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Phylogenomic analyses of rare Neotropical lineages reveal the independent loss of antennal rami in railroad-worm beetles (Coleoptera: Phengodidae)

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Abstract

Phengodidae, or railroad-worm beetles, are bioluminescent soft-bodied beetles with flight capable adult males and highly paedomorphic larviform females. They are well accepted as part of the "lampyroid" clade within Elateroidea, and their suprageneric relationships have been recently studied using a phylogenomic approach. However, the placement of taxa currently classified in the subfamily Penicillophorinae remained untested. Penicillophorinae form an assemblage of morphologically modified, rarely collected Neotropical genera that are unique among Phengodidae. They are particularly characterized by their moniliform, serrate or uniramose antennae, in contrast to the typically bipectinate antennae present in all other members of the family. To investigate the phylogenetic position of Penicillophorinae, we implemented a low-coverage whole genome sequencing approach to produce genomic data for *Acladocera* and *Walterius*, two out of five genera classified in this subfamily. The resulting phylogenomic analyses confirmed the monophyly of Phengodidae; however, Penicillophorinae were not found as a monophyletic group. Our results recovered the topology: Cydistinae + (Phengodinae + (*Acladocera* + (Cenophenginae + Mastinocerinae including *Walterius*))). Therefore, we suggest that the antennal double rami were lost at least twice among Phengodidae: once in the newly circumscribed Mastinocerinae (with *Walterius*), and once in *Acladocera*, which we tentatively keep in Penicillophorinae. Further, we discuss the morphological modifications of other genera currently classified in Penicillophorinae. Future phylogenomic research should focus on clarifying the boundaries and composition of phengodid subfamilies, particularly by including additional genera from Penicillophorinae and Mastinocerinae.

Key words

Acladocera, bioluminescence, neoteny, paedomorphosis, soft-bodied beetle, *Walterius*

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1. Introduction

Phengodidae are a small soft-bodied elateroid family, currently containing approximately 300 species in 45 genera (Kundrata et al. 2019; Ferreira et al. 2024; Roza 2025; Uchima-Taborda et al. 2025). The group is present in the Americas and in West Asia, with most of its diversity concentrated in the Neotropical realm (Kundrata et al. 2019; Ferreira et al. 2024; Roza 2022; Zaragoza-Caballero and Pérez-Hernández 2014). The family is of particular interest to entomologists and evolutionary biologists due to the presence of bioluminescence, which is found in both immatures and adults (Costa and Zaragoza-Caballero 2010), and the paedomorphosis syndrome, which highly affects all known females that remain larviform in their adulthood (Costa et al. 1999; Ferreira and Ivie 2022). Based on current phylogenetic hypotheses, Phengodidae are a monophyletic family well nested within the Elateroidea, as part of the "lampyroid" clade (Ferreira et al. 2024; Kundrata 2025). Their placement in Elateroidea is corroborated by multigene Sanger-based analyses (e.g. Kundrata et al. 2014; McKenna et al. 2015) as well as by all phylogenomic studies that include the group (e.g. Zhang et al. 2018; McKenna et al. 2019; Douglas et al. 2021; Cai et al. 2022).

The phylogeny of Phengodidae has received increased attention in the last decade, with three research papers (Zaragoza-Caballero and Zurita-García 2015; Kundrata et al. 2019; Ferreira et al. 2024) and two doctoral dissertations (Quintino 2017; Roza 2022) published on the subject. The family was traditionally thought to be endemic to the New World, and divided into three subfamilies: Mastinocerinae, Phengodinae, and Penicillophorinae (e.g. Wittmer 1976; Zaragoza-Caballero and Pérez-Hernández 2014). The only published morphology-based phylogeny of the family (Zaragoza-Caballero and Zurita-García 2015), which was deemed preliminary by the authors themselves, found almost no support for most of the relationships therein recovered. Their study found subfamilies to be either polyphyletic (Mastinocerinae and Phengodinae) or nested in Telegeusidae (Penicillophorinae), outside of Phengodidae (for a synthesis, see Fig. 2 in Ferreira et al. 2024).

More recently, molecular-based phylogenies have made important additions to the family systematics and classification. These include the recognition of the Asian Cydistinae as phengodids (Kundrata et al. 2019), and the recognition and description of the fourth New World subfamily, Cenophenginae (Ferreira et al. 2024). The latter study is the latest and most comprehensive phylogenetic hypothesis for the family Phengodidae. Using an anchored hybrid enrichment (AHE) phylogenomics approach, the authors recovered four subfamilies in the following topology: Cydistinae + (Phengodinae + (Cenophenginae + Mastinocerinae)) (Ferreira et al. 2024). Despite recent advances on the high-level phylogenetics of Phengodidae, the placement of taxa currently classified in the subfamily Penicillophorinae (Figs 1–3) remains unresolved and untested using a molecular-based approach.

The Penicillophorinae are a group of morphologically modified genera that are unique among Phengodidae. Currently composed of five genera and seven species (Roza et al. 2024), they differ significantly from other Phengodidae in having moniliform, serrate, or shortly uniramose antennae, which lack the double branches present in all the other known genera of the family (Figs 1, 2). In addition to modifications of the antennae, the morphology among these genera is quite disparate. This variation includes the numbers of tentorial pits, gular sutures, and labial palpomeres, the wing venation, and the presence/absence of ventral tarsal combs on both the first pro- and mesotarsomere (see Roza et al. 2024). Variability in a number of labial palpomeres and a wing venation (Fig. 3) might be an effect of miniaturization (Zaragoza-Caballero and Pérez-Hernández 2014; Roza et al. 2024).

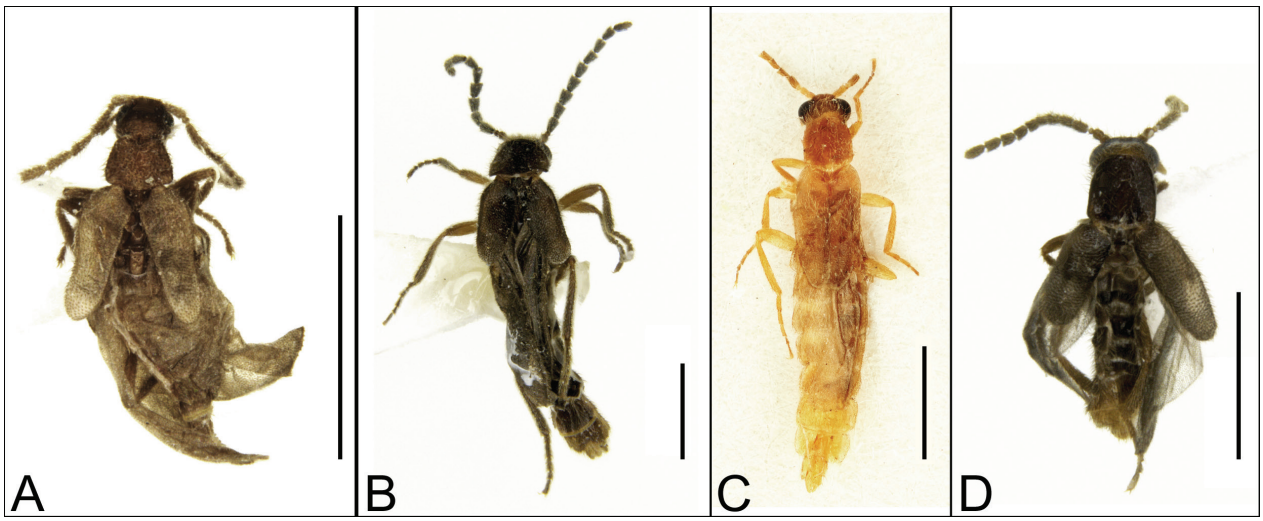

Figure 1. Genera traditionally placed in Penicillophorinae. **A** *Penicillophorus* sp. (Venezuela), **B** *Acladocera* sp. (Puerto Rico), **C** *Adendrocera flavula* Wittmer, 1976 (Guatemala) (modified from Roza et al. 2024), **D** *Walterius* sp. (Belize). Scale bars: 1 mm.

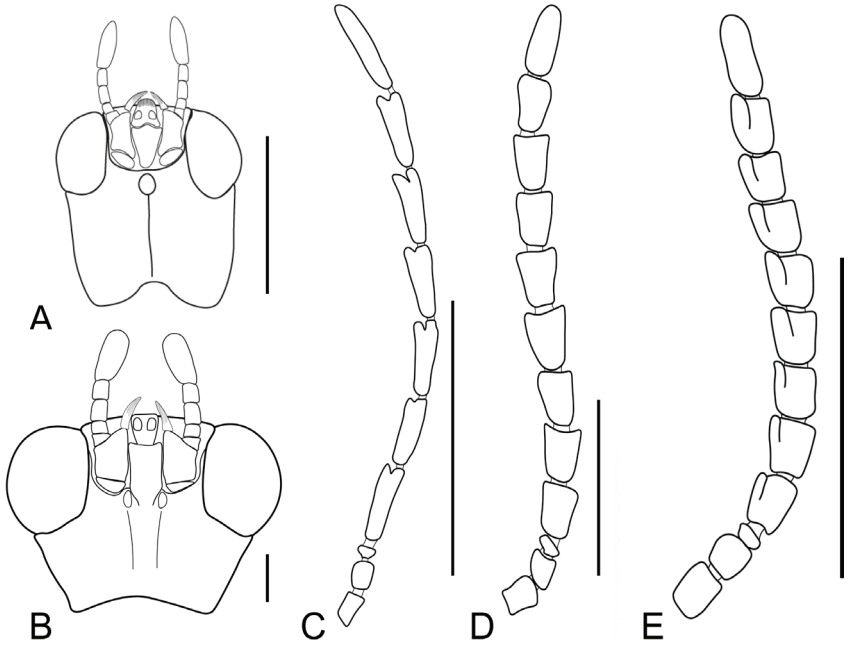

Figure 2. Morphology of genera traditionally placed in Penicillophorinae. **A**, **B** Head ventral: **A** *Penicillophorus* sp. **B** *Acladocera hispaniola* Wittmer, 1981. **C**–**E** Right antenna: **C** *Penicillophorus* sp., **D** *Acladocera* sp., **E** *Walterius* sp. Scale bars: 0.2 mm (A, B), 0.5 mm (C–E).

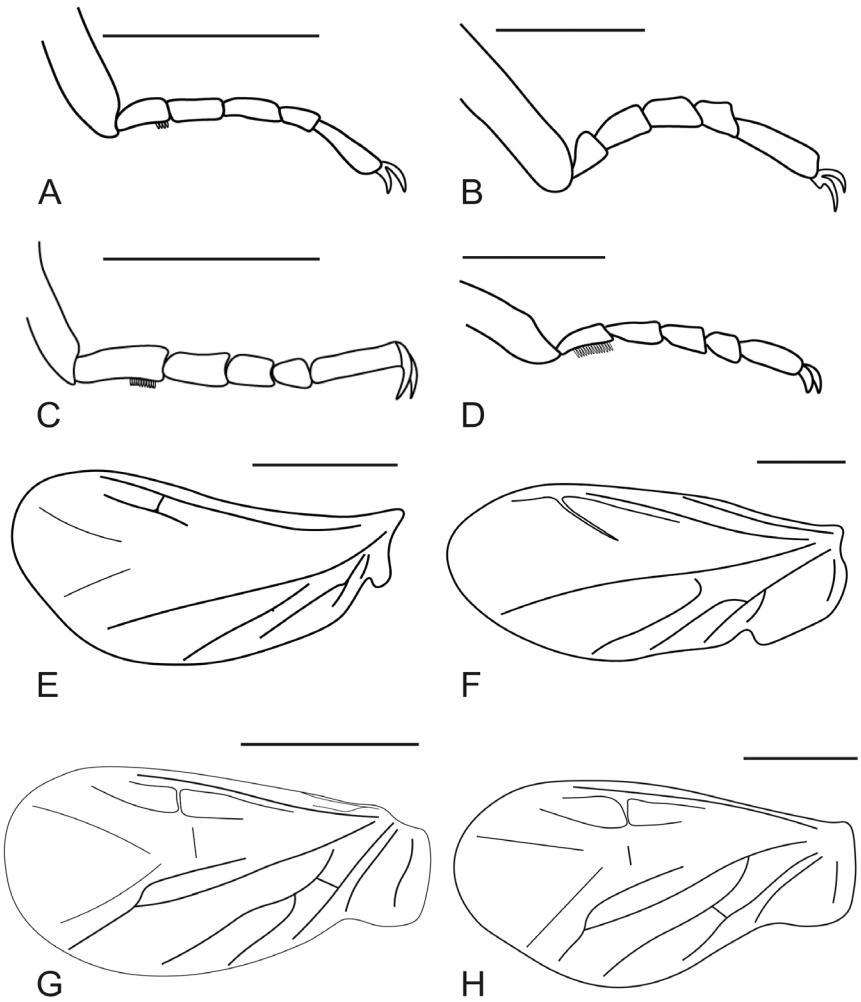

Figure 3. Morphology of genera traditionally placed in Penicillophorinae. **A**–**D** Right protarsus: **A** *Penicillophorus* sp., **B** *Acladocera* sp., **C** *Adendrocera carmelita* Roza et al., 2024 (modified from Roza et al. 2024), **D** *Walterius* sp. **E**–**H** Left hind wing: **E** *Penicillophorus* sp. **F** *Acladocera* sp. **G** *Adendrocera flavula* Wittmer, 1976 (modified from Roza et al. 2024), **H** *Walterius* sp. Scale bars: 0.2 mm (A–D), 0.5 mm (E–H).

Previous morphological phylogenetic assessments of Phengodidae considered Penicillophorinae to be outside the family, forming a clade with Telegeusidae (currently in Elateroidea: Omethidae) (Zaragoza-Caballero and Zurita-García 2015), or well nested within Phengodidae: Mastinocerinae (Quintino 2017; Roza 2022). However, those hypotheses obtained low statistical support (i.e. nodal support) or no support at all and were based mainly on homoplastic features, such as antennomere shapes and the absence or reduction of some hindwing veins. Representatives of Penicillophorinae are rarely collected, and only known from their type series. To date, there is no published molecular data for any member of this subfamily. The recent collection of taxa currently classified as Penicillophorinae by MFG and JH allowed the DNA extraction and production of the first genomic data for two representatives of Penicillophorinae. We took this opportunity to test their phylogenetic placement within Phengodidae.

2. Material and Methods

2.1. Taxon sampling, identification, and morphology

Our taxon sampling included a total of 13 terminals, of which five (including *Acladocera* Wittmer, 1981 and *Walterius* Zaragoza-Caballero, 2008) were newly sequenced. For full information on the analyzed dataset, see Table S1. In the present study we included fewer terminals than Ferreira et al. (2024) did; given the high level of missing data in the dataset by Ferreira et al. (2024), we opted for a smaller but more complete gene-sampled approach to test the phylogenetic position of Penicillophorinae genera. Therefore, we used a straightforward and simple procedure of phylogenomic subsampling (see Edwards 2016; Koch 2021). With this procedure, we have obtained much more sequence data due to the quality of the specimens (i.e. specimens which yielded more genomics data, both in volume and quality, see Table S1 and the sequencing method.

The two Penicillophorinae samples were initially identified by their collectors as *Acladocera* sp. (SDEIC003) (by JH), and as *Walterius* sp. Zaragoza-Caballero, 2008 (SDEIC035) (by MFG). Their identifications were later corroborated by ASR and MFG based on current literature on Phengodidae (Wittmer 1976; Zaragoza-Caballero and Pérez-Hernández 2014; Roza et al. 2024), and by direct comparison with type specimens of *Acladocera hispaniola* Wittmer, 1981 and *Walterius caballeroae*. Sampled *Acladocera* sp. was collected in the Dominican Republic, and will be deposited in the Natural History Museum, London, The United Kingdom. *Walterius* sp. was collected in Belize, and will be deposited in the Naturéum - Muséum cantonal des sciences naturelles, Lausanne, Switzerland. The morphological terminology follows Costa and Zaragoza–Caballero (2010) and Roza (2023). For hind wings, we follow Lawrence et al. (2021). The examined hind wings were glued on cards and permanently placed under each respective specimen. Photographs and measurements were taken with the Leica DFC450 and Application Suite CV3 software. The photographs were edited using Adobe Photoshop CC, and the figure plates and drawings were designed with Adobe Illustrator CC.

Our final datasets (50-NT, 70-NT, 50-AA, and 70-AA, see results section) consisted of 13 samples. Ingroup consisted of 10 taxa representing all five currently recognized subfamilies of Phengodidae: Cydistinae (*Microcydistus minor* (Bolívar and Pieltain, 1913)); Phengodinae (*Zarhipis integripennis* (LeConte, 1874)); Penicillophorinae (*Acladocera* sp. and *Walterius* sp.); Cenophenginae (*Cenophengus debilis* LeConte, 1881); and Mastinocerinae (*Brasilocerus oberthuri* (Pic, 1955), *Cephalophrixothrix* sp., *Distremocephalus opaculus* (Horn, 1895), *Oxymastinocerus peruanus* (Wittmer, 1956), and *Phrixothrix hirtus* E. Olivier, 1909). We adopted the Phengodidae classification proposed by Ferreira et al. (2024). As outgroup, we sampled one Sinopyrophoridae (*Sinopyrophorus schimmeli* Bi and Li, 2019; as the most distant outgroup, = root), one Lampyridae [*Lamprohiza splendidula* (Linnaeus, 1767)], and one Rhagophthalmidae [*Menghuoius giganteus* (Fairmaire, 1888); as a representative of the sister-group to Phengodidae].

2.2. DNA extraction, sequencing, and genome assembly

Genomic extractions followed Ferreira et al. (2024). Extractions were done using the DNeasy Blood and Tissue kit (Qiagen, Germantown, MD, USA). The manufacturer's protocol for tissue samples was followed with a prolonged overnight (10–14 hours) lysis period. The final DNA was eluted in buffer EB after a 10-min incubation. This step was repeated twice, with 30 µl each time, for a final elution volume of 60 µl. Extractions were done in the molecular laboratory at the Senckenberg Deutsches Entomologisches Institut (SDEI). The full genomic extractions are permanently deposited at -80 °C at the DNA and tissue collections at the SDEI.

Quality control, normalization, library preparation, and sequencing of samples were done by Novogene Genomics (Munich, Germany). A DNA library of 350 bp insert size was constructed for the sample and sequenced in a NovaSeq X Plus Series with a pair-end 150 bp sequencing strategy. About 10Gb reads were obtained from each sample.

Sequence reads were initially processed using the CAPTUS pipeline (Ortiz et al. 2023): raw reads were adapter-trimmed and filtered in two consecutive rounds using BBDUK from BBTOOLS (Bushnell 2022; Ortiz et al. 2023), and de novo assembled using MEGAHIT (Li et al. 2015), with CAPTUS default preset "CAPSKIM," which is optimized for hybridization capture or genome skimming data (or a combination of both) (Ortiz et al. 2023).

2.3. Ortholog search

After initial data quality control and genome assembly, our ortholog search was conducted using CAPTUS (Ortiz et al. 2023). We used CAPTUS modules "extract" and "align" to identify orthologous genes from the dataset used to produce the custom Elateridae Ortholog Target Enrichment Baits ("ElaterBaits"), which were originally presented in Douglas et al. (2021). This ortholog database was originally designed to be used for Elateridae (Douglas et al. 2021), but it was also successfully used for Phengodidae by Ferreira et al. (2024). CAPTUS pipeline was implemented following Ortiz et al. (2023) and CAPTUS tutorial available at https://edgardomortiz.github.io/captus.docs/tutorials/assembly/basic (last access on 9 September 2025).

Since we did not implement an in vivo hybridization strategy for data acquisition (see the previous section), instead of using the "Bait-100-85_Elateridae.fasta" as our reference file for the ortholog search in CAPTUS, we used the original file "Elateridae_Submission.fasta" used for their probe design. Full details about the production of the "ElaterBaits" are available at Douglas et al. (2021), and original files can be found at https://github.com/AAFC-BICoE/Elateridae-ortholog-baitset (last access on 9 September 2025).

CAPTUS extract step was performed in our previously assembled genomes. We implemented the CAPTUS "Nuclear proteins" search strategy. This approach uses Scipio (Hatje et al. 2011) to perform protein extractions and automatically correct frameshifts for downstream analyses (see details in Ortiz et al. 2023). During the "align" step, CAPTUS extracted the copies (hits) of markers found in assembly files and ranked them by their similarity to the reference sequences (Ortiz et al. 2023). Sequences were then aligned with MAFFT Version 7 (Katoh and Standley 2013), and paralogs filtered using CAPTUS naive method and informed method (see Ortiz et al. 2023 for details). After paralogs were filtered, alignments were trimmed with ClipKIT (Steenwyk et al. 2020).

2.4. Phylogenomic analyses

Phylogenetic relationship reconstruction and other tests were performed on four versions of our multiple sequence alignments (MSAs) composed of loci with ≥50% or ≥70% of taxa present ("completeness"). For determining the completeness of each dataset, we used Geneious 11.1.5. The multiple sequence alignments were concatenated using AMAS (Borowiec 2016). The phylogenetic analyses were conducted under two tree-building methods (optimality criteria) as follows: 1) concatenated analyses performed using the maximum likelihood (ML) method (Felsenstein 1973, 1981) via IQ-TREE2 software (Minh et al. 2020a), and 2) a summary method of species tree inference from gene trees, modeled under the multispecies coalescent model (MSCM; Pamilo and Nei 1988; Rannala and Yang 2003) via ASTRAL-Pro2 software (Mirarab et al. 2014; Zhang and Mirarab 2022), with input gene trees being reconstructed individually under ML via IQ-TREE2 software.

Phylogenetic analyses were performed in four datasets (Table 1), i.e. at the nucleotide level 50-NT (50% completeness matrix) and 70-NT (70% completeness matrix), and amino acid level 50-AA (50% completeness matrix) and 70-AA (70% completeness matrix). All analyses carried out with these datasets were partitioned by locus. We determined the evolutionary models for the partitioned and individual gene trees analyses (MFP + MERGE command of IQ-TREE2) via ModelFinder software (Kalyaanamoorthy et al. 2017). The resulting trees were visualized via the FigTree 1.4.0 (Rambaut 2012) and iTOL: Interactive Tree Of Life (Letunic and Bork 2016) software packages.

The nodal support in ML analyses was measured through the following metrics: the "ultrafast" bootstrap (UFBoot; Hoang et al. 2018), the Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT; Guindon et al. 2010), and the Bayesian-like transformation of aLRT (aBayes; Anisimova et al. 2011). For coalescent ASTRAL analyses, supports were calculated as local posterior probabilities (Local PP; Mirarab et al. 2014; Zhang and Mirarab 2022). Nodes with support values above ≥95% / ≥0.95 (for UFBoot, aBayes, and Local PP) and above ≥80% (for SH-aLRT) were considered strongly supported (Guindon et al. 2010; Anisimova et al. 2011; Hoang et al. 2018). Support measures were computed via IQ-TREE2 software, except for the Local PP, calculated via ASTRAL-Pro2 software.

Concordance factors are important tools in phylogenomics. These measures help to summarize information from different gene trees and sites that arise due to the boundary between reticulate and divergent evolution (Baum 2007). Two concordance factors are widely used in phylogenomics studies: 1) the gene concordance factor (gCF; Baum 2007; Minh et al. 2020b), which computes the proportion of individual gene trees that support each specific node, corrected for variable terminal coverage; and 2) the improved version of the site concordance factor (sCF; Minh et al. 2020b; Mo et al. 2023), which computes the proportion of alignment sites concordant with each specific node in a maximum likelihood framework using the probability distributions of ancestral states at internal nodes. Although concordance factors have been interpreted as measures of statistical support of nodes, Lanfear and Hahn (2024) indicated that this interpretation is not adequate.

The concordance factors (gCF and sCF) are more properly defined as "descriptors of topological variation" and "estimates of biological parameters," in which they can be used to measure the proportion of the dataset for which a given split/clade is considered true (see Lanfear and Hahn 2024). In this interpretation, concordant factors are complementary measures in relation to nodal support measures in a phylogenomic context; they can provide important information regarding the predictive power of the inferred phylogenetic species tree that may not be clearly contained in regular statistics regarding node support (Minh et al. 2020b; Lanfear and Hahn 2024). Since,

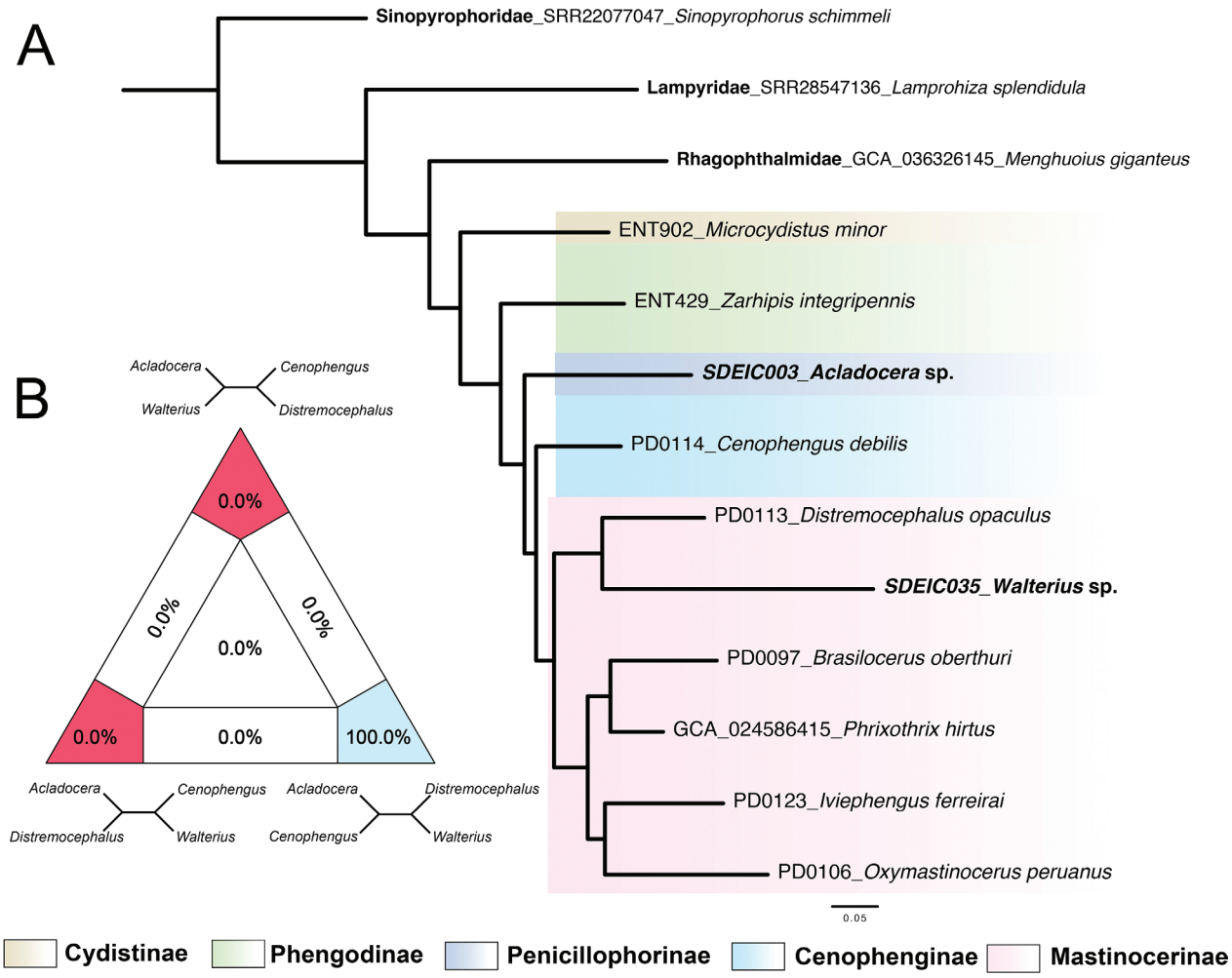

Figure 4. A Phylogenomic hypothesis for Phengodidae. All topologies rendered the same topology with the analyses performed at the nucleotide level 50-NT (50% completeness matrix) and 70-NT (70% completeness matrix) and amino acid level 50-AA (50% completeness matrix) and 70-AA (70% completeness matrix). All branches are supported with maximum support levels for the Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT), the Bayesian-like transformation of aLRT (aBayes), and the "ultrafast" bootstrap (UFBoot). **B** four-cluster likelihood mapping (FcLM) test of alternative phylogenetic hypotheses showing the placement of *Acladocera* and *Walterius* in relationship to other Phengodidae groups.

by definition, it is not adequate to interpret concordance factors as measures of nodal support, we cannot define threshold levels of significance for the gCF and sCF, as we do for SH-aLRT, aBayes, UFBoot, and Local PP. Therefore, in this study, we do not present gCF and sCF values.

2.5. Four-cluster likelihood mapping

To further investigate the statistical support of alternative hypotheses, in which the sampled Penicillophorinae genera *Acladocera* and *Walterius* clustered together, we applied the method of four-cluster quartet-likelihood mapping analysis (FcLM; Strimmer and von Haeseler 1997; Nieselt-Struwe and von Haeseler 2001). We evaluated the position of the following genera a) *Acladocera*, b) *Walterius*, c) *Cenophengus*, and d) *Distremocephalus*. Using this procedure, we properly tested three competing hypotheses: 1) (*Acladocera* + *Walterius*)-(*Cenophengus* + *Distremocephalus*); 2) (*Acladocera* + *Distremocephalus*)-(*Walterius* + *Cenophengus*); and 3) (*Acladocera* + *Cenophengus*)-(*Walterius* + *Distremocephalus*). In this method, hypothesized groups are organized into quartets (four-terminal sets), representing a simplified topology of the relationships to be tested. This procedure outputs a two-dimensional simplex plot that displays the statistical support of competing hypotheses via the proportions of quartets recovered from each possible topology or even inconclusive relationships.

This analysis was designed to directly assess the phylogenetic signal of all tested datasets (nucleotides, amino acids, 50% and 70% completeness), which are supporting the putative non-monophyly of *Acladocera* and *Walterius* in the analyses. At the same time, we tested the current putative topology regarding the subfamilies Penicillophorinae, Cenophenginae, and Mastinocerinae (Ferreira et al. 2024). With this procedure, we included all possible relationships, regarding the two sampled Penicillophorinae genera and their putative most closely related subfamilies. Four-cluster quartet likelihood mapping analyses were performed using IQ-TREE2 software.

Table 1. Statistical support for internal branches measured for each phylogenetic reconstruction based on nucleotide (NT) and amino acid (AA) datasets with 50% and 70% completeness. Support levels for maximum likelihood (ML) analyses refer to the Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT; in %), the Bayesian-like transformation of aLRT (aBayes); and the "ultrafast" bootstrap (UFBoot, in %). Support levels for coalescent ASTRAL analysis refer to the local posterior probabilities (Local PP).

Tree/ Analysis	**Supports**	**(Rhagophthalmidae + Phengodidae)**	**Phengodidae**	**(Phengodinae + (*Acladocera* + (Cenophenginae + Mastinocerinae including *Walterius*)))**	**(*Acladocera* + (Cenophenginae + Mastinocerinae including *Walterius*))**	**(Cenophenginae + Mastinocerinae including *Walterius*)**	**Mastinocerinae including *Walterius***	**(*Oxymastinocerus* + *Cephalophrixothrix*)**
01-ML-50-AA	SH-aLRT / aBayes/ UFBoot	100/1/100	100/1/100	100/1/100	100/1/100	100/1/100	100/1/100	100/1/100
02-ML-70-AA	SH-aLRT / aBayes/ UFBoot	100/1/100	100/1/100	100/1/100	100/1/100	100/1/100	100/1/100	100/1/100
03-ML-50-NT	SH-aLRT / aBayes/ UFBoot	100/1/100	100/1/100	100/1/100	100/1/100	100/1/100	100/1/100	100/1/100
04-ML-70-NT	SH-aLRT / aBayes/ UFBoot	100/1/100	100/1/100	100/1/100	100/1/100	100/1/100	100/1/100	100/1/100
05-ASTRAL-50-AA	Local PP	1	1	1	1	0.7109	0.9996	0.8796
06-ASTRAL-70-AA	Local PP	1	1	1	1	0.6642	0.9997	0.9064
07-ASTRAL-50-NT	Local PP	1	1	1	1	1	1	1
08-ASTRAL-70-NT	Local PP	1	1	1	1	1	1	1

3. Results

3.1. Ortholog search, individual loci recovered, and datasets assembly

After ortholog search with CAPTUS, our loci recovery levels ranged from 839 (*Zarhipis integripennis*) to 2239 (*Lamprohiza splendidula*) from each sample or archived data source (Table S1). After processing and concatenation of single-locus alignments with AMAS, our final datasets consisted of the following number of loci, bp, and degree of missing data: 50-NT (2180 loci, 1.300.931 bp, degree of missing data: 2.59%–84.65%), 70-NT (1697 loci, 1.011.955 bp, degree of missing data: 1.76%–81.45%), 50-AA (2180 loci, 433.626 aa, degree of missing data: 2.59%–84.66%) 70-AA (1697 loci, 337.309 aa, degree of missing data: 1.76%–81.46%). The final datasets are available in File S1.

3.2. Phylogenetic analyses and four-cluster likelihood mapping

Rhagophthalmidae were recovered as a sister-group of Phengodidae, and Phengodidae as a monophylum in all analyses, with maximum support. All analyses (Fig. 4; Table 1; File S1) rendered identical topologies in the following conformation: Cydistinae + (Phengodinae + (*Acladocera* + (Cenophenginae + Mastinocerinae including *Walterius*))). All maximum likelihood analyses recovered all branches with maximum support, and ASTRAL analyses recovered almost all groups with maximum support (see details below). The monophyly of the two sampled genera traditionally placed in Penicillophorinae, i.e. *Acladocera* and *Walterius*, was rejected in all analyses. *Acladocera* was recovered in a basal position in relation to Cenophenginae + Mastinocerinae including *Walterius* in all analyses, with maximum support. The group Cenophenginae + Mastinocerinae including *Walterius* was recovered in all analyses (Local PP of 50-AA-ASTRAL = 0.7109; Local PP of 70-AA-ASTRAL= 0.6642; all other supports at maximum level). Mastinocerinae including *Walterius* were recovered monophyletic in all analyses, with almost maximum support (Local PP of 50-AA-ASTRAL = 0.9996; Local PP of 70-AA-ASTRAL= 0.9997; all other supports at maximum level). Inside Mastinocerinae, *Walterius* was recovered as a sister-group of *Distremocephalus* in all analyses, always with maximum support. These relationships were supported by the exploration of the phylogenetic signal content (i.e. a proportion of quartets recovered) via a four-cluster likelihood mapping using both nucleotide and amino acid datasets. The reduced quartet (*Acladocera* + *Cenophengus*)-(*Walterius* + *Distremocephalus*) presented maximum support (100%) for both nucleotide and amino acid datasets (Fig. 4).

4. Discussion

After the most comprehensive phylogenomic hypothesis for Phengodidae by Ferreira et al. (2024), the monophyly and phylogenetic placement of rarely collected and morphologically modified Penicillophorinae remained the biggest challenge in the family's systematics (Kundrata 2025). Although placed outside the Phengodidae by the morphology-based analysis of Zaragoza-Caballero and Zurita-García (2015), Penicillophorinae were further treated as phengodids by most authors (Roza 2022, Ferreira et al. 2024, Roza et al. 2024). Here, using a phylogenomic approach, we confirmed that *Acladocera* and *Walterius*, both hitherto classified in Penicillophorinae, are indeed phengodids. However, their monophyly was rejected in all of our analyses (Fig. 4; File S1). Although these two genera share simplified, non-bipectinate antennae, their morphology otherwise varies considerably.

Our analyses recovered *Acladocera* as a sister-group of Cenophenginae + Mastinocerinae, and *Walterius* nested inside Mastinocerinae. These results are corroborated by morphology. *Acladocera* has two gular sutures (Fig. 2B) and the hindwing vein CAS (Fig. 3F), features shared with Cydistinae and with Cenophenginae. Besides that, *Acladocera* does not have the characteristic ventral tarsal combs that occur in several genera across the diverse Mastinocerinae. *Walterius*, on the other hand, has a single gular suture and lacks the CAS vein. It also has the ventral tarsal combs on both the first pro- and mesotarsomere (Fig. 3D), a state shared with several New World Phengodidae (Zaragoza-Caballero and Pérez-Hernández 2014; Roza 2022). In this context, it makes sense that *Walterius* is more closely related to Mastinocerinae than to *Acladocera*.

The placement of the remaining Penicillophorinae (i.e. *Adendrocera* Wittmer, 1976, *Penicillophorus* Paulus, 1975, and *Tarsakanthos* Zaragoza-Caballero, 2008), although not investigated in our study, can be hypothesized based on their morphology. *Adendrocera* and *Penicillophorus* are more similar to *Walterius* than to *Acladocera*, based on a number of characters like the presence of a single gular suture, ventral tarsal combs on the first pro- and mesotarsomere, although of variable length in relation to the tarsomere (Figs 3A, C, D), and by the presence of an open radial cell, with a long vein r3 parallel to the radial cell (Figs 3E, G, H). There are some differences between *Walterius* and two above-mentioned genera, like the presence of two tentorial pits in *Walterius* (one tentorial pit in *Adendrocera* and *Penicillophorus*), and the shortly uniramose antennae in *Walterius* (despite being described as moniliform by Zaragoza-Caballero 2008), with the rami short and stout, closely positioned in relation to the antennomere (Fig. 2E; serrate in *Adendrocera* and serrate to uniramose, with rami small, formed by an emargination of the serrate apex in *Penicillophorus*; Fig. 2C).

Acladocera, in turn, has the most aberrant morphology when compared with other genera currently placed in the Penicillophorinae. The only similarity with *Adendrocera* and *Penicillophorus* is in the antennae. Otherwise, *Acladocera* has two gular sutures (Fig. 2B), no tarsal combs (Fig. 3B), and absent hind-wing radial cell (Fig. 3F). The remaining genus in Penicillophorinae, *Tarsakanthos*, is most likely not a phengodid (ASR and MAI pers. observ.), and its systematic placement will be treated in detail elsewhere. To sum up, it is probable that *Adendrocera* and *Penicillophorus* are more related to *Walterius* or other Mastinocerinae genera, which would prompt the subfamily Penicillophorinae to be considered a synonym of Mastinocerinae.

The antennae of Phengodidae are typically bipectinate or biflabellate, i.e. with most antennomeres having a pair of short or long rami. Such antennae occur in all phengodid subfamilies but Penicillophorinae, and were also present on the only fossil phengodid recently described from the Cretaceous Burmese amber (Roza et al. 2023). Further, biramose antennae are also known in Rhagophthalmidae (the sister-group of Phengodidae; Kundrata et al. 2022) and in Cretophengodidae, which are putative extinct relatives of Phengodidae known only from Burmese amber (Li et al. 2021). Our results confirmed that antennal simplification (i.e. loss or reduction of the plesiomorphic double rami) happened at least twice in the evolutionary history of Phengodidae, and we cannot rule out the possibility that future analyses including *Adendrocera* and *Penicillophorus* show even more cases. This is not surprising, since rami modifications are rather common in the highly diverse Mastinocerinae: fused rami are found in several species of *Akamboja* Roza et al., 2017 and *Euryopa* Gorham, 1881 (Wittmer 1996; Coelho et al. 2024), the enlargement of certain antennomeres and simultaneous loss of rami are found in *Eurymastinocerus* Wittmer, 1976 and *Euryognathus* Wittmer, 1976 (Wittmer 1976), and the presence of a third ramus, attached apically on the antennomere body, is present in all species of *Paraptorthodius* Schaeffer, 1904 and some species of *Phrixothrix* Olivier, 1807 (e.g. *Phrixothrix vivianii* Wittmer, 1996) (Zaragoza-Caballero 1999; Wittmer 1996). Therefore, the antennal simplification of *Walterius* only contributes to a larger record on antennal variation in Mastinocerinae. It should be noted that rather extreme variability in the shape of antennae among closely related taxa is a common phenomenon in many other soft-bodied elateroids, including Rhagophthalmidae (Kundrata et al. 2022) or Elateridae: Drilini (Kundrata & Bocak 2019).

As we failed to sequence *Penicillophorus* in our study, the subfamily status of Penicillophorinae remains dubious, and the only change we proposed in this study is the transfer of *Walterius* to Mastinocerinae. Future studies including more Penicillophorinae (especially the type genus, *Penicillophorus*) and a broader sampling of other subfamilies, in particular more Mastinocerinae genera, are necessary to understand and redefine the boundaries and composition of these subfamilies, and further elucidate the phylogenetic history of the family Phengodidae.

5. Conclusions

With a low-coverage whole genome sequencing approach, we successfully produced genomic data for two species of the highly morphologically modified genera traditionally placed in Penicillophorinae (*Acladocera* sp. and *Walterius* sp.) allowing us to test their phylogenetic placement within Phengodidae. Penicillophorinae were consistently recovered as a non-monophyletic group, rendering the following relationship: Cydistinae + (Phengodinae + (*Acladocera* + (Cenophenginae + Mastinocerinae including *Walterius*))). Although we did not have access to the DNA-grade material of the type genus of Penicillophorinae, we were able to test the placement of other superficially similar genera within the broader context of Phengodidae phylogenomics. These results suffer from the absence of the type genus of the Penicillophorinae, which is morphologically quite divergent from the two genera included here (Figs 2–3). Therefore, the only taxonomic change proposed in our study is the transfer of *Walterius* to Mastinocerinae. Discovery of a DNA-grade specimen of that genus remains a priority. Future research is essential to clarify the limits of Penicillophorinae and other phengodid subfamilies, especially Mastinocerinae.

6. Declarations

Author contributions. Conceptualization: FFB, ASR, VSF, RK. Funding acquisition: ASR, JRMM, VSF. Data curation: ASR, VSF, LSD. Formal analysis: FFB, VSF. Investigation: FFB, ASR, JRMM, MFG, JH, LSD, MAI, VVB, VSF, RK. Methodology: FFB, LSD, VSF. Writing—original draft: FFB, ASR, VSF, RK. Writing—review & editing: FFB, ASR, JRMM, MFG, JH, LSD, MAI, VVB, VSF, RK. Project administration: FFB, ASR, VSF, RK. Validation: FFB, ASR, VSF, RK. Visualization: ASR, VSF.

Competing interests. The authors declare that they have no conflicts of interest in relation to this work.

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8. References

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Supplementary Material 1

Table S1

Authors: Barbosa FF, Roza AS, Mermudes JRM, Geiser MF, Hodecek J, Dey L-S, Ivie MA, Vega-Badillo V, Ferreira VS, Kundrata R (2025)
Data type: .pdf
Explanation notes: Taxon sampling of terminals included in the present study, including taxon and GenBank accession codes, and information on recovered loci.
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.
Link: https://doi.org/10.3897/asp.83.e164315.suppl1

Supplementary Material 2

File S1

Authors: Barbosa FF, Roza AS, Mermudes JRM, Geiser MF, Hodecek J, Dey L-S, Ivie MA, Vega-Badillo V, Ferreira VS, Kundrata R (2025)
Data type: .zip
Explanation notes: Nucleotide and amino acid sequences, partition files, and phylogenetic trees.
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