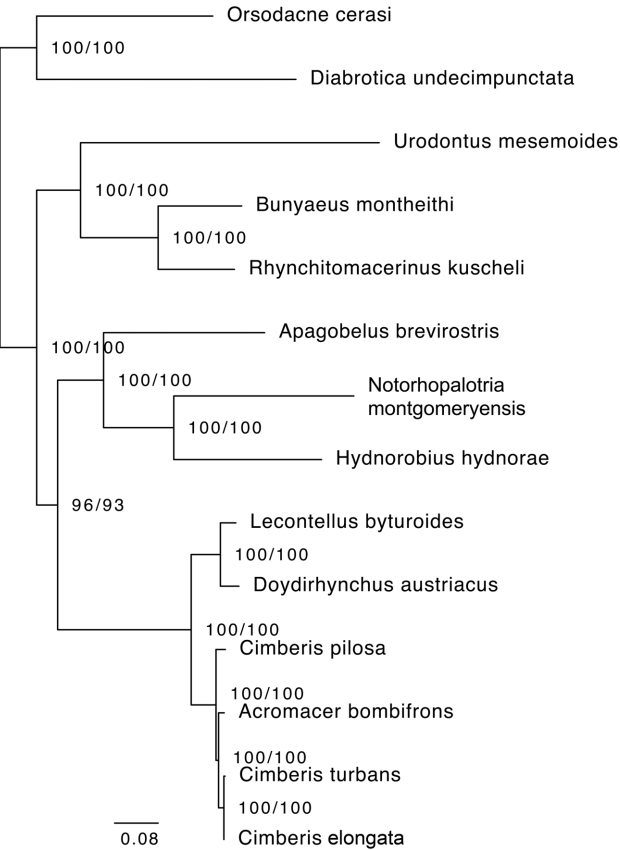
Fig. S1. Topology generated by maximum likelihood (ML) analysis of the NT dataset for Cimberididae partitioned by gene and codon position. Numbers at nodes are SH-aLRT (left) and UFboot (right).
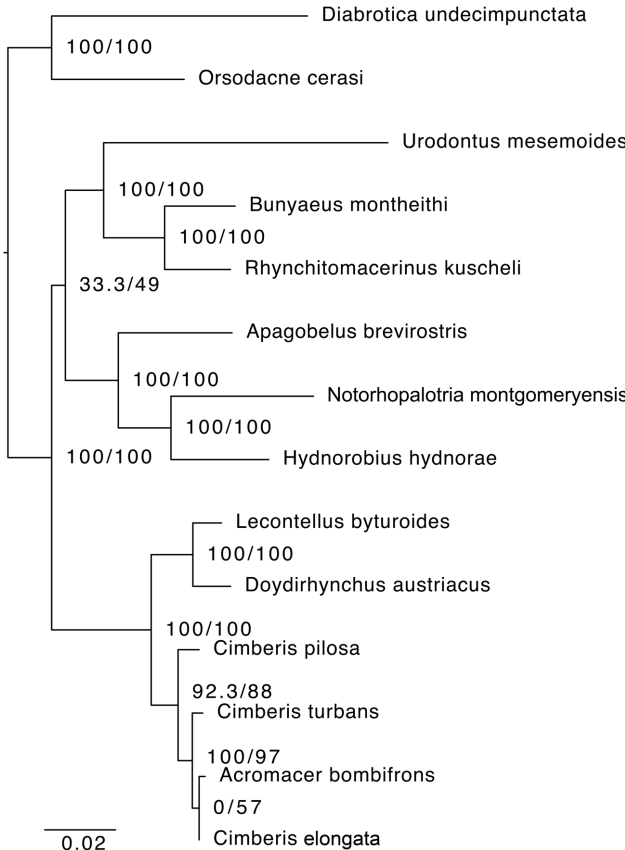
Fig. S3. Topology generated by maximum likelihood (ML) analysis of the AA dataset for Cimberididae partitioned by gene. Numbers at nodes are SH-aLRT (left) and UFboot (right).
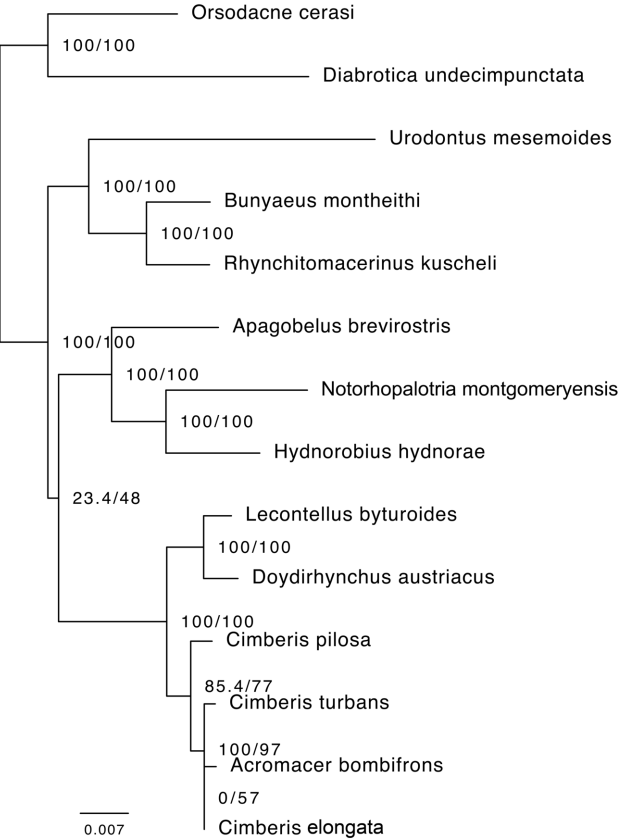
Fig. S2. Topology generated by maximum likelihood (ML) analysis of the NTDegen dataset for Cimberididae partitioned by gene. Numbers at nodes are SH-aLRT (left) and UFboot (right).