UNIVERSITY OF CALIFORNIA
RIVERSIDE

Systematic Research on Heteroptera With Emphasis on Dicrotelini (Reduviidae: Harpactorinae) and Nearctic Phylinae (Miridae)

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Entomology

by

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March 2023

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Acknowledgements

I would like to acknowledge the past and current members of the Heteropteran Systematics Lab for all of their help and guidance along this journey.

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Exploring the morphology of Dicrotelini Stål, 1859 (Hemiptera: Reduviidae: Harpactorinae)

Abstract

Dicrotelini Stål, 1859 are a small tribe of the assassin bug subfamily Harpactorinae Amyot and Serville, 1843 that is restricted to the Australian and Oriental regions. Little is known about their biology and ecology, and specimens are rare in collections. Despite drastic morphological differences, molecular phylogenies have recovered Dictrotelini as sister taxon to Ectinoderini Stål, 1859, the Old World resin bugs, that are also part of the Harpactorinae. The placement of Dictrotelini among the early diverging lineages of Harpactorinae is surprising, given their superficial similarity to Harpactorini Amyot and Serville, 1843 and other Higher Harpactorinae. In depth morphological documentation of Dictrotelini is critical to further evaluate the phylogenetic position of this small tribe. Here, we document the external morphology, male genitalia, and female external and internal genitalia of two undescribed, micropterous species of Dicrotelini from Thailand using macrophotography and scanning electron microscopy. Dicrotelini lack several characters that are putative synapomorphies of Higher Harpactorinae, corroborating the phylogenetic placement based on molecular data.

Introduction

Dicrotelini Stål, 1859 are a small and little studied group of assassin bugs (Heteroptera: Reduviidae) in the subfamily Harpactorinae that are diagnosed by an enlarged, anteriorly projecting clypeus, among other features (Malipatil, 1988). Known

from the Australian and Oriental regions, this tribe currently contains 13 genera, of which many are monotypic or contain relatively few species (Erichson, 1842; Stål, 1859; Breddin, 1900; Miller, 1954; Malipatil, 1988; Cai, 1995; Tomokuni and Cai, 2002; Lu et al. 2006). Dicrotelini are relatively rare in collections, and nothing is known about their biology or ecology. Miller (1954) speculated that Dicrotelini may spend part of their life cycle under loose bark of dead trees based on their general habitus. Stål (1859) first described the taxon Dicrotelida for *Dicrotelus* Erichson, 1842 from Tasmania and *Nyllius* Stål, 1859 from mainland Australia and provided a diagnosis for this group. Translated from Latin, this diagnosis indicates that these two genera are set apart from other Reduviidae by the "antenna bent at an angle with first segment enlarged; head between and anterior to antennae produced; ocelli obsolete; anterior coxae close, posterior somewhat distant; last abdominal segment divided; anterior femora incrassate, ventrally with spines; foretibiae shorter than forefemora". Lethierry and Severin (1896) first recognized this taxon as part of the Harpactorinae and Miller (1954) treated it as a tribe, even though he did not add to the original diagnosis by Stål (1859), nor did he designate a type genus. Since then, the 13 genera that share most of the above listed characters have either been referred to as a tribe (Malipatil, 1988; Cai, 1995; Tomokuni and Cai, 2002; Lue et al., 2006; Weirauch et al. 2014; Zhang et al., 2016) or treated as part of a broadly conceived Harpactorinae that also includes Rhaphidosomini Distant, 1904, but not the remaining tribes of Harpactorinae (Maldonado, 1990). Davis (1969) in his comparative morphological synopsis of Harpactorinae did not mention Dicrotelini, suggesting that he may have considered them to be part of the Harpactorini. Dicrotelini were also missing

from the morphology-based phylogeny of Reduviidae by Weirauch (2008) and the first molecular phylogenies of the group (Weirauch and Munro, 2009; Hwang and Weirauch, 2012).

In a molecular phylogenetic study focused on Harpactorinae and Bactrodinae, Zhang et al. (2016) for the first time included species of *Nyllius*, *Henricohahnia* Breddin, 1900, and an undescribed species of Dicrotelini from Thailand that may belong to *Hsiaotycoris* Lu, Zhao, and Cai, 2006. These taxa were recovered as monophyletic, and surprisingly, given the strikingly dissimilar morphology, as sister taxon to the Oriental harpactorine tribe Ectinoderini Stål, 1859, the Old World resin bugs, which comprise the genera *Amulius* Stål, 1865, and *Ectinoderus* Westwood, 1843 (Fig. 1). Together with the Apiomerini Amyot and Serville, 1843, and Bactrodinae Stål, 1862, both restricted to the New World, these taxa form the sister group to the Higher Harpactorinae that in addition to the paraphyletic Harpactorini Amyot and Serville, 1843, also includes the monophyletic tribes Rhaphidosomini, Diaspidiini Miller, 1959, and Tegeini Villiers, 1948. In Zhang et al. (2016), the Higher Harpactorinae were strongly supported, and while low support among Bactrodinae, Apiomerini, Ectinoderini, and Dicrotelini makes the placement of Dicrotelini somewhat tentative, it is clear from this analysis that they are not part of the Higher Harpactorinae. Harpactorine tribes and Bactrodinae have quite distinctive morphology as documented by Davis (1969) and morphological data have been used to infer relationships among some of them (Weirauch, 2008). However, Dicrotelini were excluded from these two studies and their morphology has remained largely undocumented beyond taxonomic descriptive purposes.

The aim of this study is to use macrophotography and scanning electron microscopy to document, the external and genitalic morphology of two undescribed species of Dicrotelini from Thailand. Morphology will be interpreted in the context of published information on other groups of Harpactorinae. This study aims to be a first step towards combined molecular and morphological phylogenetic analyses of Harpactorinae that will aid in reclassifying this group to better reflect current phylogenetic information.

Material and Methods

Material

Two species of Dictrotelini from Thailand were examined that likely represent undescribed species of *Hsiaotycoris.* The concept of this genus will need to be revised substantially (e.g., wings are extremely reduced in both species) to accommodate these species and we refrain from describing them as part of this study. All specimens were adults collected in Doi Inthanon National Park, Thailand in 2007 using Malaise traps (see Supplementary Table 1 for specific locality information). Species 1 “brown” was represented by one female and two male specimens. Species 2 “red” was represented by one female and five male specimens. Specimens examined in this study are deposited at the University of California, Riverside (UCR) Entomological Research Museum, California, USA.

Methods

Specimens were examined under a Nikon SMZ1000 dissecting microscope and imaged using a Leica Z16 APO imaging system with Leica Application Suite (LAS) v4.3 software. Composite images were generated using Zerene Stacker v1.04. Four specimens

were also mounted onto stubs using conductive carbon adhesive tape and imaged using a Hitachi TM4000PlusE II Tabletop scanning electron microscope (SEM) at the UCR Microscopy and Imagine Core Facility. Images in Fig. 9 are from iNaturalist (www.inaturalist.org). Photographs were edited and plates assembled in Adobe Photoshop CS6.

For genitalic examination, the abdomens of one male and one female of both species were removed and placed in warm ~10% KOH until tissue was dissolved. Male genitalia were dissected (i.e., the aedeagus removed from the pygophore) and pygophore, parameres, and aedeagus were imaged. Female abdomens (for examination of dorsal abdominal glands) and internal genitalic structures (for examination of lateral spermathecae and other internal structures) were stained with Chlorazol Black E Stain and examined in glycerol on microscope slides using a Nikon Eclipse 80i light microscope equipped with a Nikon Y-IDT drawing tube. Genitalic dissections are stored in genitalic vials and reassociated with the voucher specimens. Morphological terminology mainly follows that of Weirauch (2008) and Forero and Weirauch (2012).

Abbreviations

anf, antennifer; apl, anterior pronotal lobe; asp, abdominal spiracle; bc, bursa copulatrix; bpa, basal plate arms; bpb, basal plate bridge; bpe, basal plate extension; bt, basal tooth of claw; ca, carina; cl, clypeus; DAG 1-3, dorsal abdominal glands 1-3; dms, dorsomedian sensillum; dps, dorsal phallothecal sclerite; ds, dorsal connexival suture; fe, femur; ge, gena; go, glandular opening; goa, gonapophysis; goc, gonocoxa; gop, gonoplac; hc, honeycomb patterning surrounding DAG; is, intersegmental membrane;

ios, interocular sulcus; L2-4, labial segment 2-4; lp, lateral process of metanotum; lr, labrum; ls, lateral spermatheca; lsp, longitudinal sulcus of anterior pronotal lobe; lts, lateral tufts of setae on DAG; max, maxillary stylets; md, mandible; mdb, mandibular plate; mesepm, mesepimeron; mtn, metanotum; mo, median oviduct; mseps, mesepisternum; mteps, metepisternum; mxp, maxillary plate; ppl, posterior pronotal lobe; pr, parempodial seta; prem, proepimeron; pres, proepisternum; pss, prosternal stridulitum; res, reservoir; rec, receiving canal; S8, sternite 8; sac, saccule; sbc, sclerotized part of bursa copulatrix; sc, scutellum; spur, protibial spur; st, struts; synt, syntergite; T, tergum; tar, tarsomere; tbs, trichobothria socket; tib, tibia; vs, ventral connexival suture; wp, wing pad.

Results

The habitus of Species 1 and 2 is shown in Figure 2. Both species are micropterous and have a slender, elongated head, relatively small thorax, and ovoid abdomen in both sexes. The following morphological description is based on both males and females, given sexual dimorphism is minimal.

Head (Figs 2–4): Body covered with light-colored, thin setae. Anteocular area shorter than postocular, with distinct, curved posteriad interocular sulcus (Fig. 3A). Head almost parallel-sided in dorsal view (Figs 2A, G, 3A). Dorsal part of clypeus extended into large process that surpasses ventral portion of clypeus and labrum in lateral view, ventral surface of process straight and dorsal side curving downward anteriorly (Figs 3A, C, 4A). Labial segment 2 (first visible) short, not reaching anterior margin of eye in lateral view, with macrosetae on large tubercles; labial segment 3 long and straight; labial segment 4

slightly shorter than segment 2 and tapering (Figs 3C, E, 4A). Mandible with ridges dorsally, but without ridges on other surfaces (Fig. 4C); maxillae largely obscured by mandible. Eyes globular, protruding laterally with posterior margin slightly sinuous in lateral view (Figs 2H, 3C, 4A). Ocelli absent. Postocular region tapering to neck (Figs 3A, 4A). Antennal scape stout and with macrosetae on large tubercles (Fig. 2D, E), pedicel subequal in length to head, with row of pedicellar trichobothria (Figs 2I, 4B), each surrounded by a membranous area. Prepedicellite and preflagelloid sclerotized (Fig. 2F). Basi- and distiflagellomeres together longer than pedicel, distiflagellomere shortest antennal segment.

Thorax including wing pad and legs (Figs 3–5): Thorax in dorsal view dominated by anterior pronotal lobe, with posterior pronotal lobe, meso- and metathorax narrow (Figs 2, 3B, D). Anterior pronotal lobe large (Fig. 3B), dorsal and lateral surfaces with macrosetae on large tubercles (Figs 3F, 4E). Anterior pronotal lobe with smooth, subrectangular sections delineated by ridges with macrosetae on large tubercles (Fig. 3B). Longitudinal sulcus of anterior pronotal lobe wide anteriorly, narrowing posteriorly (Fig. 3B). Posterior lobe of pronotum reduced and rugose centrally (Figs 2D, 3B). Forefemur strongly incrassate compared to mid- and hindfemora (Fig. 2B, F). Forefemur longer than foretibia, macrosetae on large spine-like processes dorsally and ventrally (Figs 2C, F, 4A, 5A, B). Foretibia with macrosetae on large tubercles along mesal surface. Foretibial comb on prominent spur (Fig. 5A). Foretrochanter and posterior tip of tibia with dense pads of setae (Fig. 2I, 5A, B). Setae on apex of foretibia contacts setae on foretrochanter when tibia is held opposed to femur (Fig. 5B). Mid- and hindtibia setose, with smaller

pads of setae at apex of tibia. Tarsi 3-segmented (Fig. 6A). Pretarsus with large basal tooth on claw (Fig. 5C). Claw and basal tooth with ridges dorsally, claw also with ridges on mesal ventral surface (Fig. 5C). Paired, long, parallel parempodial setae (Fig. 6B). Dorsomedian sensillum externally peg-like, located medially at junction between claw and basal claw tooth (Fig. 5D). Small wing pads represent forewings, pads in Species 1 "red" distinctly smaller than in Species 2 "brown" (Fig. 3D). Hindwings absent. Metanotum with lateral process (Fig. 3B). Scutellum distally with carina along margin (Fig. 3D). Rugose sculpture on dorsolateral surface of metepimeron possibly suggesting presence of Brindley's gland (Fig. 4F go?).

Pre-genital abdomen (Figs 4–6): Dorsal (Fig. 4D, 6C) and ventral (Fig. 5F) connexival sutures well developed. Spiracles 2-7 on mediosternite adjacent to ventral connexival suture. Ostioles of dorsal abdominal glands 1-3 (DAG) surrounded by honeycomb patterned cuticle (Fig. 5E). DAGs with trapezoidal membranous reservoir with large, but relatively few glandular units (Fig. 6F). Each DAG with paired lateral tufts of setae placed on tergite anterior to DAG (Figs 6D, E), smaller in Species 2 than in Species 1.

Genitalia (Figs 7–8): ***Male:*** Sternite 8 with spiracle on sclerite (Fig. 7F). Pygophore (segment 9) rounded with constriction laterally near middle, posterior margin rounded without median process., ventrally rounded with numerous setae (Figs 7B, C, E). Parameres short and slender, slightly bent near apex, apex rounded with sparse setae at apex (Fig. 7A, D). Phallus with basal plate triangular with bridge thinner than arms (Figs 7G, J); long, narrow basal plate extension (Fig. 7H, I); dorsal phallothecal sclerite without lateral arms (Fig. 7I, L); struts extending to distal part of dorsal phallothecal

sclerite and distally fused (Fig. 7H, K). ***Female:*** External: Tergum 8 with posterior margin rounded in dorsal view. Tergites 9 and 10 vertical and fused, forming syntergite 9/10, but delineated by invagination (Fig. 8A, B). Gonocoxae 8 slender, dorsally rectangular tapering to a point ventrally curving around gonapophysis 8. Gonapophysis 8 large, triangular with ridged surface (Fig. 8A, B). Gonoplacs represented by median sclerite. Internal: Subrectal and vermiform glands absent. Bursa copulatrix with central area more heavily sclerotized (Fig. 8C). Paired lateral spermatheca inserted on median oviduct (Fig. 8C). Egg (dissected from bursa copulatrix): Elongated, ovoid with slight upturn at sealing bar (Fig. 8D). Operculum with submedial circular carina (Fig. 8E, F). **Coloration:** Species 1 "brown" sexually dimorphic with male darker brown than female (Fig. 2). Species 2 "red" without sexual dimorphism in coloration. Both species have pronotum patterned with darker colored grooves between lighter, subrectangular sections (Fig. 3B). Pronotal longitudinal sulcus of pronotum lighter. Forefemur with dark stripes. Foretibia and mid and hindlegs with scattered dark markings. Connexival sutures darkened (Fig. 6A). Abdomen with scattered, irregular scattered light and dark markings.

Discussion

To date, documentation of dicroteline morphology is scant and has been largely limited to descriptions of new species (Erichson, 1842; Stål, 1859; Breddin, 1900; Miller, 1954; Malipatil, 1988; Cai, 1995; Tomokuni and Cai, 2002; Lu et al. 2006). In the current study, we aimed to further the morphological documentation of Dicrotelini to set the stage for future combined morphological and molecular phylogenetic studies. Dicrotelini show a diverse range of morphological characters, many of which superficially resemble

Higher Harpactorinae. Davis (1969) compiled a list of characters for Harpactorinae and other Reduviidae and interpreted them as apomorphic or plesiomorphic for Harpactorinae, although without conducting formal phylogenetic analyses. He examined and discussed Harpactorinae, Bactrodinae, Apiomerinae, Ectinoderinae, Rhaphidosominae, Tegeinae, Phonolibinae, and Diaspidiinae as part of the "Harpactoroid Complex". Based on his morphological assessment he synonymized all except Bactrodinae with Harpactorinae and recognized them as tribes of Harpactorinae (Fig. 1). Dicrotelini were not mentioned or assessed. Davis (1969; Fig. 1) further suggested that the three tribes known for the use of resin for prey capture, the "resin bugs" Ectinoderini, Diaspidiini, and Apiomerini, were monophyletic and sister to Bactrodinae + (Rhaphidosomini, Tegeini, and Harpactorini). The first published phylogenetic analysis of Reduviidae based on morphological characters included five of the harpactorine tribes and found resin bugs to be paraphyletic, with Ectinoderini recovered as sister group to the remaining Harpactorinae and Diaspidiini as close relatives of Harpactorini and Tegeini (Weirauch, 2008; Fig. 1). However, that study lacked representatives of Bactrodinae, Rhaphidosomini, and Dicrotelini. The molecular phylogenetic hypothesis by Zhang et al. (2016) included all tribes of Harpactorinae, including, for the first time in any analysis, Dicrotelini. The term "Higher Harpactorinae" was coined for the well supported clade formed by Harpactorini, Diaspidiini, Tegeini, and Rhaphidosomini.

A recent publication on the natural history and morphology of Bactrodinae (Weirauch et al. 2021) and our findings on the morphology of Dicrotelini presented above now allow us to better trace and evaluate character state transitions among the early diverging

lineages of Harpactorinae. While we refrain from a formal ancestral character state reconstruction of these characters, we are using the phylogenetic hypothesis derived from Zhang et al. (2016) as the basis for our discussion. As synapomorphies of Harpactorinae, Weirauch (2008) recovered the pedicellar trichobothria surrounded by membranes, quadrate cell in the forewing, absence of the dorsal connexival suture, reduction of vermiform gland, and absence of the metathoracic gland (node 1 in Fig. 1). The pedicellar trichobothria in Dicrotelini are also surrounded by membranes, corroborating this character as a synapomorphy of Harpactorinae (but Bactrodinae have not yet been examined). The quadrate cell is absent in Bactrodinae, which we here interpret as a secondary loss. Dicrotelini (this study), but not Ectinoderini (Weirauch 2008) possess a well-developed dorsal connexival suture, suggesting that this membrane evolved secondarily in Dicrotelini. The absence of the vermiform gland was confirmed for Bactrodinae (Weirauch et al. 2021) and Dicrotelini, suggesting that egg cement in all Harpactorinae is derived from a structure(s) other than the vermiform gland. The absence or presence of metathoracic glands are difficult to confirm based on standard dissections, and their absence remains to be confirmed for both Bactrodinae and Dicrotelini. Two additional synapomorphies, a short labial segment 2 and long and straight segment 3, were recovered as homoplastic (reversals in Harpactorini) in Weirauch (2008). New data from Bactrodinae (longer segment 2 and segment 3 straight) and Dicrotelini (short segment 2 and straight segment 3) lend further support to the hypothesis that these characters are homoplastic in Harpactorinae (additional reversal in Bactrodinae).

Weirauch (2008) reported three synapomorphies for Harpactorinae with the exclusion of Ectinoderini: a campaniform dorsomedian sensillum (as opposed to externally visible as in Ectinoderini and some other Reduviidae), loss of the ventral connexival suture (present in Ectinoderini and other Reduviidae), and a short basal plate extension on the aedeagus (long in Ectinoderini and many other Reduviidae). We here documented an externally visible dorsomedian sensillum on the pretarsus, presence of the ventral connexival suture, and relatively long basal plate extension also in Dictrotelini. The pretarsus of Bactrodinae is highly modified and the median sensillum has not been observed, but Bactrodinae also posses a ventral connexival suture and a long basal plate extension. Our interpretation of these three characters based on the phylogenetic hypothesis by Zhang et al. (2016) suggests that a peg-like dorsomedian sensillum, ventral connexival suture, and long basal plates were plesiomorphic for Harpactorinae and that the campaniform state of the dorsomedian sensillum, fused mediosternites and ventral laterotergites, and short basal plate extension may have evolved independently in Apiomerini and the Higher Harpactorinae (node 5).

According to Weirauch (2008), Higher Harpactorinae (node 5) were supported as a monophyletic group by a straight posterior eye margin, presence of a basal tooth on the claw, ridges on the claws, the dorsal phallothecal sclerite with lateral processes, presence of a subrectal gland, and the insertion of the lateral spermathecae on the bursa copulatrix. In addition, the protibial comb located on a distinct spur was recognized as supporting Harpactorini and Tegeini among the Higher Harpactorinae. Both Dicrotelini and Bactrodinae possess a basal tooth and ridges on their claws, suggesting that these

characters could be synapomorphies of Harpactorinae with reversals in Ectinoderini and Apiomerini, or that teeth and ridges are independently derived in Dicrotelini, Bactrodinae, and Higher Harpactorinae. Similarly, the fairly straight posterior eye margin in Bactrodinae, Dicrotelini, and Higher Harpactorinae could be of independent origin. The protibial spur is another potentially highly homoplastic character that in addition to Harpactorini and Tegeini also occurs in Dicrotelini (this study) and Bactrodinae (Weirauch et al., 2021). It either evolved independently in Dicrotelini, Bactrodinae, and within the Higher Harpactorinae, or its absence in the three resin bug lineages (Ectinoderini, Apiomerini, and Diaspidiini) represents secondary losses. Structures potentially homologous to the lateral processes on the dorsal phallothecal sclerite in Higher Harpactorinae have been documented for Bactrodinae (Weirauch et al. 2021), but lateral processes are absent in Apiomerini and Ectinoderini (Forero and Weirauch 2012) and we here document their absence in Dicrotelini. While homoplastic, these lateral processes can still be considered a synapomorphy of Higher Harpactorinae. We here confirm the absence of a subrectal gland in Dicrotelini and the insertion of the lateral spermathecae on the bursa copulatrix, similar to the condition documented in Bactrodinae (Weirauch et al., 2021). Both features are therefore corroborated as synapomorphies of Higher Harpactorinae.

While many of the interpretations above are ambiguous, the reconciliation of morphological observations with phylogenetic hypotheses based on molecular data requires the assumption of rampant homoplasy, especially across the early diverging lineages of Harpactorinae. Alternatively, it is plausible that published molecular

phylogenetic hypotheses do not accurately represent phylogenetic relationships among early diverging Harpactorinae. This is possible, given that support values are relatively low in the analysis by Zhang et al. (2016). However, emerging phylogenomic datasets also support Dicrotelini as sister taxon to Ectinoderini and recover this clade as sister taxon to the Higher Harpactorinae (Knyshov et al., in prep.). It is noteworthy that our fairly comprehensive morphological documentation of Dictrotelini did not uncover any putatively synapomorphic morphological characters for Dicrotelini and Ectinoderini, even though the two groups share a number of plesiomorphic features that are uncommon across Harpactorinae. Other morphological characters have not been studied widely across Harpactorinae but could potentially be phylogenetically informative. Among those are the structure of the dorsal abdominal glands and the evaporatory areas surrounding the gland openings. In addition to more extensive comparative morphological studies, combined morphological and molecular analyses should continue to test the phylogenetic position of the small and still enigmatic Dicrotelini.

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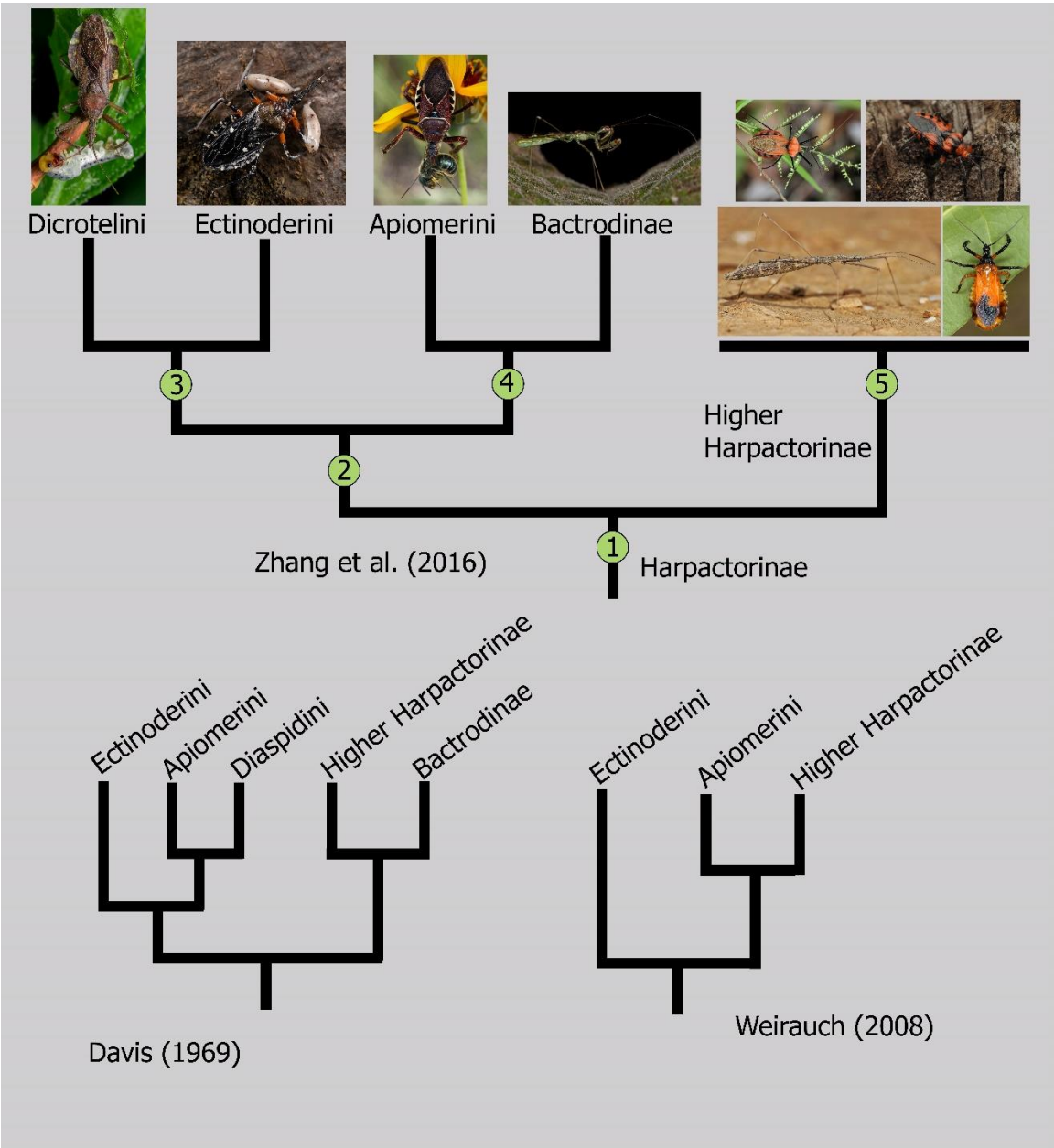


Fig 1. Proposed hypotheses of relationships among tribes of Harpactorinae. Tree topologies shown from the morphological studies by Davis (1969) and Weirauch (2008) and the molecular study by Zhang et al. (2016). Node numbers on Zhang et al. (2016) are referred to in the text. Photographs (left to right): Dicrotelini (*Tapirocoris limbatus* ©Janus Olajuan Boediman), Ectinoderini (*Amulius* sp. ©Chien Lee), Apiomerini (*Apiomerus spissipes* ©Abigail Rector), Bactrodinae (*Bactrodes misionensis* ©Vinícius Rodrigues de Souza); Higher Harpactorinae, top row: Harpactorini (*Montina scutellaris* ©Dr. Alexey Yakovlev) and Tegeini (*Tegea atropicta* ©Cynthia Chan), bottom row: Rhaphidosomini (*Rhaphidosoma* sp. ©Alfred Daniel) and Diaspidiini (*Cleontes* sp. ©Gernot Kunz).

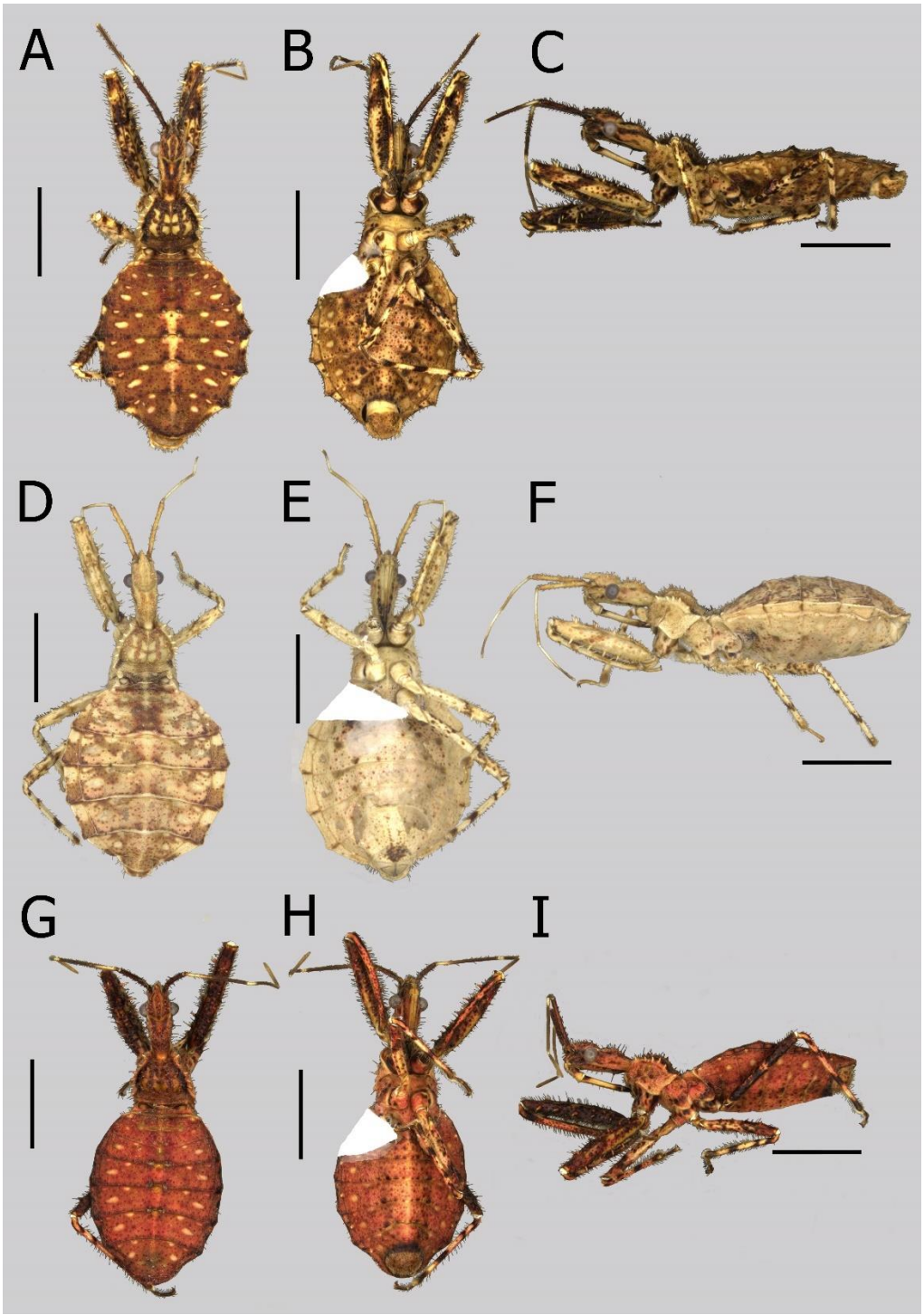


Fig 2. Habitus of two undescribed species of Dicrotelini from Thailand. (A-C) Species 1, male. (D-F) Species 1, female. (G-I) Species 2, male. (A, D, G) Dorsal view. (B, E, H) Ventral view. (C, F, I) Lateral view. Scale bars = 3 mm.

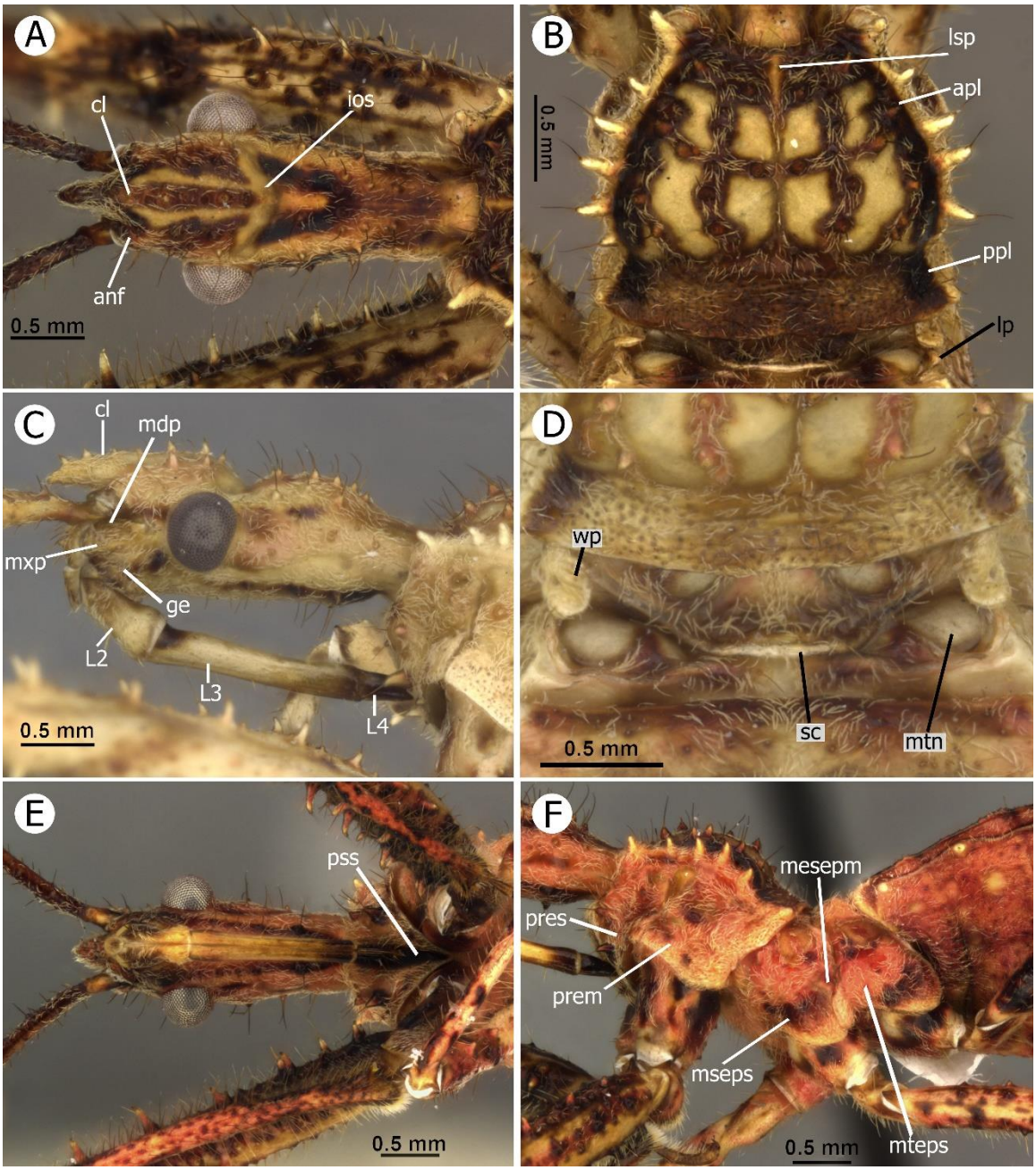


Fig 3. Details of head and thorax. (A-B) Species 1, male. (C-D) Species 1, female. (E-F) Species 2, male. (A) Dorsal, (C) lateral, and (E) ventral views of head. (B) Dorsal view of pronotum. (D) Dorsal view of posterior margin or pronotum, meso- and metanotum. (F) Lateral view of thorax. anf, antennifer; apl, anterior pronotal lobe; cl, clypeus; ge, gena; ios, interocular sulcus; L2-4, labial segment 2-4; lp, lateral process on metanotum; lsp, longitudinal sulcus of anterior pronotal lobe; mdp, mandibular plate; mesepm, mesepimeron, mseps, mesepisternum; mteps, metepisternum; mtn, metanotum; mxp, maxillary plate; ppl, posterior pronotal lobe; prem, proepimeron; pres, proepisternum; pss, prosternal stridulitum; sc, (meso-)scutellum; wp, wing pads of forewing.

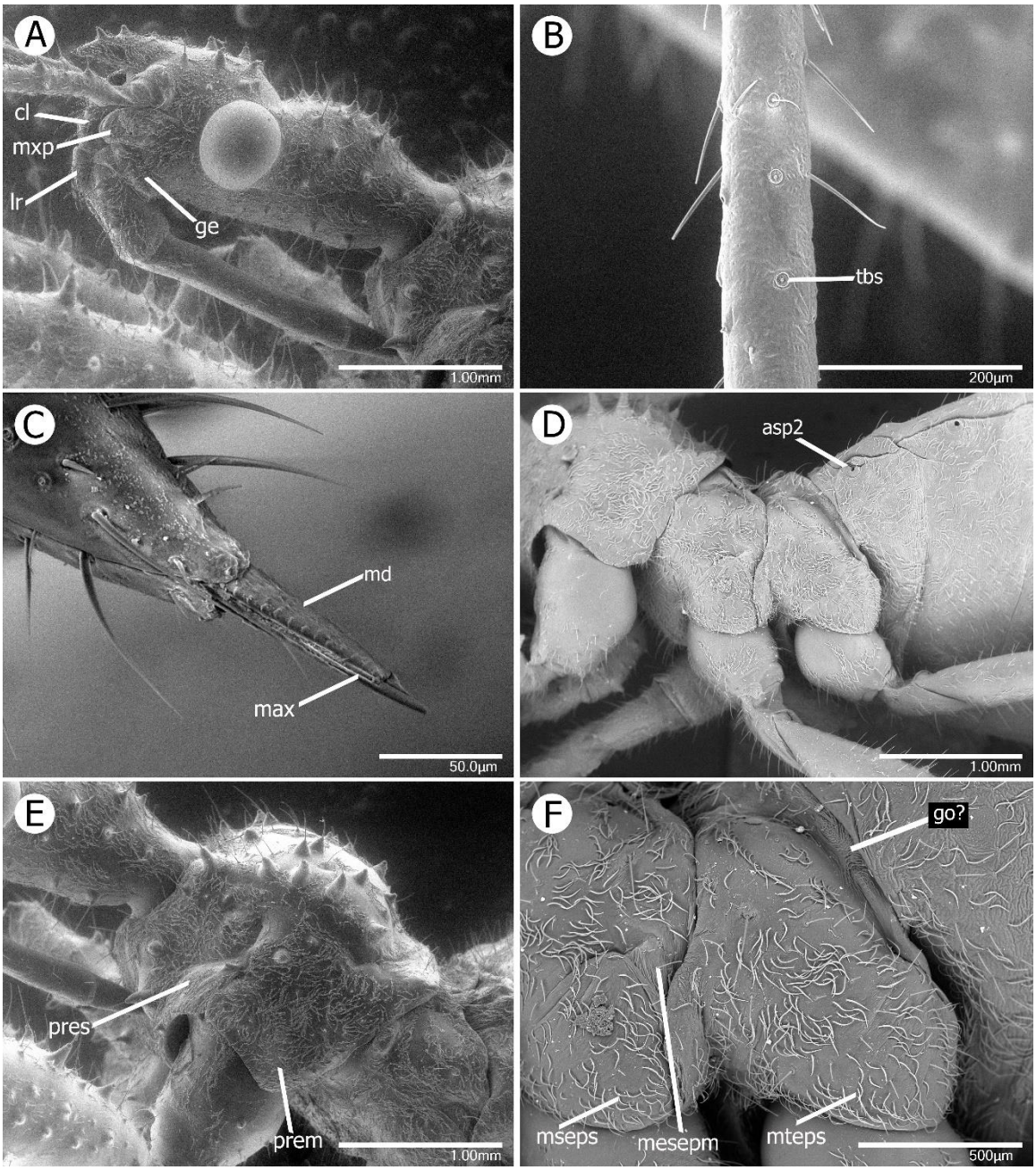


Fig 4. Details of head and thorax of Species 1, SEM. (A) Head lateral view, showing c. (B) trichobothria on pedicel. (C) Close-up on labium tip with mandible and stylets protruding. (D) lateral view of thorax. (E) Lateral view of pronotum. (F) Lateral view of meso-metanotum. asp2, abdominal spiracle 2; cl, clypeus; ge, gena; go?, possible glandular opening; lr, labrum; max, maxillary stylets; md, mandible; mesepm, mesepimeron; mseps, mesepisternum; mteps, metepisternum; mxp, maxillary plate; pres, proepisternum; tbs, trichobothria socket.

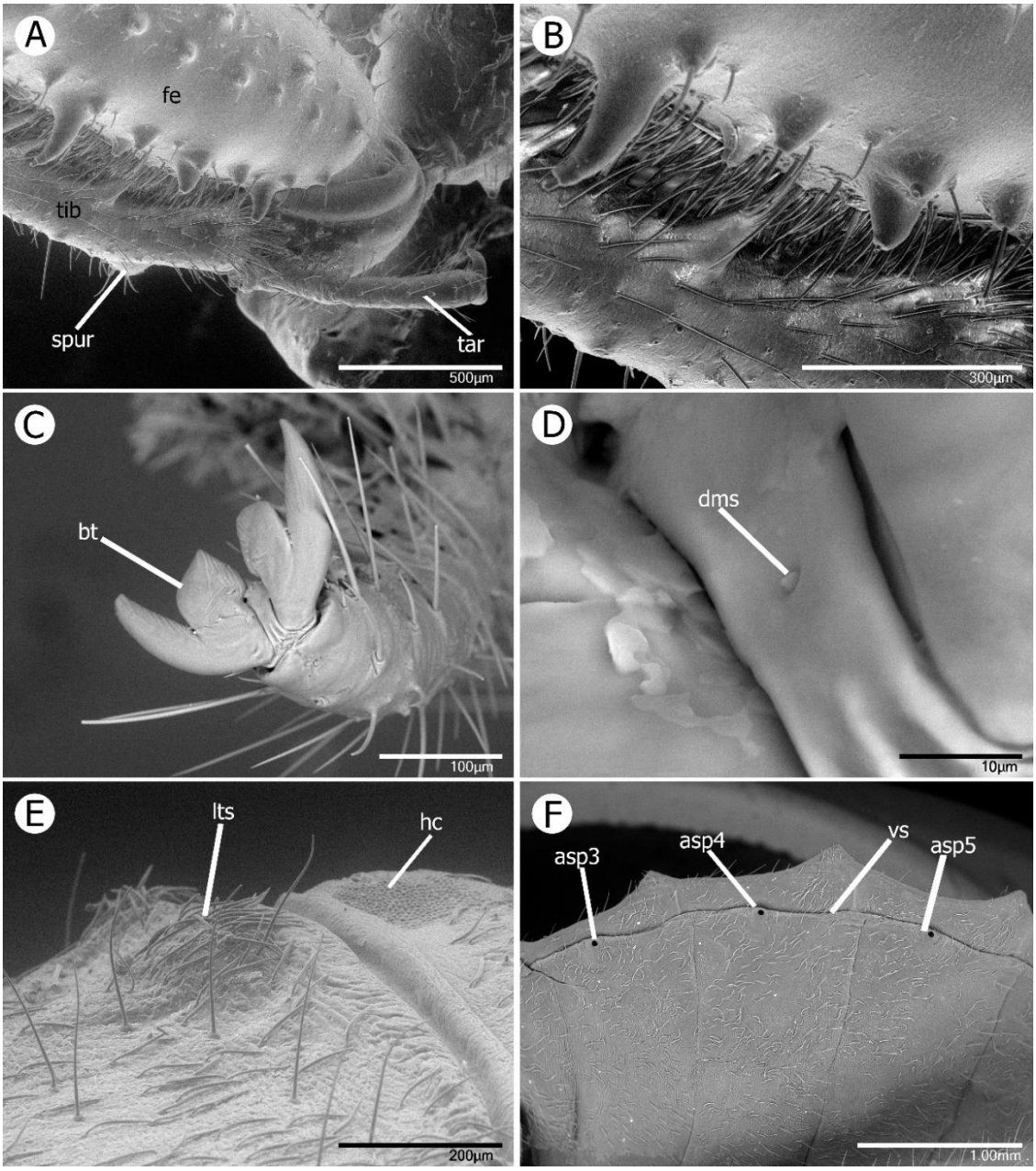


Fig 5. Details of legs and abdomen of Species 1, SEM. (A) Foreleg. (B) close-up on tibia and femur setae. (C) Pretarsus of foreleg. (D) Close up on dorsal medial sensillum. (E) Dorsolateral view of dorsal abdominal gland. (F) Ventral connexival suture. asp3-5, abdominal spiracle 3-5; bt, basal tooth of claw; dms, dorsomedian sensillum; fe, femur; hc, honeycomb patterning on DAG; lts, lateral tufts of setae; spur, protibial spur; tar, tarsomere; tib, tibia; vs, ventral connexival suture.

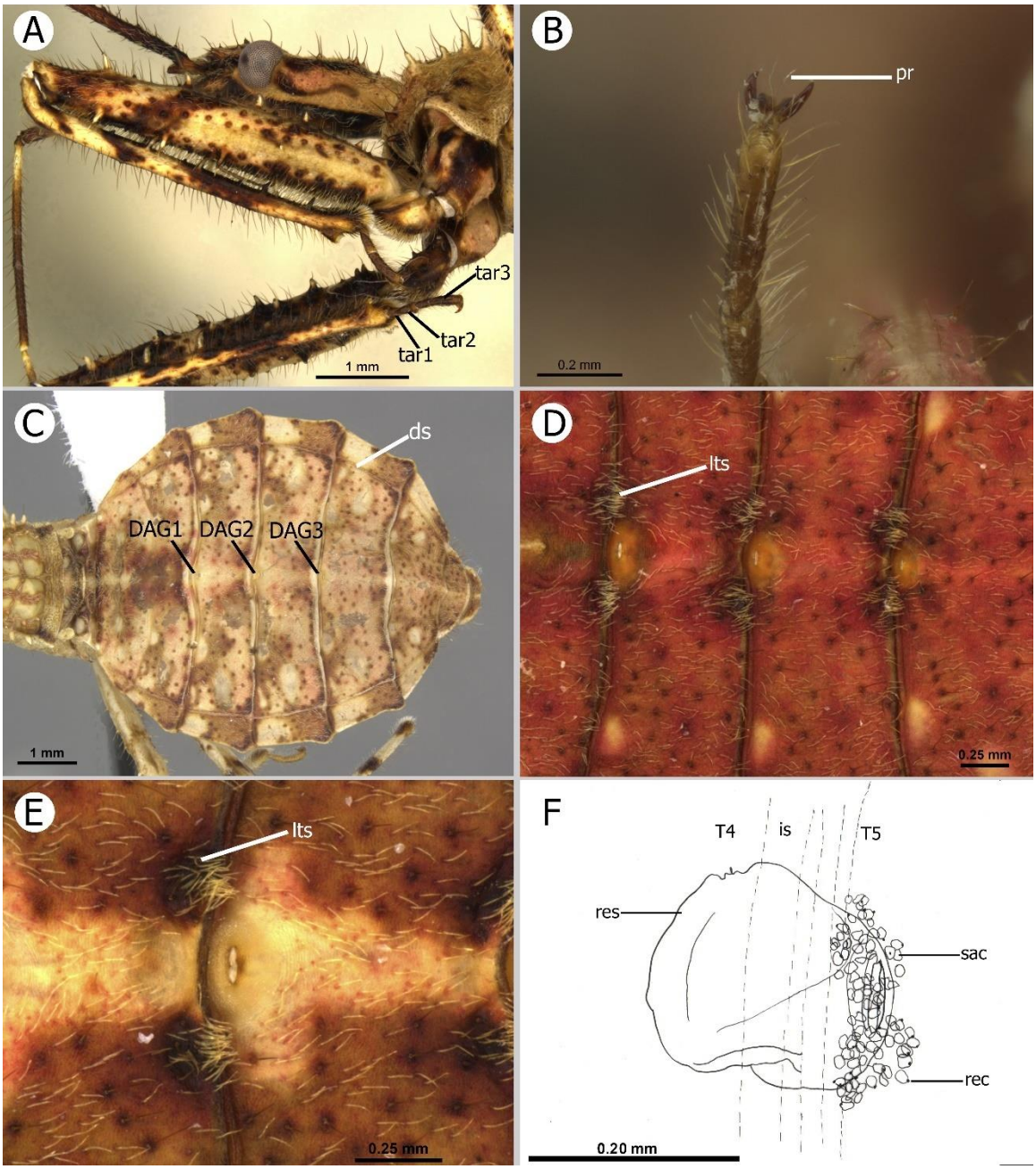
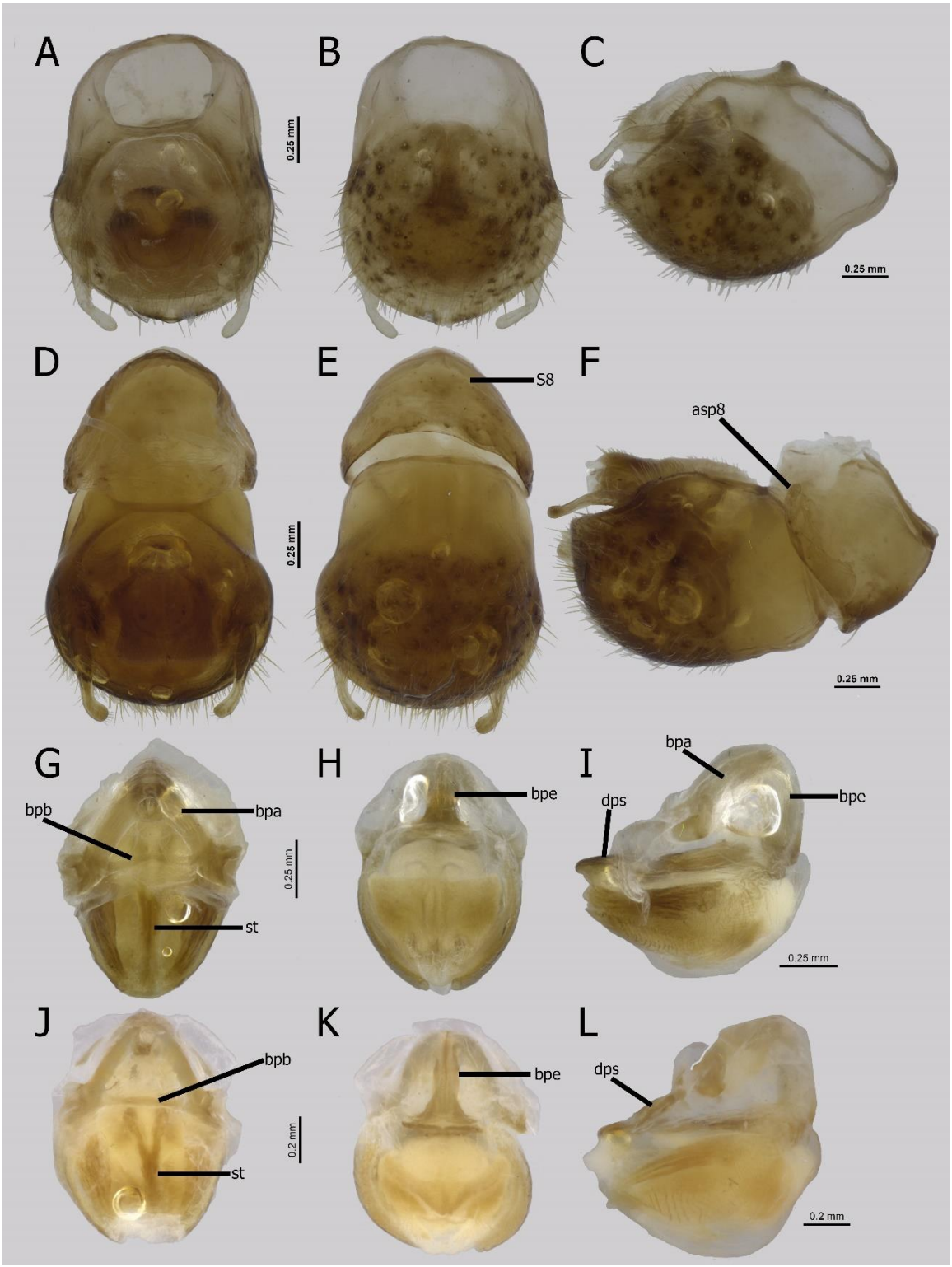


Fig 6. Details of leg and abdominal structures. (A, E) Species 1, male. (B, D) Species 2, male. (C) Species 1, female. (F) Species 2, female. (A) Lateral view of foreleg. (B) Parempodia on foreleg. (C) Dorsal view of abdomen. (D) Dorsal abdominal glands. (E) Close-up on dorsal abdominal glands. (F) Internal structure of dorsal abdominal glands. DAG1-3, dorsal abdominal glands 1-3; ds, dorsal connexival suture; is, intersegmental membrane; lts, lateral tufts of setae; pr, parempodial seta; rec, receiving canal; res, reservoir; sac, saccule; T4-5, tergite 4-5, tar1-3, tarsomere 1-3.


A
B
C
0.25 mm
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S8
F
asp8
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K
bpe
L
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0.2 mm

Fig 7. Male genitalia. (A-C, G-I) Species 1. (D-F, J-L) Species 2. (A-F) Pygophore. (G-L) Phallus. (A, D, G, J) Dorsal view. (B, E, H, K) Ventral view. (C, F, I, L) Lateral view. asp8, abdominal spiracle 8; bpa, basal plate arms; bpb, basal plate bridge; bpe, basal plate extension; dps, dorsal phallothecal sclerite; S8, sternite 8; st, struts.

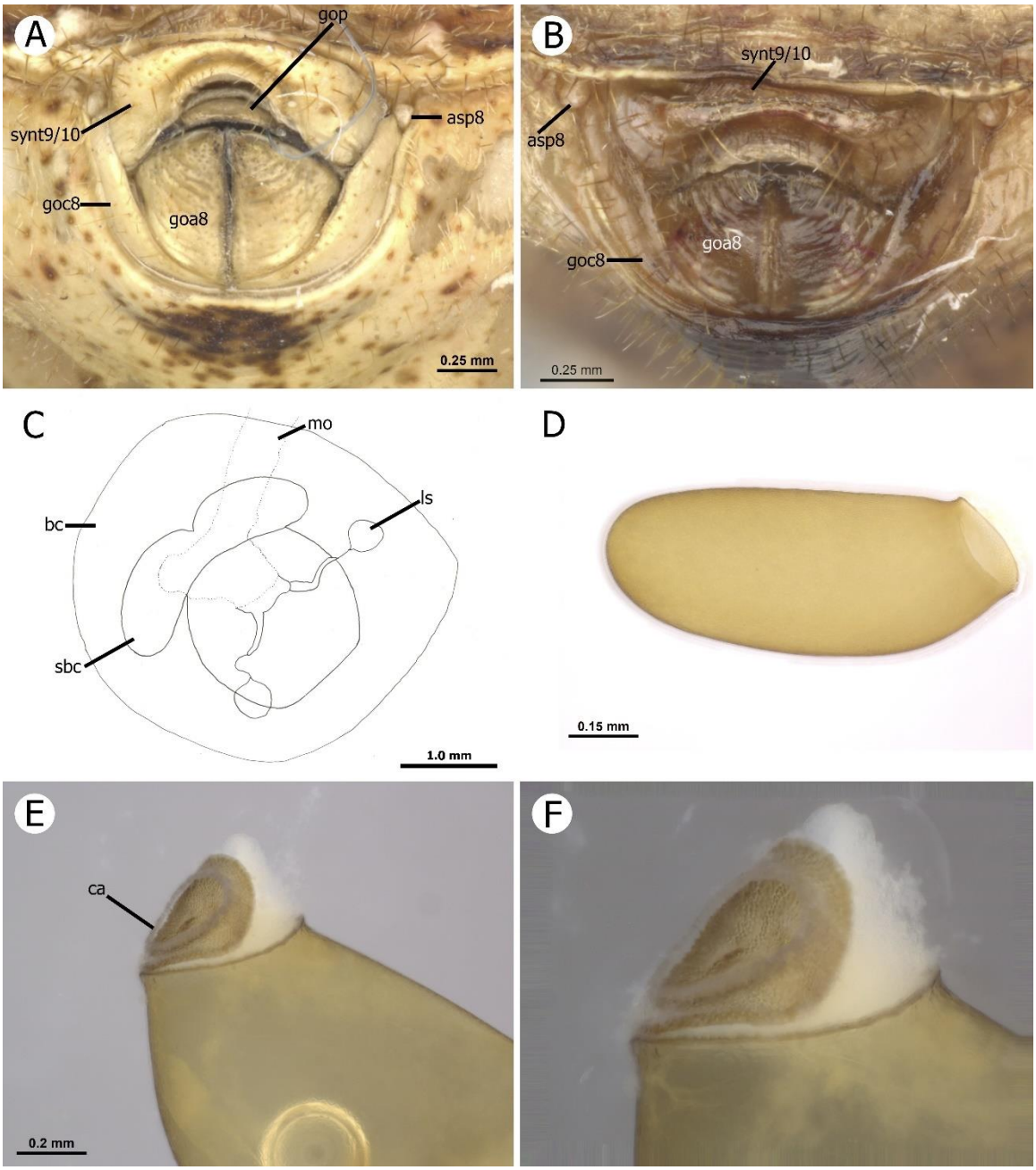

Fig 8. Female genitalia and eggs. (A, C, E-F) Species 1. (B, D) Species 2. (A-B) female genitalia in caudal view. (C) Internal genitalia. (D) Lateral view of egg. (E-F) Egg with operculum. asp8, abdominal spiracle 8; bc, bursa copulatrix; ca, carina; goa8, gonapophysis 8; goc8, gonocoxa 8; gop, gonoplac; ls, lateral spermatheca; mo, median oviduct; sbc, heavily sclerotized area on bursa copulatrix; synt9/10, syntergite9/10.

Reconstructing host plant repertoire and timing of evolution of phyline plant bugs (Hemiptera: Miridae: Phylinae)

Abstract

The diversity of phytophagous insects is often attributed to the success of land plants in the framework of ecological speciation. Several hypotheses have been proposed to explain host plant driven insect diversification in a phylogenetic context and have mostly been explored using Lepidoptera. We posit that Miridae are a great system to examine these hypotheses, because they are one of the largest primarily phytophagous insect families and include many species with narrow host repertoire. Focusing on the species-rich Phylinae (>2,700 spp.), we generate the most taxon-rich phylogeny published to date and for the first time estimate divergence times and trace the evolution of host-plant associations across the group. Focusing on two clades of oak-associated phylines, we further examine if diversification in these insects and their hosts coincided or if the insects tracked their hosts. We find that Phylinae diverged from their orthotyline sister group before the end of the Cretaceous, tribal-level taxa diversified throughout the Paleogene, and diversification within genera mostly occurred in the Neogene. Host plant repertoire reconstructions at the family level show that transitions from monophagy to polyphagy are more common than the reverse. We reconstructed the ancestral phyline host as Malpighiales, followed by Asterales throughout most of the deep splits. Species-level divergences in the two oak-associated clades are shallower than those in oaks,

suggesting that they tracked their hosts. While only the first step towards testing hypotheses on ecological speciation of plants and insects, our study shows that Phylinae are a suitable system to further explore these questions.

Introduction

At least half of all described insect species are phytophagous and many more are plant associated. Plants have therefore been hypothesized to have had a large effect on the evolution of many lineages of insects (Grimaldi & Engel 2005). Consequently, exploring how insect-plant associations may have driven the diversification of insects in the framework of ecological speciation has been of interest to many entomologists (Ehrlich & Raven 1964, Janz & Nylin 2008, Hardy & Otto 2014). Many phytophagous insects have a narrow host repertoire, feeding on one or few usually closely related species of plants (Kergoat et al. 2017). This specialization can drive ecological speciation as insect populations are divided among plant species and over generations evolve adaptations to their new host plants. Using phylogenetic and comparative phylogenetic methods, it is now possible to trace patterns of host plant associations and diet repertoire, examine niche conservatism, and determine if insects coevolve with their hosts or if they track them (Kergoat et al. 2017). Host plant associations have been reconstructed to establish the host association for the common ancestor of a particular group (e.g., Cognato et al. 2021). Similarly, diet repertoire has been examined through the different degrees of monophagy (i.e., feeding on one species, family, or order of host plants) and polyphagy (feeding on more than one species, family, or order of host plants) (Peterson et al. 2020).

Observations on different groups have resulted in the formulation of several hypotheses to explain plant-insect evolution. The so-called Escape and Radiate hypothesis (Ehrlich & Raven 1964) assumes phylogenetic host conservation and cospeciation (Janz 2011). The prediction of cospeciation can be tested by comparing divergence-dated phylogenies of insects and their host plants. In contrast, speciation in many insect clades appears to lag behind the diversification of their host clades, a phenomenon that has been referred to as host tracking and is not compatible with the Escape and Radiate hypothesis (Kergoat et al. 2017). Examining lability in the host plant repertoire while estimating diversification rates for monophagous and polyphagous clades has been used to test two additional hypotheses, the Oscillation and the Musical Chairs hypotheses, mostly using lepidopteran systems (Jousselin and Elias n.d.). In larval Nymphalidae, few genera are polyphagous, but different genera are found on a wide range of different host plant families and orders (Nylin et al. 2014). Host plant switching and diet repertoire expansions were shown to have driven speciation in this group, providing evidence for the Oscillation hypothesis (Nylin & Wahlberg 2008, Janz et al. 2006, Hardy 2017). Larval Pieridae use a small number of host plant families, show few host plant transitions, and patterns across lineages appear to support elements of both the Escape and Radiate and Oscillation hypotheses (Braga et al. 2018, 2021). Other systems that have been examined are Coleoptera (Becerra 2003, Hunt et al. 2007) and Diptera (Nyman et al. 2010, Joy & Crespi 2012) but few studies have focused on non-holometabolous groups including Hemiptera.

Plant-insect evolution research on Hemiptera has largely centered on Sternorrhyncha. This includes a study on aphids that determined speciation to be driven by geographic isolation rather than host switching (Jousselin et al. 2013, Meseguer et al 2015). In contrast, scale insects show host plant driven speciation and host use in armored scale insects is phylogenetically conserved, with more specialized species being generally less abundant than less-specialized species (Lin et al. 2015, Peterson et al. 2020). Hardy et al. (2016) found that diversification rates in scale insects increase with increased host repertoire, suggesting that diversification is driven by drift rather than adaptive specialization. In Psylloidea, host switching appears to account for the wide range of host plants used and hosts taxa are spread across the angiosperm phylogeny (Ouvrard et al. (2015). Given its species richness and predominant phytophagy, we postulate that Miridae, the plant bugs, may be a good group to further explore putative host plant driven speciation in Hemiptera and insects in general.

Miridae are one the most diverse families of insects with > 11,350 species in 7 subfamilies (Cassis & Schuh 2012; Schuh & Weirauch, 2020). Feeding strategies in this group include predation, phytophagy, and omnivory. Their economic importance therefore ranges from plants pest to biological control agents. The ancestral feeding strategy of Miridae has been debated controversially as either phytophagous or predatory (Wheeler 2001). Early evidence based on the functional morphology of mouth parts, physiology of salivary proteins, and the observation that predatory Isometopinae were recovered as earliest diverging lineage within Miridae seemed to suggest that the common ancestor was predatory (Goodchild 1952, 1966, Schuh & Štys 1991; Schuh &

Slater 1995). However, Jung and Lee (2011) reconstructed the most recent common ancestor of Miridae as phytophagous, a hypothesis corroborated by Weirauch et al. (2018).

Mirid-host plant associations are well-documented in the taxonomic literature (summarized in the Online Systematic Catalog of Plant Bugs: Schuh 2002-2013) and specimen records in the Arthropod Easy Capture Specimen Database (514,582 records for Miridae) (https://research.amnh.org/pbi/locality/). About ~60% or mirid species with known host association are monophagous in the strict sense, i.e., they feed on a single host plant species (Cassis & Schuh 2012). Many of the remaining species feed on restricted clades of plants (i.e. one or two plant families), with relatively few species showing rampant polyphagy (i.e., feeding on multiple plant families or orders). A synopsis of plant hosts at the order level shows that mirid-host associations are clustered in particular orders, in particular Asterales, Fabales, Fagales, Rosales, Lamiales, and Caryophalyes (Cassis & Schuh 2012). In addition, our understanding of plant evolution has accelerated during the past decade, with divergence dated phylogenies now available for many groups that include important mirid hosts (Hipp et al. 2017, Li et al. 2019). To interpret the vast amount of available host-plant data for Miridae in a phylogenetic context, divergence-dated, comprehensive phylogenies of Miridae are critical, but currently largely unavailable.

The plant bug subfamily Phylinae contains >2,700 species in all biogeographic regions with the highest biodiversity in Mediterranean climates (Fig. 1) (Schuh & Menard 2013). Most Phylinae are phytophagous, but the group includes a few predatory

and obligatory omnivorous species (Wheeler 2001). Phytophagous phylines tend to feed on plant flower buds and inflorescences (Wheeler 2001). Obligate omnivorous species feed on small insects such as aphids and insect eggs, but also feed on their host plants when prey insects are scarce (Wheeler 2001). Phylinae are currently divided into 9 tribes (Hallodapini Van Duzee, 1916, Decomiini Schuh & Menard 2013, Phylini Douglas and Scott, 1865, Pilophorini Douglas and Scott, 1876, Leucophoropterini Schuh, 1974, Nasocorini Reuter, 1883, Exaeretini Puton, 1875, Cremnorrhini Reuter, 1883, Semiini Knight, 1923). The tribes are split into 9 subtribes (Cremnorrhini: Cremnorrhina Reuter, 1883 and Coatonocapsina Schuh & Menard, 2013, Phylini: Keltoniina Schuh & Menard, 2013, Phylina Douglas & Scott, 1865, Oncotylina Douglas & Scott, 1865, Semiini: Exocarpocorina Schuh & Menard, 2013, Semiina, Knight, 1923, Leucophoropterini: Tuxedoina Schuh & Menard, 2013, and Leucophoropterina Schuh, 1974). The phylogenetic placement of Phylinae within Miridae is uncertain as either sister taxon to all remaining Miridae (Jung & Lee 2011) or to Orthotylinae (Menard et al. 2014). Phylogenetic hypotheses for Phylinae are better developed than for any of the other mirid subfamilies (Knyshov et al. 2019, Menard et al. 2014, Schuh & Menard 2013). However, only a small proportion (155 spp.) of described species is currently represented in these studies. The only published divergence dating analysis for Miridae recovered Phylinae as sister to all remaining Miridae and estimated that divergence to ~225 MYA, in the Triassic (Jung & Lee 2011). This very old estimate conflicts with divergence dating analyses across Heteroptera that estimated the origin of Miroidea to the Jurassic (168 MYA to 195 MYA) (Ye et al. 2022, Johnson et al. 2018, Li et al. 2012). We hypothesize

that the very old estimate in Jung and Lee (2011) may be due to the use of controversial fossil calibrations that have been reevaluated only recently (Schuh & Weirauch 2020).

Only a few studies have so far attempted to optimize host data on morphological phylogenies (e.g., Schuh and Weirauch, 2010). The phyline diet repertoire ranges from monophagous (e.g., *Larinocerus balius* Froeschner on *Scutellaria mexicana* (Torr.) A.J. Paton) to polyphagous (e.g., *Pilophorus discretus* Van Duzee on various species of Asteraceae, Rosaceae, Euphorbiacae, and Fabaceae). Monophagous and polyphagous species sometimes even occur within a given plant bug genus (e.g., *Plagiognathus* Fieber). In contrast, certain phyline lineages appear to exhibit niche conservatism. Examples are the eight Nearctic genera referred to as the "orange oak bug" [OOB] clade (Oncotylina) and the distantly related Nearctic genus *Tuxedo* Schuh (seven species) (Tuxedoiina) that are generally associated with oaks [*Quercus* L.] (Schuh, 2004; Weirauch 2006a, b; Knyshov et al., 2019). Distributions and host associations are well-documented for these two clades and they have been robustly sampled in recent phylogenetic hypotheses. *Quercus* (oak), a Holarctic genus of Fagaceae, is known for their unique host associations with cynipid wasps (Buffington et al. 2020). Recent studies have estimated the phylogeny and diversification of North American *Quercus* (Hipp et al. 2017, Grivet et al. 2006, Hauser et al. 2017). This makes oak-associated Nearctic plant bugs good systems to evaluate the prevalence of cospeciation versus host tracking.

The aims of this study are threefold: we increase taxon-sampling of phylogenetic hypotheses for Phylinae, estimate the first divergence dating analysis while using well-vetted fossils, and reconstruct host associations and diet repertoire for the first time across

the subfamily. Focusing specifically on the two oak-associated clades, we test if the evolution of oaks and oak-associated plant bugs coincided or was the result of host tracking.

Materials and Methods:

Taxon sampling

This study builds on the most recently published phylogenetic dataset for Phylinae (Knyshov et al. 2019) by adding 25 outgroup (i.e., non-phyline) terminals representing 25 species and 27 ingroup terminals representing 17 genera. Our dataset now includes 193 ingroup and 31 outgroup terminals. Outgroups are broadly sampled across mirid subfamilies (Mirinae, Orthotylinae, Deraeocorinae, Bryocorinae, Cylapinae, Isometopinae), but also include Tingidae and Thaumastocoridae to better represent Miroidea. Ingroup sequences were newly generated for this study and additional outgroup sequences are derived from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (Table S1).

New ingroup taxa were collected by the authors in 2021, were previously collected by the Weirauch lab, or are derived from passive trap samples donated by Mike Irwin. Species were identified using male genitalic dissections combined with locality and host plant information. Habitus images of voucher specimens were captured using a Leica imaging system. Each specimen was given a unique specimen identifier (USI) label that connects a specimen to a record in the Arthropod Easy Capture (AEC) database (https://research.amnh.org/pbi/locality/index.php). Voucher images are linked to AEC records and publicly accessible via the Heteroptera Species Pages

(http://research.amnh.org/pbi/species). Table S1 provides GenBank accession numbers and other information for molecular vouchers.

DNA extraction, primers, PCR amplification and sequencing

The abdomen was used for non-destructive DNA extraction, which was performed using the Qiagen DNeasy® Blood & Tissue kit following the manufacturer protocols (Knyshov et al. 2019). After extraction, the abdomen was placed in ethanol to be later dissected. Five gene regions [16S rDNA, 18S rDNA, 28S D2 rDNA, 28S D3-5 rDNA, cytochrome oxidase I (COI)] were sequenced. Table S2 lists the PCR conditions and primers used. Sera-mag Speedbeads were used to purify amplicons prior to sequencing following manufacturer protocols. Cleaned PCR product was sanger sequenced at Retrogen, Inc. (San Diego, CA).

Sequence alignment and phylogenetic analyses

Forward and reverse sequences were assembled in Geneious 11.0.2 (https://www.geneious.com/). Sequences were manually checked for errors and trimmed at primer regions. We used the Basic Local Alignment Search Tool (BLAST) to rule out contamination (https://blast.ncbi.nlm.nih.gov/). Sequences were aligned using MAFFT (Katoh & Standley 2013) and the E-INS-I algorithm. Protein encoding sequences (COI) were translated into amino acids to check for stop codons. The five regions were concatenated in SequenceMatrix v1.7.8 (Vaidya et al. 2011). A Maximum Likelihood (ML) phylogeny was reconstructed using RAxML-HPC BlackBOx (8.2.12) (Stamatakis 2014) with four partitions (16S, 18S, 28S, and COI) on the CIPRES Scientific Gateway. Parameters were set to default, including running 1000 rapid bootstrap iterations (BS).

Divergence dating analyses

Divergence times were estimated using either a starting tree (Fig. S3), no starting tree (Fig. S4), or a fixed tree analysis using the best tree from the maximum likelihood analysis and four fossil calibrations (Fig. 10). We used best practices to select four fossil species and determine their phylogenetic placement to accurately calibrate nodes (Parham et al. 2012). We compared the description of *Reuteroscopus carvalhoi* (Maldonado & Poinar 1995) from Dominican amber (15-20 MYA; Iturralde-Vinent & Macphee 1996) to diagnostic features listed in Schuh & Menard (2013) and used it to constrain Keltoniina. Our assessment is based on the prominent hemelytral coloration similar to other Keltoniina and especially *Reuteroscopus* together with the long, prominent setae on the hemelytron intermixed with light colored setae (Schuh & Menard 2013). *Hallodapomimus elektrinus* (Herczek and Popov 2015) from Baltic Amber (34-48 MYA; Seyfullah et al. 2018) was used to calibrate Hallodapini. *Sinaldocader ponomarenkoi* (Golub & Popov 2008) is estimated to date to 48-89 MYA (International Chronostratigraphic Chart 2022) and was used to constrain Tingidae. This fossil is confidently placed within Tingidae based on the areolate hemelytra as well as the venation seen in modern members of the family and the punctate pronotum. Because of the lack of phylogenetic hypotheses for Tingidae, we did not place the fossil within a subfamily and instead used it to provide a minimum age for the family. Finally, *Miridoides mesozoicus* (Becker-Migdisova & Popov 1963) from the late Jurassic and dated at 150-170 MYA was used to calibrate the most recent common ancestor of Miridae + Tingidae. While this fossil was originally described in Orthotylinae, a

placement later confirmed by Herczek and Popov (2001), Schuh & Weirauch (2020) confirm that this species belongs to the Miridae, but emphasize the lack of evidence for subfamily-level placement.

Divergences were estimated using BEAST 2.6.3 (Bouckaert et al. 2014). A relaxed log normal molecular clock model was selected for all four partitions (16S, 18S, 28S, and COI). BEAUTi (2.6.3) was used to make an XML control file. Models were selected via ModelFinder (Kalyaanamoorthy et al., 2017). We ran a Markov Chain Monte-Carlo (MCMC) algorithm for 200,000,000 generations and logged trace and tree files every 5000 generations. For the fixed tree we ran six independent MCMC runs simultaneously. Tracer v1.7.2 (Rambaut et al. 2018) was used to confirm that stationary distribution was reached and to check that the effective sample size (ESS) was greater than 200 for most parameters. After confirming the convergence, we used LogCombiner v2.6.7 specifying a 10% burnin for each tree file, we thinned the trees resampling at 250,000. Trees were then summarized in TreeAnnotator v2.6.3 using 'Maximum clade credibility tree' and 'Common Ancestor heights' options. The tree was then displayed in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) with age in millions of years. The result from the fixed tree analysis is shown in Fig. 10, because neither the "starting tree" nor the "no starting tree" analyses converged.

Reconstruction of ancestral host plant

Host plant information was assembled from multiple sources (Table S1) including our own observations. We followed the APG IV to place families into orders (Chase et al. 2016). Despite monophagous mirid species often being host specific at the species- or

genus level, we coded hosts at the family and order levels to better visualize common patterns (Cassis & Schuh 2012). To reconstruct the evolution of ancestral hosts across Phylinae, we coded a data set that included 53 plant families belonging to 28 orders. Plant families and orders were coded in separate ancestral character state analyses with 53 and 28 character states, respectively. Polyphagous taxa were coded as polymorphic. Since certain Phylinae are predators, our analyses included a separate state for "predatory". Hosts for several species in our phylogenetic hypothesis are unknown and were coded as such. A separate data set coding monophagy (at the family level) and polyphagy was used to reconstruct evolutionary patterns between different diet repertoires and to determine the number and directionality of transitions. Ancestral character state reconstructions were performed in Mesquite 3.70 (Maddison & Maddison 2011) based on the best tree derived from the maximum likelihood analysis. Host associations were reconstructed using likelihood and host repertoire using parsimony because likelihood is unable to reconstruct polymorphic data.

Results

Phylogenetic analyses

Only some general results and relationships deviating from past analyses are outlined below and we place some focus on genera placed in a phylogenetic hypothesis for the first time. Thaumastocoridae were well supported as sister taxon to Tingidae and Miridae (>80% bootstrap [BS]) (Fig. 10, S1). "Orthotylinae" were recovered as paraphyletic, with one lineage as poorly supported (<60% BS) sister taxon to Phylinae. Phylinae were monophyletic and well-supported (100% BS). Within Phylinae, the tribe Hallodapini was

monophyletic (72% BS) and well-supported as the sister group to the remaining Phylinae (100% BS). Cremnorrhini is paraphyletic with Coatocapsina (Cremnorrhini) diverging from the remaining Phylinae before Cremnorrhina but with low support (<60% BS). Keltoniina (Phylini) were recovered nested within Cremnorrhina, though weakly supported (<60% BS). Oncotylina are monophyletic, but in Menard et al. (2014) and our analysis this subtribe also includes *Atractotomus cercocarpi* Knight. However, *Atractotomus* Fieber is classified as Nasocorini (Schuh and Menard, 2013) and all other species of *Atractotomus* group together, so we suspect that this unexpected phylogenetic placement is due to misidentification. Our analysis also provides the first test of the phylogenetic placement for several genera found in the southwestern United States including California that so far had not been included in phylogenetic analyses. Surprisingly, *Oncotylus* Fieber, the type genus of Oncotylina, was placed as the sister genus to *Keltonia* Knight + *Pseudatomoscelis* Poppius within Keltoniina, rendering Oncotylina sensu Schuh and Menard (2013) paraphyletic. Within Oncotylina, *Ceratopsallus* Schuh was placed as sister taxon to *Phymatopsallus* Knight (>80% BS), consistent with the phylogenetic hypothesis proposed by Schuh (2006). The mistle-toe feeding species of *Viscacoris* Weirauch was found to be nested within *Oligotylus* Van Duzee (>80% BS), where species overwhelmingly feed on Rhamnaceae. *Vanduzeephylus* Schuh and Schwartz was recovered as sister genus to *Europiella* Reuter and an unknown genus and species of Oncotylina (67% BS). The chamise-feeding species of *Adenostomocoris* Schuh and Schwartz included in our analysis is placed within Nasocorini where it renders *Megalopsallus* Knight paraphyletic.

Divergence dating analyses

The BEAST2 divergence dating results based on the fixed maximum likelihood tree are shown in Fig. 10. The chronogram with all terminal taxa labeled and 95% highest posterior density (HPD) node bars annotated is provided as Figure S2. Our analysis estimates that Miridae diverged from Tingidae in the Jurassic, around 154 MYA (145.6-203.2 MYA). Phylinae diverged from other Miridae in the Late Cretaceous, about 75 MYA (58-91 MYA). Phyline tribes emerged within the Paleocene and Eocene, ranging from ~64 MYA (Hallodapini; 50.1-78.5 MYA) to ~41 MYA (Leucophoropterini; 40.8 MYA (31.7-50.5 MYA). Semiini diverged from its sister group Leucophoropterini + Pilophorini ~37 MYA (33.3-53 MYA). This young age of Semiini is noteworthy, because Semiini include taxa from distant biogeographic regions, including the Nearctic, Australian, and Afrotropical regions. The oak-associated Nearctic OOB clade diverged from the remaining Oncotylina ~34 MYA (25.7-42.8 MYA), while diversification in the second oak-associated clade, *Tuxedo*, started marginally later ~30 MYA (19.3-37.4 MYA). The Nearctic genus "*Tuxedo*" is rendered paraphyletic by *Pseudophylus* Yasunaga that occurs in the eastern Palearctic; the divergence of *Pseudophylus* from its Nearctic sister taxa *Tuxedo bincintus* Van Duzee and *Tuxedo cruralis* Van Duzee was estimated to be in the Miocene, ~12 MYA (5.6-19.2 MYA). Within the Holarctic genus *Plagiognathus* several divergences between Palearctic and Nearctic taxa were dated to in between 5.8-29.8 MYA.

Reconstruction of host plant associations

Reconstructing the evolution of host plant orders and families: Our maximum likelihood reconstruction of plant orders determined the ancestral host of Phylinae to be Malpighiales (Fig. 11A). Host associations for several species of Hallodapini and Decomiini are unknown, making host reconstructions ambiguous; however, an association with Malpighiales appears to have been retained in at least some species in both groups. The most recent common ancestor (MRCA) of Phylinae (Fig. 11A, node 1) except Hallodapini and Decomiini was reconstructed as having been associated with Asterales, and this association was maintained along part of the backbone of Phylinae. Nasocorini (Fig. 11A, node 2) use a wide range of host orders, including Fabales, Fagales, Rosales, and Ephedrales, amongst others. Hosts for several Coatocapsina (Fig. 11A, node 3) are unknown and those represented feed on Asterales and Caryophylales. Several species of Cremnorrhina and Keltoniina (Fig. 11A, node 4) feed on Asterales that was also reconstructed as host for the MRCA of this clade. The MRCA of Oncotylina (Fig. 11A, node 6) was ambiguously reconstructed as associated with Asterales or Fagales; it is therefore ambiguous if the association with Fagales seen within the clade formed by *Psallus* Fieber and related taxa and the one in OOB is due to a common or independent colonization events. Other lineages within Oncotylina are dominated by Asterales-feeding taxa. Semiini (Fig. 11A, node 7) were shown to feed on a range of plant orders and included one of the rare transitions from phytophagous to predatory (*Tytthus* Fieber). The MRCA of Semiini was reconstructed as being associated with Fabales. Host associations within the Leucophoropterina (Fig. 11A, node 10) and

Pilophorini (Fig. 11A, node 8) included a broad range of host orders, reflected in frequent host transitions and ambiguity regarding the ancestral hosts in both clades. In contrast, the MRCA of Tuxedoina was associated with Fagales, with one switch to Rosales and Malvales, amongst others, within the clade. The family-focused reconstruction of host associations (Fig. S5) is largely congruent with reconstructions of host orders, with a few exceptions. For example, the ancestral host for the Pilophorini at the order level is ambiguously reconstructed as Fabales or Asterales, but unambiguously as Fabaceae in the family-level reconstruction.

Reconstructing the evolution of monophagous and polyphagous diet repertoires: Our parsimony reconstruction of diet repertoire at the host plant family level found up to 41 transitions between monophagous and polyphagous taxa of Phylinae, depending on optimization (Fig. 11B). We found 25 unambiguous transitions from mono- to polyphagous and only three unambiguous changes from poly- to monophagous. Taking ambiguously optimized changes into account, transitions from monophagous to polyphagous occurred between 25 and 37 times within Phylinae in contrast to only between three and 14 transitions from mono- to polyphagous. The ancestor of Phylinae (Fig. 11B, node 1) was reconstructed as monophagous. While diet repertoire expansions and contractions are ubiquitous across Phylinae, we detected a prevalence for either narrow or broad diet repertoires in certain clades. As examples, within the Keltoniina (Phylini) (Fig. 11B, node 11) and Leucophoropterina (Fig. 11B, node 10) polyphagy is more prevalent than monophagy. In contrast, within Oncotylina (Phylini) (Fig. 11B, node 6), monophagy is more common than polyphagy, especially within the OOB clade, but

polyphagy is more widespread within the earliest diverging lineage of Oncotylina that comprises *Plagiognathus* and the *Phymatopsallus* group.

Discussion

Combining new sequence data with data from GenBank (Table S1), our phylogenetic hypothesis represents the most taxon-rich phylogeny of Phylinae published to date. Given the overlap in taxa and the fact that the same gene fragments were used, it is unsurprising that our hypothesis is broadly congruent with published studies (Knyshov et al. 2019, Gordon 2017, Menard et al. 2014). The monophyly of Phylinae and of some tribes are highly supported, but many of the deeper nodes remain poorly supported, a situation that will likely only be improved by generating and analyzing taxon-rich phylogenomic datasets. Our hypothesis shows Thaumastocoridae as sister taxon to Tingidae + Miridae with high support, corroborating published studies (Weirauch et al. 2018, Gordon 2017). The placement of Phylinae as sister taxon to part of "Orthotylinae" is consistent with some published results but requires further testing. Within Phylinae, Hallodapini were paraphyletic in the study by Knyshov et al. (2019); however, our analysis corroborates results by Menard et al. (2014) that recovered that tribe as monophyletic and we suspect that our increased outgroup sampling may have contributed to this outcome. With respect to genera that were not previously included in molecular phylogenetic analyses, most taxa were recovered within the clades predicted by the classification and diagnostic features provided by Schuh & Menard (2013). An interesting exception is *Oncotylus*, the type genus of Oncotylina, that our analysis found deeply nested within Keltoniina. Future studies should include additional taxa to further investigate this outcome. Similarly,

several newly added genera rendered existing genera paraphyletic. Given that even most modern taxonomic revisions of phyline genera were carried out without molecular phylogenetic analyses, we suspect that some additional revisionary work may be necessary, but also point out that future phylogenetic analyses should include additional species and more substantial molecular datasets.

Our divergence estimation found Miroidea to have evolved in the Late Jurassic, which is consistent with some previous findings based on fossil-calibrated analyses as well as the fossil record (Ye et al. 2022, Johnson et al. 2018, Li et al. 2012, Wang et al. 2016). Most published time-calibrated phylogenies have included few Miridae and several omitted Thaumastocoridae (Johnson et al. 2018, Li et al. 2012, Wang et al. 2016, Guilbert et al. 2014, Cassis & Schuh 2012). The divergence of Miridae from Tingidae reported in different studies ranged from the Permian (~277 MYA) to the Cretaceous (93 MYA) (Johnson et al. 2018, Jung and Lee 2011, Li et al. 2012, Wang et al. 2016). Our estimate for that divergence is Late Jurassic (~154 MYA), more than 100 MYA younger than the result in Jung and Lee (2011) but is close to the 138 MYA estimate reported in Ye et al. (2022). Ye et al. (2022), though not primarily focused on Miridae, included all subfamilies of Miridae and used a combination of Sanger and high-throughput-sequencing for their analyses. The strikingly different estimate between Jung and Lee (2011) and our analysis is best explained by the different placement of *Mirioides mesozoicus* to either calibrate Orthotylinae (Jung & Lee 2011) or Miridae + Tingidae (our analysis). *Mirioides mesozoicus* is considered the oldest mirid fossil but cannot be placed within any of the extant subfamilies because of the lack of diagnostic features (Weirauch

and Schuh 2020). For the same reason, estimates for Phylinae are drastically different between our analysis (Late Cretaceous; 74 MYA) and Jung and Lee (2011) (Triassic; 225 MYA). The relatively young overall ages derived from our analysis also shows that diversification within Semiini occurred starting in the Paleogene, suggesting that the disjunct distributions of Semiini in the Palearctic, Nearctic, Afrotropical, and Australian regions are derived from dispersal rather than vicariance events. Finally, many large genera diversified starting in the Miocene, including *Oligotylus, Plagiognathus*, *Psallus*, and *Megalopsallus*. This is in contrast to *Reuteroscopus* where diversification within the genus only started in the Pleistocene. Better taxon sampling and more robust phylogenies will be critical to further evaluate these results.

Most angiosperm orders evolved in the Cretaceous (Li et al. 2019), before Phylinae diverged from other Miridae according to our new hypothesis. Our estimated origin of Phylinae (Late Cretaceous; 74 MTY) and reconstruction of ancestral host as Malpighiales is consistent with the origin of this plant order in the Mid-Cretaceous (Li et al. 2019). Based on available evidence, diversification in most plant families colonized by Phylinae predated the diversification of the plant bugs (i.e., Rosales (~111 MYA, Magallón et al. 2015), Fabales (~105 MYA, Li et al. 2019), Fagales (~95 MYA, Larson-Johnson 2015), Asterales (~92 MYA, Magallón et al. 2015)), suggesting that cospeciation with their host plants has likely played a minor role in the diversification of Phylinae. While the great majority of Phylinae feed on angiosperms, several lineages have colonized Pinales (see *Pilophorus*, *Psallovius*, *Parapsallus* Fig.3A), a group that evolved during the Early Cretaceous (Ran et al. 2018). Overall, the evolution of host associations in Phylinae is

dominated by pervasive transitions between different host orders in most clades, similar to evolutionary host patterns in larval nymphalids (Nylin et al. 2014). Nevertheless, some lineages show some degree of niche conservatism, e.g., species of *Pilophorus* on Pinales or *Europiella* species on Asterales.

Diet repertoire evolution in Phylinae is quite labile, with numerous transitions between polyphagy and monophagy. This pattern contradicts the Musical Chairs hypothesis that predicts diet repertoire to be fairly conserved (Hardy & Otto, 2014; Hardy, 2017). In addition, transitions are heavily biased towards shifts from monophagous to polyphagous compared to the reverse. This pattern is not consistent with the prediction of the Oscillation hypothesis that speciation events should be associated with transitions from polyphagous to monophagous species (Hardy & Otto 2014). With regard to host plant coevolution versus host plant tracking in oak-associated plant bugs, our study suggests that oak-associated plant bugs tracked their hosts rather than co-evolved with them. Recent studies on *Quercus* have determined that even though the red and white oaks colonized the California Floristic province separately, both diversified in the Miocene and giving rise to several endemic species (Grivet et al. 2006, Hauser et al. 2017, Hipp et al. 2017). While the earliest speciation events within the OOB clade and *Tuxedo* fall within the diversification period of *Quercus*, the majority of the species level divergences within the OOB and *Tuxedo* occurred after that period.

In conclusion, our study provides first glimpses into the evolution of host plant associations in Phylinae but is hampered by the lack of support across many nodes and suboptimal taxon sampling. Future studies should build on taxon- and data-rich

phylogenetic hypotheses that would allow phylogenetic comparative analyses to test concepts surrounding geographic and ecological diversification in this diverse lineage of phytophagous insects.

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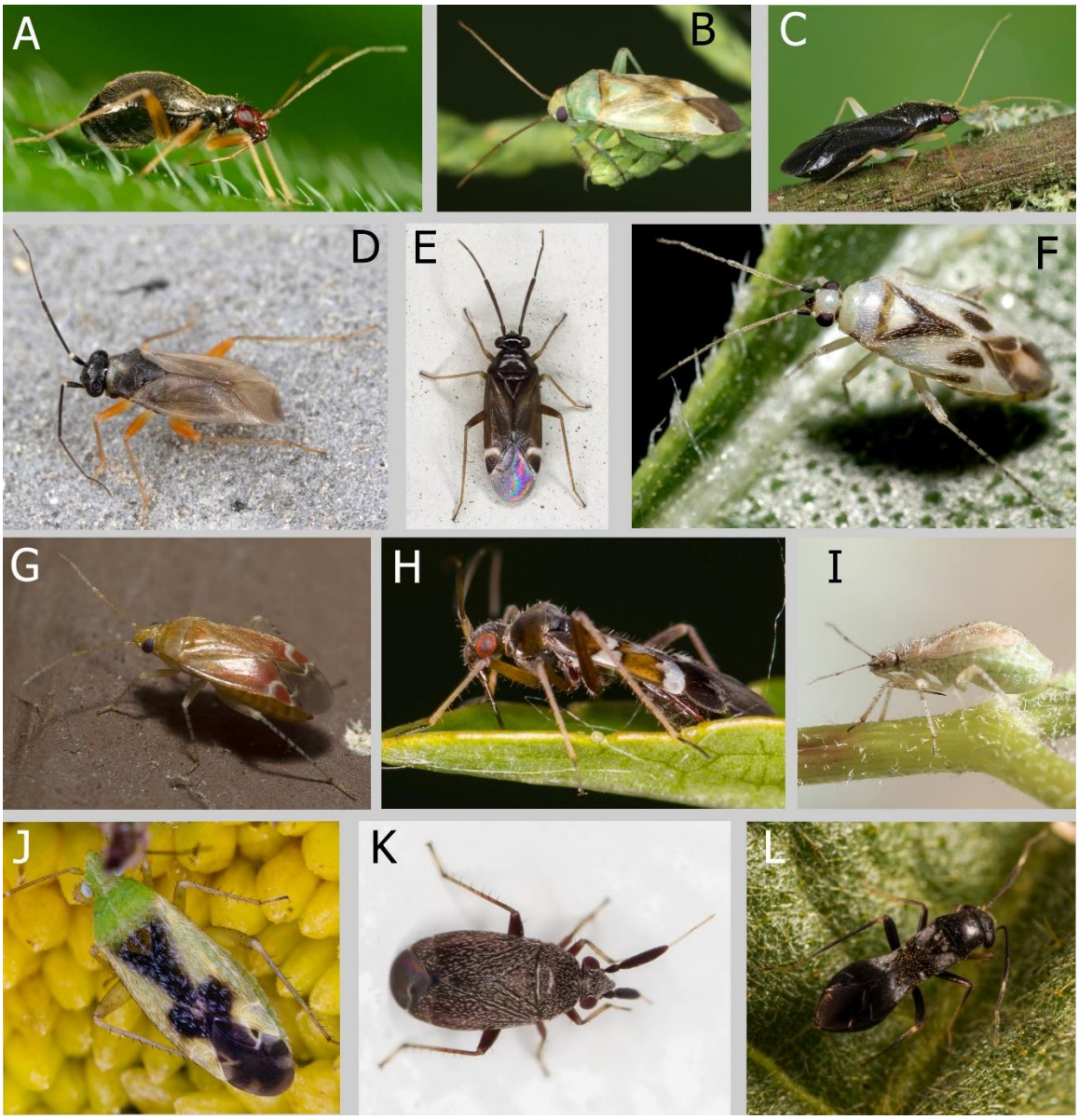

Fig. 9: Diversity of Phylinae. A. Phylina (*Orthonotus rufifrons* © Gilles San Martin) B. Exaeretini (*Tuponia mixicolor* © Gernot Kunz) C. Phylina (*Phylus coryli* © Gilles San Martin) D. Semiini (*Tytthus pygmaeus* © Gernot Kunz) E. Tuxedoiina (*Tuxedo flavicollis* © Gary McDonald) F. Oncotylina (*Plagiognathus albatus* © Matthew A. Mulvey) G. Oncotylina (Orange Oak Bug © Matthew A. Mulvey) H. Hallodapini (*Systellonotus triguttatus* © Murtazin Shamil) I. Semiini (*Hoplomachidea consors* © Andrew Meeds) J. Keltoniina (*Reuteroscopus ornatus* © Lee Alloway) K. Nasocorini (*Atractotomus mali* © Murtazin Shami) L. Pilophorini (*Pilophorous typicus* © Stefan Obenauer, Hong Kong).

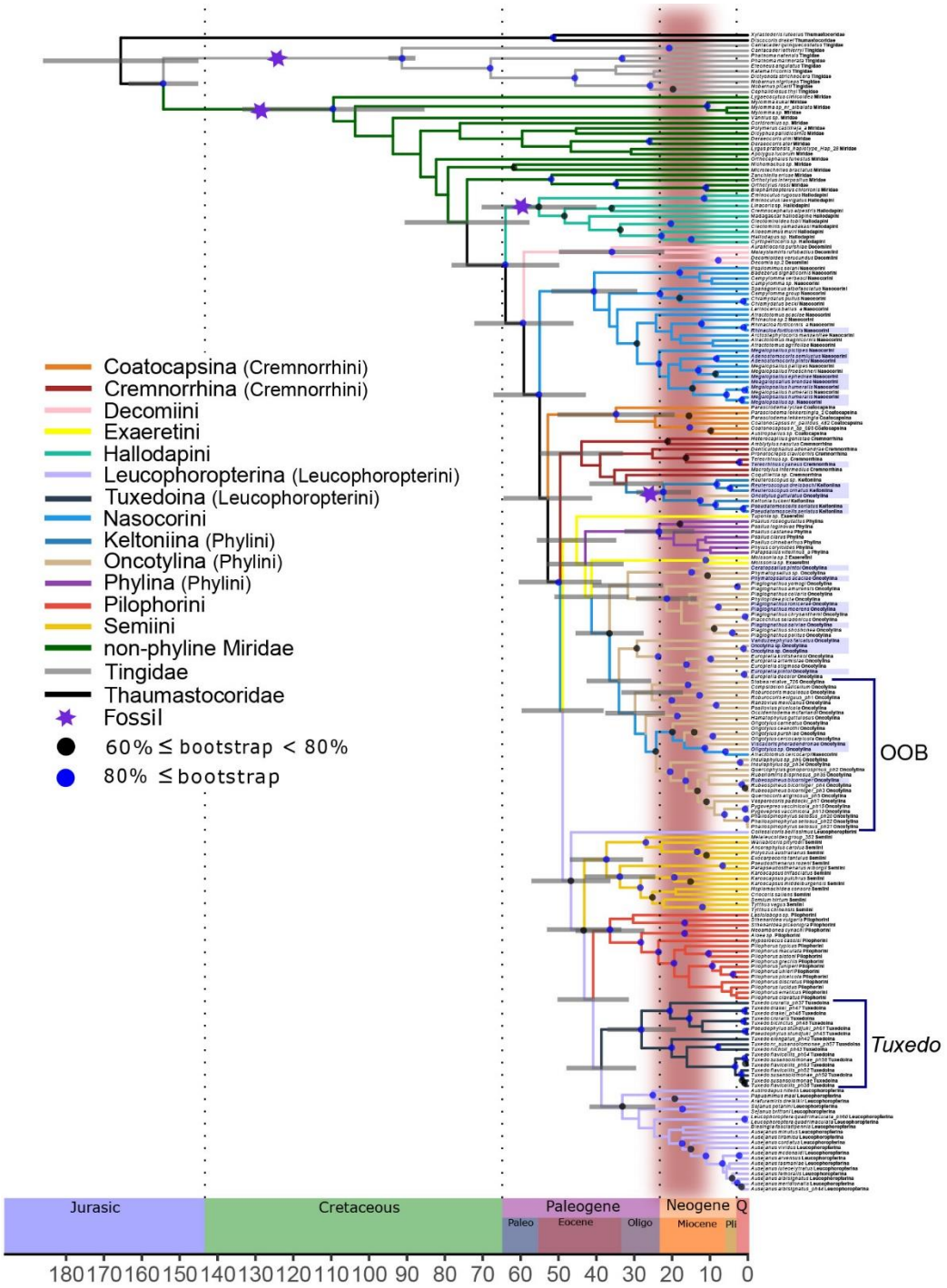
Coatocapsina (Cremnorrhini)
Cremnorrhina (Cremnorrhini)
Decomiini
Exaeretini
Hallodapini
Leucophoropterina (Leucophoropterini)
Tuxedoina (Leucophoropterini)
Nasocorini
Keltoniina (Phylini)
Oncotylina (Phylini)
Phylina (Phylini)
Pilophorini
Semiini
non-phyline Miridae
Tingidae
Thaumastocoridae
Fossil
60% ≤ bootstrap < 80%
80% ≤ bootstrap
OOB
Tuxedo
Jurassic
Cretaceous
Paleogene
Neogene
Q
Paleo
Eocene
Oligo
Miocene
Pli
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0

Fig. 10: Estimated divergence times of Phylinae. Results of Beast analysis with a the fixed RAxML tree. Grey bars denote 95% posterior probability at major nodes. Red bar is approximately the diversification of *Quercus* in Southern California (Hipp et al. 2017). Blue and black node dots represent bootstrap from the RAxML tree. Purple stars represent fossil calibration points. Names highlighted in lavender were sequenced and added in this study. Paleo= Paleocene, Oligo= Oligocene, Pli= Pleistocene, Q= Quaternary period.

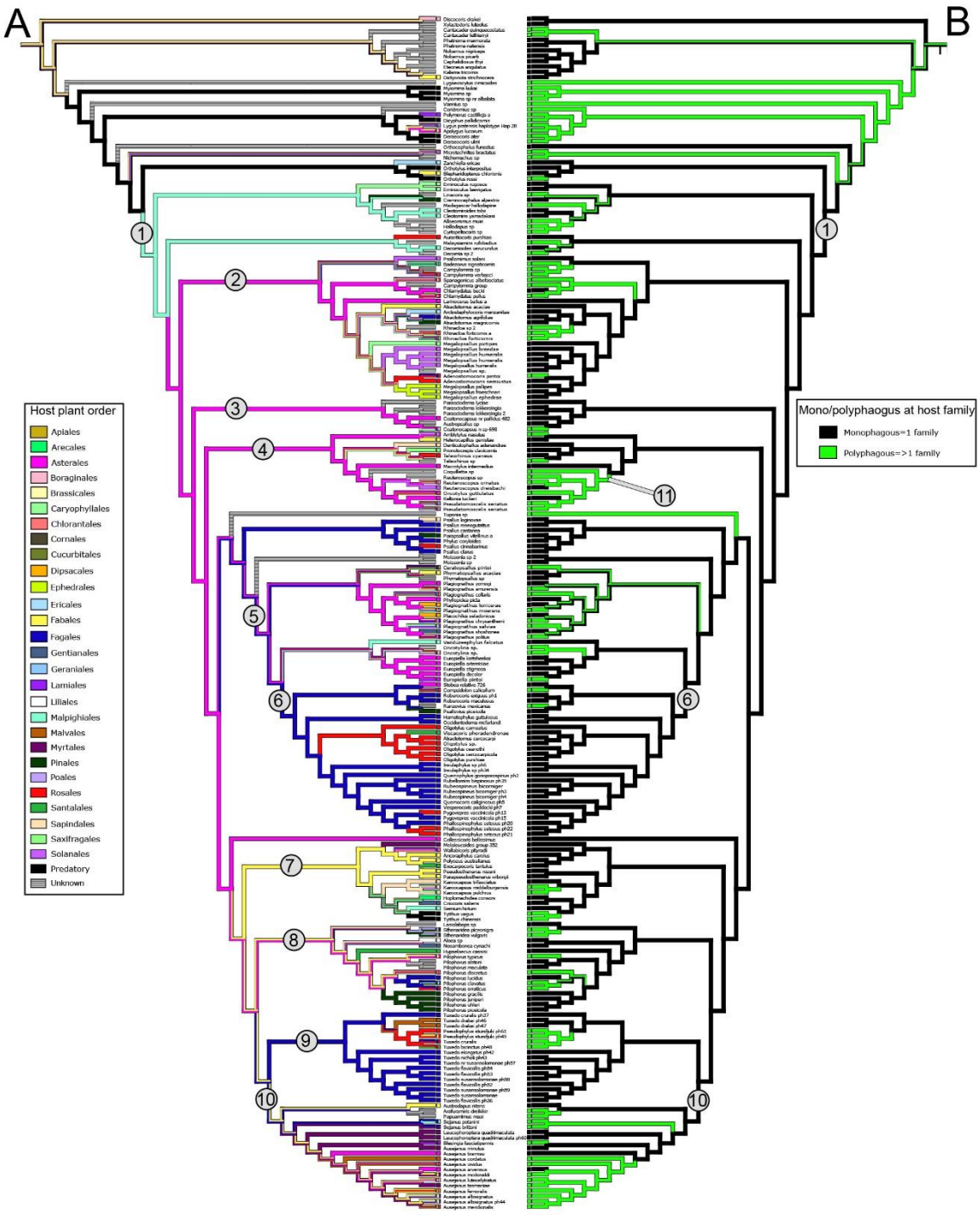

Fig. 11: Ancestral state reconstruction of hosts on the maximum likelihood tree. A) Parsimony reconstruction of host plants at the order level. B) Likelihood reconstruction of states as monophagous (feeding on one host family) or polyphagous (feeding on more than one host family). These trees are not correlated.