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Ultraconserved elements reconstruct the evolution of Chagas disease-vectoring kissing bugs (Reduviidae: Triatominae)

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Abstract. Triatominae are the largest radiation of haematophagous species within the true bugs (Hemiptera) and the sole vectors of *Trypanosoma cruzi* Chagas, the causative agent of Chagas disease, a neglected human disease that affects millions. We used a combined ultraconserved element (UCE) and ribosomal dataset from ethanol-preserved and pinned specimens in a museomics approach to elucidate the phylogenetic relationships among triatomines, including taxa of four of the five tribes with substantial representation of the Old World fauna. We conclude that: (i) Triatominae are monophyletic and *Opisthacidius* Berg is their predatory sister taxon; (ii) the three large genera (*Rhodnius* Stål, *Triatoma* Laporte and *Panstrongylus* Berg) are not monophyletic; (iii) Triatomini fall into nine well-supported clades, only two of which are identical in composition to previously recognized groups; and (iv) the Old World clade is deeply nested within Triatomini. Ancestral character state reconstructions of specific character traits provide insights into the evolutionary history of Triatominae.

Introduction

The haematophagous Triatominae are reputedly the most infamous and destructive group of Heteroptera, the true bugs (Hemiptera) (Schuh & Slater, 1995). Also known as kissing bugs, Triatominae comprise about 150 extant species that are all capable of transmitting *Trypanosoma cruzi* Chagas to mammals including humans (Schofield & Galvão, 2009; Bargues *et al.*, 2017; Monteiro *et al.*, 2018). This protozoan blood parasite causes Chagas disease in humans, one of the major neglected tropical diseases affecting most Latin American countries (WHO, 2010, 2015), which was recently acknowledged as an emerging disease in southern U.S.A. (Garcia *et al.*, 2014). Despite successful control programs in many countries, Chagas disease is not only a concern in endemic areas, but due to increased mobility of humans around the globe is now considered a worldwide public health problem (Hotez *et al.*, 2012; Lidani *et al.*, 2019). Due to their epidemiological importance, kissing bug genetics, physiology, biology and ecology have been extensively investigated during the past century, ranging from studies on trypanosome-triatomine interactions (Melo *et al.*, 2020), microbial associates that support their haematophagous diet (Rodríguez-Ruano *et al.*, 2018), chemical ecology (May-Concha *et al.*, 2018) and niche evolution (Ceccarelli *et al.*, 2020). Although there is now substantial knowledge on the biology and ecology of the major human disease vectors (Ibarra-Cerdeña *et al.*, 2009, 2014), many of the minor vectors remain poorly understood, and insights into the ecology of many kissing bug species in their natural environments are limited (Georgieva *et al.*, 2017). Importantly, a robust and comprehensive phylogeny across Triatominae is still

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unavailable, hindering studies underpinned by the need for an evolutionary framework.

Triatominae are classified into five tribes with 15 genera (Schofield & Galvão, 2009; Justi *et al.*, 2016; Bargues *et al.*, 2017; Justi & Galvão, 2017). They represent one of the smaller subfamilies of assassin bugs (Reduviidae), which itself represents the largest nonphytophagous clade of Hemiptera with approximately 7000 described species worldwide (Putshkov & Putshkov, 1985; Maldonado Capriles, 1990; Schuh & Slater, 1995; Weirauch *et al.*, 2014). Triatominae are most species-rich in the Neotropical region, with only 17 species recorded from the Nearctic and 14 from tropical areas of the Old World (Gorla *et al.*, 1997; Hypsa *et al.*, 2002; Ramsey *et al.*, 2015). Reduviidae are typically predators of other insects and arthropods that they capture using an astonishing diversity of predatory behaviours (Zhang *et al.*, 2016). The evolutionary origin and timing by ancestral Triatominae from arthropod predation to mammalian blood-feeding remain controversial, with phylogenetic relationships among (and within) the five tribes poorly understood (Otálora-Luna, 2015; Bargues *et al.*, 2017; Monteiro *et al.*, 2018; Weirauch, 2021).

Triatominae were traditionally assumed to be monophyletic (Usinger, 1943, 1944; Lent & Wygodzinsky, 1979) and this hypothesis is supported by some modern phylogenetic analyses (Hypsa *et al.*, 2002; Justi *et al.*, 2016; Georgieva *et al.*, 2017), whereas others found the group to be paraphyletic (Hwang & Weirauch, 2012; Zhang *et al.*, 2016) or polyphyletic (Schofield & Dolling, 1993; Schofield, 2000; de Paula *et al.*, 2005; Schofield & Galvão, 2009; Bargues *et al.*, 2017) (reviewed by Monteiro *et al.*, 2018 and Weirauch, 2021). The polyphyly hypothesis has lost favour recently and all Triatominae have been shown to belong to a clade of Reduviidae that also comprises Stenopodainae and the *Zelurus* Hahn clade of Reduviinae. Still, these datasets were ill-suited to elucidate Triatominae monophyly relative to the *Zelurus* group, specifically the genus *Opisthacidius* Berg and potentially closely related genera. The close relationship between *Opisthacidius* and Triatominae is intriguing, because while species of *Opisthacidius* are predators based on the structure of their mouthparts, at least one species is known to live in nests of the red-rumped cacique (Icteridae) (Lent & Wygodzinsky, 1956). This suggests that the common ancestor of *Opisthacidius* and Triatominae may have been associated with vertebrate nests (Hwang & Weirauch, 2012), corroborating a hypothesis by Schofield and colleagues that envisioned the transition to haematophagy by Triatominae was derived originally from free-living to nest-dwelling predators and then to vertebrate feeding nest-dwellers (Schofield & Dujardin, 1999).

Although several studies published during the past two decades have addressed aspects of phylogenetic relationships within Triatominae, most were limited in taxonomic scope and sampling, or were based on relatively small molecular or morphological datasets. Much of this research focused on species-level relationships within species groups or genera (Monteiro *et al.*, 2000; Marcilla *et al.*, 2002; Tartarotti *et al.*, 2006), and few studies have investigated tribal (de Paula *et al.*, 2007; Gil-Santana, 2014; Justi *et al.*, 2014) or subfamilial relationships (Hypsa *et al.*, 2002; de Paula *et al.*, 2005; Justi *et al.*, 2016; Georgieva *et al.*, 2017). All previous studies have relied either on morphology (Gil-Santana, 2014) or small numbers of mitochondrial (e.g., COI, CytB, 16S, 12S) and/or nuclear (e.g., ITS, 18S, 28S) genetic loci. Nevertheless, these studies have advanced our understanding of evolutionary relationships of Triatominae beyond the early hypotheses by Lent & Wygodzinsky (1979). For instance, none of the three large and epidemiologically important genera *Rhodnius* Stål, *Panstrongylus* Berg and *Triatoma* Laporte are monophyletic. *Rhodnius* is known to be rendered paraphyletic by *Psammolestes* Bergroth and several species of *Panstrongylus* are more closely related to *Triatoma* species than the broader *Panstrongylus* (Justi *et al.*, 2016). In addition, the large genus *Triatoma* is paraphyletic with respect to most other genera of Triatomini (*Dipetalogaster* Usinger, *Eratyrus* Stål, *Linshcosteus* Distant, *Paratriatoma* Barber, in addition to several genera recognized by some authors, but not others: *Meccus* Stål, *Mepraia* Mazza, Gajardo & Joerg, and *Nesotriatoma* Usinger) (Justi *et al.*, 2016; Georgieva *et al.*, 2017). Although some authors have doubted that Rhodniini and Triatomini are monophyletic, there is currently no evidence for these hypotheses (see Monteiro *et al.*, 2000; Justi *et al.*, 2014). However, because of suboptimal taxon sampling that has largely omitted representatives of the three small tribes Alberproseniini, Bolboderini and Cavernicolini, published analyses have failed to provide stringent tests of these hypotheses. Within Triatomini, the *dispar* group (*sensu* Justi *et al.*, 2016) has been recovered as sister to all remaining Triatomini, the species-rich *infestans* group appears to comprise a number of mostly nonmonophyletic complexes and subcomplexes, and likewise, the monophyly of complexes and subcomplexes within the *rubrofasciata* group is questionable (Justi *et al.*, 2016; Georgieva *et al.*, 2017). Most species groups within *Triatoma* are based on concepts by Usinger and co-authors (Usinger, 1944; Usinger *et al.*, 1966) that were subsequently expanded to accommodate species outside of the Nearctic and Caribbean regions by Lent & Wygodzinsky (1979) and others. The current composition of species groups, complexes and subcomplexes was summarized by Schofield & Galvão (2009), Dujardin & Schofield (2004) and Justi & Galvão (2017). However, most of these groups were poorly characterized from the start, and little progress has been made since to provide morphologically diagnostic features for many of them. In addition, species groups in *Triatoma* are still applied under the assumption of *Triatoma* monophyly and have thus rarely considered species in related genera (e.g., *Paratriatoma*, *Dipetalogaster*). While beyond the scope of the present study, a comparative assessment of morphological characters interpreted using the robust phylogenetic framework presented here will be critical to better align the classification of Triatomini with their evolutionary history.

Although the aforementioned hypotheses are fairly well corroborated by published datasets, others are less so, mostly because of insufficient taxon sampling and/or low branch support. Both Cavernicolini and Bolboderini have been recovered as close relatives of Rhodniini (Patterson & Gaunt, 2010; Justi *et al.*, 2016; Zhang *et al.*, 2016; Georgieva *et al.*, 2017), but their exact relationships are uncertain. Also, although Bolboderini are monophyletic based on morphology-based analyses

(Gil-Santana, 2014), this hypothesis remains to be tested using molecular datasets. In addition, *Rhodnius* and *Psammolestes* are consistently found to form three well-supported, monophyletic species groups (*pictipes*, *pallescens* and *prolixus* + *Psammolestes* groups), but relationships among them remain unresolved (Monteiro *et al.*, 2000; de Paula *et al.*, 2005, 2007; Justi *et al.*, 2016; Georgieva *et al.*, 2017). Within Triatomini, relationships beyond the divergence of the *dispar* group are poorly understood and many are weakly supported in published analyses. Open questions include the monophyly and phylogenetic position of the *rubrofasciata* and *infestans* groups as recognized by Justi & Galvão (2017), as well as the monophyly of, and relationships among, species complexes and subcomplexes. Of particular interest is the assumed monophyly of the 'Old World group' of Triatomini, which if corroborated, would imply a single dispersal event from the New World to the Old World (Schofield, 2000; Hypsa *et al.*, 2002; Aguilera-Uribe *et al.*, 2020). This group comprises seven described species of *Triatoma* that are endemic to the Oriental and Australian regions, the circumtropical *Triatoma rubrofasciata* (De Geer) and the small genus *Linshcosteus* Distant that is confined to the Indian subcontinent. Although this Old World group has long been assumed to be monophyletic (Gorla *et al.*, 1997), phylogenetic tests of this hypothesis have so far only included representatives of *Linshcosteus*, the circumtropical *T. rubrofasciata* and a single species of endemic Oriental *Triatoma*, *Triatoma migrans* Breddin (Hypsa *et al.*, 2002; Aguilera-Uribe *et al.*, 2020).

Using the most comprehensive dataset assembled to date with respect to both data (ultraconserved elements (UCE) + ribosomal (rDNA) loci) and taxonomic coverage (~106 ingroup species, representing ~71% of the described species-level diversity), we present a topologically robust and densely sampled phylogeny for Triatominae. Using DNA sequence data from UCE and ribosomal loci, we evaluate established tribes, genera and species groups, and examine aspects of the evolutionary history of Triatominae by reconstructing morphological and colour features with potential adaptive significance.

Material and methods

Exemplar sampling

Two datasets were generated, one consisting of the 74 terminals (69 ingroup and 5 outgroups) for which UCE data were obtained (dataset 1), the other combining UCE for these specimens with existing and newly acquired bycatch rRNA ribosomal data (dataset 2) for a total of 176 terminals (169 ingroup and 25 outgroup taxa). This dataset includes 142 newly collected ribosomal sequences and data obtained for the first time from historical specimens collected as long ago as 1931 of rarely collected species (Tables S1, S3, S4). The two datasets include 52 and 106 putative species of Triatominae, respectively. Voucher information for exemplars, including voucher depositories, is presented as Table S1. Specimens were identified using a combination of taxonomic keys (Lent & Wygodzinsky, 1979) and authoritatively identified specimens deposited in major natural history museums. Specimens derived from the Weirauch lab were imaged and databased using the PBI instance of the Arthropod Easy Capture database.

DNA amplification and sequencing

We extracted DNA from 70 specimens representing 12 genera and 53 species that were recently collected or were deposited in museum collections (Table S1). We used either a Qiagen DNeasy kit, Qiagen QIAquick PCR Clean Up kit, or a Phenol Chloroform Isoamyl extraction method (Table S3) for all samples. The concentration and quality of DNA were assessed on a Qubit 2.0, fragment analyser and a 1.5% agarose gel. Samples with intact, high molecular weight were fragmented on a Bioruptor UCD-300 sonication device (Diagenode Inc., Denville, NJ, USA). We varied the number of cycles of 30 s on/30 s off from 2 to 9 based on the DNA quality. Fragmented DNA was run on a 1.5% agarose gel showing a size range from 200 to 1000 bp.

Libraries were prepped following Kieran *et al.* (2019). Briefly, we used a KAPA Hyper Prep Kit (Kapa Biosystems, Inc., Wilmington, MA, USA) with Universal TruSeq compatible adaptor stubs and Illumina TruSeq compatible dual-indexed primers with modified 8 bp indexes (Glenn *et al.*, 2019) to construct the libraries. Post-PCR product was cleaned using Sera-Mag magnetic beads (Thermo-Scientific, Waltham, MA, U.S.A.) and quantified with Qubit 2.0. The samples were combined in equimolar amounts based on library size, quality and taxonomic relatedness, for 11–17 samples per 500 ng pool. Pools of libraries were then enriched using previously designed (Faircloth, 2017) and tested (Kieran *et al.*, 2019) Hemiptera v1 UCE baits. We used a myBaits kit (Arbor Biosciences, Ann Arbor, MI, USA) following the manufacturer's protocol. Enriched libraries were sequenced on an Illumina HiSeq 3000 using paired-end 150 bp reads (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA).

Sequence processing

Sequenced data were processed using PHYLUCE v1.6.1 (Faircloth, 2016) incorporated software. Adaptors and low-quality bases were removed using Illumiprocessor (https://github.com/faircloth-lab/illumiprocessor). Reads were assembled using Trinity v1 r20140717 (Grabherr *et al.*, 2011). Raw reads from each UCE capture dataset were searched using the program MITObim v.1.9.1 (Hahn *et al.*, 2013) to map reads to 16S mitochondrial rRNA, 18S and 28S nuclear rRNA bait sequences from a representative species of Triatominae as in Simon *et al.* (2019). The resulting contigs were aligned using the MAFFT v.7 E-INS-i algorithm (Katoh *et al.*, 2018) and manual trimming of apparent misassembled or duplicated segments was conducted before realignment. We also removed any sequences that showed evidence of index-swapping or cross-contamination based on individual genes trees constructed using RAxML v.8 (Stamatakis, 2014) on the CIPRES web server (Miller *et al.*, 2012). NCBI Sequence Read Archive accession numbers are provided in Table S3.

Sequence alignment and phylogenetic analyses

We aligned UCE loci using MAFFT (Katoh & Standley, 2013), changing the max divergence to 40% and trimmed with GBLOCKS (Castresana, 2000; Talavera & Castresana, 2007). We created 60 and 85% data matrices for downstream analyses. As previously performed (Kieran *et al.*, 2019) we incorporated UCE loci from transcriptome data for nontriatomine reduviids for species where a transcriptome was available (Table S1). We performed a maximum likelihood (ML) phylogenetic analysis using RAxML v8.1.20 (Stamatakis, 2014) with the 60 and 85% UCE data matrices. We used the best of 20 ML trees, followed by 100 bootstrap replicates, using the GTRGAMMA model on concatenated loci (60%, 85% matrix), and partitioned by locus (85% matrix). Partitions were determined using SWSC-EN (Tagliacollo & Lanfear, 2018) for UCE data and PartitionFinder2 (Lanfear *et al.*, 2017). Using all recovered UCE loci, we performed a genetree analysis using ASTRAL-III v5.6.1 (Zhang *et al.*, 2018). To extend taxonomic coverage for a densely sampled phylogenetic hypothesis, we added 16S rDNA, 18S rDNA and 28S rDNA from GenBank (Table S1, Table S) and extracted from our UCE enrichment data to the 85% UCE matrix and performed RAxML partitioned and unpartitioned analyses following the procedures outlined above. We also performed a Bayesian analysis with MrBayes v3.2.6 (Ronquist *et al.*, 2012), using partition results from PartitionFinder2. We conducted two independent runs using default settings of one cold and three heated chains for 4 million Markov chain Monte Carlo (MCMC) generations sampling every 1000 with 25% burnin. Alignment files, scripts and configuration files are available from Dryad (https://doi.org/10.5061/dryad.37pvmcvh4).

Ancestral character state reconstruction of selected morphological characters

We reconstructed and visualized transitions for eight morphological features including traits that may be involved in antipredator strategies based on the ML tree derived from the partitioned analysis that includes 106 species of Triatominae. The dorsal abdominal glands (DAG) 1–3 are the main antipredator or defensive glands of immature true bugs (Schuh & Slater, 1995), but are absent in Triatominae. The loss of DAG 3 (categorical character #2) was previously recovered as a synapomorphy for Triatominae and relatives (Weirauch, 2008) and we here test this hypothesis. It is unclear if the loss of DAGs 1 and 2 also predates the evolution of Triatominae or if it occurred in the MRCA of Triatominae (categorical character #1). The metatarsal brush (categorical character #3) that occurs in first instar immatures of some, but not all Triatominae, is associated with camouflaging behaviours (Zeledón *et al.*, 1973; Weirauch, 2006a) and likely represents another antipredatory strategy. Many predatory Reduviidae feature a tibial attachment structure on the fore- and mid-legs, the fossula spongiosa, that is used during prey capture (Zhang *et al.*, 2016). Many Triatominae also have a fossula spongiosa, but it is usually better developed in males than in females and is thought to be used during mating (Lent & Wygodzinsky, 1979) or locomotion (Miller, 1942). The presence and absence of the fossula spongiosa were here optimized for males (categorical characters #4 and 5) to investigate transitions across Triatominae. Triatominae show an array of colour patterns ranging from uniform and potentially cryptic, to contrasting and putatively aposematic (Fig. 1). To explore colour pattern evolution across Triatominae, we encoded the colour pattern of the corium (categorical character #6). To investigate overall body length and shape evolution across Triatominae, we coded total body length and the ratio of greatest abdominal width to total length as a proxy for body shape (continuous characters #1 and #2).

Character descriptions and matrices for the continuous (Matrix 1) and categorical (Matrix 2) characters are provided as Files S1 and S2. See Table S7 for measurements and ratios for continuous characters. Measurements (males only) are from the literature (Table S7; see references). Where ranges were provided in the original source, we used the median as a proxy for our reconstructions. Several ingroup taxa were coded as not applicable (NA) in Table S7; we were unable to deduce measurements and ratios for these taxa from the literature, males are unknown, or the original description or voucher specimens were unavailable. Ancestral character state reconstructions are based on the UCE+ ribosomal dataset and were performed in Mesquite v 3.61 (Maddison & Maddison, 2019) using the parsimony criterion (minimizing the number of steps of character change given the tree and the data) for both categorical and continuous characters as outlined in http://www.mesquiteproject.org. Specifically, we used the options of 'trace', 'reconstruct ancestral states' and 'parsimony ancestral states'.

Results

UCE recovery

We generated an average of 4 242 725 raw paired-end reads per sample with 89.23% passing filter (Table S3) and recovered a total of 2544 UCE loci with a range of 273–1943 per sample (mean = 1470, median = 1613.5) with an average of 44.65% on-target. A total of 1539 and 341 UCE loci per 60 and 85% data matrix were obtained, respectively (Table S6). The average amount of missing data for the 85% matrix was half (11.74%) of the 60% matrix (22.12%) (Table S3).

Phylogenetic hypotheses

The UCE-only and UCE + ribosomal 60 and 85% matrices as well as partitioned and unpartitioned analyses using RAxML of the concatenated matrix, ASTRAL gene tree approaches and MrBayes analyses produced overall consistent topologies

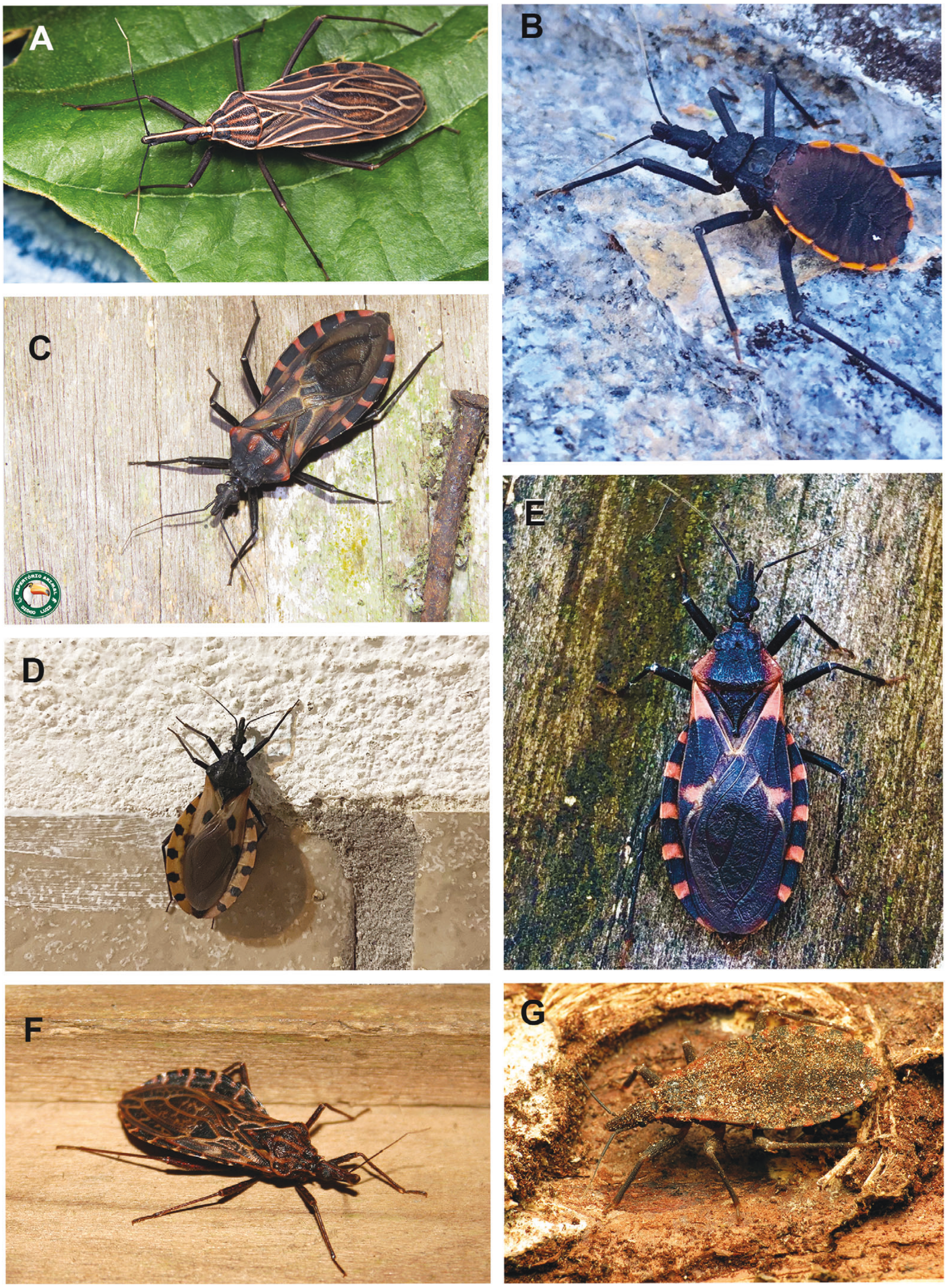

Fig. 1. (A) *Rhodnius barretti* (photo by Eerika Schulz); (B) *Dipetalogaster maximus* (photo by Bill Levine); (C) *Panstrongylus megistus* (photo by Diego Luiz); (D) *Triatoma dimidiata* (photo by Carlos N. Ibarra-Cerdeña); (E) *Triatoma sanguisuga* (photo by James O. Palmer); (F) *Triatoma dispar* (photo by William Flaxington); (G) *Triatoma sanguisuga*, partially camouflaged immature (photo by Katja Schulz). [Colour figure can be viewed at wileyonlinelibrary.com]

and support values (Table S2; Figs 2, 3, S1–S6). Although the majority of nodes were supported in all or most analyses, mostly with absolute or near-absolute support values, several conflicting or less-well-supported nodes suggest that a small number of deeper-level relationships within Triatominae will require further testing using expanded datasets.

All but one of the analyses recovered Triatominae as monophyletic with high or absolute branch support values (Table S2).

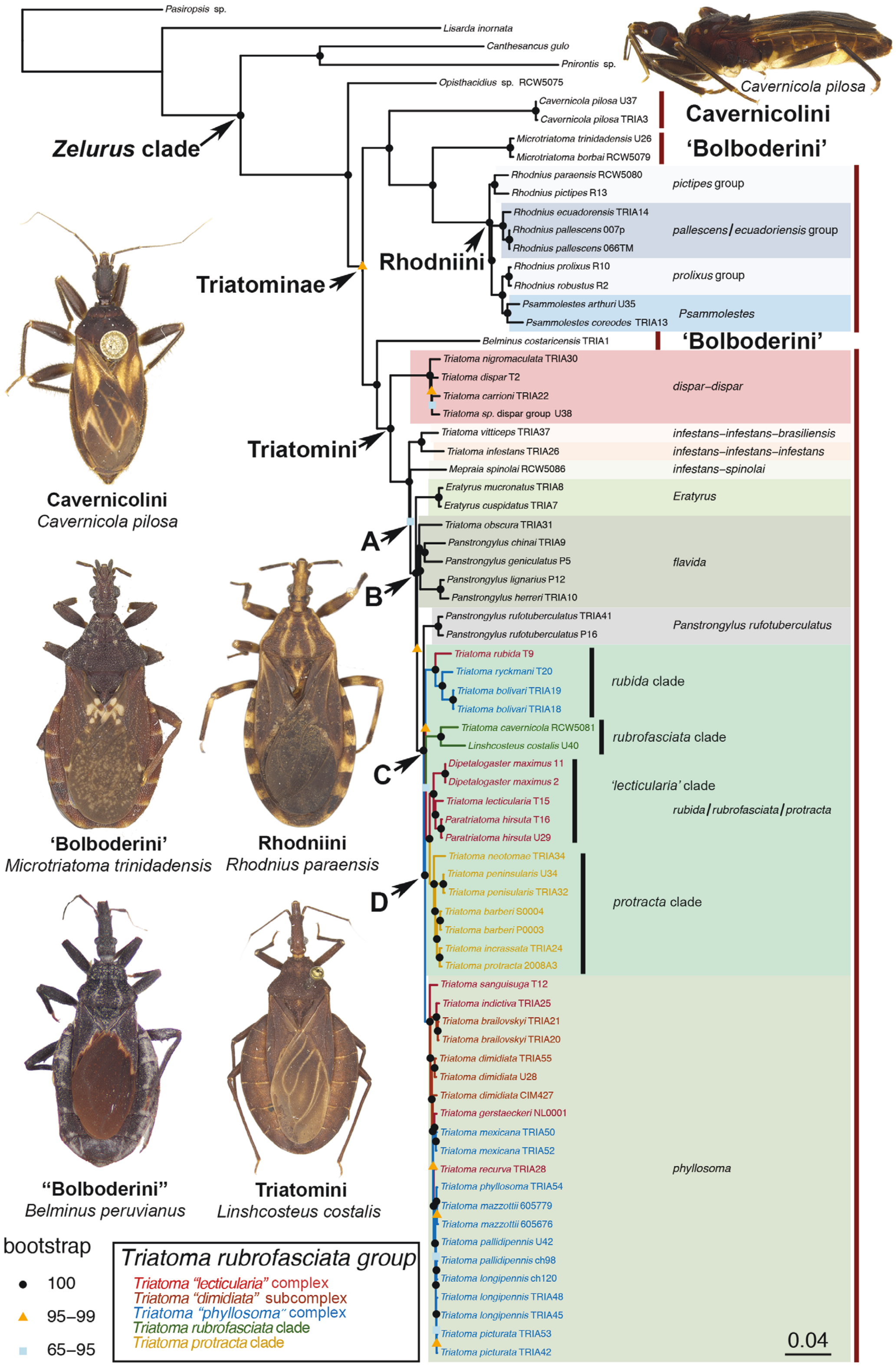

Fig. 2. Phylogenetic hypothesis of Triatominae based on the best likelihood tree derived from the partitioned analysis of the 85% matrix of the UCE-only dataset. Habitus photographs show selected voucher specimens. The classification follows Schofield & Galvão (2009) and Justi & Galvão (2017). Names on the right side for Rhodniini and Triatomini refer to species groups, complex and subcomplexes (i.e., group–complex–subcomplex), unless indicated otherwise, capital letters next to nodes are explained in the Figure 3 legend. [Colour figure can be viewed at wileyonlinelibrary.com]

The reduviine *Opisthacidius* was recovered as the sister taxon to Triatominae with similarly high support in all analyses except one. In the single conflicting topology (ASTRAL; Fig. S3), *Opisthacidius* was inferred to be the sister to the Cavernicolini + *Microtriatoma* + Rhodniini clade, rendering Triatominae paraphyletic.

In all eight analyses, Triatominae were recovered a well-supported dichotomy between (Cavernicolini (*Microtriatoma* ['Bolboderini'] + Rhodniini)) and (*Belminus* Stål ['Bolboderini'] + Triatomini); both subclades also recovered with strong support. 'Bolboderini', represented by three species in two genera, were polyphyletic, whereas Cavernicolini (100%), Rhodniini (100%) and Triatomini (five analyses with 100%; three remaining with 96, 94 and 50%) were monophyletic. Within the (Cavernicolini (*Microtriatoma* ['Bolboderini'] + Rhodniini)) clade, *Psammolestes* rendered *Rhodnius* paraphyletic in all analyses and was recovered as sister taxon to or nested within the *prolixus* group of *Rhodnius*. The three previously recognized species groups of Rhodniini were recovered in all analyses, receiving full support in the UCE-only and UCE + ribosomal MrBayes analyses, with support values in the high 90% in the remaining analyses. The *pallescens* and *prolixus* groups were strongly supported as sister taxa in all analyses (five with 100%, remaining 97–99%), with the *pictipes* group as sister lineage to that clade.

Within Triatomini, we consistently recovered nine fully or highly supported clades (*dispar*, *infestans*, *spinolai*, *flavida*, *phyllosoma*, *rubida*, *rubrofasciata* and *protracta* clades plus *Eratyrus*). Among these are the *dispar* clade (all 100%) that corresponds to the *dispar* group of Schofield & Galvão (2009). Although most *infestans* group species formed a moderately well-supported clade in two of the UCE + ribosomal analyses (80 and 82%; only two taxa included in the UCE-only analyses, all with 100%), the *spinolai* complex of the *infestans* group was always a separate lineage from our *infestans* clade and *Triatoma tibiamaculata* (Pinto) (*brasiliensis* subcomplex of *infestans* group and complex) was recovered as part of what we refer to as *flavida* clade that also comprises most *Panstrongylus* spp. Only the *infestans* complex was monophyletic, whereas all remaining subcomplexes (*brasiliensis*, *rubrovaria*, *sordida*, *matogrossensis* and *maculata*) are paraphyletic or polyphyletic (Fig. 3).

The *spinolai* complex, sometimes also treated as the separate genus *Mepraia*, was recovered as monophyletic in the UCE + ribosomal analyses (88–100%; only one species included in the UCE-only analysis). We refer to the monophyletic group comprising the *spinolai* clade and all remaining Triatominae as clade A (Fig. 2). The two species of *Eratyrus* included in all analyses were always fully supported sister taxa, and together with the remaining Triatomini represent clade B (Fig. 2). All species of *Panstrongylus* Berg except *Panstrongylus rufotuberculatus* (Champion) were recovered in a clade with species of the *flavida* group (sometimes treated as the separate genus *Nesotriatoma*) in both UCE-only and UCE + ribosomal analyses; in the UCE + ribosomal analyses, this clade in addition included *T. tibiamaculata* (*infestans* group). We refer to this monophyletic group as the *flavida* clade; it was recovered with full support in all UCE-only analyses with values in the UCE + ribosomal analyses ranging between 76 and 100%. *Panstrongylus rufotuberculatus* was excluded from this clade in all analyses and always represented the sister to the *phyllosoma*, *protracta*, *rubrofasciata* and *rubida* clades (100% in UCE-only, 93–100% in UCE + ribosomal analyses); we refer to the five lineages together as clade C (Fig. 2).

The remaining four clades (clade D; Figs 2, 3) were fully supported in almost all analyses (see Table S2). Our *phyllosoma* clade (fully supported in all analyses) combined most species of the *lecticularia* and *phyllosoma* complexes, neither of which was monophyletic in our analyses, but excluded *Triatoma bolivari* Carcavallo, Martinez & Pelaez, *Triatoma rubida* (Uhler) and *T. ryckmani* Zeledon & Ponce. We refer to these three species together as *rubida* clade (fully supported in seven analyses, one with 99%). Our clade E comprised the *rubida* + *protracta* + *rubrofasciata* clades (Figs 2, 3).

The *rubrofasciata* complex *sensu* Schofield & Galvão (2009) that we here refer to as *rubrofasciata* clade was fully supported in all but two analyses, which received values of 97 and 98%; this clade comprised the two (UCE + ribosomal) or one (UCE-only) included species of *Linshcosteus* Distant, the cosmopolitan *Triatoma rubrofasciata* and the three (UCE + ribosomal) or one (UCE-only) Old World endemic species of *Triatoma* included in our analyses. Support for the *protracta* clade was similar (full for six analyses, 98 and 99% for the remaining two); in addition to species of the *protracta* complex *sensu* Schofield & Galvão (2009), it included *Dipetalogaster maximus* (Uhler), *Paratriatoma hirsuta* Barber and two species classified in the *lecticularia* complex, *Triatoma indictiva* Neiva and *Triatoma lecticularia* (Stål).

Although the *protracta* + *rubrofasciata* clade received full support in two of the analyses (Table S2), others did not recover this clade, or only with low support. The *rubida* + *protracta* + *rubrofasciata* clade was overall much better supported (between 80 and 100%) but was not recovered in the ASTRAL analysis. Similarly, although clade B, comprising *Eratyrus*, the *flavida* clade, *P. rufotuberculatus* and the *rubrofasciata* group, was highly or fully supported in most analyses, it was not inferred in the ASTRAL analysis. Even less well supported was clade A that included the above-mentioned clade plus the *spinolai* clade that was found in only five of the eight analyses, all with low to moderate support.

Ancestral character state reconstructions

Evolution of body length and shape. Parsimony reconstruction of male body length on the preferred UCE + ribosomal topology (Fig. S7; Files S1 and S2) inferred that the ancestor of Triatominae was of medium size (~18 mm) and that body size along the backbone of the phylogeny remained relatively stable, ranging between ~20 and 26 mm. Small body size (15 mm or less; blue shades) evolved several times independently, namely in *Cavernicola* (~11 mm in the most recent common ancestor [MRCA] of included species of that genus), *Microtriatoma*

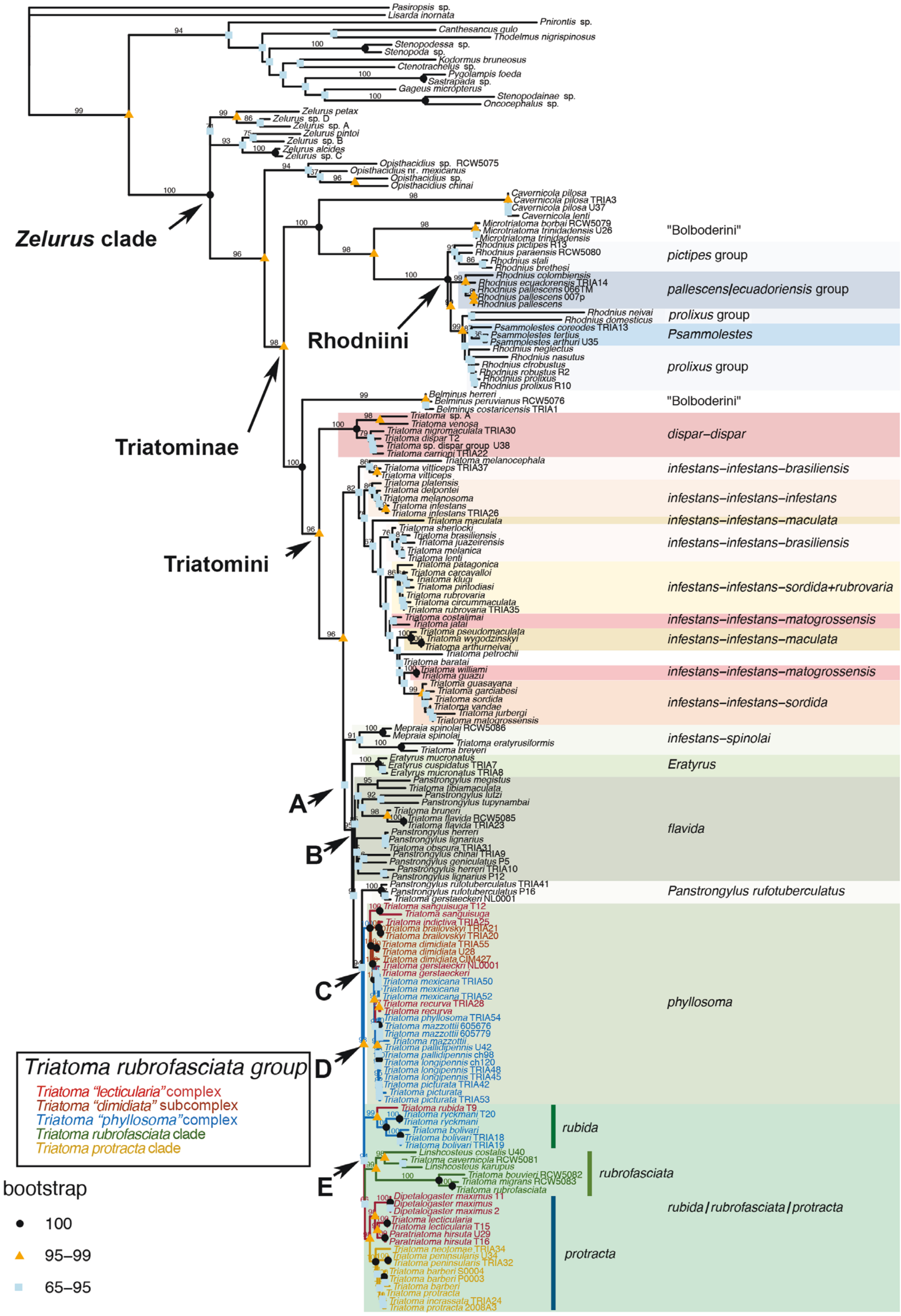

Fig. 3. Phylogenetic hypothesis of Triatominae based on the best likelihood tree derived from the partitioned analysis of the 85% matrix of the combined UCE and ribosomal dataset. Groups/complexes/subcomplexes are after Schofield & Galvão (2009) and Justi & Galvão (2017). Clade A: *spinolai* clade + *Eratyrus* + *flavida* clade + *P. rufotuberculatus* + *phyllosoma* clade + *rubida* clade + *protracta* clade + *rubrofasciata* clade; Clade B: Clade A less *spinolai* clade; Clade C: *P. rufotuberculatus* + *phyllosoma* clade + *rubida* clade + *protracta* clade + *rubrofasciata* clade; Clade D: Clade C less *P. rufotuberculatus*. Clade E: *rubida* clade + *protracta* clade + *rubrofasciata* clade. Names on the right side for Rhodniini and Triatomini refer to species groups, complex and subcomplexes (i.e., group–complex–subcomplex), unless indicated otherwise. [Colour figure can be viewed at wileyonlinelibrary.com]

(~8 mm in MRCA) and *Psammolestes* (~15 mm in MRCA) among the Rhodniini and relatives and in *Belminus* (~8.5 mm in MRCA), *Triatoma peninsularis* (~11 mm in MRCA) and *P. hirsuta* (~13.2 mm in MRCA) among Triatomini and allies. Very large body size (>30 mm; red shades) evolved independently in *D. maximus* and part of the *phyllosoma* clade. Body shape (abdominal width to total length) in males is fairly conserved in Triatominae (Fig. S7). More slender body shapes are prevalent in *Rhodnius*, the *dispar* clade of Triatomini, some species within the *infestans* clade, the *spinolai* clade, *Eratyrus* and within the *rubrofasciata* and *protracta* clades.

Evolution of antipredator features, fossula spongiosa and colour pattern. Reconstructions for categorical characters are provided in Files S1–S3. Figure 4 shows reconstructions of the fossula spongiosa (character #4). The loss of dorsal abdominal glands (DAGs) 1 and 2 (character #1) is a previously unrecognized morphological synapomorphy of *Opisthacidius* + Triatominae, whereas DAG 3 was already lost in the MRCA of the clade including Triatominae, Stenopodainae and the *Zelurus* group of Reduviinae (character #2). We reconstructed a metatarsal brush to have evolved in the MRCA of Triatomini, with subsequent losses in part of the *protracta* clade, in *T. sanguisuga* and *T. indictiva* (character #3). Reconstructions of a fossula spongiosa on fore- and mid-legs (character #4; Fig. 4) and hind legs (character #5) of males indicate that a fore- and mid-leg fossula spongiosa was present in the MRCA of Triatominae, with between eight and 12 losses within the subfamily, and that the rare hind leg fossula is restricted to part of ‘Bolboderini’ (*Microtriatoma*). Corial colour patterns in Triatominae were shaped by frequent evolutionary transitions (character #6; Fig. S8). Our reconstructions suggest that the MRCA of Triatominae had a corium that featured either large contrasting patches or was dark with small pale patches. Although patterns along the backbone of Triatominae are fairly conserved (e.g., corium with large contrasting patches), they are homoplastic overall, with multiple transitions between most states.

Discussion

We here present the first robust phylogenetic hypothesis for Triatominae. Although several of the well-supported major results are consistent with previously published results based on morphology or small molecular datasets (e.g., nonmonophyly of the three species-rich and medically important genera; species groups of *Rhodnius*), others are novel. Among these is the polyphyly of the small tribe ‘Bolboderini’ and support for and relationships among most clades within Triatomini.

Triatominae monophyly

Although recent phylogenetic analyses of Triatominae have shown no support for the notion that blood-feeding Reduviidae may be derived from multiple predatory ancestors (Schofield, 2000; de Paula *et al.*, 2005; Bargues *et al.*, 2017), evidence for monophyly (Hypsa *et al.*, 2002; Justi *et al.*, 2016; Georgieva *et al.*, 2017) or paraphyly (Hwang & Weirauch, 2012; Zhang *et al.*, 2016) of the group has remained tentative. In topologies supporting the paraphyly hypothesis, predatory species of *Opisthacidius* and possibly other *Zelurus* clade Reduviinae are nested within Triatominae, either as sister to Rhodniini (Hwang & Weirauch, 2012) or to Triatomini (Zhang *et al.*, 2016). All prior analyses were limited by the small number of closely related outgroups and poor sampling of early diverging taxa within Triatominae, and none of the results were particularly well supported. Our analyses strongly support Triatominae as monophyletic in all analyses (Table S2), with *Opisthacidius* as sister. Although not tested in our analyses, diagnostic morphological features of kissing bugs including the unusual mobility of the labium, small mandibular plates and specialized mandibular and maxillary stylets are therefore likely synapomorphies for Triatominae.

Bolboderini polyphyly

Likely the most surprising result of our analyses is the polyphyly of Bolboderini, a small tribe comprised of four genera and 13 species (Gil-Santana, 2014). Our results place *Microtriatoma* (two species sampled) nested within Cavernicolini and Rhodniini, whereas *Belminus* is the sister taxon to Triatomini. Previously published molecular analyses included only one species of *Microtriatoma* that, consistent with our result, was recovered as closely related to Rhodniini (Patterson & Gaunt, 2010; Georgieva *et al.*, 2017). In contrast, the morphology-based analysis by Gil-Santana (2014) included representatives of all four genera (*Belminus*, *Bolbodera*, *Microtriatoma* and *Parabelminus*) and recovered the tribe as monophyletic, with *Zelurus* + Triatomini as sister clade. Gil-Santana (2014) listed a process on the antennifer, the length and shape of the maxillary plates, and the granulose integument of the forefemora as synapomorphies for Bolboderini. Our topology suggests that these features may have evolved independently in *Microtriatoma* and *Belminus*. Relationships among genera in Gil-Santana (2014) differed from those proposed by Lent & Wygodzinsky (1979), but the monophyly of Bolboderini had never been seriously questioned. Nevertheless, support for the placement of *Microtriatoma* and *Belminus* in our current analyses is unequivocal. We were unable to obtain sequence data for *Bolbodera* and *Parabelminus*. Lent & Wygodzinsky (1979) recovered *Parabelminus* and *Microtriatoma* as sister taxa, with *Belminus* as the next closest relative, whereas Gil-Santana (2014) recovered (*Microtriatoma* +(*Parabelminus* + [*Bolbodera* + *Belminus*])). *Bolbodera*, *Belminus* and *Parabelminus* share a similar habitus with distinctly elongated head, and we speculate that *Bolbodera* and *Parabelminus* may be closely related to *Belminus*. Together with the phylogenetic position of *Alberprosenia* (Alberproseniini), the phylogenetic relationships of *Bolbodera* and *Parabelminus* genera remain important topics for future research.

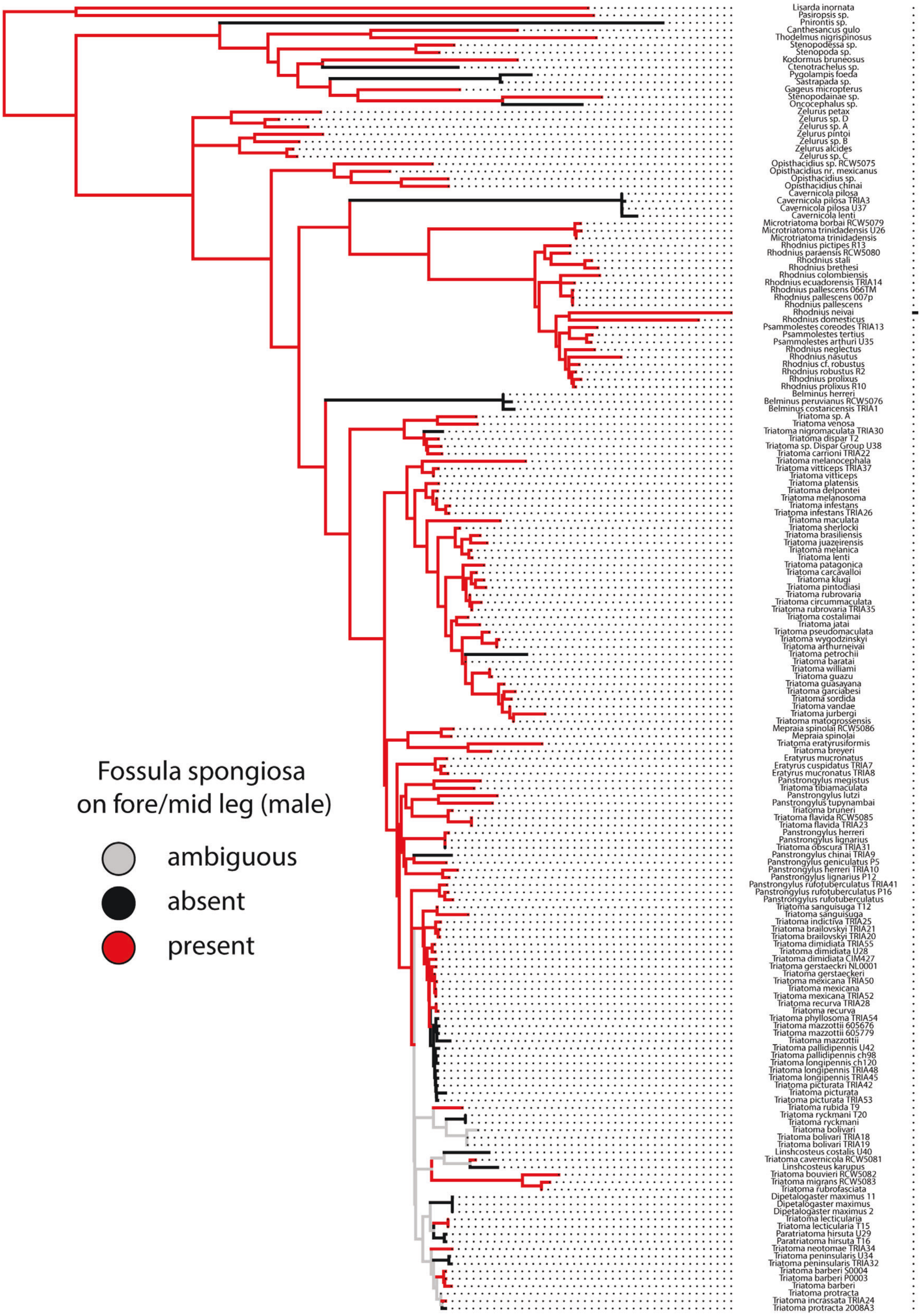

Fig. 4. Ancestral character state reconstruction of the fossula spongiosa in males. [Colour figure can be viewed at wileyonlinelibrary.com]

Paraphyly of *Panstrongylus, Rhodnius* and *Triatoma*

The three species-rich genera, *Panstrongylus*, *Rhodnius and Triatoma*, were recovered as paraphyletic in previous molecular analyses (Monteiro *et al.*, 2000; de Paula *et al.*, 2005; Justi *et al.*, 2016) and this result is here corroborated. *Psammolestes* is always nested within *Rhodnius* and *Panstrongylus* is paraphyletic with respect to the *Triatoma flavida* group and clade D in our analyses. Several monotypic or species-poor genera have long been recognized because of their distinctive, autapomorphic morphology and/or disjunct biogeographic distribution: *Dipetalogaster* is diagnosed by large size, unique structure of the connexiva and a labial flask-like organ; *Eratyrus* has long antennae among other features; the Indian genus *Linshcosteus* is diagnosed by the short labium and loss of the prosternal stridulitrum; and *Paratriatoma* by the small body size and abundance of setae (Lent & Wygodzinsky, 1979). Our analyses confirm that these lineages are nested among various clades of species classified as *Triatoma*. The situation is similar for species groups or complexes that are recognized as separate genera by some authors, but not by others, i.e., the *spinolai* complex (genus *Mepraia*), *flavida* group (genus *Nesotriatoma*) and *phyllosoma* group (genus *Meccus* Stål). Although all of these putative genera are recovered as monophyletic in our analyses, sometimes with the inclusion of additional taxa (i.e., the *flavida* group and part of *Panstrongylus*) a global taxonomic revision of Triatomini based on molecular and morphological evidence is essential to produce a meaningful classification that reflects phylogenetic relatedness. Although some of the shallower nodes still lack full or very high support, we argue that our combined UCE + ribosomal analyses provide a robust molecular framework for such a revision.

Rhodniini and relatives

Triatominae are basally split into two major, fully supported clades in all analyses, one combining Cavernicolini, Rhodniini and *Microtriatoma* ('Bolboderini', in part), with the second consisting of Triatomini and *Belminus* ('Bolboderini', in part). A close relationship of Rhodniini and Cavernicolini had been reported by some authors (Hwang & Weirauch, 2012; Justi *et al.*, 2016), but in contrast to these analyses, we recover *Microtriatoma* as the sister to Rhodniini, similar to the poorly supported result in the only published analysis that included representatives of all three tribes (Georgieva *et al.*, 2017). Although Rhodniini have long shown to be monophyletic, relationships among the three species groups in the tribe remain controversial. The *pictipes*, *pallescens* and *prolixus* groups (including *Psammolestes*) were proposed based on a combination of molecular and geographic distributional data (*pictipes* and *prolixus* species occur east of the Andes, *pallescens* species west of the Andes) and similar to other species groups, complexes and subcomplexes in Triatominae, morphological diagnoses have not yet been published. The three groups have typically been recovered as monophyletic in molecular analyses, albeit with varying support, but relationships among them remain unclear: either the *pictipes* (Georgieva *et al.*, 2017) or the *pallescens* (de Paula *et al.*, 2005; Justi *et al.*, 2016) groups are recovered as the sister to the *prolixus* group that also includes *Psammolestes*, whereas other analyses have supported the *pallescens* and *pictipes* groups as sister taxa (Abad-Franch *et al.*, 2008). Although not all species of *Rhodnius* are included in our analyses, representation from each of the species groups is substantial and their relationships are highly supported. We therefore corroborate that the *prolixus* (including *Psammolestes*) and *pallescens* groups are sister taxa, with the *pictipes* group as their closest relative.

Clades within Triatomini

Among the nine well-supported clades within Triatomini recovered in our analyses, only some correspond to previously recognized groups, complexes or subcomplexes. As in Justi *et al.* (2016), the *dispar* clade (sometimes referred to as *venosa* complex; Carcavallo *et al.*, 2000) is associated with mesic forests in Central and north-western South America and is recovered as sister to all remaining Triatomini. The South American *infestans* clade only partially aligns with the *infestans* group *sensu* Schofield & Galvão (2009). It includes the *spinolai* complex, which is polyphyletic in our analyses, with the *spinolai* clade being more closely related to the remaining Triatomini (clade A), and *T. tibiamaculata* recovered as part of the *flavida* clade. According to our results, all *infestans* complexes and subcomplexes except the *infestans* subcomplex need revision. This statement also applies to species of the *flavida* clade that, in our analyses, also includes several species of *Panstrongylus*. However, *P. rufotuberculatus* falls outside of this clade and is sister to clade C of the Triatomini. The original concept of the *flavida* group comprised several species from the Caribbean (Carcavallo *et al.*, 2000), whereas *T. tibiamaculata* is known from Brazil, and species of *Panstrongylus* occur across Central and northern South America. Finally, our concept of the *spinolai* clade includes species from Chile and Argentina sometimes accommodated in the separate genus *Mepraia* (*Triatoma spinolai* Porter and *Triatoma gajardoi* Frias, Henry and Gonzalez) together with the *breyeri* complex (*Triatoma breyeri* Del Ponte and *Triatoma eratyrusiformis* Del Ponte). This clade is sister to *Eratyrus* (Central and northern South America) and the remaining species of Triatomini that are mostly restricted to Central and North America, as well as the Old World.

The *rubrofasciata* group *sensu* Schofield & Galvão (2009) is highly supported across all analyses (our clade D), whereas relationships within this group are only partially reflected by current complex and subcomplex assignments. Justi *et al.* (2016) recovered the Old World clade as sister to their monophyletic *lecticularia* and *phyllosoma* groups, and it formed the sister taxon to the *protracta* clade in the analysis by Aguilera-Uribe *et al.* (2020). In our analyses, the *phyllosoma* clade (see Tables S2, S5 for our concepts of these clades) forms the sister group to the *rubida*, *rubrofasciata* and *protracta* clades. Support for these clades and their relationships is high in the UCE-only analyses, and they are well supported in the UCE + ribosomal topologies, which is consistent with results by Aguilera-Uribe *et al.* (2020). We posit that these relationships will still require some further scrutiny and inclusion of morphological data into combined analyses before a phylogeny-based reclassification should be attempted.

Our Old World *rubrofasciata* clade comprises *Linshcosteus*, the three endemic Old World species of *Triatoma* included in our analyses (*Triatoma cavernicola* Else & Cheong, *Triatoma bouvieri* Larrousse and T. *migrans*) in addition to the circumtropical *T. rubrofasciata*. The Old World Triatominae are therefore monophyletic, corroborating the hypothesis proposed by Aguilera-Uribe *et al.* (2020) and Hypsa *et al.* (2002) and contrasting with Schofield (2000) who had proposed separate Old World invasions of the MRCA of *Linshcosteus* and of the endemic *Triatoma* species. Although we do not perform divergence dating and historical biogeographic analyses as part of the present study, relationships within clade D suggest that the Old World was colonized only once by species of Triatominae. The origin of *T. rubrofasciata* in either the Oriental or Neotropical regions has been debated even in recent publications (Dujardin *et al.*, 2015a,b). Given that *T. rubrofasciata* is nested among endemic Oriental species of *Triatoma* and *Linshcosteus*, we propose that this species has likely originated in the Oriental region and from there spread across all circumtropical regions, making its occurrence in the Neotropical region a secondary colonization.

Evolution of morphological features and colour patterns

Body size and shape and colour patterns evolved plastically across Triatominae. We speculate that this plasticity mirrors those observed in host associations (Georgieva *et al.*, 2017; Weirauch, 2021) and may be a reflection of how different habitats and microhabitats may have shaped this group. Our analyses also provide first insights into the evolutionary history of several morphological features that are likely associated with specific behaviours, including antipredator strategies, mating and locomotion. Despite differences in fine structure (Weirauch, 2007) and function as attachment structure involved in prey capture (Zhang *et al.*, 2016), mating (Lent & Wygodzinsky, 1979) or locomotion (Miller, 1942), our analysis is the first to corroborate based on a comprehensive phylogeny that the fossula spongiosa in nontriatomine Reduviidae and in kissing bugs is homologous. We further show that the fossula is conserved across most lineages of Triatominae, with losses concentrated in clade D. Our reconstruction presents a framework to investigate the behavioural and ecological context of these attachment structures across kissing bugs. Similarly, a metatarsal brush likely associated with camouflaging behaviours and thus antipredator strategies in first instar immatures in many species of Triatominae (Zeledón *et al.*, 1973; Weirauch, 2006a) is here shown to have evolved early during the evolution of Triatomini, with multiple losses, in particular within the *rubrofasciata* group. Our ancestral character state reconstructions of the three dorsal abdominal glands (DAGs 1–3), the defence glands of immatures that are retained into the adult stage in most Reduviidae (Weirauch, 2006b) showed that although DAG3 was already lost in a more distant common ancestor, DAGs 1 and 2 were reduced in the MRCA *Opisthacidius* + Triatominae. Based on Hwang & Weirauch (2012), the transition from free living to nest-dwelling assassin bugs likely occurred in the MRCA of *Opisthacidius* + Triatominae, and the loss of the defence glands in the immatures may therefore have coincided with the invasion of more cryptic and secluded microhabitats. Although investigating the morphological, physiological and behavioural transitions from predatory assassin bug to blood-feeding kissing bug and adaptations to various habitats and host associations across Triatominae is beyond the scope of this study, we here provide the phylogenetic framework and some preliminary insights that hopefully will encourage future studies.

Conclusion

Our hypothesis is maximally taxon-inclusive and is based on a UCE+ ribosomal dataset made possible by the incidental capture of high-copy rRNA and mitochondrial genes in UCE capture samples, combined with legacy Sanger data derived from GenBank. This phylogeny resolves most of the controversially discussed big open questions in kissing bug systematics, providing strong evidence that Triatominae are monophyletic, and that part of the *Zelurus* clade (Reduviinae) are their closest predatory relatives. We show that the analysis of UCE data – a type of data currently underutilized in hemipteran phylogenetics – results in a strongly supported topology for this assassin bug subfamily. UCEs are relatively easy and cost-effective to produce and can be generated from pinned museum specimens. We posit that complementing the current dataset to include all species of Triatominae should be possible in the not too distant future. We also show that combining datasets of UCEs and ribosomal loci, while resulting in less support for certain nodes, produced a robust and taxonomically inclusive phylogenetic framework. However, we acknowledge that the overarching phylogeny of Triatominae is not resolved until several additional taxa are included in hypotheses, most critically the two genera of ‘Bolboderini’ not sampled in our analysis and the monotypic tribe Alberprosenini. In the meantime, our phylogenetic hypotheses provide a sound foundation for studies that could test proposed timelines for the evolution of Triatominae or investigate the historical biogeography of the group with focus on the Old World clade and the hypothesized secondary colonization of the New World by *T. rubrofasciata*.

Supporting Information

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

Fig. S1. Best likelihood tree of the unpartitioned analysis of the 60% UCE-only matrix.

Fig. S2. Best likelihood tree of the unpartitioned analysis of the 85% UCE-only matrix.

Fig. S3. ASTRAL analysis of UCE data.

Fig. S4. Best likelihood tree of the unpartitioned analysis of the 85% UCE + ribosomal matrix.

Fig. S5. MrBayes 85% UCE-only.

Fig. S6. MrBayes 85% UCE + ribosomal.

Fig. S7. Ancestral character state reconstruction of male body length (left) and body shape (ratio of greatest abdominal width to total body length; right) across Triatominae.

Fig. S8. Ancestral character state reconstruction of colour patterns of the corium.

Table S1. Classification for taxa sampled for the UCE and ribosomal datasets following Schofield & Galvão (2009) and Justi & Galvão (2017), clade assignments according to Justi *et al.* (2016) and the present study, and reference to supplemental table with accession numbers.

Table S2. Bootstrap support percentage (Figs S1–S4) or posterior probability (Figs S5, S6) for critical nodes in the eight different analyses. Abbreviations: cl: clade; cn: conflicting node; gr: group; na: not applicable (only one taxon); U ML 85 P: UCE-only partitioned likelihood of 85% matrix; U + R ML 85 P: UCE and ribosomal dataset partitioned likelihood of 85% matrix; U ML 60 UP: UCE-only partitioned likelihood of 60% matrix; U ML 85 UP: UCE-only partitioned likelihood of 85% matrix; U AS: UCE-only Astral; U + R ML 85 UP: UCE and ribosomal dataset unpartitioned likelihood of 85% matrix; U MrB 85: UCE-only MrBayes of 85% matrix; U + R MrB 85: UCE and ribosomal MrBayes of 85% matrix. Clades A-D are shown in Figs 1, 2. Clade A: *spinolai* clade + *Eratyrus* + *flavida* clade + *P. rufotuberculatus* + *phyllosoma* clade + *rubida* clade + *protacta* clade + *rubrofasciata* clade; Clade B: Clade A less *spinolai* clade; Clade C: *P. rufotuberculatus* + *phyllosoma* clade + *rubida* clade + *protacta* clade + *rubrofasciata* clade; Clade D: Clade C less *P. rufotuberculatus*. Clade E: *rubida* clade + *protracta* clade + *rubrofasciata* clade.

Table S3. Voucher information for UCE taxa sequenced for this study, including collection event, depository, preservation method, and DNA extraction method as well as SRA accession numbers, and read and contig statistics. Acronyms for depositories: AMNH – American Museum of Natural History; UNAM – Universidad Autonoma Mexico; UCR – University of California, Riverside.

Table S4. GenBank accession numbers for ribosomal data for the combined UCE and ribosomal analyses.

Table S5. Expanded classification for taxa sampled for the UCE and ribosomal datasets, including group, subgroup, and complex assignment of Triatomini based on Schofield & Galvão (2009) and other sources.

Table S6. Matrix statistics for the 60% UCE, 85% UCE, and 85% UCE + ribosomal datasets, including number of taxa, loci, sites, and informative sites.

Table S7. Measurements and ratios for continuous characters.

File S1. Ancestral character state reconstruction of selected morphological characters. Character descriptions and results.

File S2. Nexus file including matrices 1 (continuous) and 2 (categorical) for morphological character ancestral state reconstructions.

File S3. Ancestral character state reconstruction of characters 1–3 and 5.

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Data availability statement

NCBI Sequence Read Archive accession numbers are provided in Table S3. Alignment files, scripts, and configuration files are available from Dryad (https://doi.org/10.5061/dryad.37pvmcvh4).

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