

# The phylogeny of ceutorhynchine weevils (Ceutorhynchinae, Curculionidae): Mitogenome data improve the resolution of tribal relationships

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## Abstract

Ceutorhynchinae Gistel are a diverse weevil subfamily of almost worldwide distribution and considerable economic importance. Nevertheless, the classification of Ceutorhynchinae and their phylogenetic relationships are not yet fully resolved. Here, we sequenced the mitogenomes of 54 ceutorhynchine species. Phylogenetic analyses by maximum likelihood and Bayesian inference were performed on a dataset of 13 protein-coding and two ribosomal genes. All analyses recovered three well supported clades A–C. A principal component analysis shows that codon usage differs considerably between these clades, indicating a compositional asymmetry in ceutorhynchine mitogenomes. This increased the challenge of resolving the early relationships among the three clades. The resolution of the later diversification was more robust, and the resulting topologies were largely compatible with each other and with the current taxonomic classification. Exceptions are the genera *Micrelus* Thomson, which is transferred from the tribe Ceutorhynchini to Egrriini Pajni and Kohli (new position) and *Amalus* Schoenherr, which is transferred to Phytobiini Gistel (new position). *Amalini* Wagner 1936 is a junior synonym of Phytobiini Gistel 1848 (syn. n.). *Coeliodini* Lacordaire (new status), a tribe previously regarded as junior synonym of Ceutorhynchini, is re-established. Our analyses also clarified the difficult assignments of taxa to the tribes Scleropterini Schultze and Phytobiini. All taxa with the ability to jump as adult beetles belong to clade B, which comprises the tribes Cnemogonini Colonnelli, Hypurini Schultze, Mecysmoderini Wagner and Phytobiini. With dense taxon sampling and appropriate analytical methods, mitogenome data provide a phylogeny well suited to improve the traditional classification of this neglected and species-rich taxon.

## KEYWORDS

base composition bias, Bayesian inference, maximum likelihood, mitochondrial DNA, phytophagy, weevil classification

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## INTRODUCTION

Weevils of the subfamily Ceutorhynchinae Gistel comprise a highly diverse group with about 1400 described species, many of which are of economic importance. In general, ceutorhynchine species are either monophagous, that is, tied to a single species or genus of host plants, or oligophagous, that is, using different species of host plants that belong to the same genus or at least the same family of plants (Colonnelli, 2004; Morris, 2008; Rheinheimer & Hassler, 2010). Their host plant associations determine if a species is relevant as an agricultural pest or a beneficial biocontrol agent. Several species of *Ceutorhynchus* Germar (e.g., the cabbage seedpod weevil (*C. obstructus* (Paykull)), the cabbage stem weevil (*C. pallidactylus* (Marsham)) and rape stem weevil (*C. napi* Gyllenhal)) are pests of cultivated species of *Brassica* spp., such as oil-seed rape (*B. napus* L.) and cabbage (*B. oleraceae* L.) in Central Europe (Juran et al., 2011; Williams, 2010). In contrast, some ceutorhynchine species take a beneficial role targeting invasive plant species, for example, *Phrydiuchus tau* Warner against the Mediterranean sage (*Salvia aethiops* L.) (Villegas, 2007), *Trichosirocalus horridus* (Panzer) against the plumeless thistle (*Carduus acanthoides* L.) (Kok & Mays, 1991) and the milfoil weevil *Euhrychiopsis lecontei* (Dietz) against the Eurasian water-milfoil (*Myriophyllum spicatum* L.) (Creed & Sheldon, 1993).

The Ceutorhynchinae has traditionally been of subfamily status (Alonso-Zarazaga & Lyal, 1999; Colonnelli, 2004). Lately, they were sunk into Baridinae Schoenherr (Oberprieler et al., 2007), and respectively, into Conoderinae Schoenherr (Prena et al., 2014). However, no evidence was provided for these new placements, which are also in conflict with a recent genomic phylogeny of weevils (Haran et al., 2023; Shin et al., 2018). Thus, in the following, we maintain their traditional status as a distinct subfamily of unknown relationships within the Curculionidae.

To date, Ceutorhynchinae is divided into 11 tribes and shows an almost worldwide distribution and occurs in a great variety of habitats (Colonnelli, 2004). Species of the genus *Neoxyonyx* Hoffmann are adapted to feed on *Ephedra* L. plants and live in desert and semi-desert habitats (Colonnelli, 1995). Several genera of the tribe Phytobiini Gistel are even adapted to an aquatic lifestyle, such as *E. lecontei* (Dietz) and *Eubrychius velutus* (Beck), with both larvae and adults spending most of their life submerged in freshwater (Newman et al., 2006). Curiously, their highest diversity is in the temperate zones of the Palearctic, followed by the Nearctic and southern Africa. Tropical regions are conspicuously less speciose. Each of these zoogeographic regions is dominated by one particular tribe (Korotyaev, 2008). First attempts to subdivide Ceutorhynchinae into natural groups focused on the Palearctic fauna (Schultze, 1902; Wagner, 1938). The largest Palearctic tribe Ceutorhynchini Gistel comprises about 900 species in 80 genera (Colonnelli, 2004). Other tribes, such as Hypurini Schultze (8 genera), Scleropterini Schultze (12 genera) and Phytobiini (9 genera), are less species-rich, while two others are monobasic, that is, Mononychini LeConte (with the single genus *Mononychus* Germar, comprising nine species) and Amalini Wagner (only represented by *Amalus scortillum* (Herbst)). The current composition of the tribes Scleropterini and Phytobiini is controversial, and some species may need to be excluded (Korotyaev, 2006). Originally, the Oxyonychini (Colonnelli) and

Coeliadini Dalla Torre and Hustache have been identified as natural groups by their particular host associations, but since no diagnostic morphological characters could be identified, they were later included in Ceutorhynchini (Colonnelli, 1984, 1995, 2004). The Oriental region is dominated by the tribe Mecysmoderini Wagner, comprising 120 species in total. Of these, 25% were discovered only in the past decade, indicating that the true diversity of this group may be severely underestimated to date (Colonnelli, 2004; Korotyaev, 2018, 2019; Korotyaev & Glikman, 2018; Lu et al., 2018). The current classification of the ceutorhynchine fauna of the Oriental, Afrotropical, and Nearctic regions was largely proposed by Colonnelli since the 1980s, by revisions and proposals of new tribes, that is, Lioxonychini Colonnelli (sub-Saharan Africa, 11 species; Colonnelli, 1984, 2004), Hypohypurini Colonnelli (sub-Saharan Africa, South Asia and Australia; Colonnelli, 2004) and Cnemogonini Colonnelli (predominant tribe of the Nearctic, 122 species; Colonnelli, 1979).

The phylogenetic relationships among ceutorhynchine tribes are currently not well understood (Korotyaev, 2006; Prena et al., 2014). So far, there are only two molecular studies on the phylogeny of Ceutorhynchinae. The first, directed at inferring the tribal affiliations of the genus *Wagnerinus* Korotyaev from East Asia, used a dataset of mitochondrial ribosomal RNA comprising 26 species of the tribes Ceutorhynchini, Scleropterini and Egrini Pajni & Kohli (Kato et al., 2006). In this study, neither Ceutorhynchini nor Scleropterini appeared to be monophyletic. This result was later corroborated by Letsch et al. (2018), based on nuclear and mitochondrial sequence data of 235 species, mainly from the Palearctic. *Micrelus* Thomson + *Cyphosenus* Schultze were found as sister group to all other tribes. The remaining ingroup was divided into two major clades, one mostly represented by the tribe Ceutorhynchini and the second a heterogeneous assemblage of species representing the majority of other tribes. However, support for all of these relationships were less robust than desired, especially along the backbone of the tree.

In the present study, we use a mitogenomic approach to infer the phylogenetic relationships of Ceutorhynchinae and verify the current composition of the tribes using a dataset of 54 newly sequenced mitogenomes. Although mitogenome sequence data have proven to be a powerful tool in the systematics of other weevils (Gillett et al., 2014; Haran et al., 2013), as well as other beetles (Timmermans et al., 2016) and other insect orders (e.g., Neuropterida; Wang et al., 2017, Palaeoptera; Song et al., 2019, Mantodea; Liu et al., 2023), their resolution of deeper phylogenetic splits may be limited, and their application require caution of a potential analytical impediment caused by substitutional saturation, base composition heterogeneity and rate variation among sites and taxa (Li et al., 2015; Sheffield et al., 2009; Song et al., 2010; Talavera & Vila, 2011; Timmermans et al., 2016).

## MATERIALS AND METHODS

### Taxon sampling and DNA extraction

A total of 54 mitogenomes were newly sequenced in this study. A total of 9 out of currently 11 recognized tribes of Ceutorhynchinae

(sensu Colonnelli, 2004) are represented in this selection, 7 of them by their type genus. Representatives of the two missing tribes and two missing type genera could not be obtained despite our best efforts. DNA samples representing 33 species were obtained from the Molecular Weevil Identification Project (MWISchütte et al., 2023). Specimens representing 23 species were freshly collected between 2013 and 2022 in Israel, Poland, Japan, Austria and Germany. The DNA of freshly collected material was extracted by a non-destructive method with the Analytik Jena innuPREP® DNA Micro Kit and the 658 bp long DNA-Barcoding fragment at the 5'-end of the Cytochrome c oxidase subunit (COI) gene was amplified (see Gottsberger et al., 2021). Morphological identifications were confirmed by blasting the COI gene fragment in the online identification tool of BOLD systems (Barcode of Life Database: <http://www.boldsystems.org>). Voucher specimens for all newly sequenced taxa are deposited at the Department of Botany and Biodiversity Research, University of Vienna. In addition, 4 mitogenomes of Ceutorhynchinae and 13 weevil outgroup species were downloaded from the NCBI GenBank (Table S1).

### Library preparation, sequencing and mitogenome assembly

Extracted DNA was quantified with a Qubit® 3.0 Fluorometer using the high sensitivity kit (Invitrogen; Thermo Fisher Scientific, Inc.). Genomic DNA libraries were prepared with the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® (New England Biolabs), and the fragmentation step was limited to between 5 and 10 min to achieve an optimal average fragment length of approximately 300 bp. An optimal adapter concentration was determined to avoid adapter dimer formation, and the genomic DNA was then amplified according to the manufacturer's instructions by using the Multiplex Oligos for Illumina® Dual Index Primers Set E7600S (New England Biolabs). The resulting libraries were quantified with a Bioanalyzer® to determine the fragment size distribution (2100 Bioanalyzer Instrument; Agilent Technologies, Inc.). Sequencing was performed by Novogene on the Illumina NovaSeq PE150 platform. The paired-end sequencing data underwent quality control and exclusion of low quality reads by using the FASTP v0.23.4 (Chen et al., 2018) and FASTQC v0.12.1 (Andrews, 2010) software before proceeding to further phylogenetic analysis.

Preliminary mitochondrial genomes were assembled from the trimmed and cleaned whole genome sequencing (WGS) short-reads using MEGAHIT v1.2.9 (Li et al., 2016) and BLAST 2.14 implemented in the MitoFinder pipeline v1.4.1 (Allio et al., 2020), with the complete *Ceutorhynchus obstrictus* (Marsham) mitogenome sequence (NC\_045101; Lee et al., 2019) as reference and the invertebrate genetic code for mitochondria. Mitochondrial tRNAs were annotated with ARWEN v1.2 (Laslett & Canbäck, 2008). In Geneious Prime 23.1.1., reads were mapped to the mitogenome contigs to check for indels and ensure completeness and circularization. Finally, complete mitogenome sequences were annotated using the MITOS2 webserver (Bernt et al., 2013; Donath et al., 2019). Subsequently, the boundaries

of protein coding genes (PCGs), tRNA and rRNA were determined by comparisons with annotated mitogenomes of the weevil genus *Trigonopterus* Fauvel (Narakusumo et al., 2019) as references. The newly generated mitogenome sequences were submitted to GenBank (see Table S1 for accession numbers).

For PCGs, we calculated the relative synonymous codon usage (RSCU), to infer a potential bias in codon usage among different genes and weevil groups. The RSCU is the ratio of the observed frequency of a codon to a uniform usage of all synonymous codons (Sharp & Li, 1986). A codon that is used less or more frequently than expected will have an RSCU value below or above 1, respectively. The calculations of the RSCU per PCG were performed in R (R Development Core Team, 2019) using the package 'seqinr' (Charif & Lobry, 2007). To infer a potential codon bias between different weevil groups, we applied a principal component analysis (PCA) based on the RSCU values obtained for each codon using the R package 'stats'. The PCA visualizes the clustering of the data by taxonomic groups.

### Sequence alignment and phylogenetic analyses

Protein coding sequences were each translated into amino acids and aligned with the M-Coffee algorithm (Wallace et al., 2006) as implemented in the T-Coffee webserver (Di Tommaso et al., 2011). Subsequently, the amino acid alignments were applied as templates to align the corresponding nucleotide sequences using the PAL2NAL v14 program (Suyama et al., 2006). Ribosomal RNA sequences were aligned with the L-INS-i algorithm as implemented in the MAFFT webserver (Katoh et al., 2017; Katoh & Standley, 2014).

Regions of low quality in all single alignments were identified and excluded with the program Aliscore v2.076 (Misof & Misof, 2009). For the PCGs, Aliscore was applied on the amino acid level, using the following parameter settings: sliding window size was four positions, gaps were treated as ambiguous characters and the number of possible pairwise comparisons was maximized. Subsequently, the identified random or ambiguous similarities were masked using Alicut v2.3 (Kück et al., 2010). The perl script *alinuc.pl* was then used to apply the masking scheme to the nucleotide data (Peters et al., 2017). The masked alignments of the different genes were concatenated with AMAS v0.96 (Borowiec, 2016). In total, two different concatenation matrices were compiled: (1) combined amino acid sequences of 13 PCGs and 2 rRNA genes and (2) combined nucleotide sequences of 13 PCGs and 2 rRNA genes.

Maximum likelihood (ML) in IQ-TREE (Chernomor et al., 2016; Nguyen et al., 2015) and Bayesian inference (BI) in PhyloBayes (MPI version 1.8c; Lartillot et al., 2013) were employed to conduct tree searches. For ML tree reconstruction of the nucleotide dataset, an a priori partition scheme with PCGs separated into codon positions and rRNAs separated into single partitions were created. This scheme comprised a total of 41 partitions. Subsequently, the best-fitting partition scheme and nucleotide substitution model for each partition were estimated using the ModelFinder algorithm (Kalyaanamoorthy et al., 2017) in IQ-TREE. Tree search was performed by

10 independent ML analyses increasing the number of stop iterations (–stop 200), an increased maximum number of iterations to stop (–nm 5000) and the ‘bnni’ option (Hoang et al., 2018) to reduce the risk of overestimating branch support. Branch support values were estimated using 1000 ultrafast (UF) bootstrap repeats (Minh et al., 2013). For the amino acid dataset, the partitioning and model scheme and tree search were applied in a similar manner, using genes as individual partitions.

For increased taxon coverage, we also conducted ML analyses on an extended dataset that included *cox1* and rRNA sequences for 17 additional ceutorhynchine species from GenBank (see Table S2). For these analyses, the additional sequences were included in the dataset of the combined nucleotide sequences of 13 PCGs and 2 rRNA genes and tree reconstructions were conducted as outlined before, using the best model schemes, as derived from the mitogenome analyses.

BI analyses of the mitogenome datasets were applied in PhyloBayes that implements the CAT mixture models, developed to better account for across site heterogeneities in sequence evolution and to reduce potential effects of compositional and mutational bias (Lartillot et al., 2007). For all analyses, the CAT-GTR model was used, which allows rate variation across sites under the GTR model and constant positions were included, as their exclusion might affect likelihood calculations (Whelan & Halanych, 2017). For each dataset, two independent chains with a minimum of 10,000 cycles with one tree sampled for each generation were conducted, and the convergence of model parameters and tree space with the PhyloBayes tools *tracecomp* and *bpcomp* were evaluated using a 20% burn-in. Convergence of topologies was ensured by calculating the maximum difference (maxdiff) in the bipartition frequencies of the two chains.

## RESULTS AND DISCUSSION

### Genome characterization

The complete mitochondrial genomes (mitogenomes) examined herein ranged from 16,353 to 19,696 bp in length. Their structure conforms to the general pattern found in other beetles and most metazoans, containing 13 PCGs, 22 transfer RNAs (tRNAs), 2 subunits of ribosomal RNA (rRNA) and a control region (CR; Bernt et al., 2013; Cameron, 2014a, 2014b). In all the species examined, the non-coding region is divided into two parts by the *trnI* gene. This division into CR1 (between *rrnS* and *trnI* genes) and CR2 (between *trnI* and *trnQ*) is presumably a derived character of Curculionoidea (Narakusumo et al., 2019). Control regions vary considerably in their length among different taxa (Figure 1a,b). A short non-coding region between *trnS2* and *nad1* was found in the following species: *Auleutes epilobii* (Paykull) (Figure 1a), *Coelioderes nigrinus* (Hong & Woo), *Hesperorrhynchus phytobioides* (Wollaston), *Mecysmoderes ater* Hustache, *Mogulonoides radula* (Germar), *Phrydiuchus topiarius* (Germar) and *Xenysmoderes consularis* (Pascoe). While this includes all species of the tribe Mecysmoderini of the dataset, the other species appear unrelated. As shown

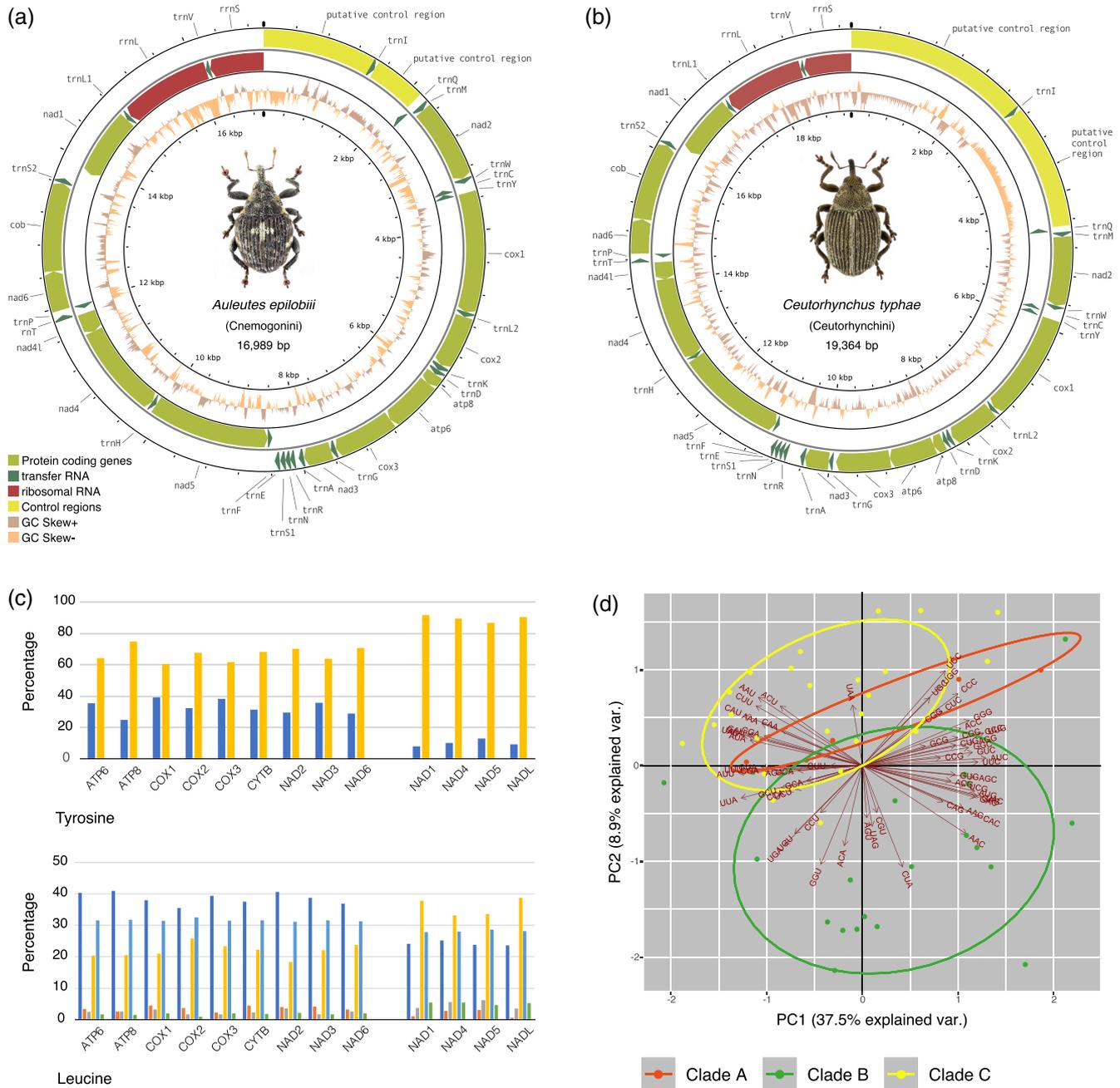
by Haran et al. (2013) for the genus *Ceutorhynchus*, most species also exhibit a non-coding region in the ARNSEF tRNA cluster. This insertion greatly varies in length, from only eight base pairs in *M. ater* to 500 bp in *Ceutorhynchus asper* Bedel. This length variability shows no phylogenetic pattern. Representatives with short as well as longer insertions occur in all tribes and some species even do not show an insertion at all.

Analyses of RSCU values recovered differential codon usage among PCGs as well as between different weevil groups. The relative codon usage measured among genes on the forward and reverse strand, respectively, shows large differences in the preferred codon on each strand (Figure 1c), as already shown for Curculionidae (Haran et al., 2013) and Coleoptera in general (Pons et al., 2010).

### Phylogenetic reconstructions

This pattern is also reflected by the partitioning scheme for the ML tree reconstruction analyses, as inferred with ModelFinder in IQ-TREE, where genes encoded on the forward and reverse strand, respectively, are assigned to the same partitions (Table S3). Partitioning of the dataset according to codon positions and strands has been shown to contribute to the performance of tree reconstruction in beetles, besides the application of site-heterogeneous models (Gillett et al., 2014; Haran et al., 2013; Timmermans et al., 2016). All tree reconstructions based on the site-heterogeneous CAT model in BI analyses and specific partitioning schemes in ML analyses recovered largely consistent relationships among Ceutorhynchinae, which formed three main clades A–C. However, the earlier nodes connecting the three clades remain unresolved, that is, only weakly supported in all ML tree reconstructions (extended dataset bootstrap support and mitogenome nt and aa dataset bootstrap support ( $BS_{\text{ext/nt/aa}} = 71/63/63$ ; cf. Figures 2, S1 and S2).

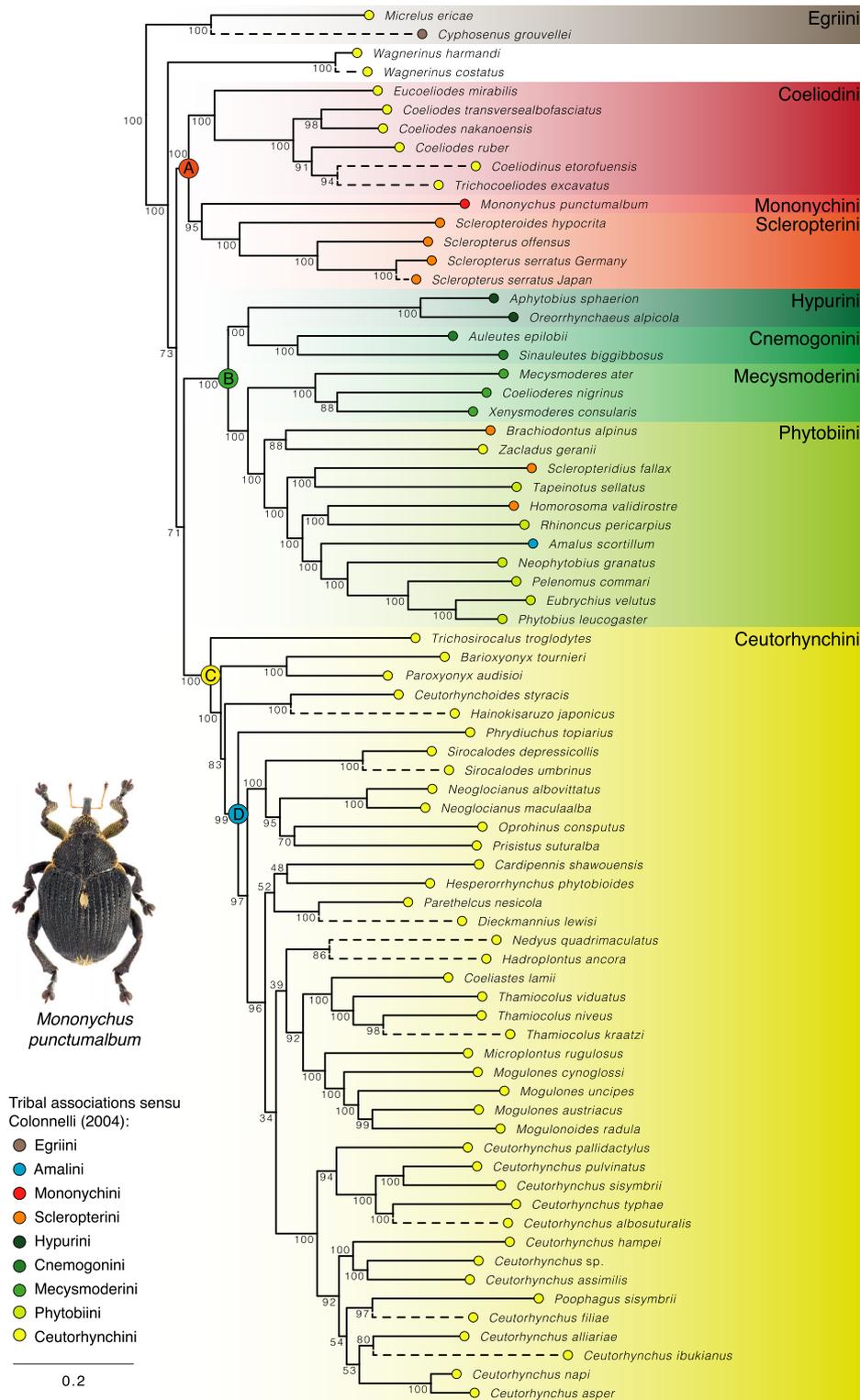
The PCA based on the RSCU calculations among different weevil groups shows that codon usage differs considerably between clades A and C on one side and clade B on the other, indicating a compositional asymmetry in ceutorhynchine mitogenomes. Heterogeneity in base composition across taxa has been reported as a confounding factor in tree reconstruction (Jermini et al., 2004; Lockhart et al., 1994), which can potentially cause ‘long-branch attraction’ (LBA) artefacts in phylogenetic reconstruction (Felsenstein, 1978), if unrelated taxa are grouped together. Since the divergence in base composition only occurs between clades A–C, it should not affect the phylogenetic reconstruction within each of these clades. However, the different codon usage in the individual clades leads to lineage-specific heterogeneous substitution patterns in the protein-coding genes of clades A–C. Similar to substitution saturation (multiple substitutions at the same site), these differences can blur the phylogenetic signal and therefore preclude a reliable reconstruction of relationships among these groups (Sheffield et al., 2009). Nevertheless, the value and usability of mitogenomic data for weevil phylogenetics could be clearly demonstrated, as most of the tribal relationships gained considerably higher support compared to an earlier one based on only



**FIGURE 1** (a) Map of the mitochondrial genome of *Auleutes epilobii*. (b) Map of the mitochondrial genome of *Ceutorhynchus typhae*. Protein coding genes are light green, rRNAs are red and tRNAs are dark green. Genes encoded on the heavy and light strands are shown outside and inside the circular gene map, respectively. Transfer RNA genes are labelled with their one-letter amino acid codes. Beetle images by M. E. Smirnov, 1993. (c) Codon usage on forward and reverse strands. The percentage of codon usage for two amino acids, tyrosine and serine, on the forward (eight genes) and reverse (four genes) strands, as examples of twofold and fourfold degenerate amino acids. (d) Principal component analysis based on the RSCU values of all codons. Clades are differentiated by colour as shown in the legend, and the arrows show the used codons.

four gene fragments (Letsch et al., 2018). However, the inferred compositional asymmetry between the three major clades A–C illustrates the limitations of mitogenome data. If heterogeneous patterns exist in the groups of interest, some relationships are hard to resolve, even with methods reducing the deleterious effects of compositional and mutational biases.

All our analyses consistently supported ( $BS_{\text{ext}/\text{nt}/\text{aa}} = 100$ , posterior probability ( $pP_{\text{nt}/\text{aa}} = 1.00$ ) *Micrelus ericae* (Gyllenhal) as sister to a clade comprising all remaining representatives of the ingroup. Its close relationship to *Cyphosenus* in the extended analyses indicates that *Micrelus* rather belongs to the tribe Egrini. This pattern corroborates the position of *Micrelus* inferred by Letsch et al. (2018). *Wagnerinus*



**FIGURE 2** Maximum likelihood tree inferred from the extended taxon sampling with combined nucleotide sequences of 13 PCGs and 2 rRNA genes using IQ-TREE. Node numbers show bootstrap support values. Coloured circles at species name indicate tribal associations as proposed by Colonnelli (2004). Lineages indicated by dashed lines are represented by COI/rRNA alone. Image of *Mononychus punctumalbum* by M. E. Smirnov, 1993.

represents the next splitting clade, but its position as a sister group to the major clades A–C, constituted by the remaining taxa, appears only weakly supported in both ML tree reconstruction analyses ( $BS_{\text{ext/nt/aa}} = 73/65/53$ ; cf. Figures 2; S1 and S2).

The remaining taxa are subdivided into three monophyletic clades that are strongly supported among all tree reconstructions ( $BS_{\text{ext/nt/aa}} = 100$ ,  $pP_{\text{nt/aa}} = 1.00$ ). Clade A consists of the tribes Coeliadini and Mononychini, as well as the genus *Scleropterus* (and *Scleropteroides* in

the extended dataset) that belong to the tribe Scleroterini. Mononychini (represented by *Mononychus punctumalbum* (Herbst)) is consistently recovered as sister taxon to Scleroterini, but this relationship was not significantly supported. In contrast to the analyses by Letsch et al. (2018), Coeliadini are clearly separated from Ceutorhynchini in this study.

Clade B consists of Cnemogonini + Hypurini as sister group to Mecysmoderini plus a clade consisting mainly of Phytobiini. The latter also includes *Scleropteridius* Otto, *Tapeinotus* Schoenherr and *Homorosoma* Frivaldszky, which were previously seen as part of Scleroterini, as well as *Amalus* Schoenherr and the genus *Zacladus* Reitter, which was regarded as a member of Ceutorhynchini by Colonnelli (2004), (Wagner, 1938: Coeliadini). These patterns are congruent with the phylogeny of Letsch et al. (2018), but now with more robust support. Moreover, these results emphasize the obvious problems to diagnose and delineate the tribes Scleroterini and Phytobiini (Korotyaev, 2006). Clade B further comprises all species that are able to jump as adult beetles, that is, Mecysmoderini: *Mecysmoderes* Schoenherr (Yoshitake, 2005); Cnemogonini: *Auleutes* Dietz; Phytobiini: *Neophytobius* Wagner, *Pelenomus* Thomson, *Phytobius* Schoenherr, *Rhinoncus* Schoenherr, *Homorosoma* and *Amalus* (Furth & Suzuki, 1992). These taxa do not form a monophyletic group, but the limited taxon sampling of our study precludes a comprehensive analysis of the evolution of the jumping organ. It could be an ancestral trait in this group, or may have evolved independently in different lineages, as shown for Alticini Newman (Ge et al., 2011).

Clade C comprises the remaining species and is largely congruent with the Ceutorhynchini as defined by Colonnelli (2004) excluding the genera *Wagnerinus*, *Zacladus* and *Micrelus*. Within this clade, the deep splits are well supported, corroborating the results of Letsch et al. (2018), with the genus *Trichosirocalus* Colonnelli as sister group to all other Ceutorhynchini. The next splitting clade comprising the genera *Barioxyonyx* Hustache and *Paroxyonyx* Hustache represents species feeding on Gnetales (*Ephedra* L.). Similar to Coeliadini, this unique host-plant association in beetles has led to the original designation of this group as a separate tribe Oxyonychini Hoffmann, 1956 (Korotyaev, 1998). However, our results corroborate its inclusion into Ceutorhynchini, as already proposed by Colonnelli (1984).

Within the 'core Ceutorhynchini' as defined by Letsch et al. (2018), the deep relationships remain unresolved, as the positions of the genera *Parethelcus* Dieckmann, *Cardipennis* Korotyaev and *Hesperorrhynchus* Peyerimhoff vary among all analyses (cf. Figure S1). This pattern supports the hypothesis of a rapid radiation of early 'core Ceutorhynchini' in the Early Eocene (Letsch et al., 2018). Such rapid radiations can be problematic in phylogenetic analyses: during the short time periods in which they take place, only few characters can accumulate to infer phylogenetic relationships. The long time since the origin of these lineages also increases the possibility that their signal is masked by later substitutions (Whitfield & Kjer, 2008).

In 'core Ceutorhynchinae', our analyses also support the prevalence of conserved host plant associations, meaning that closely related species feed on plants of the same family (Letsch et al., 2018; Rheinheimer & Hassler, 2010). We could infer monophyly of the

genera (1) *Ceutorhynchus* + *Poophagus* Schoenherr (nested in *Ceutorhynchus*), living on Brassicaceae Burnett; (2) *Mogulones* Reitter + *Mogulonoides* Colonnelli (nested in *Mogulones*), living on Boraginaceae Juss. and (3) *Coeliastes* Weise + *Thamiocolus* Thomson, living on Lamiaceae Martinov.

## Taxonomic implications

In many speciose clades of organisms, a relative paucity of stable morphological characters precludes phylogenetic reconstruction, as well as a satisfactory diagnosis of species and higher categories by morphological characters (Erpenbeck et al., 2006; Jörger & Schrödl, 2013; Tessler et al., 2022). Therefore, the question of how information from the growing body of molecular data and molecular phylogenies is best incorporated into the current classification, which has developed over the past centuries, is highly relevant. We believe that in the absence of conflicting morphological characters, careful adjustments to the classification of a group should be made, as long as the support from molecular phylogenetic analyses appears sufficiently robust.

The genus *Micrelus* was recovered with strong support as part of Egrini in all our analyses (herein; Letsch et al., 2018). A close resemblance of *Micrelus* to Egrini has already been implied by Voss (1962) with the description of several African species of *Micrelus*. For these species, he erected a new genus *Paregrius* Voss, as closely related to the genus *Egrius* Pascoe (Colonnelli, 1984). The genus *Paregrius* is now considered a subjective synonym of *Micrelus* (Alonso-Zarazaga & Lyal, 1999). Since there is no conflicting evidence, we transfer *Micrelus* from the tribe Ceutorhynchini Gistel to Egrini Pajni and Kohli (new position). This is also supported by the biogeographic focus of both *Micrelus* and Egrini in Africa (Colonnelli, 2004).

*Amalus* was found deeply nested in Phytobiini, which is consistent with its Polygonaceae hosts. The number of six articles of its antennal funicle is also found in its sister-clade, comprising *Eubrychius* Thomson, *Neophytobius*, *Pelenomus* and *Phytobius*. The shape of its rostrum is somewhat atypical of Phytobiini, but this is most likely a character reversal. The present evidence appears sufficient to transfer *Amalus* to Phytobiini (new position).

Coeliadini Dalla Torre and Hustache are here re-established as a distinct tribe (new status). This tribe was first introduced in Dalla Torre and Hustache (von Dalla Torre & Hustache, 1930) and further substantiated by Wagner (1938). Later, Coeliadini were regarded as a junior synonym of Ceutorhynchini Gistel (Alonso-Zarazaga & Lyal, 1999; Colonnelli, 1995). However, their host association with trees of the families Fagaceae and Betulaceae is very distinct from Ceutorhynchini, which generally feed on herbaceous plants (Colonnelli, 2004; Rheinheimer & Hassler, 2010). Their conspicuous apex of the meso- and metatibia (Wagner, 1938) with an ascending apical comb and their transverse elytral squamose patterns also support a separation from Ceutorhynchini.

*Wagnerinus* has previously been accommodated in Scleroterini (Korotyaev & Hong, 2004), and a close relationship to some members of this tribe was recovered by Letsch et al. (2018). In contrast, Kato

et al. (2006) inferred *Wagnerinus* as sister to Ceutorhynchini, Scleopterini and Mecysmoderini, whereas Colonnelli (2004) regarded it a member of Ceutorhynchini. None of these relationships gained high support, and the position of *Wagnerinus* remains unresolved. Herein, it is one of the two earliest branches of Ceutorhynchinae, with the caveat that there is only weak ML support for its sister group relationship with all other Ceutorhynchinae besides Egriini. Based on this contradictory evidence and the fact that it is obviously an important distinct lineage, we suggest that *Wagnerinus* is best placed in Ceutorhynchinae without tribal assignment, but ‘incertae sedis’ pending further clarification.

The recovered composition of the Phytobiini indicates that several genera originally included in Scleopterini should be transferred to Phytobiini, that is, *Tapeinotus*, *Homorosoma*, *Brachiodontus* and *Scleropteridius*, as well as *Amalus* (previously in their own tribe Amalini) and *Zacladus* (previously included in Ceutorhynchini). Although support for these placements is robust, we refrain from making any formal taxonomic changes at this point. An extended study on Phytobiini comprising a greater number of taxa, integrating also an analysis of morphological characters, should be done before proposing substantial rearrangements of their classification. Similarly, the genera *Amalorrhynchus* Reitter, *Poophagus* and *Drupenatus* Reitter appear to be deeply nested in the genus *Ceutorhynchus* (Letsch et al., 2018), making them candidates to be put into synonymy. This would also be supported by their host associations with Brassicaceae. However, there are morphological characters defining all these genera, and only *Poophagus* was part of our current dataset. On the other hand, these morphological differences from other *Ceutorhynchus* species, concerning especially the well-developed squamiform body vestiture, may easily be related to the water-depending lifestyle common to all these three genera. Therefore, we refrain from making any changes here. However, future studies comprising a larger number of *Ceutorhynchus* species should include *Amalorrhynchus*, *Poophagus* and *Drupenatus* and then decide if they are best treated as members of a single more inclusive genus *Ceutorhynchus*.

## CONCLUSION

Our new mitogenome dataset of 54 species of ceutorhynchine weevils provided deeper and new insights into the evolution and classification of this taxonomically neglected taxon. The phylogenetic resolution was superior to our previous analysis based on four markers (Letsch et al., 2018), although uncertainties remain about the early nodes of the tree’s backbone. The resulting topologies were largely compatible with the current taxonomic classification. Some differences need to be reconciled by correcting the existing classification. With enough evidence, this is reciprocal illumination (Hennig, 1966), not circular reasoning. A larger taxon sampling with more representatives of Nearctic and African groups, that is, Cnemonini, Egriini and Lioxyonychini, could further improve resolution of the deeper relationships within Ceutorhynchinae. However, it must be kept in mind that the differences in codon usage between the

clades, indicating compositional asymmetry, will not disappear with a larger taxon selection. Ideally, nuclear genomic data become available to clarify the remaining mysteries of early ceutorhynchine diversification.

## AUTHOR CONTRIBUTIONS

**Harald Letsch:** Conceptualization; investigation; funding acquisition; writing – original draft; writing – review and editing; visualization; validation; methodology; software; formal analysis; project administration; data curation; supervision; resources. **Sonja Vukotić:** Writing – original draft; writing – review and editing; software; data curation; formal analysis. **Brigitte Gottsberger:** Writing – review and editing; software; formal analysis; data curation. **Ariel Leib Leonid Friedman:** Writing – review and editing; resources. **Marek Wanat:** Writing – review and editing; resources. **Franziska Beran:** Writing – review and editing; data curation; formal analysis. **Konrad Fiedler:** Writing – original draft; writing – review and editing; resources. **Alexander Riedel:** Writing – original draft; writing – review and editing; conceptualization; methodology; data curation.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available. Sequences used in the analyses are available in GenBank (Tables S1 and S2). Alignments used for tree reconstruction analyses are provided in Zenodo data repository <https://doi.org/10.5281/zenodo.10598614>.

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### SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Figure S1.** Tree reconstruction results of the 71 taxa mitogenome dataset. Maximum likelihood tree inferred from combined nucleotide sequences of 13 PCGs and 2 rRNA genes using IQ-TREE. Node numbers show bootstrap support values.

**Figure S2.** Tree reconstruction results of the 71 taxa mitogenome dataset. Maximum likelihood tree inferred from combined amino acid

sequences of 13 PCGs and 2 rRNA genes using IQ-TREE. Node numbers show bootstrap support values.

**Figure S3.** Tree reconstruction results of the 71 taxa mitogenome dataset. Bayesian inference tree inferred from combined nucleotide sequences of 13 PCGs and 2 rRNA genes using PhyloBayes. Node numbers show posterior probability values.

**Figure S4.** Tree reconstruction results of the 71 taxa mitogenome dataset. Bayesian inference tree inferred from combined amino acid sequences of 13 PCGs and 2 rRNA genes using PhyloBayes. Node numbers show posterior probability values.

**Table S1.** Taxon sampling of the mitogenome dataset including NCBI Genbank accession.

**Table S2.** NCBI Genbank accession numbers of taxa added by COI and rRNA sequences.

**Table S3.** ML tree reconstruction partitioning and model scheme.

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