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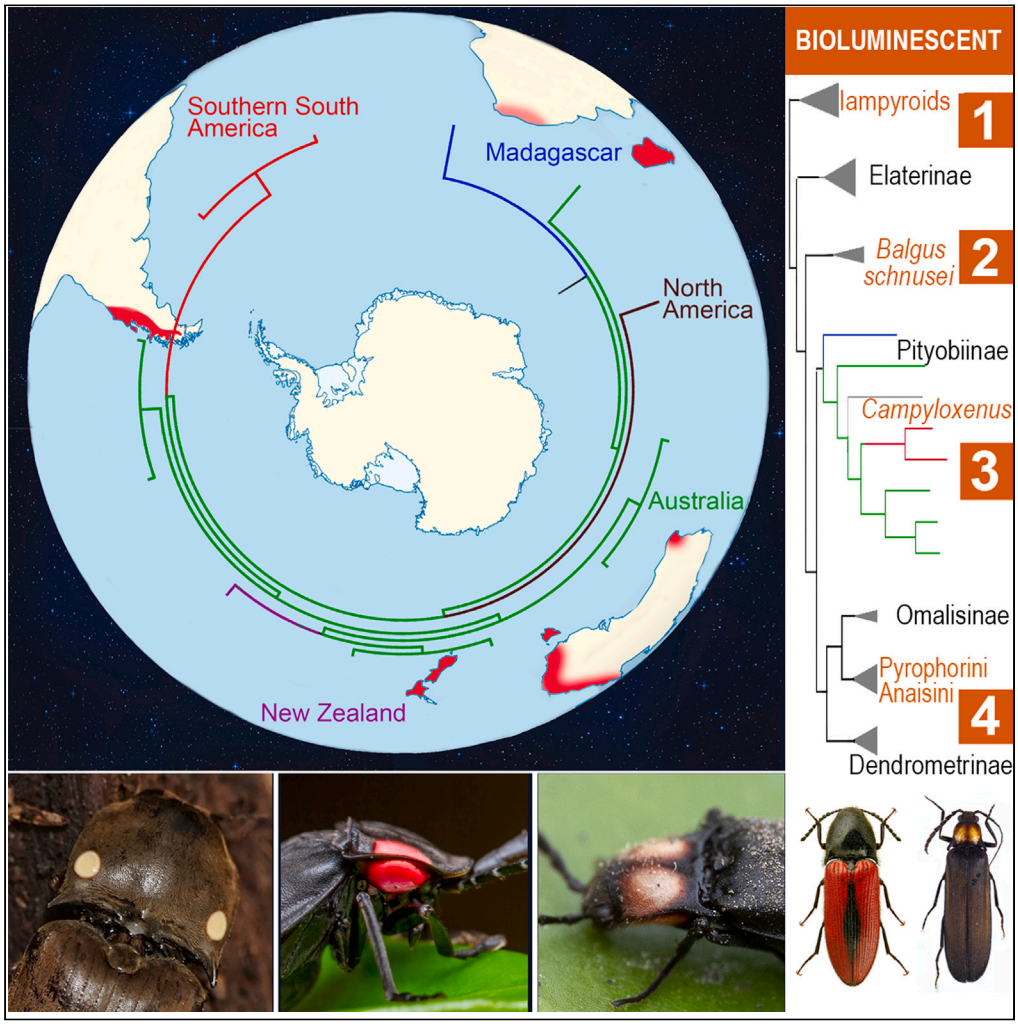

Michal Motyka, Dominik Kusy, Elizabeth T. Arias-Bohart, Seth M. Bybee, Ladislav Bocak

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Highlights

We studied bioluminescence and relationships among Gondwanan click beetles

The newly hypothesized clade of elaterid Gondwanan lineages contains ancient groups

Campyloxenus unveils a separate, recent origin of bioluminescence in elateroids

Motyka et al., iScience *26*, 108440
December 15, 2023
https://doi.org/10.1016/j.isci.2023.108440

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Enigmatic *Campyloxenus*: Shedding light on the delayed origin of bioluminescence in ancient Gondwanan click beetles

Michal Motyka,[1,4] Dominik Kusy,[1,4] Elizabeth T. Arias-Bohart,[2] Seth M. Bybee,[3] and Ladislav Bocak[1,5,*]

SUMMARY

Gondwanan elaterids, previously thought to be unrelated, include bioluminescent *Campyloxenus* earlier placed in bioluminescent Pyrophorinae. Genomic data suggest close relationships between Gondwanan groups. We maintain Morostomatinae and Hapatesinae and redefine Pityobiinae with Nearctic Pityobiini, Gondwanan Parablacini stat. nov., Campyloxenini stat. nov., and Tibionemini trib. nov. Their ancestors putatively underwent differentiation in Gondwana during the Cretaceous separation of southern continents. In contrast with their age, extant groups are species poor. *Campyloxenus* represents a recent origin of bioluminescence, no older than ~53 my. Its large pronotal lanterns differ from Pyrophorini and resemble color patches of sympatric beetle co-mimics. This discovery highlights the fourth or fifth origin of bioluminescence in Elateroidea, alongside the lampyroid clade, click beetles Pyrophorini, *Alampoides* and *Coctilelater* in Anaissini (Pyrophorinae), and *Balgus schnusei* (Thylacosterninae). While our phylogenetic findings illuminate the phylogenetic aspects, the complete story awaits further field observations and in-depth genomic analyses of biochemical pathways used by bioluminescent elateroids.

INTRODUCTION

Elateridae stands as the largest family within the Elateroidea, boasting over 10,000 species distributed worldwide. Approximately twenty elaterid subfamilies have been recognized.[1–6] The subfamilies Elaterinae, Agrypninae, and Dendrometrinae hold thousands of species and have truly global distribution. In contrast, some subfamilies are somewhat limited in their diversity, being monogeneric or containing only a few genera with a handful of species. In this study, our focus centers on five such species-poor, morphologically diverse, and predominantly Gondwanan subfamilies: Hapatesinae, Pityobiinae, Morostomatinae, Campyloxeninae, and Parablacinae.[1,2] Our research delves into the origins, relationships, and zoogeography. Additionally, our investigation aims to shed light on the evolutionary origins of bioluminescence in click beetles as one of the Gondwanan lineages, *Campyloxenus*, possesses pronotal light-producing lanterns.

Phylogenetic relationships and classification

The actual classification stems from a prolonged period of taxonomic instability, a result of discrepancies between morphological and, later, short-fragment molecular analyses.[1,2,7–9] Morostomatinae was established by Dolin[10] and encompasses several originally dendrometrine genera from Madagascar. Hapatesinae, proposed recently by Kusy et al.,[11] groups two initially dendrometrine genera.[12,13] Costa[14] created the monogeneric Campyloxeninae, excluding *Campyloxenus* Fairmaire & Germain from Agrypninae. While its exact position relative to other click beetle subfamilies remained unclear, its status as a separate subfamily was rarely questioned.[7] Throughout the years, the concepts of these subfamilies have remained clear, and their generic composition has shown stability.

On the contrary, the boundaries of Pityobiinae have undergone substantial shifts over time. Initially, Hyslop[15] established the monogeneric Pityobiini as a Nearctic group within Agrypninae. It was only much later that the group began to expand, with additional taxa altering the perception of pityobiines and their placement.[7,16] Crowson[17] hypothesized a connection between *Metablax* Candèze from New Zealand and North American *Pityobius* LeConte. Subsequently, Dolin[18] expanded the pityobiines to include Australian *Parablax* Schwarz and South American *Tibionema* Solier. Calder[16] added further Australian genera, and, more recently, Arias-Bohart & Elgueta[19] described *Sharon* Arias-Bohart & Elgueta from Chile. Eventually, the parablacine genera were transferred to a separate subfamily, Parablacinae,[2] causing Pityobiinae to shrink to only *Pityobius* and *Tibionema*. Nonetheless, *Pityobius* and *Tibionema* exhibit distinct morphological characteristics, warranting further investigation into their relationship.[3]

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https://doi.org/10.1016/j.isci.2023.108440

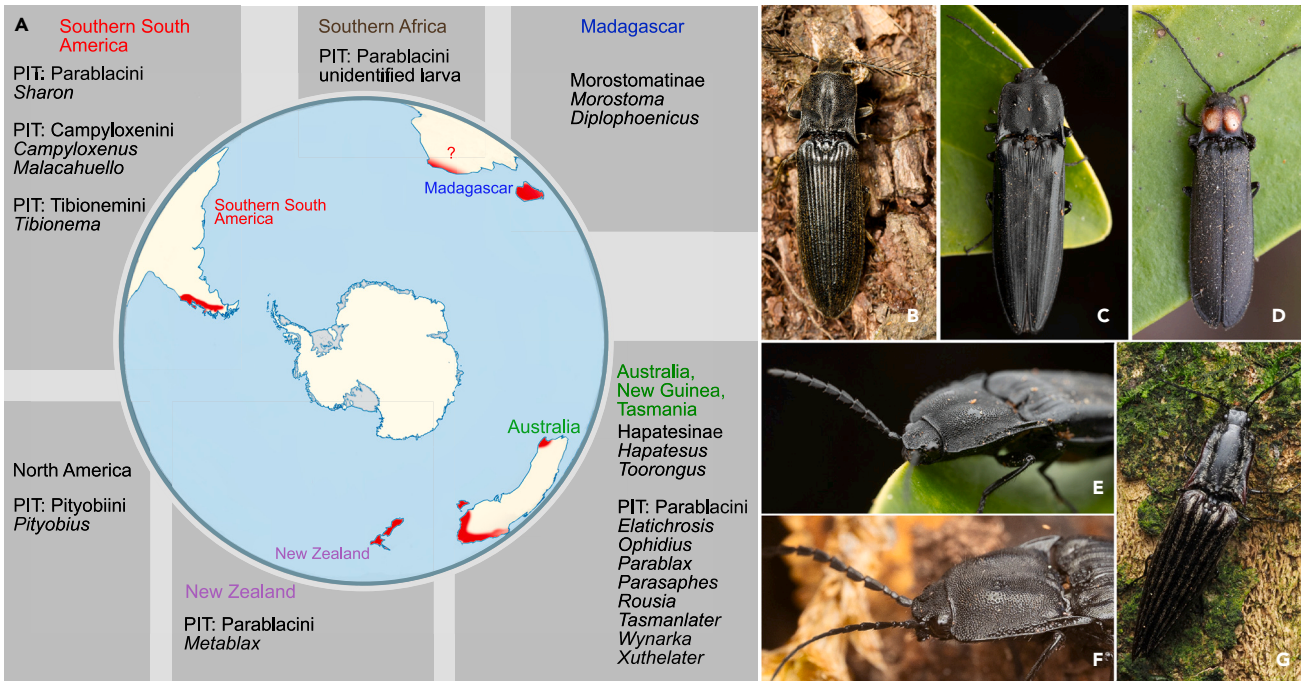

Figure 1. Distribution and diversity of focal groups

(A) The modern distribution of focal subfamilies in the Southern Hemisphere (distribution of Nearctic *Pityobius* not shown). Representatives of pityobiine lineages in nature: (B) *Pityobius anguinus* (photo by Chris Rorabaugh, USA, FL), (C, E, and F) *Tibionema abdominalis* (photo by Matías Gargiulo, Chile), (D) *Campyloxenus pyrothorax* (photo by Matías Gargiulo, Chile), and (G) *Metablax* sp. (photo by Lance Wakefield, New Zealand). Abbreviation. PIT – Pityobiinae; constituent tribes spelled in full.

Based on morphology (Figures 1B–1E), the relationships among these subfamilies remained uncertain. Previous studies proposed various positions for Pityobiinae, suggesting it as a sister to the rest of Elateridae (including Eucnemidae and Throscidae), closely related to Agrypninae or Oxynopterinae (now Dendrometrinae: Oxynopterini), and potentially included within Dendrometrinae.[7,9,16,20,21] Molecular studies on Gondwanan groups were limited initially. Morostomatinae was placed as a sister to Agrypninae,[22] a sister to Dendrometrinae, Negastriinae, and Cardiophorinae,[2] or as the next branching event after the separation of Hapatesinae + Pityobiinae.[11] Kundrata et al.[2] proposed Parablacinae as a separate subfamily based on its redefinition. *Pityobius* was hypothesized as a sister to the Lissominae + Thylacosterninae clade, and Parablacinae formed a clade of four genera following Hemiopinae + Oestodinae and preceding *Panspoeus guttatus* Sharp. A mitogenomic analysis merged *Hapatesus*, *Parasaphes*, and *Tibionema* into a single clade,[11] and the latest phylogenetic study on click beetles reaffirmed these relationships when other taxa were unavailable.[3] Thus, all analyses support the distinctiveness of these click beetles and their early origin, although their exact mutual relationships remain contentious.

Zoogeography

The poor representation of rare and endemic Gondwanan lineages in phylogenetic studies has been a long-standing problem of beetle phylogenetics.[23–25] Concerning click beetles, Douglas et al.[3] also called for including the Southern Hemisphere's fauna in the analyses. The Gondwanan groups are species poor, yet they are essential for understanding the click beetle evolution due to their ancient origins and the high taxonomic ranks in the earlier studies.[1]

Apart from *Pityobius*, all genera within these families exhibit a Gondwanan distribution (Figure 1A). Morostomatinae is endemic to Madagascar and comprises eight genera, including *Morostoma* Candèze and *Diplophoenicus* Candèze. Hapatesinae, with two genera, is predominantly found in Australia, with some species recorded in New Guinea.[9,11–13] Parablacinae is mainly distributed in Australia and New Zealand (Figure 1A), with eight genera, while one genus, *Sharon,* was recently described from South America[2,9,26] (Figure 1G). Pityobiinae *sensu* Douglas et al.[3] includes two genera, *Pityobius* from North America (Figure 1B) and *Tibionema* from South America (Figures 1C, 1E, and 1F). Two monotypic Campyloxeninae genera are known from Chile and Argentina[26] (Figure 1D).

Various earlier published topologies suggested that the Gondwanan groups are distantly related. It kept open the possibility that the Gondwanan fauna is highly phylogenetically diverse. Elaterids are indeed ancient enough to exist before the Gondwana breakup. The abundant fossil record can be traced back to the Triassic of Britain,[27] the Jurassic of Kyrgyzstan and China,[28,29] the Upper Cretaceous of the Jinju Formation,[30–33] Cretaceous deposits in Russia,[34] and Mid-Cretaceous amber.[35] Although some Mesozoic elaterids were transferred to other families, it is widely accepted that click beetles were diverse already in the Late Jurassic/Early Cretaceous and predate the split of Gondwana.

Pityobiinae is also present in the fossil record,[36] and *Cretopityobius* Otto was reported from Cenomanian Burmese amber, which contains both Gondwanan and Laurasian fauna. Therefore, pityobiines are ancient enough to consider the hypothesis of the ancestor in Gondwana when it separated from the northern continents.[37]

Bioluminescence

Recently, the bioluminescence of elaterids has been a hotly debated topic.[38–41] The luciferin-luciferase reaction, interactions in predator-prey systems, chemoecology of protective compounds, and genes critical in beetle bioluminescence were studied by various authors.[42–46] However, disagreements persist among systematists regarding the phylogenetic relationships of crucial lineages.[3,4,47,48] Notably, some recent studies discussed only the origin of bioluminescence in Pyrophorini and did not comment on non-pyrophorine groups.[38,41] We assert that a comprehensive exploration of the phylogenetic origins of bioluminescence is imperative to provide a robust foundation for further genomics research.

All bioluminescent elateroids are concentrated in the elaterid-lampyroid clade proposed by Kusy et al.[4] Over 2,000 bioluminescent species form the lampyroid group consisting of Sinopyrophoridae, Lampyridae, Phengodidae, and Rhagophthalmidae. According to Kusy et al.'s 4,200 ortholog phylogeny,[4] this clade emerges as a sister to elaterids. Alternatively, the clade Lampyridae + Phengodidae + Rhagophthalmidae occupies the terminal position in elaterids when *Sinopyrophorus* was absent in the analysis.[3] Under both scenarios, it is an ancient group with no later than an early Cretaceous origin of a bioluminescent most recent common ancestor.[39] The subsequent clade of bioluminescent taxa encompasses Pyrophorini in Agrypninae and eventually Anaissini (only *Alampoides* Schwarz and *Coctilelater* Costa). The latter tribe involves *Alampoides* known for bioluminescent larvae,[49] yet its relationship to Pyrophorini necessitates further confirmation. The age of the Pyrophorini clade would be notably younger compared to the lampyroid clade if lampyroids are regarded as elaterids' sister group,[4] while it could approximate contemporaneity if the lampyroid clade is positioned terminally within click beetles.[3] *Balgus schnusei* (Heller) stands as the solitary bioluminescent species within the otherwise non-luminescent *Balgus* Fleutiaux of the Lissominae-Thylacosterninae clade.[50,51] *Balgus schnusei* possibly represents the most recent origin of bioluminescence in Elateroidea.

Campyloxenus, with its orphan position in the elaterid classification,[14] is the last potential candidate for an independent bioluminescence origin. The sole known species within *Campyloxenus* is named "*pyrothorax*."[52] Although it is unlikely that the species epithet's creators had observational data and no subsequent information on its luminescence has been reported in the literature, *C. pyrothorax* Fairmaire & Germain has always been designated a bioluminescent click beetle. Its bioluminescence initially contributed to its placement within Agrypnini, a tribe that houses most bioluminescent genera of Elateridae.[7,52,53] Costa,[14] upon revising pyrophorine genera, identified morphological distinctions warranting establishing a distinct subfamily. However, the closest relatives of Campyloxeninae have never been hypothesized.[1] A non-luminescent *Malalcahuello* Arias-Bohart was recently described within Campyloxeninae.[26]

In this study, we undertake the first assessment of the placement of bioluminescent *Campyloxenus*, which has, at times, been linked closely with other bioluminescent elaterids.[7,54] With the newly shotgun-sequenced elaterid genomes from Gondwanan continents, our objective also addresses fundamental zoogeographic inquiries, including the possibility of a shared ancestor among Gondwanan elaterid lineages and their estimated age. The analyses also aim to improve click beetle classification as relationships of Gondwanan lineages are poorly understood. The incomplete and highly unstable click beetle classification negatively affects further research in bioluminescence and other interesting evolutionary phenomena.

RESULTS

Molecular analyses

We shotgun-sequenced eight click beetle taxa and extracted orthologs for phylogenetic analyses. The AliStat completeness of the 66-ortholog dataset was 0.79 (Figure S1). The SymTest analyses unveil a high compositional heterogeneity among click beetle in the nucleotide (NT) dataset but low in the amino acid (AA) dataset (Figure S2). The aligned orthologs were analyzed with maximum likelihood (ML) and coalescent approaches to identify relationships among Gondwanan subfamilies, *Pityobius*, and other click beetles. Half of the analyses (i.e., ML analyses at the AA level, partitioned and unpartitioned, and the ASTRAL analysis at the AA level) suggest the clade of Nearctic *Pityobius* (Pityobiinae: Pityobiini) and four traditional subfamilies with Gondwanan distribution—*Diplophoenicus* (Morostomatinae) *Tibionema* (currently Pityobiinae), *Hapatesus* (Hapatesinae), *Elatichrosis*, *Parasaphes*, *Rousia* (currently Parablacinae), and *Campyloxenus* (currently Campyloxeninae) (Figures 2A and S3–S6). This clade is deeply rooted, and it is a sister to the Omalisinae + Dendrometrinae + Agrypninae clade in most analyses. Alternatively, *Diplophoenicus* (Morostomatinae) shifts from the first split of the previously recovered clade to the base of its sister, i.e., the Omalisinae, Agrypninae, and Dendrometrinae clade (both ML analyses at the NT level; Figures 2A and S3–S5) or to the base of the *Diplophoenicus* + Dendrometrinae clade (the coalescent analysis at the NT level; Figure S5), when the base of the whole clade (local posterior probability, LPP 99) is recovered as a series of very short branches with low support (LPP values 0.5 and 0.41, the coalescent analysis at the NT level; Figure S5).

The first lineage to split was *Diplophoenicus*, and its inclusion in the recovered clade consisting of Gondwanan subfamilies and *Pityobius* was generally weakly supported in both hypothesized positions (bootstrap support, BS 53%–100%; Figures S3–S5). Given the poor support in ML and coalescent analyses, we performed a quartet likelihood mapping analysis using all 66-gene datasets at AA and NT levels to determine the most probable position of Morostomatinae (Figures 2B–2D). Both analyses preferred the sister relationships of Morostomatinae and the *Pityobius*/Gondwanan subclade, but the differences between the numbers of quartets supporting alternative topologies are low (44% versus 32% at the AA level and 42% versus 35% at the NT level). Additional tests also did not provide

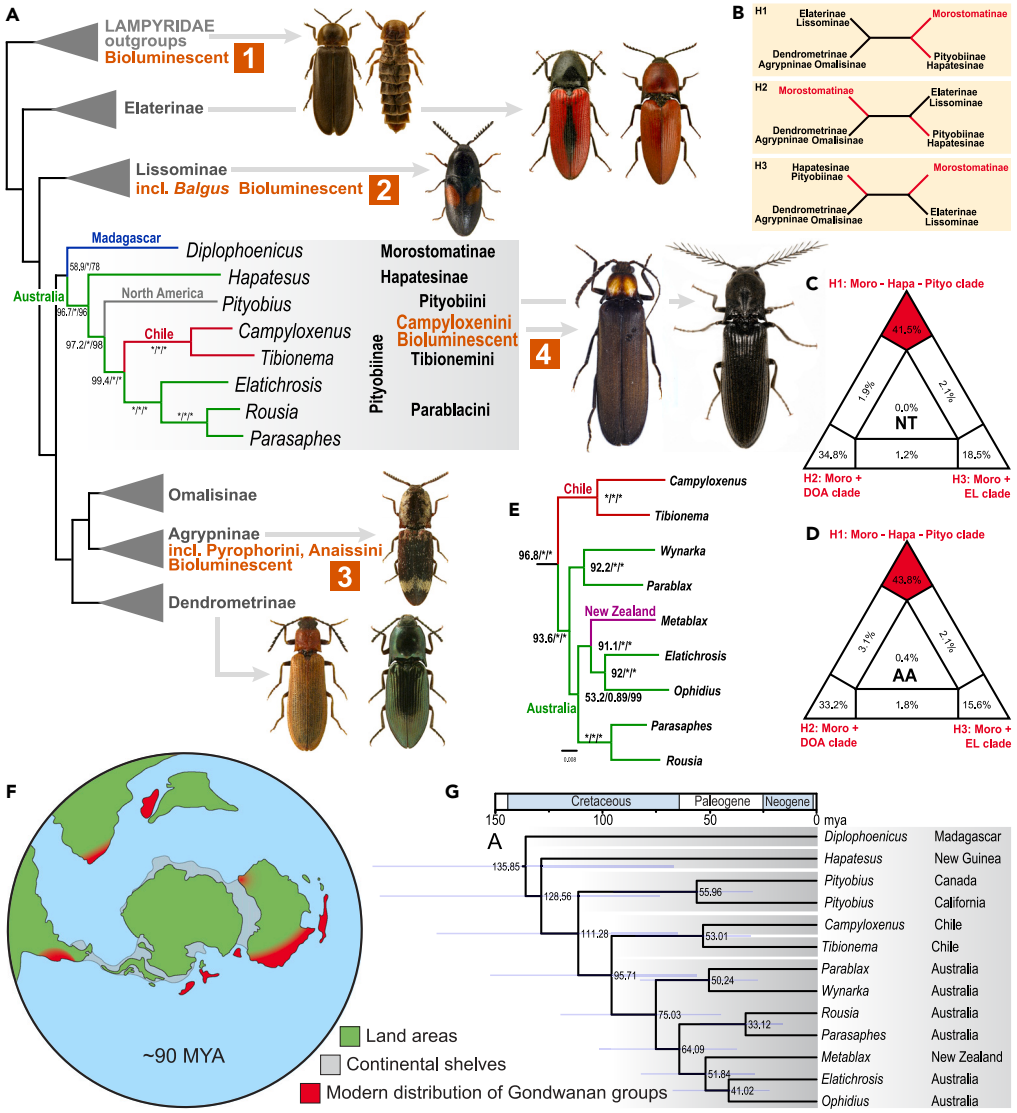

Figure 2. The results of phylogenetic analyses

(A) Topology recovered by the maximum likelihood (ML) analysis of the 66-gene dataset at the amino acid (AA) level. The numbers separated by slashes designate SH-aLRT, aBayes, and UFBoot, respectively, asterisks indicate the maximum support in Figures A and E; (B) The tested four-cluster likelihood mapping (FcLM) hypotheses; (C) The support of alternative hypotheses recovered by the nucleotide analysis of the 66-gene dataset; (D) ditto at the AA level; (E) The relationships of *Campyloxenus*, *Tibionema*, and Pityobiini genera recovered by the ML analysis of the four-fragment dataset (node numbers as in Fig. A); (F) The positions of southern continents ~90 mya with the extant distribution of Gondwanan subfamilies marked; the map courtesy of J. P. Klages/AWI; (G) Dated tree of the Gondwanan groups and *Pityobius* (all numbers designate million years ago). The light blue bars designate the 95% highest posterior density interval. Full versions of all trees are presented in supplemental information). Photographs of focal taxa were provided by L. Borowiec (European species) and C. Rorabaugh (*Pityobius*).

significant support. The p values were insignificantly higher for the alternative with Morostomatinae as a part of the clade of Gondwanan subfamilies (Table S4).

Almost all subsequent splits were recovered with full support in all analyses at the NT and AA levels and under various settings (Figures 2 and S3–S7). Only one analysis suggested the shift between serial splits of *Hapatesus* and *Pityobius* (Figure S5; the coalescent analysis at the AA level). The aberrant position got low support (LPP 55%), unlike the alternative topology.

We also expanded the earlier published 4-gene dataset[2,8,22,55] by additional samples of *Diplophoenicus*, *Pityobius*, *Hapatesus*, *Elatichrosis*, *Parasaphes*, *Rousia*, *Tibionema*, and *Campyloxenus*. The ML analyses (partitioned/unpartitioned) did not recover all focal taxa as a single clade (Figure S7). Two *Pityobius* species were recovered as a sister to the Lissominae + Thylacosterninae clade in the partitioned analysis, and other focal lineages as a sister to the Dendrometrinae + Agrypninae clade (Figure S7). The unpartitioned analysis of the same dataset yielded

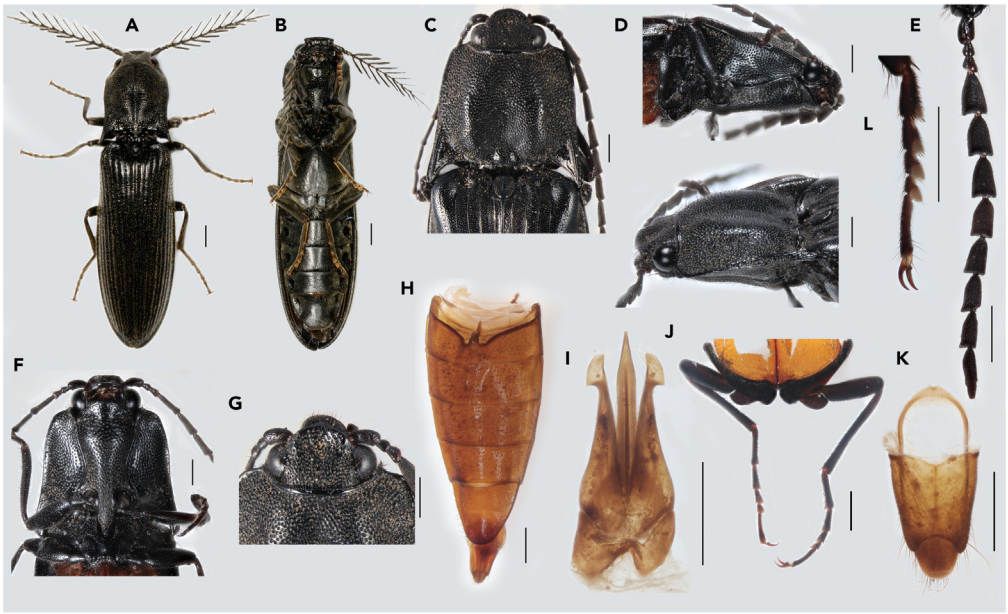

Figure 3. The morphology of Pityobiini and Tibionemini

Pityobius anguinus. (A, B) habitus dorsally and ventrally. *Tibionema abdominalis*. (C–F) Pronotum in various aspects, the arrow designates mesosternal keel; (G) Head, dorsal view; (H) antenna, male; (I) abdomen, ventral view; (J) male genitalia, (K) abdominal terminal segments, male; (L) hind legs; (M) tarsus, prothoracic leg. Scales 1 mm (A–H, J), 0.5 mm (I, K).

a large clade of focal lineages and several other subfamilies. The first subclade contained Hemiopinae, *Pityobius* spp., *Hapatesus*, and Oestodinae, while the second subclade was formed by parablacine genera, *Tibionema* and *Campyloxenus* (Figure S7). The BS values of the deepest splits were as low as 46%. The Campyloxenini + Tibionemini + Parablacini clade was recovered, and the internal relationships agree with the 66-gene topology (Figure 2A).

We estimate the first split in the clade of Gondwanan subfamilies and *Pityobius* at 136 million years ago (mya) and the subsequent splits between Australian Hapatesinae and Pityobiinae at 129 mya (Figures 2F, 2G, and S8). The separation of North American *Pityobius* from other Pityobiinae predates the continental breakup of Gondwana. North American *Pityobius* is the only taxon of these subfamilies that occurs outside the Gondwanan continents, but, with available sampling, the separation of *Pityobius* from its relatives is very ancient. There is no evidence of active dispersal routes from Australia to southeastern Asia or between Madagascar and Africa. If the dated topology is analyzed without Morostomatinae as the sister of the hapatesine-pityobiine clade (due to uncertain position), similar ages were estimated for shallower splits (Figures S8D and S8E).

Taxonomy

Subfamily Pityobiinae Hyslop, 1917, new sense.

Pityobiini Hyslop, 1917: 249.[15]

Pityobiinae Hyslop, 1917: various authors.[17,18]

Type genus: *Pityobius* LeConte, 1853.

Diagnosis

Several morphological characters support the relationships of pityobiine lineages. Notably, these features include short ball-like antennomeres 2 and 3 (Figures 3 and 4), a pronotal disc with parallel bulges (Figures 3 and 4), and the relatively short tarsomere 4 (Figures 3 and 4). Yet, these characters are not shared by all members. For example, Campyloxenini exhibits differences in the structure of antennomeres[14] (Figure 1D). Additionally, certain Protelaterinae and Agrypninae have similar relative lengths of basal antennomeres.

Remark

We uphold the classification of Morostomatinae and Hapatesinae as separate subfamilies based on their distinct morphological characteristics and congruency with earlier classifications.[1,3,11] We propose merging Pityobiini, Parablacini, Campyloxenini, and Tibionemini trib. nov. into a redefined subfamily Pityobiinae. This clade is robustly supported by phylogenomic analyses (Figures 2A and 2E), although the support from morphology is less clear. Pityobiinae retains its current rank, with Pityobiini as a nominotypical tribe comprising only *Pityobius*. Meanwhile, Parablacinae and Campyloxeninae are downranked to tribes within Pityobiinae. To address the non-monophyly of Pityobiinae *sensu*

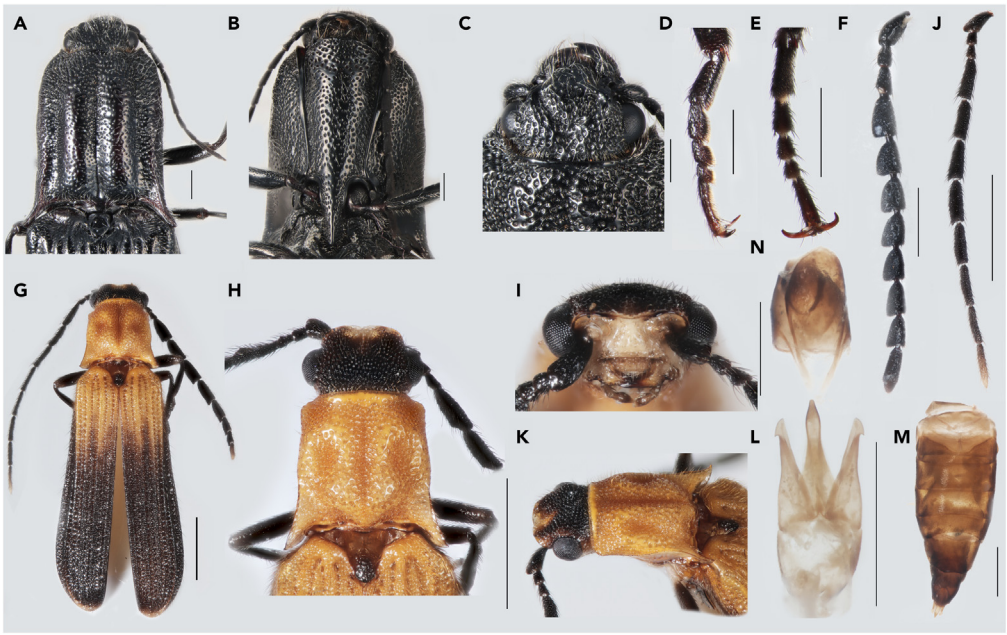

Figure 4. The morphology of Parablacini

Elatichrosis sp. (A, B) pronotum, dorsal, and ventral view; (C) head, dorsally; (D, E) tarsus, prothoracic leg, lateral and ventral view; (F) antenna, female. *Rousia* sp. (G) habitus; (H, K) pronotum, dorsal, and dorsolateral views; (I) head, frontal view, (J) antenna, male; (L) male genitalia; (M) abdomen, ventral view. Scales 1.0 mm (Figures A–K, M), 0.5 mm (Figures L, N).

Kundrata et al.[2] and Douglas et al.[3] (Figures 2A and 2E), we introduce a new tribe, Tibionemini trib. nov. The generic composition and species richness of genera are given in Table 2.

Tribe Pityobiini Hyslop, 1917: 249,[15] **new sense**.

Type genus: *Pityobius* LeConte, 1853.

Remark

Pityobius and *Tibionema* are morphologically distinct (Figures 1A, 1C, and 4), have a long evolutionary history (*Pityobius* split from relatives at 111 mya; Figure 2G), and occur in distant regions (Figure 1A). We propose to render Pityobiini into a monogeneric tribe and handle *Tibionema* in a tribe of its own (see in the following).

Parablacini Kundrata, Gunter, Douglas & Bocak, 2016, **stat. nov.**

Parablacinae Kundrata, Gunter, Douglas & Bocak, 2016: 299.[2]

Type genus: *Parablax* Schwarz, 1906.

Remark

The phylogenomic analyses revealed that three parablacine genera form a subterminal monophyletic group within the Gondwanan clade. This clade diverges after *Pityobius* but before the *Campyloxenus* + *Tibionema* clade (Figures 2A and 2E). As a result of these findings, we propose downranking Parablacinae to Parablacini stat. nov.

Based on the genomic analysis and shared morphological characters, we propose transferring *Rousia* and *Elatichrosis* from Dendrometrinae to Pityobiinae: Parablacini. The decision to make these transfers is supported by the presence of similar ball-like antennomeres 2 and 3 (Figures 3 and 4) and concealed labrum (Figures 3 and 4) in these genera, akin to the Chilean Parablacini and at least one group of *Metablax* from New Zealand. *Rousia* also shares certain features with other Parablacini, such as a poorly defined anterior margin of the scutellum. Notably, Calder[9,56] did not include *Elatichrosis* and *Rousia* in the widely defined Pityobiini, encompassing other parablacine genera. The potential reason for Calder's decision to retain some parablacines in Dendrometrinae was the presence of other genera with partially similar morphology in this subfamily, including related genera that were later transferred to Hapatesinae.[11] Moving forward, the placement of several other genera, such as *Paracrepidomenus* Schwarz, 1906, warrants further investigation.

Distribution

Australia and New Zealand harbor a rich diversity of Parablacini, currently comprising eight genera with twenty-three species[56] (Table 2). However, we suspect that the biodiversity inventory for Australian Parablacini is still far from complete. In addition to the Australian diversity, there is a single parablacine genus, *Sharon*, known from the southern part of the Neotropical region.[26,57]

Table 1. The list of newly sequenced and analyzed genomic data (taxonomic placement proposed in the present study)

Voucher	Species	Subfamily	Tribe	Geographic origin
G19011	*Diplophoenicus* sp.	Morostomatinae		Madagascar
G20007	*Hapatesus tropicus*	Hapatesinae		New Guinea
G21014	*Elatichrosis* sp.	Pityobiinae	Parablacini	Australia
G19006	*Parasaphes* sp.	Pityobiinae	Parablacini	Australia
G21031	*Rousia* sp.	Pityobiinae	Parablacini	Australia
G20012	*Tibionema abdominalis*	Pityobiinae	Tibionemini	Chile
G21037	*Pityobius murrayi*	Pityobiinae	Pityobiini	USA
G21036	*Campyloxenus pyrothorax*	Pityobiinae	Campyloxenini	Chile

Campyloxenini Costa, 1975, **stat. nov.**
Campyloxeninae Costa, 1975: 114.[14]
Type genus: *Campyloxenus* Fairmaire & Germain, 1860.

Remark

Our analyses provide compelling evidence supporting the placement of *Campyloxenus* within the pityobiine clade, where it emerges as a sister to *Tibionema* (Figures 2A and 2E). Based on relationships shown in Figures 2A, 2E, and S3–S7, we propose downranking Campyloxeninae to a tribe within Pityobiinae. Arias-Bohart[26] extensively discussed the morphological distinctiveness of the two constituent genera. *Malalcahuello* exhibits a well-sclerotized cuticle, and *Campyloxenus* displays a more soft-bodied nature resembling fireflies. Adult female light emission was observed by Isai Madríz; reliable information on pupae and larvae is unavailable.

Tibionemini Motyka, Kusy, Arias, Bybee et Bocak, new tribe
urn:lsid:zoobank.org:pub:91027F71-C0E4-4746-A440-DBD9C1894D6A.
= Tibionemini Ulrich, 1988 (*in litt.*),[58] unavailable name.
Type genus. *Tibionema* Solier, 1851.

Diagnosis

Tibionema has a medium-to-large body, flat pronotum, and elytra (Figure 1C), distant longitudinal bulges in the pronotum, and acutely prominent posterior angles of the pronotum (Figure 3C). The prosternum is narrow, and the prosternal process is long and slender (Figure 3F). The lateral edges of the pronotum are complete (Figure 3E). There is a sharp keel between mesocoxae (Figure 3D). Antennae are 11-segmented and serrate; the scapus is parallel-sided, antennomeres 2 and 3 are short and ball like, and antennomeres 1–3 are almost bare and shining (Figure 3E). The abdomen has five visible segments, a short intercoxal process, a straight posterior margin of the penultimate segment, and a triangular, apically rounded last visible abdominal segment. Internal abdominal segments consist of long and narrow penultimate and small ultimate sternites (Figure 3K). *Tibionema* and *Pityobius* differ in the number of antennomeres (twelve in *Pityobius* versus eleven in *Tibionema*); the posterior pronotal angles bent toward the body in *Tibionema* (also in *Hapatesus* and *Oxynopterus*), but not in *Pityobius* (Figure 3A). *Tibionema* has a prominent intercoxal keel in the mesosternum (Figure 3) that has not been observed in other Pityobiinae. *Campyloxenus* differs in much longer antennomere 3. The larva and pupa of *T. abdominalis* were described by Angulo.[59]

Remark

Tibionemini trib. nov. is proposed due to the refuted monophyly of Pityobiinae *sensu* Kundrata et al.[2] and Douglas et al.[3] (Figure 2A, 2E, and 2G). Following the principles of phylogenetic systematics, preserving the subfamily ranks for Parablacinae and Campyloxeninae would necessitate assigning the same rank to *Tibionema*, ultimately increasing the number of subfamilies. The morphological divergence between *Pityobius* and *Tibionema* was recognized. Due to the sparse sampling, *Tibionema* was recovered as a sister to Parablacinae by Kusy et al.[11] and a sister to *Pityobius* by Bi et al.[60] Here, with parablacines and *Campyloxenus* analyzed, the two genera are recovered in distant positions (Figures 2A and 2E).

DISCUSSION

The phylogeny of Gondwanan click beetles

The internal relationships among click beetle subfamilies have been a subject of active debate, with investigations spanning morphological to phylogenomic analyses.[2–9,11,14,18,21,61,62] However, several subfamilies from the Southern continents (Figure 1A) have yet to be included in data-rich molecular analyses. In this study, we examine the relationships of newly sequenced sixty-six orthologs for Morostomatinae, Hapatesinae, and Pityobiinae, including Pityobiini, Parablacini, Campyloxenini, and Tibionemini (see taxonomy for revised ranks; Table 2). This gene-rich dataset represents the best currently available sampling of species-poor Gondwanan click beetles (Table 1).

Our analyses frequently placed *Pityobius* and all Gondwanan groups within a single clade (Figures 2 and S3–S7). Still, the position of Morostomatinae remains uncertain, either as a sister to the hapatesine-pityobiine clade or as a subsequent serial split in the tree, i.e., a sister to the

Table 2. The overview of tribes, genera, diversity, and distribution of Pityobiinae

Tribe/Genus	Type species	Distribution	spp.
Pityobiini Hyslop, 1917			2
Pityobius LeConte, 1853	*Pityobius anguinus* LeConte, 1853	USA: E. & W., Canada: Manitoba	2
Parablacini Kundrata et al., 2016			38
Elatichrosis Hyslop, 1921	*Chrosis exarata* Candèze, 1863	Australia	13
Metablax Candèze, 1869	*Elater acutipennis* White, 1846	New Zealand	5
Ophidius Candèze, 1863	*Ophidius elegans* Candèze, 1863	Australia: NSW, QLD	4
Parablax Schwarz, 1906	*Metablax trisulcatus* Schwarz, 1903	Australia, Tasmania	9
Parasaphes Candèze, 1882	*Parasaphes elegans* Candèze, 1882	Australia: QLD	1
Rousia Calder, 1996	*Rousia dumbrellium* Calder, 1996	Australia: NSW, QLD	1
Sharon Arias-Bohart & Elgueta, 2015	*Asaphes amoenus* Philippi, 1861	Chile	2
Tasmanelater Calder, 1996	*Tasm. pelionensis* Calder, 1996	Tasmania	1
Wynarka Calder, 1986	*Wynarka sylvestre* Calder, 1986	Australia: NSW, VIC, ACT	1
Xuthelater Calder, 1996	*Xuthelater moppiensis* Calder, 1996	Australia: NSW	1
Campyloxenini Costa, 1975			2
Campyloxenus Fairm. & Germ., 1860	*C. pyrothorax* Fairm. & Germ., 1860	Southern Chile, Argentina	1
Malalcahuello Arias-Bohart, 2015	*Mal. ocaresi* Arias-Bohart, 2015	Southern Chile	1
Tibionemini trib. n.			1
Tibionema Solier, 1851	*Tibionema rufiventre* Solier, 1851	Chile	1

NSW – New South Wales, QLD – Queensland, VIC – Victoria, ACT – Australian Capital Territory.

Agrypninae and Dendrometrinae clade (Figures S3–S7). We observed that all focal groups form a clade in 66-gene ML/NT (partitioned and unpartitioned) and ASTRAL (coalescent) analyses at the AA level. Alternatively, we recovered a paraphylum at the ML/NT and ASTRAL/NT analyses of the same dataset (Figures 2 and S3–S7). The four-cluster likelihood mapping (FcLM) analyses show only slightly higher probabilities for the close relationships of Morostomatinae and other Gondwanan groups (Figures 2A–2D). The monophyly of the clade should be reinvestigated in the future with denser sampling and a larger dataset to handle better biological and analytic factors causing pervasive uncertainty in the topologies.[63]

However, the monophyly of Hapatesinae + Pityobiinae *sensu lato* was robustly supported by all genomic analyses (Figures 2 and S3–S6). The only ambiguity in the internal relationships was the relative position of *Pityobius* (Pityobiinae: Pityobiini) and *Hapatesus* (Hapatesinae). Five of the six analyses recovered Hapatesinae as a sister to the redefined Pityobiinae (≥95% in 66-gene ML analyses, Figure 3). While the ASTRAL analysis at the AA level deviates and suggests a switch between *Hapatesus* and *Pityobius*. However, the statistical support is considerably lower (LPP 0.55, Figure S5B). Therefore, we favor Hapatesinae as a sister to the redefined Parablacinae. The congruent relationship between *Hapatesus* and *Pityobius* + *Tibionema* was suggested in the mitogenomic and genomic studies when other taxa were unavailable.[3,11] The morphological distinctiveness of *Hapatesus* justifies the subfamily rank for Hapatesinae.[11]

Based on the preferred topology, we redefine Pityobiinae, which now contains (a) Pityobiini (monogeneric; *Pityobius* was previously part of Pityobiinae[2,3]), (b) Parablacini (nine genera, earlier in Pityobiinae *sensu lato*[9,18] and Parablacinae[2]), (c) Campyloxenini (*Campyloxenus* and *Malalcahuello*, earlier in Agrypninae, later Campyloxeninae[14,26]), and (d) Tibionemini, new tribe (monogeneric, *Tibionema* has been placed in Pityobiinae[3]). We reject the distant position of *Pityobius* and parablacine genera[2,22] and the sister position of *Pityobius* and other Elateridae.[21] Furthermore, we demonstrate that the earlier proposed concept of Pityobiinae consisting of *Tibionema* and *Pityobius* was an artifact of sparse sampling.[3] With the revised taxonomy, the redefined Pityobiinae remains a species-poor subfamily, comprising 13 genera and 43 described species (Table 2).

Further, we expanded and reanalyzed the short-fragment dataset published by earlier authors.[2,22,55,62] Even with additional taxa, the separate positions of *Pityobius* and parablacines are again recovered (Figures 2E and S7). As these lineages represent ancient splits, the information from rRNA genes and mtDNA fragments is likely insufficient for robust analyses. The instability of the backbone is evident from very low statistical support in earlier studies,[2,60] and the present reanalysis of short DNA fragments (Figure S7). While some researchers still follow relationships recovered by short-fragment analyses,[48] we prefer detailed analyses of large multigene datasets.[63] The topologies recovered from such analyses can be tested for the biological and methodological sources of systematic errors. The strictly tested topologies provide a more robust basis for classification than a short-fragment analysis without subsequent tests (Figures 2A and S3–S6).

Zoogeography

Elateridae is found on all continents except Antarctica.[1] The large subfamilies, Elaterinae, Dendrometrinae, and Agrypninae, encompass most described genera and species and exhibit a truly global distribution. In contrast, the lineages studied here are species-poor and mostly confined to small geographic ranges on the continents that formed Gondwana (Figure 1A).

The split of Morostomatinae is estimated at ~136 mya at the time of high connectivity between the Gondwanan continents (Figure 2G). It corresponds with the opening of the South Atlantic at 135 mya.[37] However, Morostomatinae does not occur in continental Africa and India despite the connectivity between these regions and Madagascar during the Cretaceous.

Pityobiini originated ~111 mya. It is the sole modern lineage of the focal clade occurring in the Nearctic region. Yet, its Cretaceous representative *Cretopityobius* has been reported from Burmese amber (Cenomanian).[36] These findings suggest that Pityobiini was once more widespread and persisted only in North America. It is worth noting that Cenomanian Burmese amber is considerably younger than click beetle subfamilies,[24,39] and the fauna includes both Gondwanan and Laurasian taxa. Campyloxenini and Tibionemini originated at ~96 mya, i.e., when South America became isolated from Africa, but earlier than it lost its connection with Antarctica and Australia.[37] The distribution of Parablacini in the southernmost part of South America, New Zealand, and Australia can be attributed to vicariance and Cretaceous connectivity through temperate forests in Antarctica.[64] Their diversification started shortly after the opening of the Tasman Sea, 80 mya. A more extensive sampling would be necessary to delve deeper into this question as South American Parablacini has not been analyzed (Figure 2E).

The modern distribution suggests that the Gondwanan click beetles might face limitations in crossing open seas. For instance, the seas between Australia and southeastern Asia appear to be an impermeable barrier for Australian pityobiine and hapatesine genera, even with the availability of the Wallacea steppingstone connection since the Miocene.[65] Similarly, Morostomatinae has not crossed the Mozambique strait and remains endemic to Madagascar. Thus far, there is no reliable record of the old Gondwanan click beetles from continental Africa. However, Ulrich[58] reported a putative pityobiine larva from Southern Africa. Adult specimens have not yet been collected. Few species in restricted ranges raise macroevolutionary questions regarding diversification and extinction rates. There is no evidence of the dispersal capability of Gondwanan lineages and their ability to undergo rapid speciation. Despite being a very ancient lineage with ample opportunities to colonize new territories during the Cenozoic and Quaternary periods, the Gondwanan lineages still bear the signature of the breakup of the southern continents.

Bioluminescence in click beetles: Exploring the Campyloxenus case

Since the dissolution of the traditional cantharoid clade,[55] our understanding of the origins of bioluminescence in elateriform beetles has progressively advanced.[38,39,41,42,66] Most bioluminescent elateroids are soft bodied (~2,000 spp; fireflies and railworm beetles, Lampyridae, Phengodidae, and Rhagophthalmidae). Jointly with clicking Sinopyrophoridae, they constitute a lampyroid clade *sensu* Kusy et al.[4] It represents an independent origin of bioluminescence, leading to the most extensive radiation of luminescent elateroids.

In contrast, other light-emitting elateroids exhibit pronounced sclerotization and the clicking mechanism (Elateridae, ~100 species), and they are currently placed into three subfamilies. Most are concentrated in Agrypninae, particularly Pyrophorini in current classification, i.e., including Hapsodrilina, Nyctophyxina, and Pyrophorina (the second origin of bioluminescence[7,14,54]). The larva of *Alampoides* Schwarz (Pyrophorinae: Anaissini) is luminescent and might eventually represent an additional, third, origin if distant relationships of Anaissini and Pyrophorini are confirmed.[14,49] Currently, *Coctilelater* Costa is also placed in Anaissini but in a separate position from *Alampoides*.[14] As *C. sanguinicollis* (Candèze) was originally placed in *Pyrophorus*, it should be a bioluminescent species. The genus-level analysis is needed for a detailed investigation of the origins and, eventually, losses of bioluminescence in Agrypninae. To be conservative, we provisionally hypothesize a single origin of bioluminescence in Agrypninae as we do not have sufficiently dense sampling to investigate possible origins and losses of bioluminescence in this group.

Balgus schnusei, currently placed in Thylacosterninae, represents evidence for the third origin of bioluminescence in Elateroidea[50,51] (Figure 2A). The bioluminescence of this taxon was observed by G. L. Tavakilian in Saul, French Guyana.[51] There is no information available on later observations and the larva of *Balgus schnusei*.

The presence of luminous patches and recent observation of the bred specimen by Isai Madríz (personal communication) substantiate the bioluminescence of *Campyloxenus* adults. Isai Madríz bred a *Campyloxenus* larva to the imago and did not observe its luminescence. The larva of *Campyloxenus'* closest relative, at adult stage non-luminescent *Malalcahuello* Arias-Bohart, is unknown. Genomic data analyses unequivocally identify *Campyloxenus* as the fourth well-documented origin of bioluminescence in click beetles (Figures 2A and 2E). We adopt the Elateridae concept introduced by Kusy et al.,[4] which is based on a thorough analysis of 4,200 orthologs and corroborated by multiple tests exploring the sources of signal and conflicts and supporting the monophyly of click beetles. In this sense, we hypothesize four origins of bioluminescence in Elateroidea.

Alternatively, *Sinopyrophorus* will represent the additional, fifth, origin if it is hypothesized as related to Oestodinae and Hemiopinae.[60] Nonetheless, we ardently favor its membership in the lampyroid clade recovered by the phylogenomic analyses.[4] The *Sinopyrophorus* + Oestodinae + Hemiopinae clade asserted by Bi et al.[60] and recently defended by Lawrence et al.[48] is based on analyzing a notably incomplete dataset (completeness ~25%). This dataset was *ad hoc* assembled from newly sequenced *Sinopyrophorus* (mitogenome, rRNA genes) and previously published short DNA fragments.[8,22,55] These data were merged with publicly available mitogenomes of elaterids.[67,68] The statistical support for the (*Oestodes*(*Hemiopus*, *Sinopyrophorus*)) clade was very low (the whole clade BS 13%, and *Hemiopus* + *Sinopyrophorus* BS 41%). The backbone of Elateridae also lacks robust support (the five deepest splits having BS values 15%–49%). The dataset was insufficient for the reliable recovery of deep splits.

An additional report on the bioluminescence referred to *Omalisus* (Omalisinae)[69,] and it was revived by Beutel.[70] The possibility of bioluminescent *Omalisus* was definitively rejected by Burakowski[71] and other authors who frequently collected this species.[72,73]

Undeniably, the bioluminescence in Elateroidea is ancient. Fossil evidence includes a firefly fossil in Cenomanian Burmese amber,[74] while recent dating analyses place the terrestrial luminescence origin in the Early Cretaceous.[39] Our dating analysis suggests the delayed origin of

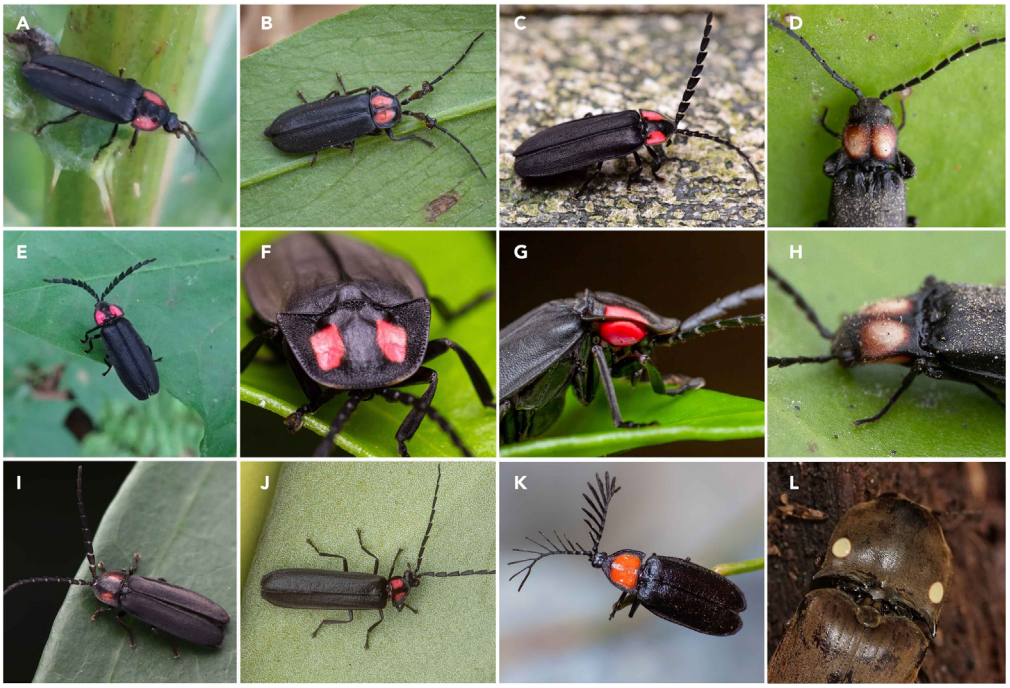

Figure 5. The general appearance of elateroid species

(A) *Pyropyga nigricans* (USA, photo by Erin Moore, USA); (B) *Dysmorphocerus dilaticornis* (Chile, photo by Claudio Maureira, Chile); (C) *Lucidina vitalisi* (China, Zhenjiang, photo by Fan Gao, Nanjing); (D, H) *Campyloxenus pyrothorax*, (Chile, photo by Matías Gargiulo); (E) *Cladodes ater* (Chile, photo by Michael Weyman); (F, G) *Pyratocnema* sp. (photo by Matías Garguilo, Chile); (I) *Lucidina* sp., (China, photo by rosefan2 from inaturalist.org); (J) *Pyractonema* sp. (Chile, photo by Matías Gargiulo); (K) *Vesta cincticollis* (Chile, photo by Claudio Maureira); (L) *Pyrophorus* sp. (Ecuador, photo by M. Motyka).

luminescence in *Campyloxenus*, even without including its sister genus *Malalcahuello* (Figures 2G and S8). Our estimate at 53 mya stands as a maximum age, considering that non-luminescent *Malalcahuello* must have separated from *Campyloxenus* later, as both belong to Campyloxenini. We assume *Campyloxenus* might be somewhat younger than the origin of bioluminescent Pyrophorini (the origin of Pyrophorinae was estimated at 133.7–91.7 mya[39]). It is conceivable that *Balgus schnusei* became bioluminescent even later than *Campyloxenus*, as it is the sole bioluminescent species within its genus. Based on phylogenomic analyses, we can conclude that elateroids' bioluminescence originated independently at least on four occasions and at different times. *Campyloxenus*, as a relatively young taxon, could be a promising model for further studies if new material is available for breeding and *Malalcahuello* is available for analyses. Additional bioluminescence origins and eventual losses can be recovered if Pyrophorinae phylogeny is studied in detail.

The positions, sizes, and shapes of luminous lanterns exhibit substantial variability among click beetles. *Campyloxenus* has only prothoracic luminous lanterns like Pyrophorini: Nyctophyxina (Figures 5D and 5H), and the abdominal spots common in Pyrophorina are absent. Pyrophorini, e.g., *Pyrearinus* Costa and *Nyctophyxis* Costa, and *Campyloxenus* also share the position of lanterns at the posterior pronotal margins instead of lateral ones.[14] *Campyloxenus'* spots differ in diffuse boundaries and larger sizes (Figures 5D and 5H). Their color is orange to pinkish, unlike Pyrophorini (Figure 5L), and somewhat reminds those of *Nyctophyxis ocellatus* (Germar). The large lanterns are visible on the dorsal and ventral sides of the pronotum (Figures 5D and 5H). Our investigation into additional material from regions inhabited by *Campyloxenus* unveils its resemblance to certain Chilean fireflies (Lampyridae: Lampyrinae, Cladodinae, Figure 5E) and soldier beetles (Cantharidae: Dysmorphocerinae; Figure 5B). The diurnal lampyrine fireflies commonly coexist and share leaves with click beetles (Figures 1 and 5). Lampyrine fireflies are unpalatable. They contain steroidal pyrones called lucibufagins, structurally akin to the cardiotoxic bufadienolides found in toads and cardenolides in plants.[43,75] Lucibufagins protect them against spiders, toads, birds, and other predators. Similarly, soldier beetles (Figure 5B) potentially possess a chemical defense[76,77] and often participate in Batesian mimicry rings.[78]

Campyloxenus, like many other small insects, is a potential prey for small birds, spiders, and assassin bugs. Interestingly, this species has a black-colored body and elytra and bright spots on the pronotum, like some other beetles known for their unpalatability (Figure 5). This color pattern is widely distributed and shared by multiple genera and species of similar fireflies and soldier beetles. As unpalatable, phenotypically similar, but unrelated species, they potentially form a Müllerian mimicry ring.[79] Interestingly, the black-red colored unpalatable species are not closely copied by Batesian mimics in Asia and North America, possibly due to the unusual coloration of pronotal patches (Figure 5). The remarkable resemblance between *Campyloxenus* and unpalatable fireflies is an exception. *Campyloxenus* does not occur in masses, and there is no indication that it is unpalatable. Therefore, it should be a Batesian mimic. Further, *Campyloxenus* differs from other click beetles in a weakly sclerotized cuticle akin to fireflies. It might be a coincidence as it is the only true bioluminescent click beetle with weaker cuticle sclerotization.

Alternatively, it might indicate parallel processes leading to the loss of sclerotization in bioluminescent elateroids relying on a warning signal instead of an escape reaction. Other click beetles also readily imitate the sympatrically occurring unpalatable beetles; among them is *Rousia* sp., resembling *Microtrichalus* spp. and *Trichalus* spp. net-winged beetles in Queensland[78] (Figure 4G).

The phenotypic resemblance of *Campyloxenus* and diurnal fireflies (Figure 5) and a shared color signal potentially aiming at diurnal visual predators do not have anything in common with its luminescence. Diurnal lampyrids usually do not emit light or only low-intensity light.[42] Moreover, the diurnal lampyrids emit light of different wavelengths from lanterns positioned in terminal abdominal ventrites.[54,80] Consequently, *Campyloxenus* acquired the ability to produce light without any direct ecological context related to interactions with fireflies in South American ecosystems, where *Campyloxenus* is indigenous (Figure 1). Given the relatively recent emergence of luminescence in *Campyloxenus* (*Malalcahuello* is its non-luminescent sister taxon[26]), it is plausible that some bioluminescent Pyrophorini sympatrically occurred in the area where *Campyloxenus* developed its luminescence although only *Phanophorus* and *Nyctophix* share the range now (see zoogeography section). While the presence of bioluminescent taxa might not be the trigger for *Campyloxenus'* light-emitting capability, it could contribute to the subsequent selection of a more potent signal for protection against potential predators after dusk.[81]

Conclusions

Most phylogenetic studies of beetles have densely sampled faunas of Europe and Northern America, and this was also the case for Elateridae when several Australian, African, and Southern American subfamilies were omitted.[3,23] In this study, we investigated the relationships between Gondwanan subfamilies of click beetles. Given their morphological disparity and ancient origins, it is appropriate to retain earlier described higher-level taxa while redefining Pityobiinae, downranking Parablacinae and Campyloxeninae to tribes within Pityobiinae, and proposing Tibionemini trib. nov. These adjustments aim to uphold accepted family-group taxa while creating a more informative classification that reduces the number of species-poor subfamilies in Elateridae. The vicariance hypothesis following the breakup of Gondwana is the most plausible scenario for the early diversification of these click beetles. The new delimitation of the ancient morostomatine-hapatesine-pityobiine clade offers insights into the group's zoogeography, underscoring the necessity for robust and extensively sampled phylogenies to formulate zoogeographic hypotheses effectively.

Likewise, preceding phylogenetic analyses have still incompletely elucidated the origins of bioluminescence in click beetles. The placement of *Campyloxenus* within a non-luminescent Pityobiinae clade signifies a minimum of the fourth instance of bioluminescence origin within Elateroidea. Our estimation suggests that *Campyloxenus'* luminescent ability is significantly younger than that of the lampyroid clade and Pyrophorini. Additionally, we propose that *Campyloxenus* is a Müllerian mimic of sympatrically occurring unpalatable fireflies and soldier beetles (Figure 5) and could evolve its bioluminescence in the presence of other bioluminescent click beetles.

Limitations of the study

The sampling is limited by the accessibility of the properly fixed material of rare and narrowly endemic species for the genomic analyses. We conservatively count only a single origin of bioluminescence in Agrypninae, as earlier morphology-based phylogenies and classification proposed variable relationships among constituent tribes and subtribes, and we do not have new data. In the future, the robust reconstruction of bioluminescence in Agrypninae should be based on a much denser sampling of the whole subfamily. It is also possible that further bioluminescent taxa will be discovered. Conversely, further observations are needed to confirm the single published report of bioluminescence in *Balgus schnusei*. New ecological data are also needed to investigate the bioluminescence of larvae. Due to a limited fossil record, we depend on a single Pityobiini record from the Burmese amber when considering the possibility that the group was widespread in the Holarctic region. The discussed extinct taxon is morphologically distinct, and further research might question its relationships.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108440.

ACKNOWLEDGMENTS

R. Bilkova is cordially acknowledged for her assistance in the laboratory. L. Borowiec (Poland), F. Gao (China), M. Gargiulo (Chile), C. Maureira (Chile), E. Moore (USA), C. Rorabaugh (USA), and L. Wakefield (New Zealand) generously provided their photographs for this publication. We are obliged to J. P. Klages for providing a map of the 90 mya South Pole. The study was funded by the GACR 22-35327S awarded to L.B., D.K., and M.M.

AUTHOR CONTRIBUTIONS

M.M., D.K., E.T.A.-B., S.M.B., and L.B. conceived and designed the study; D.K. and M.M. conducted phylogenetic analyses; L.B. drafted the manuscript; E.T.A.-B. collected the critical taxa and provided field observations; L.B. and E.T.A.-B. analyzed morphology; L.B. and M.M. prepared illustrations; L.B. coordinated the study; L.B., M.M., and D.K. obtained the funding; all authors commented on the drafts and gave the final approval for publication.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: September 8, 2023
Revised: October 31, 2023
Accepted: November 9, 2023
Published: November 14, 2023

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Diplophoenicus sp.	Madagascar	G19011
Hapatesus tropicus	New Guinea	G20007
Ophidius sp.	Australia	G21014
Parasaphes elegans	Australia	G19006
Rousia sp.	Australia	G21031
Tibionema abdominalis	Chile	G20012
Pityobius anguinus	USA	G21037
Campyloxenus pyrothorax	Chile	G21036
Chemicals, peptides, and recombinant proteins		
Ethanol	Litolab	Cat# 221430216100
Proteinase K	Thermo Fisher Scientific	Cat# EO0491
AE buffer	QIAGEN	Cat# 67563
Critical commercial assays		
MagAttract HMW DNA extraction kit	QIAGEN	Cat# 67563
Qubit 2.0 Fluorometer	Thermo Fisher Scientific	Cat# Q32850
Deposited data		
Analyzes dataset	Mendeley Data https://doi.org/10.17632/sh7s4jzhmz.1	
Analyzes dataset	Figshare data, V1, https://doi.org/10.6084/m9.figshare.24316531	
Software and algorithms		
Photoshop v.6.0		https://www.adobe.com/products/photoshop.html
Helicon Focus v.8.2.2		https://www.heliconsoft.com/software-downloads/
fastp v.0.21.0	Chen et al. 2018[82]	https://github.com/OpenGene/fastp
FastQC v.0.11.9		https://github.com/s-andrews/FastQC
SPAdes v.3.13.1	Bankevich et al., 2012[83]	https://github.com/ablab/spades
Augustus	Stanke & Waack, 2003[84]	http://augustus.gobics.de/binaries/
BUSCO v.5	Manni et al., 2021[85]	https://busco.ezlab.org/
OrthoDB v.9.1		https://www.orthodb.org/
Orthograph v.0.6.3	Petersen et al., 2017[86]	https://mptrsen.github.io/Orthograph
summarize_orthograph_results.pl	Petersen et al., 2017[86]	https://github.com/mptrsen/Orthograph/tree/master
Geneious v.7.1.9		https://www.geneious.com/download/
TRANSALIGN		http://malde.org/~ketil/biohaskell/transalign/
MAFTT v.7.407	Katoh & Standley, 2013[87]	https://mafft.cbrc.jp/alignment/software/linux.html
AliStat v.1.7		https://github.com/thomaskf/AliStat
SymTest v.2.0.49		https://github.com/ottmi/symtest
IQ-TREE v.2.2.0	Minh et al. 2020[88]	http://www.iqtree.org/
ASTRAL v.5.15.5		https://github.com/smirarab/ASTRAL
BEAST v.1.8.1	Suchard et al., 2018	https://github.com/beast-dev/beast-mcmc
TRACER v.1.7	Rambaut et al., 2018[89]	https://github.com/beast-dev/tracer
TREEANNOTATOR	Suchard et al., 2018	https://github.com/beast-dev/beast-mcmc

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Olympus SZX16 binocular microscope		https://www.olympus-lifescience.com/en/microscopes/stereo/szx16/

RESOURCE AVAILABILITY

Lead contact

Requests for further information should be directed to and will be fulfilled by the lead contact Ladislav Bocak (ladislav.bocak@upol.cz).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data reported in this paper will be shared by the lead contact upon request.
- Further information and requests for DNA data should be directed to and will be fulfilled by M. Motyka (michal.motyka@upol.cz).

DNA data are available at: Motyka, Michal; Kusy, Dominik; Bohart, Elizabeth; Bybee Seth, Bocak, Ladislav (2023), "Enigmatic Campyloxenus: Shedding Light on the Delayed Origin of Bioluminescence in Ancient Gondwanan Click Beetles," Mendeley Data, V1, https://doi.org/10.17632/sh7s4jzhmz.1 and Figshare data, V1, https://doi.org/10.6084/m9.figshare.24316531. The study contains a taxonomical act and is registered on ZooBank with the Life Science Identifier (LSID) urn:lsid:zoobank.org:pub:91027F71-C0E4-4746-A440-DBD9C1894D6A.

- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

This study did not generate new code.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The voucher specimens used in the study are deposited in the collection of the Biodiversity and Molecular Evolution, CATRIN, Olomouc, Czech Republic.

METHOD DETAILS

Data collection - Morphology

The photographs of morphological characters were taken by a Canon M6 Mark II camera attached to an Olympus SZX16 binocular microscope. Stacks were assembled using Helicon Focus software and processed in Photoshop 6.0. Vouchers were dry-mounted and deposited in the collections of Biodiversity & Molecular Evolution, CATRIN, Olomouc. Specimens were dissected as in previous studies.[11]

Data collection - Molecular data

We analysed the relationships of predominantly Gondwanan groups Morostomatinae, Hapatesinae, Pityobiinae *sensu* Douglas et al.,[3] Campyloxeninae *sensu* Costa,[14] and Parablacinae *sensu* Kundrata et al.[2] Additional five click beetle subfamilies (Elaterinae, Dendrometrinae, Omalisinae, Agrypninae, and Lissominae, 19 samples) and two subfamilies of Lampyridae (Luciolinae and Lampyrinae, two taxa) were used as outgroups. The list of newly sequenced samples is given in Table 1, and the complete list is in Table S1.

The ethanol-conserved or dry-mounted specimens were used for the DNA isolation with a Qiagen MagAttract HMW DNA extraction kit. DNA was sequenced for 8Gb per sample with Illumina NovaSeq 6000 by Novogene, Inc., Beijing, using (2 × 150 bp) paired-end mode. Raw Illumina reads were filtered with fastp v.0.21.0[82] using -q 28 -u 50 -n 15 -l 50 settings and quality checked with FastQC. The data were processed with SPAdes v.3.13.1,[83] with k-mer sizes of 21, 33, 55, 77 and 99. The resulting contigs were used to train Augustus[84] for species-specific gene models with BUSCO v.5.[85] Predicted models were used for *ab initio* gene predictions. The single-copy ortholog set was collated by searching the OrthoDB v.9.1 database[90] (Table S3). Following Kusy et al. (2018), we carried out Orthograph v.0.6.3 searches[86] with all 95 genes from Zhang et al.[91] against the single-copy ortholog set. Only 66 single-copy orthologs were used for further analyses. Next, we searched new genomic data with Orthograph targeting 66 single-copy orthologs (66NT and 66AA datasets). Terminal stop codons were removed, and internal stop codons at the translational and nucleotide levels were masked using the Perl script summarize_orthograph_results.pl.[86] Additionally, filtered reads were mapped to mitochondrial *cox1*, *rrnL*, and nuclear *LSU*, *SSU* genes using Geneious v.7.1.9. Furthermore, they were merged with earlier published nuclear and mitochondrial fragments[2] (Table S2). The protein-coding genes were aligned using Translation Align in Geneious v.7.1.9, whereas the ribosomal genes were aligned MAFTT v.7.407 using the L-INS-i algorithm.[87] All alignments were visually checked for dubiously aligned regions and outlier sequences.

The proportion of the missing data and pairwise completeness of the 66-genes dataset was computed using AliStat v.1.7. (https://github.com/thomaskf/AliStat). The deviation from stationarity, reversibility, and homogeneity (SRH) were computed using SymTest v.2.0.49 (https://github.com/ottmi/symtest). Heatmaps were generated for NT and AA datasets to visualize the pairwise completeness and deviations from SRH conditions.

We also compiled a four-gene dataset from previously published studies of our laboratory, and we additionally included new taxa listed in Table S2 representing the taxa from the Gondwanan clade (*Pityobius murrayi*, *Tibionema abdominalis*, *Campyloxenus pyrothorax*, *Elatichrosis* sp., *Rousia* sp., *Parasaphes* sp., *Hapatesus tropicus*, and *Diplophoenicus* sp. The dataset contained 185 terminals, including the outgroups. The taxa were chosen to proportionally represent the relative diversity of click beetle lineages and to assemble the dataset with high completeness.

Phylogenetic analyses

The maximum likelihood trees were analysed using IQ-TREE v.2.2.0[88] with partitioned or unpartitioned schemes and the following options: -nt AUTO -m MFP -merit BIC -gmedian -bb 10000 -alrt 10000 -abayes. The gene trees were computed using the same approach as gene-partitioned datasets. The coalescent species tree was estimated using ASTRAL v.5.15.5.[92]

To investigate how the data fit different phylogenetic scenarios of the focal taxa, we tested amino acid (AA) and nucleotide (NT) levels 66-genes datasets using IQ-TREE v.2.2.0[88] with per-site log-likelihoods calculated using -zb 100,000 -zw and -au parameters. Then, we employed the approximately unbiased AU-test,[93] the KH-test (one-sided Kishino–Hasegawa test[94]), the p-SH (p-value of the Shimodaira–Hasegawa test[95]), the p-WKH (p-value of weighted KH test), the p-WSH (p-value of weighted SH test), and c-ELW (Expected Likelihood Weight[96]), and bp-RELL (bootstrap proportion using RELL method[97]). The 66-gene NT dataset was tested to determine whether it supports *Diplophoenicus* as a sister to the Hapatesinae + Pityobiinae clade, and the 66AA dataset supports the (*Diplophoenicus* (Omalisinae (Agrypninae + Dendrometrinae)) clade.

Additionally, we tested the monophyly of the focal clade using four-cluster likelihood mapping[98] in IQ-TREE. We divided the taxa into clusters shown in Figure 2B. All 672 unique quartets were considered.

Divergence dating

We used BEAST v.1.8.1[89] to estimate the time frame of the clade diversification. We pruned the dataset. It contained the focal taxa and *cox1*, *rrnL* mtDNA, and *LSU* rRNA genes (*SSU* rRNA was omitted due to missing data). The dataset was partitioned by genes, with an unlinked site model and the HKY+I+G4 substitution model. We used the universal *cox1* rate (0.0115[99]) as the lognormal relaxed clock. Further, the second analysis used only reported *Cretopityobius pankowskiorum* Otto from Burmese amber to calibrate the split among Pityobiini and other Pityobiinae tribes under the following settings: lognormal prior distribution, initial value 95.0, log(mean) 2.0, log (Stdev) 1.0, and offset 95.0. The resulting node distribution has 102.4 median and 96.43–133.3 95% HDP. Both analyses were set to Speciation: Birth-Death Process. The analysis was run for 100 million generations, with a 10,000 sampling frequency. Tracer v.1.7[89] was used for checking a convergence. The maximum credibility tree was estimated using Treeannotator[89] after discarding the initial 50% of trees as burn-in. Due to topological uncertainty, we also estimated with the same setting the constrained topologies without Morostomatinae, as its position was incongruent with the preferred topology in some analyses.