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RESEARCH ARTICLE

Evolution of leafcutter ant myrmecophiles: *Hamotus heidiae*, a new symbiont of *Acromyrmex* colonies with undetectable hydrocarbons (Coleoptera: Staphylinidae: Pselaphinae)

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Abstract

Colonies of *Atta* and *Acromyrmex* leafcutter ants provide niches for diverse insect species, but few such organisms have evolved obligate myrmecophilous associations inside host colonies. We report a remarkable pselaphine rove beetle myrmecophile, *Hamotus heidiae* sp. nov., discovered in galleries of *Acromyrmex histrix* Latreille in lowland tropical rainforest within Tambopata National Reserve, Peruvian Amazonia. Presence of adults and putative larvae inhabiting the central nest implies an obligate relationship, with development of the beetle likely occurring inside the colony environment. Phylogenetic analysis of four genomic loci confirms that *H. heidiae* is a morphologically derived member of the New World *Hamotus*-generic complex and is tentatively placed into *Hamotus* Aubé on morphological grounds. The new species is phylogenetically distant to the genus *Attapsenius* Bruch—previously, the only pselaphine taxon known with certainty to be an obligate myrmecophile of leafcutter ants. Both *H. heidiae* and *Attapsenius* emerge from a newly recognized "Tyrine-group" within Pselaphinae—a major radiation that includes multiple independent origins of myrmecophily and termitophily. We were unable to detect cuticular hydrocarbons (CHCs) on the body surface of *H. heidiae* adults. Chemical insignificance may form part of the strategy that this myrmecophile, and possibly other pselaphines, employ to infiltrate host colonies.

Keywords Pselaphinae · Myrmecophily · Cuticular hydrocarbons · Leafcutter ants · Chemical ecology · Evolution

Introduction

Myrmecophiles—symbionts of ant colonies—have evolved to target a diversity of formicid taxa, with the greatest numbers of myrmecophiles found in association with large-colony-forming ant species (Paivinen et al. 2002; Rettenmeyer et al. 2011; Maruyama et al. 2013; Parmentier et al. 2014; von Beeren et al. 2021). Neotropical leafcutter ants of the genera *Acromyrmex* L. and *Atta* L. (Myrmicinae: Attini) play host to a rich menagerie of myrmecophiles, including over 400 species of Coleoptera that have been recorded from the often-large nests formed by these ants (Navarrete-Heredia 2001). Approximately, one third of these beetle associates belong to the rove beetles (Staphylinidae)—a family with an evolutionary predisposition to exploiting social insect colonies (Seevers 1965; Kistner 1979, 1982; Parker 2016a; Maruyama and Parker 2017; Hölldobler and Kwapich 2022; Naragon et al. 2022). Ecological relationships between rove beetles and leafcutter ant colonies remain largely uncharacterized, but typically involve staphylinid species that appear morphologically indistinct from free-living species. Moreover, most such species have predominantly been found inhabiting the nest periphery (e.g. in the ants' refuse), perhaps facultatively so in many cases. Conversely, evidence of more intimate, obligate associations has been obtained for only a small minority of staphylinids. Very few specialized taxa are known to have penetrated the nest interior and evolved to live exclusively inside

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the leafcutter colony itself (Kistner 1982; Navarrete-Heredia 2001).

Myrmecophile rove beetle lineages are phylogenetically biased towards the two largest subfamilies, Aleocharinae and Pselaphinae (Parker 2016a). Across both of these clades, however, only a single genus—the pselaphine *Attapsenius* Bruch—is known to have demonstrably evolved an obligate relationship with leafcutter ants. The two described *Attapsenius* species both occur in close association with *Atta sexdens* L., in colonies of which the beetles frequent the fungus galleries, appearing to be tolerated by host workers with which they interact physically (Eidmann 1937). Modified mouthparts for possible trophallaxis, and putative gland-associated trichomes that might transmit host-appeasement secretions, imply that *Attapsenius* is socially integrated inside the *Atta* colony (Park 1942; Kistner 1982; Parker and Grimaldi 2014; Parker 2016a). To our knowledge, all other previously described *Atta*- or *Acromyrmex*-associated Pselaphinae are isolated species within larger genera that are anatomically generalized, and otherwise commonly found in leaf litter (Park 1942; Navarrete-Heredia 2001). Given the vast ecological target size of colonies formed by *Atta* or *Acromyrmex*, the paucity of integrated myrmecophiles implies that barriers may exist to their frequent evolution.

Here, we describe a second, apparently obligate leafcutter ant myrmecophile from within Pselaphinae. The new species was collected in association with the host *Acromyrmex histrix* Latreille, and appears to belong to the genus *Hamotus* Aubé—a large, primarily Neotropical taxon comprising 99 species distributed from the southern US to Argentina (Park 1942; Hlaváč and Chandler 2005; Newton 2024). The new species differs markedly in gross morphology from all previously described *Hamotus*—a possible consequence of its symbiotic biology. To solidify its phylogenetic position and illuminate the origins of leafcutter ant myrmecophily in Pselaphinae, we present the first taxonomically comprehensive molecular phylogeny of the supertribe Pselaphitae—a major, higher taxonomic group within Pselaphinae, which encompasses both *Hamotus* and *Attapsenius* (Newton and Thayer 1995). Our attempts to extract cuticular hydrocarbons (CHCs) from the new species yielded no detectable compounds. Diminished CHC production has been documented in a variety of myrmecophiles (Lenoir et al. 2001, 2012; Witte et al. 2008; Parmentier et al. 2017; Lorenzi and d'Ettorre 2020). We hypothesize that chemical insignificance may represent a common strategy to gain colony access among myrmecophilous Pselaphinae.

Materials and methods

Specimen acquisition

The new beetle species described herein, along with its host ants, were collected in Peru with permission from the Servicio Nacional de Áreas Naturales Protegidas por el Estado, Ministerio del Ambiente, Peru (collecting permit: RES. N° 028-2013SERNANP-JEF).

Specimen imaging and taxonomic description

For habitus specimen images of the holotype, a Visionary Digital photomicrographic apparatus with Infinity optics and a Canon 60D camera were used. Montages were constructed from image stacks using Zerene Stacker (Zerene Systems, Richland, WA, USA). For confocal imaging, we employed a method described in Parker and Grimaldi (2014). Specimens were incubated in DNA extraction buffer to yield genomic DNA for sequencing (see "DNA sequencing and phylogenetic analysis" below). After digestion of soft tissues, specimens were removed, washed multiple times in 100% ethanol, and disarticulated. Temporary slide preparations of body structures were made by mounting parts in Vectashield mounting medium (Vector Labs). A Leica SP5 confocal microscope with a 488 nm laser was used to acquire autofluorescence images of structures. Image stacks were obtained with a 1 μm interval between *Z*-sections. Image stacks were collapsed in LAS AF to create maximum intensity projections. For morphological description, intact specimens from the type series were examined under a Leica M205 microscope, and disarticulated specimens under a Zeiss compound microscope. The terminology for morphological characters follows Park (Park 1942) with modifications by Chandler (Chandler 2001), except the terms "mesoventral" and "metaventral" are used instead of "mesosternal" and "metasternal" (Herman 2013).

DNA sequencing and phylogenetic analysis

Following a protocol described in Parker and Maruyama (2013), specimens from the type series were bathed in a sodium dodecyl sulfate/proteinase K-based DNA extraction buffer (Gilbert et al. 2007) for 2 days at 55 °C. DNA was first isolated via phenol/chloroform/isoamyl alcohol extraction, with the organic phase subsequently back-extracted into 50 mM Tris+ 15 mM NaCl. The two extracts were combined, extracted a second time with phenol/chloroform/isoamyl alcohol, and extracted once more with chloroform before precipitation in 100% ethanol + 3 M NaOAc + 10 μg glycogen. Purified genomic DNA was resuspended in

Tris–EDTA. Regions from two mitochondrial loci, *16s rRNA* and *cytochrome c oxidase subunit I* (*co1*) and two nuclear loci, *18s rRNA* and *28s rRNA*, were amplified and sequenced using primer sets and methods described in Parker and Grimaldi (2014). We incorporated previously published sequences from 19 pselaphine species spanning a range of tribes and supertribes (Parker and Grimaldi 2014). In addition, new sequences were obtained for a further 53 species. These include the newly described taxon as well as a comprehensive range of species spanning the tribal diversity of Pselaphitae—the supertribe to which the new taxon belongs. To test the monophyly of Pselaphitae, additional taxa were included from several tribes within the supertribe Goniaceritae. In total, lineages spanning all 6 supertribes of Pselaphinae and 25 tribes were included. Sequences are deposited in the National Center for Biotechnology Information (NCBI) database. The 72 taxa, along with NCBI accession numbers and locality data, are listed in Supplemental Table S1. Sequences from the 72 taxa were concatenated in Concatenator (Vences et al. 2022), and aligned using MAFFT (Katoh and Standley 2013), giving a total alignment of 5370 nucleotides. Data were partitioned into 6 blocks: the three *rRNA* loci, and codon positions 1–3 of *co1*. Substitution model selection, partition scheme choice and maximum likelihood inference of tree topology were performed using IQ-Tree 2 (Minh et al. 2020), with branch support estimated via 1000 ultrafast bootstrap replicates. The tree was rooted with species of the earliest-diverging Pselaphinae lineage, Faronitae (Newton and Thayer 1995; Parker 2016b). The final consensus tree with bootstrap support values is provided as File S1.

Gas chromatography–mass spectrometry analysis of CHCs

Freshly caught groups of five beetles, or individual worker ants of the smallest body size class, were placed into 2 ml glass vials and bathed in 300 µl hexane for 5 min. The specimens were then removed and the hexane dried. Before gas chromatography–mass spectrometry (GC–MS) analysis, the extract was purified by column chromatography on 100 mg silica gel (230–400 mesh, Nacalai Tesque, Inc.) using *n*-hexane. Cuticular hydrocarbons were eluted with 2 ml of *n*-hexane and evaporated under nitrogen. The residue was then dissolved in 10 µl of hexane, of which an aliquot (1 µl) was analyzed by GC–MS (GC: GC-17A, MS: QP5050, Shimadzu, Kyoto, Japan). For analysis, a DB-1MS capillary column was used (30 m × 0.25 mm; film thickness, 250 µm; Agilent Technologies, CA, USA), with helium as carrier gas at a column head pressure of 100 kPa. The injector temperature was 300 °C and the samples were injected manually in splitless mode for 1 min. The oven temperature was programmed at 80 °C for 1 min, followed by an increase by 10 °C/min to 320 °C, where it was held for 10 min. The mass spectrometer was operated in electron ionization mode with an energy of 70 eV and an ion source temperature of 300 °C. For CHC identification using retention indices (RI), normal alkane standards from *n*-C9 to *n*-C40 were analyzed by GC–MS in advance.

Results

Hamotus heidiae **Parker, new species**

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Type material

43 specimens of the type series have the following locality data:

“PERU: Madre de Dios, nr Puerto Maldonaldo, Explorer’s Inn 12°50′15.7″ S 69°17′35.2″ W 200 m 27 IX-X 2013, in *Acromyrmex* nest, J. Parker”.

A further 24 specimens of the type series were used for GC–MS analysis and have the following locality data:

“PERU: Madre de Dios, nr Puerto Maldonaldo, Explorer’s Inn 12°50′15.7″ S 69°17′35.2″ W 200 m 27 IX-X 2013, in *Acromyrmex* nest, M. Maruyama and T. Komatsu”.

Specimen deposition

1♂ holotype, 10♂10♀ paratypes: Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Peru.

1♂1♀ paratypes: National Museum of Natural History, Smithsonian Institution, Washington, DC, USA.

1♂1♀ paratypes: Field Museum of Natural History, Chicago, IL, USA.

1♂1♀ paratypes: Natural History Museum, London, UK.

1♂1♀ paratypes: Muséum d’histoire naturelle, Geneva, Switzerland.

3♂1♀ paratypes, partially or completely disarticulated, genomic DNA extracted; 6♂4♀ paratypes, ethanol storage, −80 °C: Parker Lab collection at California Institute of Technology, Pasadena, USA (Pselaphinae DNA extraction and voucher specimen reference numbers: 351, 352).

4♂8♀ paratypes: Kyushu University Museum, Fukuoka, Japan (vouchers for GC–MS).

4♂8♀ paratypes: National Museum of Nature and Science, Tsukuba, Japan (vouchers for GC–MS).

Diagnosis

The new species can be distinguished from all other described species of *Hamotus* by the following unique combination of characters: (1) body shape strongly oval, with elytral and abdominal margins forming a near-perfect curve (Fig. 1A); (2) dorsum densely setiferous, with prominent covering of golden setae that are largely posteriorly directed, and which are especially dense on abdominal tergites (Fig. 1A, B); (3) vertexal foveae (VF) present but nude and strongly reduced (Fig. 2A); (4) prominent ocular canthus (OC), protruding laterally beyond eye margins to 1/4 eye width, and with dense, posteriorly directed setae (Fig. 2A, B); (5) antennae compact, with antennomeres 1–10 wider than long (Fig. 2C); (6) male antennomere 10 with mesal concavity (Fig. 2C); (7) pronotum with median and lateral antebasal foveae (MAF and LAF) present but nude and strongly reduced (Fig. 2I); (8) tergites IV and V with prominent teeth at apical corners (Fig. 2L); (9) male protrochanter with blunt tubercle (Fig. 2M).

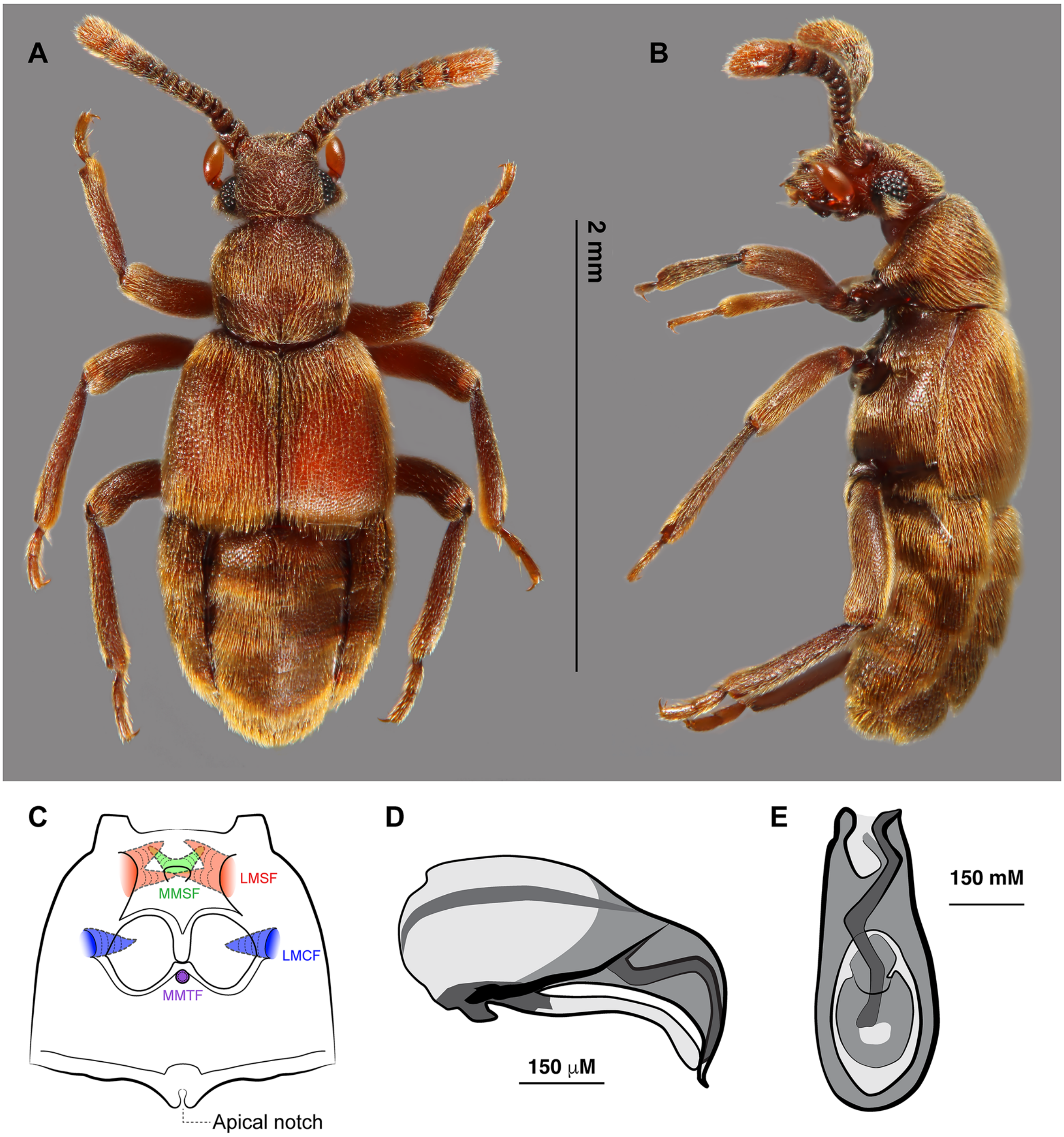


Fig. 1 *Hamotus heidiae* gen. et sp. nov. **A** Dorsal habitus of holotype male. **B** Lateral habitus of holotype male. **C** Schematic of meso-metaventrite with foveae indicated. LMSF: lateral mesoventral fovea; MMSF: median mesoventral fovea; LMCF: lateral mesocoxal fovea; MMTF: median metaventral fovea. The circular notch of the metaventral apex between metacoxae is indicated. **D** Aedeagus, lateral view. **E** Aedeagus, dorsal view

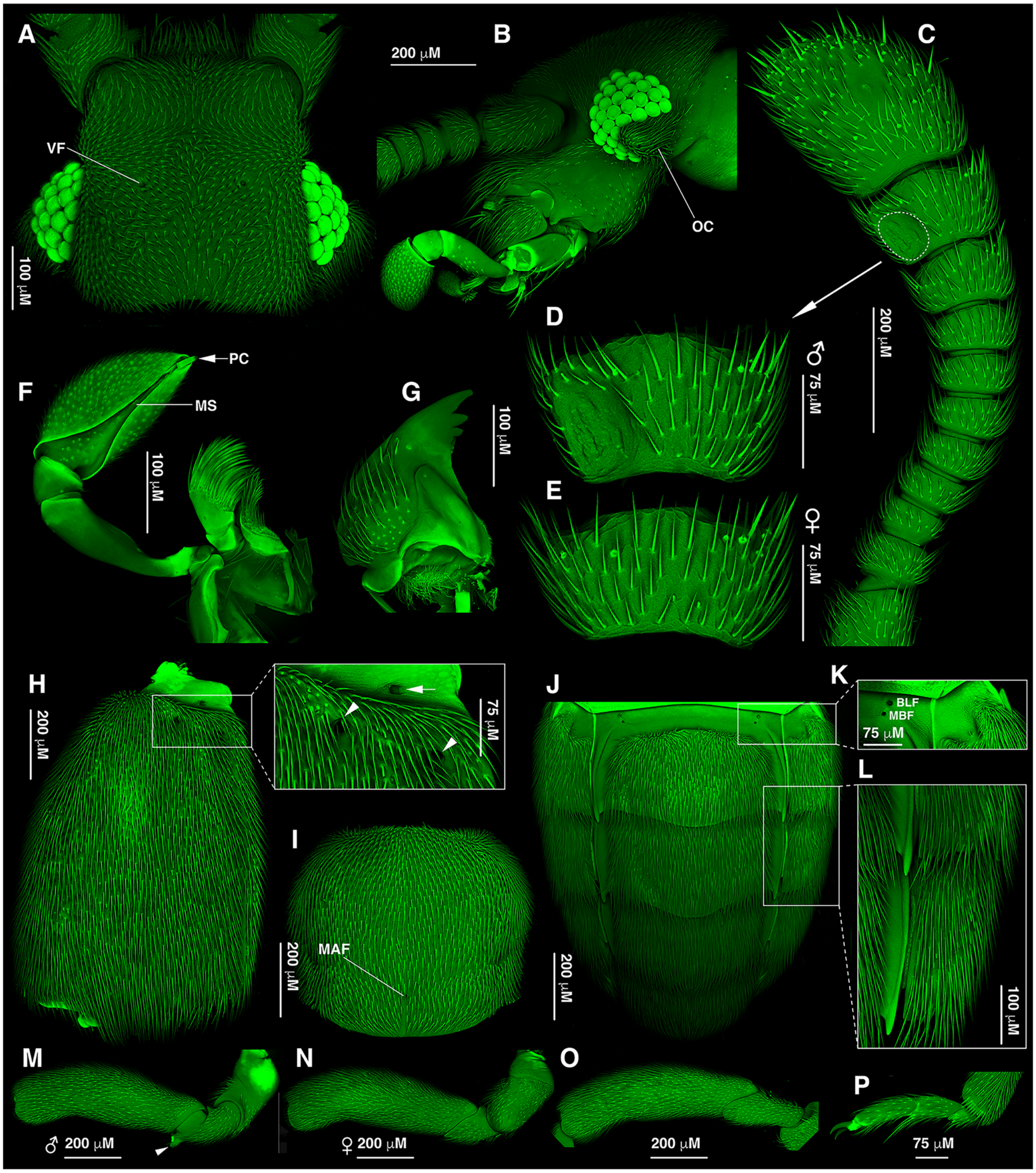


Fig. 2 *Hamotus heidiae* gen. et sp. nov. confocal images of morphology. **A** Male dorsal head. VF: vertexal fovea. **B** Male lateral head. OC: ocular canthus. **C** Male left antenna, with concavity on antennomere 10 indicated. Sexual dimorphism in antennomere 10 morphology, with concavity present in males (**D**) and absent in females (**E**). **F** Male left maxilla, showing enlarged palpomere 4 with mesal sulcus (MS) and palpal cone (PC). **G** Male right mandible. **H** Male right elytron, with magnified subbasal elytral fovea (arrow) and basal elytral foveae (arrowheads). **I** Male pronotum, with small, nude, median antebasal fovea (MAF) indicated. **J** Male dorsal abdomen. **K** Basal part of tergite IV of male, with basolateral fovea (BLF) and mediobasal fovea (MBF) indicated. **L** Lateral part of male tergites/paratergites IV and V showing teeth at apical corners of tergites. **M** Male foreleg from coxa to femur, showing blunt projection on trochanter (arrowhead). **N** Absence of trochanter projection in female. **O** Proximal part of male middle leg showing elongate mesotrochanter. **P** Male protarsus

Description

Body length 2.6–2.8 mm (Fig. 1A, B). Compact, robust body with elytra and abdomen forming near-perfect oval shape in dorsal view. Appendages thick. Entire dorsum densely setiferous, covered with long, posteriorly directed golden setae. Cuticular coloration of body and appendages dark reddish brown. Appendages densely setiferous with distally directed setae.

Head

Head length ~0.4 mm, partially covered by pronotum when in repose (Fig. 1B). Head shape transverse: width across eyes 1.4× wider than length of vertex (Fig. 2A). Vertex quadrate, relatively flat and densely setiferous (Fig. 2A). Vertexal foveae (VF) reduced but present, nude (Fig. 2A). Eye with approximately 40 facets (Fig. 2B), crescent shaped, with posterior margin interrupted by prominent, laterally protruding, densely setiferous ocular canthus (OC) (Figs. 1A, 2A, B). Dorsal antennal pits positioned directly anterior to eyes. Head venter with forked median gular foveae sharing common opening. Antennae 11-segmented, 1.1 mm in length, covered with golden setae (Fig. 2C), mounted under shelves of broad rostrum formed from apical margin of vertex (Fig. 2A, B). Antenna compact with short pedicels connecting antennomeres (Fig. 2C). Antennomere 1 elongate, 1.5× wider than long; antennomere 2 cylindrical, 1.75× wider than long; antennomeres 3–10 cylindrical, 1.6× wider than long, gradually enlarging so that antennomere 10 is twice the width and length of antennomere 3 (Fig. 2C). Male antennomere 10 with concave excavation on ventro-mesal face and scattered thick sensilla on dorsal face (Fig. 2C, D). Antennomere 11 enlarged, 1.2× wider and 2.1× longer than 10, rounded trapezoidal shape, longer ventrally than dorsally, with sparse, thick sensilla at apex (Fig. 2C). Maxilla (Fig. 2F) with elongate palpomere 2, short palpomere 3 and enlarged palpomere 4 with membranous mesal sulcus (MS) running entire length of palpomere. Apical 5th pseudosegment forming prominent palpal cone (PC) (Fig. 2F). Mandibles with low number of relatively weakly developed teeth (Fig. 2G).

Thorax

Pronotum length ~0.6 mm, strongly convex and approximately as wide as long (Figs. 1A, 2I); covered in posteriorly directed golden setae. Margins slightly wider in apical half and rounded before narrowing to base with straight margins (Figs. 1A, 2I). Small, nude lateral and median antebasal foveae present (LAF and MAF) (Fig. 2I; LAF not evident in this image). Pronotum lacking sulci. Prosternum with lateral procoxal foveae (LPCF). Meso-metaventrite less densely setiferous relative to other body regions, setae finer, aciculate (Fig. 1B) Mesoventrite with both median mesoventral fovea (MMSF) and lateral mesoventral foveae (LMSF) present and forked internally (Fig. 1C). Metaventrite with median metaventral fovea present (MMTF, Fig. 1C). Apical projection of metaventrite between metacoxae with circular notch (Fig. 1C).

Abdomen

Length of visible abdomen ~1.0 mm; abdomen at broadest point (apex of tergite IV) as broad as abdomen length (Figs. 1A, 2J). Abdomen shape oval, with margins of paratergites on visible tergites IV–VII forming continuous curve in dorsal view (Figs. 1A, 2J). Both tergites and sternites covered with dense, golden setae (Fig. 1B). Paratergites IV–XI broad, ~0.25× width of corresponding tergite widths (Figs. 1A, 2J). Tergite IV (and possibly other tergites) with basolateral foveae (BLF) and mediobasal foveae (MBF) (Fig. 2K). Apices of tergites IV and V with tooth-like projections at corners (Fig. 2L).

Elytra and flight wings

Elytron length ~0.9 mm, margins smoothly rounded, widening to apex (Fig. 2H); width across elytra equal to tergite IV width (Fig. 1A). Elytron densely setiferous, like other body regions. Elytron with one subbasal elytral fovea (SEF) and two basal elytral foveae (BEF) (Fig. 2H); lacking subhumeral foveae and any striae. Flight wings present in both sexes.

Legs

All legs with femora and tibiae relatively thickened throughout length (Fig. 1A, B). Protrochanter with ventrally projecting tubercle (Fig. 2M). Meso- and metatrochanters elongate, with distinct separation between coxa and femur (Fig. 2O). Metatrocoxae non-contiguous, separated by metaventral projection (Fig. 1C). Tarsi 3-segmented with short segment 1 and longer, subequal-length segments 2 and 3 (Fig. 2P). Tarsi with 2 tarsal claws (Fig. 2P).

Genitalia

Aedeagus with thin, elongate, symmetric, parameres (Fig. 1D). Median lobe weakly tapering apically, somewhat asymmetric at apex, dorsal diaphragm opening oval (Fig. 1E).

Sexual dimorphism

Female lacking concave excavation on antennomere 10 (Fig. 2E). Projection on protrochanter absent (Fig. 2N).

Collection and host information

All specimens were collected from a colony of *Acromyrmex* ants, which were identified as *A. histrix* Latreille (Fig. 4C). The host ant was observed to build relatively disorganized nests from mounds of litter accumulated at the base of trees.

Beetles emerged from sifting this nest material in the field, as well as from sifting fungus galleries deeper within the nest (where putative *H. heidiae* larvae were also found).

Etymology

The new species is named in honor of Dr Heidi Kay Parker, for her scientific intuition, expertise, and accomplishments, her passion for and deep knowledge of the biological world, and for her love, support, and encouragement of the first author's interest in beetles.

Taxonomic placement

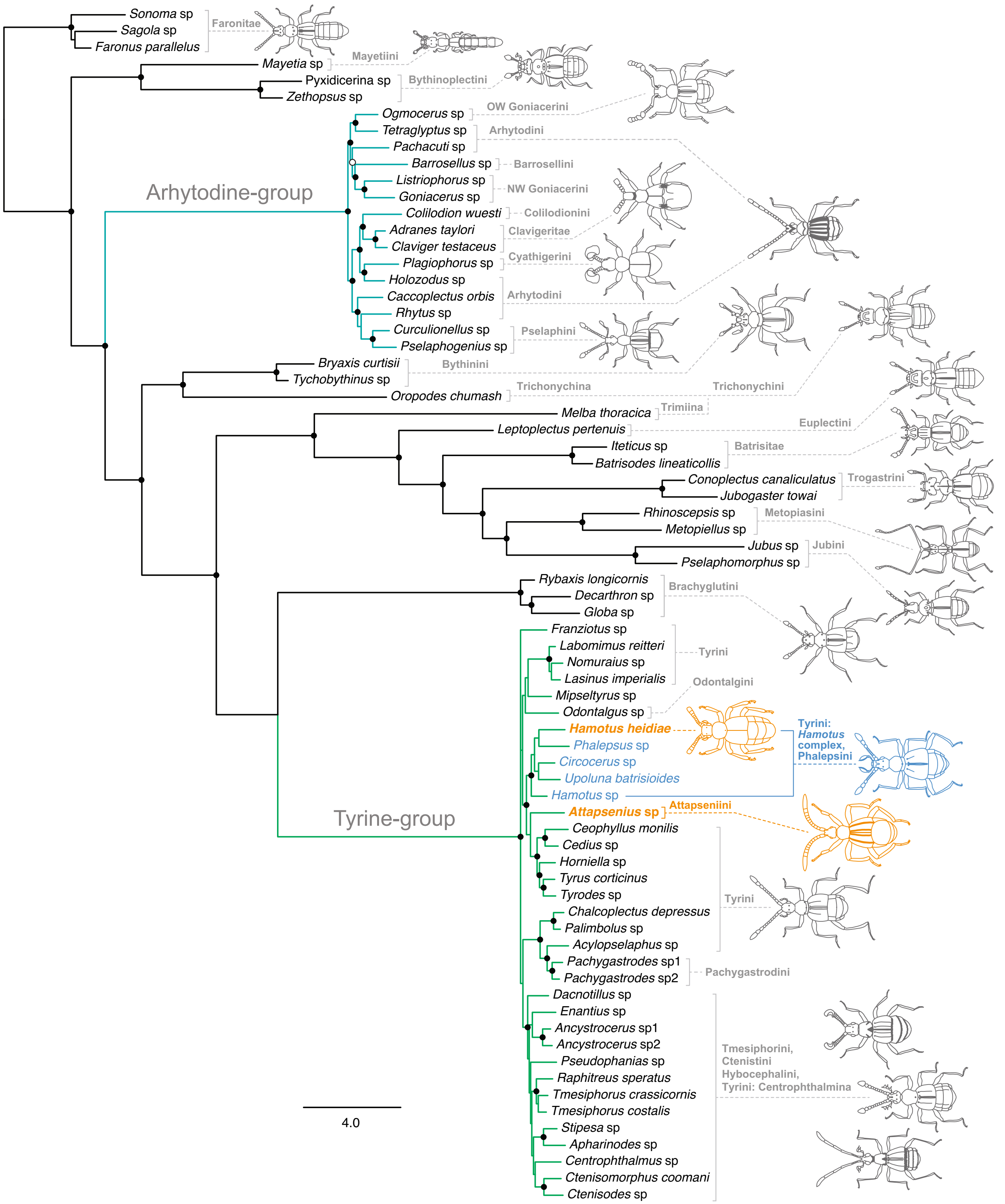
The combination of non-contiguous metacoxae separated by the metaventral apex (Fig. 1C), elongate mesotrochanters (Fig. 2O), and distinct tergites IV–VI together support inclusion of the new species within the supertribe Pselaphitae (Newton and Thayer 1995; Chandler 2001). Pselaphitae is composed of 12 tribes, 3 of which—Arhytodini, Pselaphini and Colilodionini—differ to *H. heidiae* in their possession of single tarsal claws (Lobl 2000; Hlaváč et al. 2021). Of the remaining nine tribes, eight are diagnosable with characters that are missing in *H. heidiae*, leaving a default placement of the new species in Tyrini—a heterogenous tribe comprising 769 extant described species that are currently split among four subtribes (Chandler 2001; Hlaváč and Chandler 2005). Tyrini as a group lacks putative autapomorphies, and the monophyly of the tribe itself and three of the subtribes is questionable (the fourth subtribe, Janusculina, contains a single genus, *Janusculus* Cerruti). Nevertheless, much of the diversity of Tyrini appears to resolve into a smaller set of generic complexes that likely represent real clades, even if their constituent genera may not be reciprocally monophyletic as currently delineated. It is in this context that we hypothesize *H. heidiae* is a derived lineage within the *Hamotus*-genus complex: a predominantly Neotropical group but with genera occurring in the Nearctic, Australia and Japan (Park 1942; Chandler 2001; Hlaváč and Chandler 2005).

Genera within the *Hamotus* complex possess prominent maxillary palpi in which palpomere 2 is elongate, palpomere 3 is small and triangular, and palpomere 4 is enlarged and bears a sulcus of varying length along the mesal face. The palpal form of *H. heidiae* matches that of the most speciose genus within the complex, the Neotropical and Nearctic *Hamotus* Aubé, where a sulcus extends along the entire mesal face of palpomere 4 (Fig. 2F) (Park 1942; Chandler 2001; Hlaváč and Chandler 2005). In the most recent key to World Tyrini (Hlaváč and Chandler 2005), the new species keys out to *Hamotus*, subgenus *Hamotus* Aubé. We note that an Australian *Hamotus*-complex genus, *Swan* Chandler, shares identical palpal morphology to both *Hamotus* and the new species; *Swan* further possesses prominent ocular canthi comparable to those of *H. heidiae*, as well as an apparently similar head shape and antennal form (Chandler 2001). Conversely, *Swan* lacks the broad paratergites and many mesothoracic foveae present in *H. heidiae*, and also possesses several unique character states missing from *H. heidiae*. Whether *Hamotus* is reciprocally monophyletic with respect to other genera within the *Hamotus*-complex has been questioned (Chandler 2013), and Park (1942) referred to *Hamotus* as a "stem genus" with many specialized genus-level relatives (interpreted to mean derived from within *Hamotus*). The single known species of *Swan* is termitophilous and heavily modified for this way of life. We think that both *Swan* and *H. heidiae* may represent morphologically derived, symbiotic lineages that arose convergently from a free-living, *Hamotus*-like ground plan in the Old and New Worlds, respectively. We, therefore, refrain from erecting a new genus for *H. heidiae* and instead place the new species in *Hamotus* (subgenus *Hamotus*), acknowledging that the placement is tentative.

Phylogenetic relationships of Pselaphitae and leafcutter ant myrmecophiles

The overall habitus of *Hamotus heidiae* does not obviously resemble that of any other pselaphine taxon known to us, and the new species is highly modified in comparison to all previously described *Hamotus*. We sought to place the taxon phylogenetically within Pselaphitae and used this exercise to simultaneously evaluate the placement of *Attapsenius* Bruch—the only obligate pselaphine symbiont of leafcutter ant colonies known prior to our discovery of *H. heidiae*. *Attapsenius* is currently the only genus within the tribe Attapseniini, its taxonomic isolation the result of multiple character states associated with a myrmecophilous lifestyle inside *Atta* colonies (Park 1942). These include its minute, three-segmented maxillary palpi, indicating trophic specialization on nest resources (Parker 2016a); the loss of many foveae and sulci on the body—a feature common to many inquilinous Pselaphinae (Chandler 2001; Parker and Owens 2018); an abdomen with a deep, basal excavation, flanked with setae that may function as trichomes (akin to the obligately myrmecophilous Clavigeritae) (Park 1942; Parker and Grimaldi 2014); robust antennae, with compaction of antennomeres through reduction of interconnecting pedicels, a modification commonly seen in coleopteran myrmecophiles (Parker 2016a).

Both *H. heidiae* and *Attapsenius* belong within the large supertribe Pselaphitae—a clade of 12 tribes that together include 1840 extant and 10 extinct species (Newton 2024). Inferring the relationships of *H. heidiae* and *Attapsenius* necessitated inclusion of the total tribal-level diversity of Pselaphitae. We therefore, present the first, taxonomically comprehensive molecular phylogenetic analysis



of Pselaphitae. Our analysis incorporates 43 taxa spanning Pselaphitae, along with 29 outgroups belonging to major tribes from the 5 other Pselaphinae supertribes (see Table S1 for taxon list). Using 4 molecular loci and 5370 aligned nucleotides, we determined relationships among these taxa (Fig. 3; File S1). Strikingly, our topology reveals that Pselaphitae is not monophyletic. Rather, the supertribe is split into two clades, which we informally name the

◂**Fig. 3** Phylogenetic relationships of Pselaphitae and leaf-cutter ant myrmecophiles. ML tree with 6 data partitions with the following selected models: *18s rRNA* (TNe + R4); *co1* position 1 (TIM2 + F + I + R4); *co1* position 2 (GTR + F + I + G4); *co1* position 3 (GTR + F + I + R3); *28s rRNA* (TN + F + I + R4); *16s rRNA* (GTR + F + G4). Circles at nodes indicate bootstrap percentages (BPs) estimated from 1000 ultrafast bootstrap replicates. Black circles: BP of 95–100; open circles: BP of 90–94; absence of circles: BP < 90. Major clades containing Pselaphitae taxa are colored, showing phylogenetically distinct informal "Arhytodine-" and "Tyrine-" groups. Leafcutter ant associated myrmecophiles are shown in orange, with *Hamotus heidiae* emerging within the *Hamotus* complex of genera, which also includes the tribe Phalepsini (color figure online)

"Tyrine-group" and "Arhytodine-group". These informal groups are each maximally supported but are not recovered as sister taxa in our tree. The Tyrine-group includes all tribes of Pselaphitae that, with rare exceptions (Inoue et al. 2020), possess two tarsal claws: Attapseniini, Ctenistini, Hybocephalini, Odontalgini, Pachygastrodini, Tmesiphorini, Tyrini, and we anticipate that Schistodactylini will emerge from within this group also. In contrast, the Arhytodine-group includes the three Pselaphitae tribes that possess single tarsal claws—Pselaphinae, Colilodionini and Arhytodini. The Arhytodine-group also includes the supertribe Clavigeritae, and, remarkably, three further tribes that are currently placed within the supertribe Goniaceritae: Barrosellini, Cyathigerini, and Goniacerini.

Both newly recovered informal groups descend from long stem lineages, with short internal branches indicating rapid radiations of each clade (Fig. 3). Consequently, relationships within each informal group should be regarded as provisional until more rigorously evaluated with greater genomic sampling. Nevertheless, certain relationships can be examined. Within the Arhytodine-group, Arhytodini itself is not recovered as monophyletic. Tentatively, the tribe can be interpreted as a phylogenetic backbone from which several of the most anatomically derived lineages of Pselaphinae have emerged. A close evolutionary relationship between Arhytodini, Pselaphini, and the obligately myrmecophilous Colilodionini and Clavigeritae was demonstrated in previous molecular analyses (Parker and Grimaldi 2014; Hlaváč et al. 2021). All four taxa share elongate "macrosceline" mesotrochanters (exemplified in Fig. 2O)—an important, derived character state that has historically been used to determine a taxon's membership of, or close relationship to, Pselaphitae (Raffray 1908). The close relationship of these four taxa to the tribes Barrosellini, Cyathigerini, and Goniacerini is, however, unexpected. These latter three tribes have each been considered to possess short mesotrochanters (Chandler 2001)—the plesiomorphic condition in Pselaphinae (Newton and Thayer 1995; Parker 2016b).

Barrosellini and Cyathigerini both consist of single genera—*Barrosellus* Jeannel (6 species: Afrotropical) and *Plagiophorus* Motschulsky (90 species: Afrotropical, Indomalayan, East Asian, Australian), respectively. Species in both genera are minute (<2 mm) with compact, globular bodies. In *Barrosellus*, the elytra fully cover the abdomen (Jeannel 1951), while in *Plagiophorus*, abdominal tergites IV–VI and ventrites III–VI are fused together, both anteroposteriorly and dorsoventrally (Chandler 2001). Goniacerini is a moderately large tribe of 24 genera and 99 described species, with morphologically distinctive Afrotropical and Neotropical generic complexes. Members of both complexes possess enlarged, geniculate antennae mounted on a prominent frontal rostrum; however, the Neotropical group is composed exclusively of minute, globular species, while the Afrotropical group contains some of the largest pselaphines known (up to 7.5 mm) (Leleup 1971, 1978). In our tree, Goniacerini splits polyphyletically into these zoogeographic complexes, albeit with limited taxonomic representation of each complex in our analysis. While recovery of the Arhytodine group is surprising, it is partially in agreement with a historical classification by Jeannel, who argued that Barrosellini and Cyathigerini possess atypically small macrosceline mesotrochanters, and placed them in Pselaphitae, within his "Cyathigerimorphi" group of tribes (Jeannel 1951).

Within the Tyrine group, Tyrini unsurprisingly emerges as polyphyletic, as do the three subtribes Tyrina, Somatipionina and Centrophthalmina. The *Hamotus* complex is, however, recovered with the inclusion of Phalepsini (containing the single Neotropical genus *Phalepsus*). The *Hamotus* complex indeed contains *Hamotus heidiae*, confirming our a priori assessment of the new species' likely phylogenetic placement (Fig. 3). *Hamotus heidiae* was not, however, resolved as direct sister to the other species of *Hamotus* (subgenus *Hamotus*) we included, but given the probable lack of reciprocal monophyly between *Hamotus* and other genera of the *Hamotus*-complex, we believe assignment of *H. heidiae* to *Hamotus* is judicious. Importantly, *Attapsenius* falls outside of the *Hamotus*-complex, and is distant to *H. heidiae*. We conclude that obligate leafcutter ant myrmecophily has evolved twice in Pselaphinae: once with *Atta* (*Attapsenius*), and once with *Acromyrmex* (*H. heidiae*), but both from within the Tyrine-group of Pselaphinae (Fig. 3). Lineages within the *Hamotus*-complex itself have been especially predisposed to evolving relationships with social insects, with a significant number of species discovered living in association with either ants or termites (Park 1942; Chandler and Nutting 1973; Chandler 2001).

Another major taxon recovered for the first time by our analysis is a large Tyrine-group clade consisting of the tribes Ctenistini and Tmesiphorini, from which also emerge the smaller tribe Hybocephalini and genera of the Tyrini subtribe Centrophthalmina (Fig. 3). The grouping of Ctenistini and Tmesiphorini is intuitive based on morphological features. Members of both tribes possess elaborate maxillary palpi in which palpomeres 3 and/or 4 (and sometimes palpomere 2) bear spinose tubercles or dense projections formed by clusters of setae; both tribes also share densely setose sulci surrounding the bases of the antennae (Chandler 2001). Many Tmesiphorini species, and perhaps most Ctenistini, have been found in association with ants or termites, and the two tribes include a high number of morphologically specialized inquilines, although knowledge of the biology of these taxa is scarce. In contrast to Ctenistini and Tmesiphorini, Hybocephalini possess diminutive maxillary palpi that are reduced to two or three segments. Yet, both hybocephalines and ctenistines are similarly covered in flattened, squamous setae, especially within foveae and sulci, while this setal form is absent in Tmesiphorini (Chandler 2001). Hybocephalini could be interpreted as a tmesiphorine/ctenistine lineage in which the maxillary palpi have undergone secondary reduction—a recurrent trend seen in Ctenistini (Hlaváč and Baňar 2014), although all such ctenistine genera appear to be myrmecophiles, and to our knowledge, myrmecophily has not been observed in Hybocephalini. As in the Arhytodine group, internal relationships of the Tyrine group and its constituent sub-clades clade require further resolution through greater taxon and gene sampling, as well as from the investigation of morphological synapomorphies that support these novel groupings. Resolving these parts of the Pselaphinae phylogeny is essential to retracing the evolution of myrmecophily in this staphylinid subfamily. Perhaps, the greatest concentrations of myrmecophilous and termitophilous pselaphine lineages have emerged from the Arhytodine and Tyrine groups, with a third concentration emerging from the vast tribe Batrisini.

Symbiotic biology of *Hamotus heidiae*

Hamotus heidiae is suspected to be an obligate symbiont of *Acromyrmex* leafcutter ant colonies. A series of 67 adults was collected by sifting nest material extracted from a colony of *Acromyrmex histrix* in lowland tropical forest in Tambopata National Reserve, Peru (Fig. 4A). The likely larvae of *H. heidiae* were also observed deep within the colony's fungal galleries (Fig. 4B). The recovered larvae resemble those of other genera of Tyrini closely allied to *H. heidiae* (Costa et al. 1988). Given that no other pselaphines were found within the nest, the large number of larvae we collected (~10) leads us to conclude that they indeed represent immature stages of *H. heidiae*. Unfortunately, larval specimens were lost during the export of material from Peru to the USA. Nevertheless, the presence of both adults and larvae of *H. heidiae* within the *A. histrix* nest suggests that the entire life cycle takes place within the host colony.

Superficial field observations of beetle behavior with worker ants from the disturbed *A. histrix* colony revealed limited interactions between the pselaphine and its host. One beetle was seen riding phoretically on a worker's gaster, and one possible case of brief, oral contact between a beetle and a worker was observed. The beetles were never apparently recognized and attacked by host workers. To explore the possible basis of this tolerance, we hexane-extracted chemical compounds from the beetle and host ant and performed gas chromatography mass spectrometry (GC–MS) to profile the cuticular hydrocarbons (CHCs). We recovered a clear CHC profile from *A. histrix*, including four major alkene peaks (C29, C31, C33, C35) and one linear alkane (C29), the identities of which we confirmed using chemical standards (the positions of the alkene double bonds were not determined). Strikingly, however, we were unable to detect CHCs from any of the *H. heidiae* specimens we analyzed (Fig. 4D). We interpret these data as evidence that *H. heidiae* has evolved a strongly diminished CHC profile, or lost its ability to synthesize these compounds or secrete them onto its cuticle. We venture that, as a consequence, *H. heidiae* can go largely undetected inside *A. histrix* colonies. Analogous observations of reduced or indetectable CHCs have been made in a range of social parasites (Lenoir et al. 2001; Bagnères and Lorenzi 2010; Lorenzi and d'Ettorre 2020), including other myrmecophiles (Witte et al. 2008; Lenoir et al. 2012; Parmentier et al. 2017)—a strategy termed "chemical insignificance" (Lenoir et al. 2001). We speculate that myrmecophilous pselaphines may be evolutionarily predisposed to chemical insignificance as a mechanism to gain colony entry (discussed below).

Discussion

Despite representing significant ecological targets for social parasites, colonies of leafcutter ants have seemingly provided a sink for the evolution of relatively few specialized myrmecophiles beyond those associated with nest refuse (Wasmann 1923; Kistner 1982; Waller and Moser 1990; Navarrete-Heredia 2001; Parker 2016a). The cockroach *Attaphila* Wheeler (Wheeler 1900; Nehring et al. 2016; Phillips et al. 2017; Ospina-Jara et al. 2022), and the pselaphine *Attapsenius* Bruch (Bruch 1933; Eidmann 1937) are examples of taxa that have forged unusual, obligate associations with their attine hosts and have adjusted to life within the fungus galleries. Possible explanations for the paucity of such myrmecophiles include efficient policing of colonies to exclude would-be imposter organisms; or physiological or life history traits of solitary arthropods that preclude frequent transitions into complex leafcutter societies. Here, we have added a further, putatively obligate myrmecophile—the pselaphine *Hamotus heidiae*, an associate of *Acromyrmex histrix* in Peruvian Amazonia. Much remains to be discovered about the biology of this new pselaphine, but we deduce that the entire life cycle likely takes place within the host

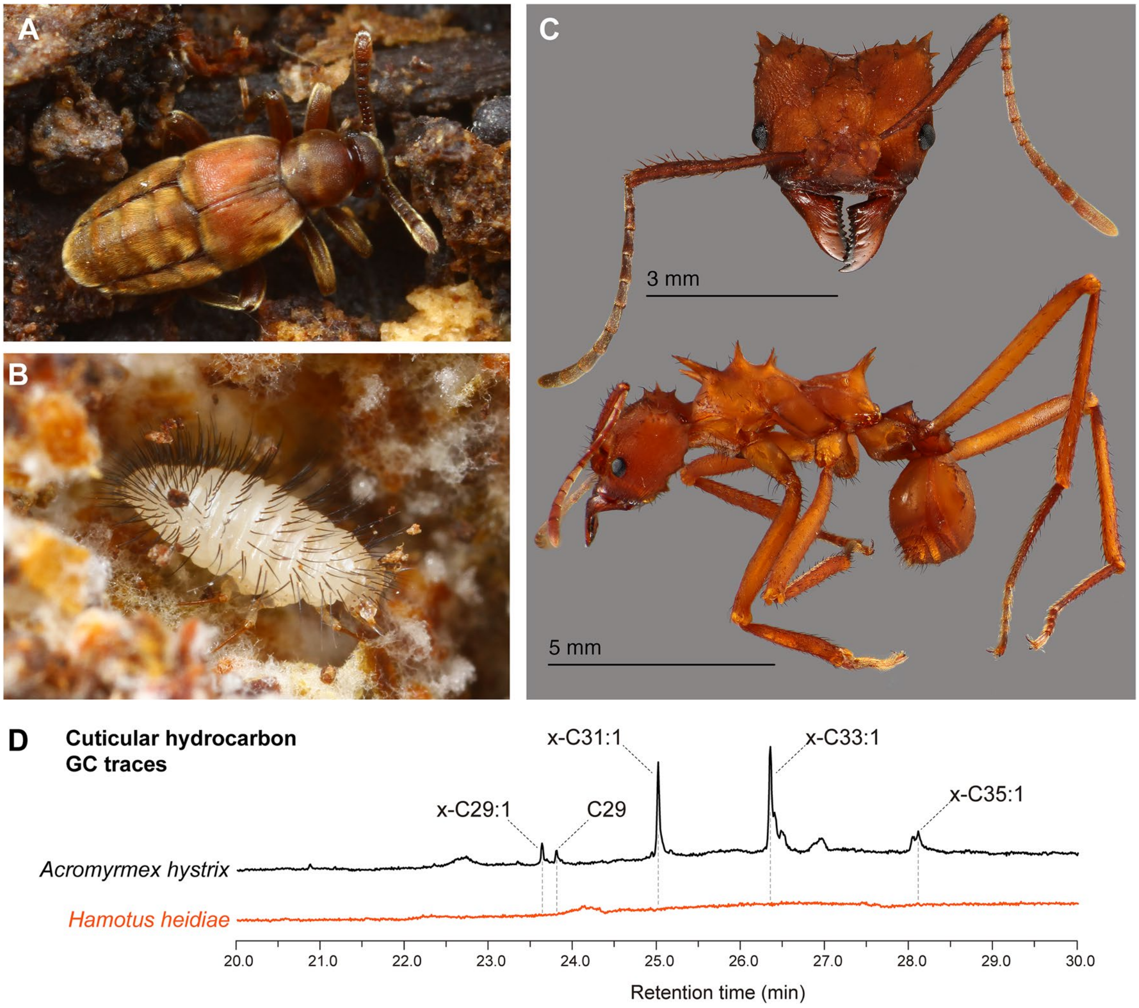


Fig. 4 *Hamotus heidiae* life cycle and cuticular hydrocarbons. **A** Adult *H. heidiae* pictured within host colony nest material. **B** Putative *H. heidiae* larva pictured within host fungus gallery. **C** Major worker of *Acromyrmex histrix*, from the host colony of the *H. heidiae* type series, **D** absence of cuticular hydrocarbons in *H. heidiae*. Top (black) gas chromatograph trace shows CHCs from an *Acromyrmex histrix* host worker. The labelled peaks were identified as *x*-nonacosene, nonacosane, *x*-hentriacontene, *x*-tritriacontene, and *x*-pentatriacontene. The lower (orange) trace shows the absence of any peaks in a hexane extract from *Hamotus heidiae*, spanning the same retention times as that of the host ant's gas chromatograph trace. Photo credit for **A**–**B**: Takashi Komatsu (color figure online)

colony. The weakly toothed mandibles of *H. heidiae* adults imply possible predation on ant brood, akin to other myrmecophilous pselaphines. Further, both sexes of *H. heidiae* appear to be flight-capable. We infer that *H. heidiae* may disperse on the wing to new nests, rather than move via trail-following, or by phoretically attaching to queens as in *Attaphila* (Phillips et al. 2017). Behavioral observations imply *H. heidiae* is not socially integrated inside colonies, and its apparent lack of CHCs suggests it lives below the ants' chemosensory radar.

The chemical ecology of *Hamotus heidiae* may embody a strategy typical of pselaphine myrmecophiles. Within Pselaphinae, direct behavioral evidence of socially integrated forms of myrmecophily exists primarily for species within Clavigeritae, all of which are thought to be obligate myrmecophiles (Donisthorpe 1927, 1999; Park 1964; Akre and Hill 1973; Leschen 1991; Cammaerts 1992, 1995, 1996). Outside of this supertribe, evidence of social interactions between pselaphines and ants has been inferred mostly indirectly, from morphological features such as trichomes and reduced mouthparts that have evolved sporadically in some lineages (genera discussed in Parker 2016a). In contrast to these scattered taxa, the majority of pselaphine myrmecophiles appear to be non-integrated with their hosts. Where behavioral observations exist, such species often go seemingly undetected by host workers and walk unmolested inside nests (e.g. Donisthorpe 1927, 1964; Park 1933, 1947; Parker and Maruyama 2013; Yin 2017). Similar non-integrated relationships have been reported for pselaphine termitophiles (Kistner 1982). Nestmate discrimination in social insects depends on the surveillance of CHC profiles (Sturgis and Gordon 2012), so the apparent invisibility of many inquilinous pselaphines to their hosts implies that these beetles either present CHCs that do not elicit appraisal, as occurs in some other social parasites (Bagnères and Lorenzi 2010; Lorenzi and d'Ettorre 2020), or that their CHCs are diminished or absent (Lenoir et al. 2001, 2012; Witte et al. 2008; Bagnères and Lorenzi 2010; Parmentier et al. 2017; Lorenzi and d'Ettorre 2020). Based on our finding that *Hamotus heidiae* produces levels of CHCs that are undetectable via GC–MS, we hypothesize that chemical insignificance may be a widespread strategy among non-integrated Pselaphinae that do not physically interact with hosts.

Achieving chemical insignificance via loss or silencing of CHC biosynthesis poses a potential challenge to the beetle's physiology. CHCs are pleiotropic compounds that function not only as pheromones but as a waxy coating that safeguards against desiccation (Sprenger and Menzel 2020; Blomquist and Ginzel 2021). In myrmecophilous aleocharine rove beetles, we have recently found similar evidence of loss of endogenous CHC biosynthesis (Wagner et al. 2024; T. Naragon, J. Parker, unpublished data). In this case, however, the beetles "groom" workers to horizontally acquire the host colony CHC profile—a behavior that achieves chemical mimicry, integrating the beetle into the host society. Importantly, grooming is also essential to safeguard against desiccation, since the groomed ant's CHCs compensate for the loss of an endogenously produced profile. Analogous grooming behavior has been documented in a diversity of socially integrated aleocharines, which die rapidly when removed from colonies, likely due to desiccation as the beetles cannot replenish their CHC supply (Akre and Rettenmeyer 1966; Akre and Torgerson 1968; Kistner and Jacobson 1990; Jacobson and Kistner 1991, 1992; Kistner 1993; Maruyama et al. 2009; von Beeren et al. 2018).

How might non-integrated pselaphine myrmecophiles, such as *H. heidiae*, counter the loss of endogenous CHCs if they do not compensate through acquisition of host-derived CHCs? We posit that the loss of hydrocarbons may be a viable strategy in pselaphines due to their remarkable integument. Relative to other rove beetle subfamilies, pselaphines are heavily sclerotized, with an exceptionally thick, chitinous cuticle. This is combined with a reduction or loss of thin, intersegmental membrane tissue between abdominal segments, as well as loss or atrophy of abdominal spiracles (Newton and Thayer 1995). These cuticular modifications may transform the integument into a more effective physical barrier against internal water loss. During the evolution of nascent interactions with social insects, we speculate that the relatively watertight integument might facilitate the evolutionary loss or reduction of CHCs. We note that losses of cuticular foveae, sulci and carinae are common among inquilinous pselaphines (Chandler 2001; Parker and Owens 2018). Reductive evolution of cuticular features may be adaptive by further reducing the exoskeleton's surface area. Whether chemical insignificance is indeed pervasive among pselaphine myrmecophiles awaits a comprehensive survey of CHC chemistry across different lineages. Understanding how chemical insignificance evolves will require investigation of the cellular mechanisms of CHC biosynthesis in myrmecophiles, and the possible role played by the unique cuticular morphology of pselaphines in enabling its evolution.

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Data availability All gene sequences used in this study are publically available at the NCBI database. All other data supporting the findings of this study are available within the paper and its Supplementary Information.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

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