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Molecular phylogenetics and biogeography of the ambush bugs (Hemiptera: Reduviidae: Phymatinae)

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ABSTRACT

The ambush bugs (Heteroptera: Reduviidae: Phymatinae) are a diverse clade of predators known for their cryptic hunting behavior and morphologically diverse raptorial forelegs. Despite their striking appearance, role as pollinator predators, and intriguing biogeographic distribution, phylogenetic relationships within Phymatinae are largely unknown and the evolutionary history of the subfamily has remained in the dark. We here utilize the most extensive molecular phylogeny of ambush bugs to date, generated from a 3328 base pair molecular dataset, to refine our understanding of phymatine relationships, estimate dates of divergence (BEAST 2), and uncover historical biogeographic patterns (S-DIVA and DEC). This taxon set (39 species of Phymatinae and six outgroups) allowed reevaluation of the proposed sister group of Phymatinae and tribal-level relationships within the group, and for the first time proposes species-level relationships within *Phymata* Latreille, the largest genus of ambush bugs (~108 spp.). Available evidence suggests that *Phymata* originated in the Neotropical region, with subsequent dispersals to the Nearctic and Palearctic regions. This study provides a framework for future research investigating the evolutionary history of ambush bugs, as well as ecological and microevolutionary investigations.

1. Introduction

The ambush bugs, Phymatinae (Hemiptera: Heteroptera: Reduviidae), are a diverse clade of charismatic, predatory true bugs that are characterized by their cryptic predatory behavior and morphologically diverse raptorial forelegs. Of the approximately 300 described species, most are diurnal and encountered on blooming vegetation where they ambush a wide range of other flower visiting insects including pollinators (Balduf, 1943; Froeschner and Kormilev, 1989). While rather small (~5 to 10 mm), these insects are capable of capturing prey many times their own size, and as generalist predators, they have been reported to alter pollinator foraging activity (Elliott and Elliott, 1991, 1994). Phymatinae exhibit sexual dimorphism and have become models to investigate microevolutionary patterns while dissecting the interactions between sexual dimorphism and sexual selection (Mason, 1986; Punzalan et al., 2008a; Punzalan et al., 2008b, 2008c, 2010; Punzalan and Rowe, 2015). A phylogenetic framework for the subfamily and well-defined species limits especially within the larger genera are currently unavailable, but critical for future ecological and evolutionary research on Phymatinae. The phylogenetic objectives of the present study are therefore threefold: to examine phylogenetic relationships to other assassin bug subfamilies, test a tribal-level phylogenetic hypothesis, and propose relationships within the largest genus of Phymatinae, *Phymata* Latreille.

Phymatinae are part of the Phymatine Complex that forms the sister group to all remaining Reduviidae, the Higher Reduviidae (Carayon et al., 1958; Davis, 1961; Hwang and Weirauch, 2012; Weirauch, 2008; Weirauch and Munro, 2009; Zhang et al., 2016). In addition to the ambush bugs, this clade is thought to include Holoptilinae, Centrocnemidinae, Elasmodeminae, and according to some hypotheses also the Hammacerinae and Phimophorinae, an assemblage of small to moderate-sized subfamilies, each comprising species with distinctive morphology and natural history (Davis, 1961; Hwang and Weirauch, 2012; Zhang et al., 2016). Carayon et al. (1958) proposed that Holoptilinae + Elasmodeminae was the sister group to Phymatinae, an association predominantly based on genitalic characters. Rédei and Tsai (2011) also noted a strong similarity in the phalli of male Holoptilinae and Centrocnemidinae (e.g., endosomal appendages not fully enclosed by the endosoma in these subfamilies, but fully enclosed in Phymatinae (Carayon et al., 1958; Davis, 1957)). Recent morphology-based or molecular hypotheses recovered either the ant-feeding specialist group Holoptilinae (Weirauch, 2008; Weirauch et al., 2011; Weirauch and Munro, 2009; Zhang et al., 2016) or Holoptilinae and the Oriental, tree-bark associated Centrocnemidinae (Hwang and Weirauch, 2012) as sister group to Phymatinae. The divergence of Phymatinae from other assassin bugs has been estimated to have occurred in the Cretaceous, approximately 114 mya, with diversification within the group starting around

Abbreviations: ML, maximum likelihood; PP, posterior probability; MRCA, most recent common ancestor

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66 mya (Hwang and Weirauch, 2012), making this subfamily distinctly older than the great majority of assassin bug subfamilies. However, relationships within the Phymatine Complex are not well explored, with analyses hampered by poor taxon sampling; Elasmodeminae and Phimophorinae have yet to be included in published phylogenetic analyses.

Representatives of the four tribes of Phymatinae (Fig. 1) occur in tropical, subtropical, and temperate regions around the world except Australia and New Zealand (Froeschner and Kormilev, 1989). These bugs are easily distinguished from other Reduviidae by their robust raptorial forelegs, fusiform distal flagellomeres, well developed bucculae, and disruptive color pattern (Weirauch et al., 2014). The monogeneric tribe Themonocorini (5 spp.) is confined to sub-Saharan Africa and members differ from other ambush bugs in possessing simple raptorial forelegs with long spines (Carayon et al., 1958; Weirauch et al., 2011). These small (∼4 mm) ambush bugs have been found in rotting masses of plant matter and nests of weaver birds, presumably stalking arthropod prey, but their natural history is otherwise unknown (van Doesburg and Jacobs, 2011). The three described genera of Carcinocorini (30 spp.), endemic to the Oriental region, are unique among Hemiptera in that they possess pincer-like chelate forelegs that superficially resemble the claws of crustaceans (Fig. 1). By far the most speciose ambush bug tribes are the Macrocephalini (20 genera; 154 spp.) and Phymatini (5 genera; 115 spp.) (Cui et al., 2003; Froeschner and Kormilev, 1989; Kormilev and van Doesburg, 1991, 1992; Rabitsch, 2004; van Doesburg and Jacobs, 2011; van Doesburg and Pluot-Sigwalt, 2007), easily recognized from other ambush bugs by their subchelate raptorial forelegs with enlarged forefemur and a slender, curved tibia that is folded against the femur (Fig. 1). Macrocephalini are largely circumtropical, but some species occur in subtropical regions. Only Phymatini have also adapted to temperate climates, with some species distributions ranging into relatively high latitudes in the Nearctic and Palearctic, although most species are endemic in tropical and subtropical areas (Cui et al., 2003; Froeschner and Kormilev, 1989; Kormilev, 1962). The vast majority of Macrocephalini and Phymatini have been described from the Americas: *Lophoscutus* Kormilev (66 spp.) and *Macrocephalus* Swederus (19 spp.), the two most speciose genera of Macrocephalini, are confined to the New World and among the 109 described species of *Phymata* (Phymatini), the largest ambush bug genus, only five are Palearctic.

Surprisingly, given the charisma of ambush bugs, the monophyly of the four tribes has remained untested and relationships between the tribes have not been investigated, beyond the hypothesis proposed by Weirauch et al. (2011). That analysis, based on a sample of only 11 Phymatinae, but including representatives of the four tribes, recovered Themonocorini as sister to Carcinocorini + Macrocephalini and Phymatini; sampling of Macrocephalini was restricted to two Neotropical genera. The lack of phylogenetic hypotheses has also hindered investigations into the biogeographical history of the subfamily. Based on the present distribution of ambush bugs, Kormilev (1962) proposed that Phymatinae originated in South America during the Late Jurassic or Early Cretaceous, and later inhabited a large range spanning across Gondwana (sans Australia) prior to its fragmentation. Kormilev (1962) suspected that Macrocephalini were the first to arise, and were later followed by Carcinocorini in the Oriental region and Phymatini in the Neotropics. This biogeographic scenario, however, did not account for the Afrotropical tribe Themonocorini which was described after Kormilev's revision of Phymatini was submitted for publication. To date, Phymatinae have not been subjected to a formal biogeographic analysis.

Similar to the situation between tribes, relationships within the two large tribes have not been investigated using cladistic methods. Three of the five genera in the tribe Phymatini are monotypic (*Anthylla* Stål, *Kelainocoris* Kormilev, and *Paraphymata* Kormilev) and *Neoanthylla* Kormilev comprises three species; all are rarely collected and we were unable to obtain specimens for the present study. We therefore focus the lower-level phylogenetic component here on the large genus *Phymata* that includes all remaining species of Phymatini, where we sampled the two speciose subgenera (of four total). These ambush bugs are commonly affiliated with open grasslands, savanna, chaparral, scrub, and desert habitats. Hwang and Weirauch (2012) estimated *Phymata* to have diverged from other Phymatinae during the Late Cretaceous, making this one of the oldest genera of assassin bugs and one with conserved morphology for potentially more than 60 mya. Despite this ancient divergence, *Phymata* exhibits a rather imbalanced distribution across the globe, with the bulk of *Phymata* diversity being restricted to North and South America (104 spp.). The five Palearctic species are morphologically similar, have been suspected to be derived from ancestors in the Americas, and are possibly close relatives of the Nearctic species *Phymata vicina* Handlirsch, *Phymata maculata* Kormilev, and *Phymata pallida* Kormilev (Handlirsch, 1897; Kormilev, 1957). These hypotheses have not been tested using formal analyses, and it is unclear when and by which route, e.g., transoceanic or via Beringia, this putative distribution expansion might have occurred. *Phymata* are less thermophilic than their kin and many species have adapted to the cool, temperate conditions found at relatively high latitudes (Kormilev, 1962), an observation that could hint to a possible Beringian dispersal route even during periods when this land bridge was dominated by temperate climates.

Within *Phymata* is a complex of ∼11 presumably closely related species and ∼15 subspecies, predominately found in the Nearctic, that we here refer to as the erosa species group (Cockerell, 1900; Evans, 1931; Froeschner and Kormilev, 1989; Handlirsch, 1897; Kormilev, 1957, 1962; Melin, 1930). Members of this complex are sexually dimorphic and many species exhibit a wide range of colorations and sizes even within the same sex. Individuals of *Phymata americana* Melin (four sspp.), *Phymata fasciata* (Gray) (five sspp.), and *Phymata pacifica* Evans (three sspp.) are commonly collected in North America, but species identification has remained a challenge and species hypotheses remain untested. Our sampling of *Phymata*

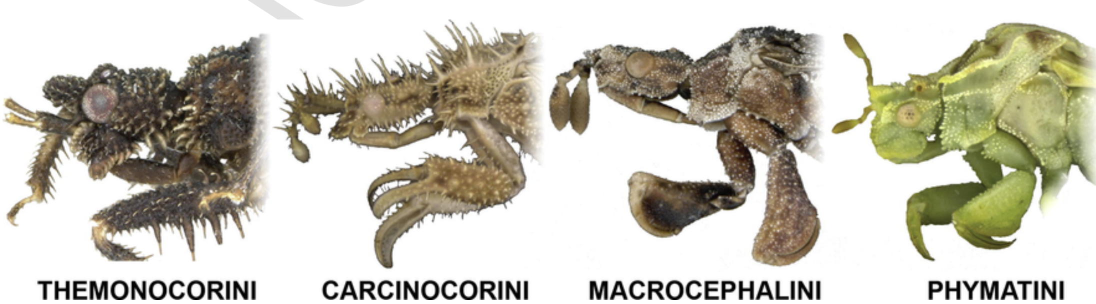

Fig. 1. Tribal-level diversity of Phymatinae and differences in raptorial foreleg morphology.

includes 17 terminals from this species complex, allowing for the first time phylogenetic insights into Nearctic ambush bugs.

We here use a molecular dataset derived from sampling six loci and 45 representatives of the Phymatine Complex to investigate phylogenetic relationships of Phymatinae to other Reduviidae (specifically Centrocnemidinae and Holoptilinae), test the tribal-level hypothesis proposed by Weirauch et al. (2011), and to reconstruct the first phylogenetic hypothesis for *Phymata*. We also performed biogeographic and divergence dating analyses to hypothesize events leading to the current disproportionate distribution of *Phymata* in the Caribbean, Nearctic, and Palearctic. Such events may include dispersal across ancient land bridges, such as the Bering land bridge and GAARlandia, or long distance oceanic dispersal. This dataset for the first time includes a Neotropical representative of the circumtropical Holoptilinae, two Old World species of Macrocephalini, and comprehensive geographic sampling of *Phymata* (Nearctic, Neotropical, and Palearctic regions), with emphasis on the Nearctic species.

2. Materials and methods

2.1. Taxon sampling and vouchering

We sampled 45 taxa across the Phymatine Complex including 39 terminals of Phymatinae, three Old and New World Holoptilinae, one member of the subfamily Centrocnemidinae, and two Hammacerinae. Elasmodeminae and Phimophorinae were not included due to lack of DNA quality material. The two species of *Microtomus* Illiger (Hammacerinae) were used to root the tree; Hammacerinae have either been recovered as sister group to all remaining Reduviidae (Weirauch, 2008), or as sister to other taxa in the Phymatine Complex (Hwang and Weirauch, 2012; Weirauch and Munro, 2009; Zhang et al., 2016). Two genera of Carcinocorini, four species of Macrocephalini (including two Old World Macrocephalini), 20 species of Phymatini, and one species of Themonocorini were sampled. Sampling of New World *Phymata* included species of the two subgenera *Phymata* (*Phymata*) Kormilev and *Phymata* (*Phymatispa*) Kormilev, the latter represented by *Phymata fortificata* (Herrich-Schäffer). The dataset also included *Phymata crassipes* (Fabricius), one of the five putatively closely related Palearctic species (Handlirsch, 1897; Kormilev, 1957, 1962). Molecular voucher specimens were designated a Unique Specimen Identifier (USI) barcode label and databased using the Plant Bug Planetary Biodiversity Inventory (PBI) instance of the Arthropod Easy Capture (AEC) Specimen Database (http://www.research.amnh.org/pbi/locality/index.php). Voucher information (USI, RCW#, Locality, Latitude/Longitude, and GenBank Accession numbers for each gene region) is available in Table S1. Dorsal and lateral habitus images of voucher specimens were generated using Leica Microsystems imaging equipment, uploaded to the AEC database, and are available online through the "Heteroptera Species Pages" (http://research.amnh.org/pbi/heteropteraspeciespage).

2.2. Molecular protocols and sequence alignment

DNA was extracted from the right hind leg using a QIAGEN DNeasy Blood and Tissue Kit following the protocol of the manufacturer. Six gene regions were amplified using PCR: two nuclear ribosomal (28S D2 and 28S D3-5), one mitochondrial ribosomal (16S), and three mitochondrial protein encoding loci (COI, COII, and CytB). PCR was conducted using primers and conditions listed in Table S2. Two primers for amplification of a region of Cytochrome Oxidase B (CytB), PhymataCytbF-363 and PhymataCytbR-943, were developed de novo for this study from existing *Phymata pennsylvanica* (Handlirsch) transcriptome sequences (Zhang et al., 2016) using Primer BLAST. Bioline SureClean Plus was used to purify PCR amplicons prior to sequencing following the standard protocol. Purified PCR amplicons were then sequenced using Sanger sequencing on an Applied Biosystems 3730xl DNA Sequencer at UCR's Institute for Integrative Genome Biology. Sequences were manually inspected, primer regions trimmed, and sequences contiged in Sequencher v4.8. All sequences were verified to rule out contamination using BLAST on the NCBI database. Sequences for each gene region were then aligned using MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/index.html). The E-INS-i refinement method was applied for its applicability to datasets with internal unalignable regions (i.e., ribosomal DNA) (Katoh and Toh, 2008). All sequence alignments were inspected in Mesquite V3.02 (Maddison and Maddison, 2015), where protein encoding sequences (COI, COII, and CytB) were translated to their corresponding amino acids to check for stop codons. Concatenation of the six gene regions resulted in a molecular data matrix totaling 3328 base pairs.

2.3. Phylogenetic analyses

PartitionFinder v1.1.1 (Lanfear et al., 2012) was used to define partitions in our partition data block file which contained 12 sets of nucleotide sites. These partition sets included 28S D2, 28S D-3, 16S, and individual codon positions for COI, COII, CytB sequences. To determine the best schemes for each set, the Bayesian information criterion (BIC) and the greedy search algorithm were applied in PartitionFinder (see Table S3). Maximum Likelihood analyses were run on the concatenated data matrix using RAxML-HPC2 (Stamatakis, 2014) on the CIPRES Science Gateway analyses. For the RAxML rapid bootstrapping parameters, we selected the general time reversible + gamma (GTRGAMMA) model (GTR = variable base frequencies, symmetrical substitution matrix) and ran 1000 bootstrap iterations. Multiple analyses were run to ensure topological convergence. A Bayesian divergence dating analysis was performed using BEAST 2 v2.3.2 (Bouckaert et al., 2014) on XSEDE, also through the CIPRES Science Gateway and an XML control file was generated in BEAUti v2.3.2 (Bouckaert et al., 2014). All trees and clock models were linked for the molecular dataset and a relaxed clock log normal clock model was selected for all 12 partitions. The substitution model settings specified for each partition are listed in Table S3.

2.4. Divergence dating calibration points

We used *Praecoris dominicana* Poinar (Holoptilinae) from Dominican amber (La Toca mine) that is estimated to be about 15–20 million years old (Iturralde-Vinent and MacPhee, 1996) as a minimum age constraint. The node representing the most recent common ancestor (MRCA) of New and Old World Holoptilinae (gray star in Fig. 3) was calibrated following Parham et al. (2012). This node was selected over neighboring nodes (i.e., Node 4, the MRCA of Centrocnemidinae and Holoptilinae) because the fossil shares more similarities with *Neolocoptiris villiersi* Wygodzinsky and Usinger, the only extant Neotropical holoptiline, than with any Old World taxa (Poinar, 1991). We applied a lognormal distribution and set a minimum hard bound age by applying an offset value of 15.0 million years. Three secondary calibration points (MRCA of Phymatinae, MRCA of Phymatinae + (Holoptilinae + Centrocnemidinae), and the root node) were selected from Hwang and Weirauch (2012), the corresponding means and sigma values (reported in Table S4) adjusted to 95% of the posterior distributions fitting within the date ranges in Hwang and Weirauch (2012), and calibrated using normal distributions and constrained monophyly for the three groups.

2.5. MCMC

We ran Markov Chain Monte-Carlo (MCMC) for 100,000,000 generations and logged trace and tree files every 1000 generations. To check for topological convergence, eight separate MCMC chains were run simultaneously. After conducting the BEAST 2 analyses, we confirmed that all parameters reached a stationary distribution and checked for convergence among each of the eight runs in Tracer v1.6.0 (Rambaut et al., 2014). Five runs converged and the three that failed and yielded low effective sample size (ESS) values were removed from the analysis. After combining the five converging runs, we verified that all important parameters had ESS values greater than 200. Using LogCombiner and by specifying a 10% burnin for each input tree file, we thinned the trees from the five converging runs and summarized them in TreeAnnotator v2.3.0. The resulting maximum clade credibility chronogram was then visualized with FigTree v1.4.2 and annotated in Adobe Illustrator v2015.1.0.

2.6. Biogeographic analyses

The 45 taxa were each assigned to one of six biogeographic regions for the analyses performed in Reconstruct Ancestral State in Phylogenies (RASP) v3.2 (Yu et al., 2015) (A = Nearctic, B = Neotropical, C = Palearctic, D = Oriental, E = Australian, and F = Afrotropical). Ancestral distributions were reconstructed using the Statistical Dispersal-Vicariance Analysis (S-DIVA) (Yu et al., 2010) and the Lagrange Dispersal-Extinction-Cladogenesis (DEC) model (Ree and Smith, 2008) in RASP. The consensus tree inferred from the BEAST 2 analysis was used to examine ancestral ranges under both models.

3. Results

3.1. Phylogenetic reconstruction

The best tree from the ML analysis is depicted in Fig. 2 and results from the divergence dating analysis in Fig. 3. Congruence in the topology between the ML and Bayesian trees is high. Bootstrap (BS) values are reported from 1000 bootstrap replicates. All nodes were recovered with posterior probability (PP) values greater than or equal to 70% (Fig. 3, Table S3). Old World Holoptilinae form a clade (BS = 78%; PP = 85%) sister to *Neolocoptiris villiersi* from French Guiana. Centrocnemidinae + Holoptilinae are sister to Phymatinae, although the sister group relationship of Centrocnemidinae and Holoptilinae is only weakly to moderately well supported (BS = 60%; PP = 94%). The monophyly of Phymatinae is strongly supported in both analyses (BS = 99%; PP = 100%) and Themono-

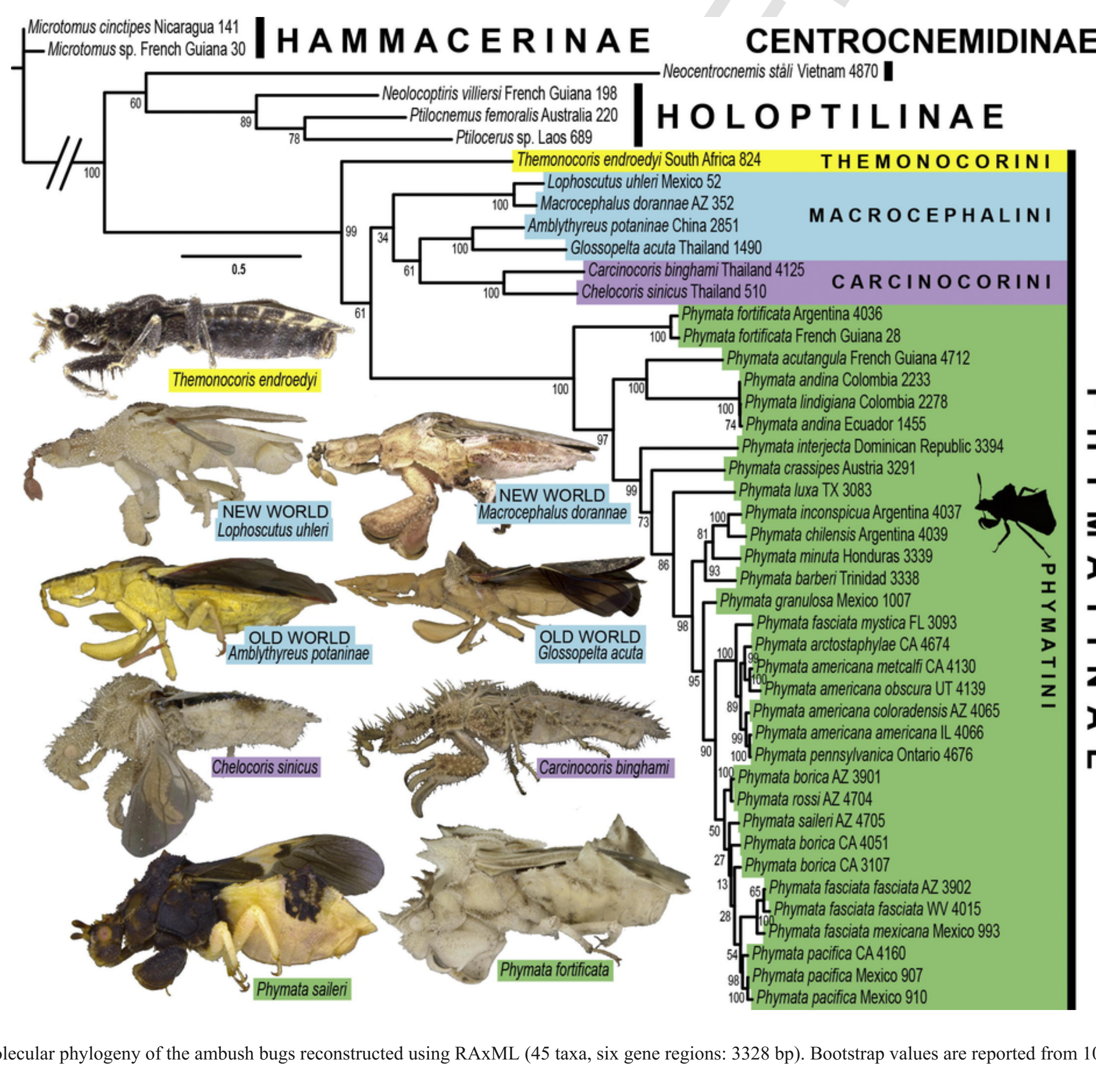

Fig. 2. Molecular phylogeny of the ambush bugs reconstructed using RAxML (45 taxa, six gene regions: 3328 bp). Bootstrap values are reported from 1000 iterations.

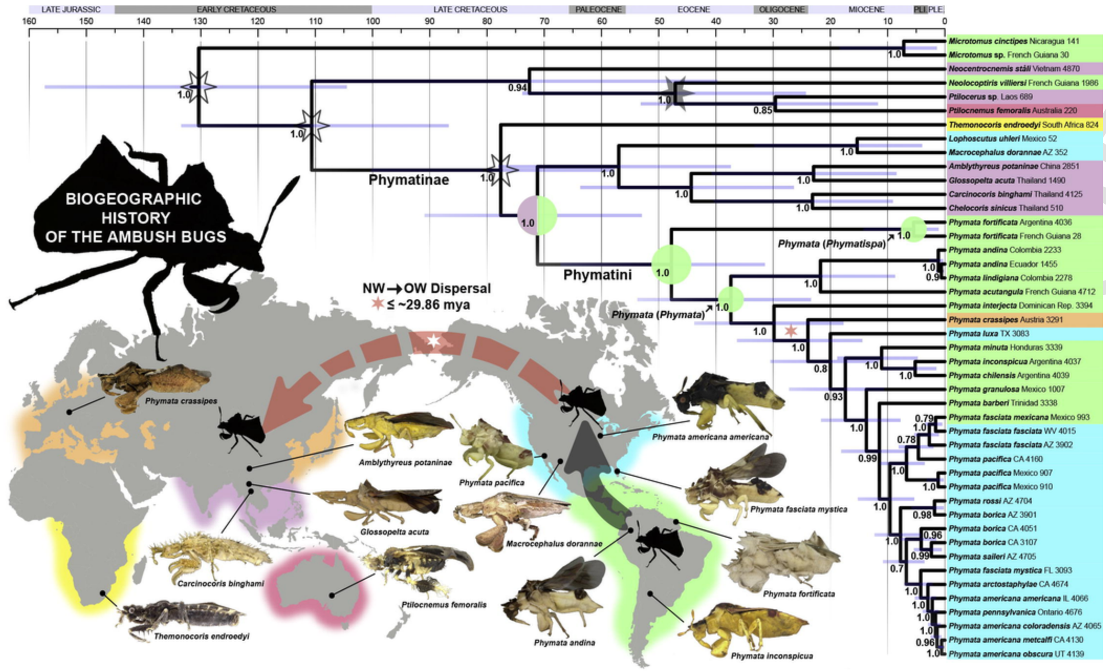

Fig. 3. Estimated ages of divergence for the Phymatine complex using BEAST 2. PP values are annotated and the 95% HPD range depicted as blue bars. White stars indicate the nodes calibrated using secondary calibrations from Hwang and Weirauch (2012) and the solid gray star indicates the fossil calibrated node. Taxa are highlighted by their biogeographic range. The red star represents the earliest estimation for which *Phymata* may have dispersed from the New World to Old World. We hypothesize that this happened across the Bering land bridge (red dashed line) or via a chance transoceanic dispersal event (not illustrated). Colored circles correspond with the biogeographic regions that mostly likely served as ancestral ranges based on results from the DEC analysis (see Table S5b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

corini was recovered as the sister group to the remaining ambush bug tribes, Carcinocorini, Macrocephalini, and Phymatinae, which form a clade (BS = 61%; PP = 100%). In both analyses, Macrocephalini was rendered paraphyletic by Carcinocorini, with the Old World Macrocephalini and Carcinocorini forming a clade (BS = 61%; PP = 100%) that is the sister group to the New World Macrocephalini ("Macrocephalini" + Carcinocorini: BS = 34%; PP = 100%). The monophyly of the tribe Phymatini is strongly supported (BS = 100%; PP = 100%), as is that of the subgenus *P.* (*Phymata*) (BS = 97%; PP = 100%).

Within *P.* (*Phymata*), several species from Northern South America (Colombia, Ecuador, and French Guiana) form a clade, while the only representative from the Caribbean is recovered as sister to all remaining species in the subgenus. The Old World *P. crassipes* is nested within the *P.* (*Phymata*) clade, as sister to the Nearctic *Phymata luxa* Evans, a number of Neotropical species ranging from Mexico to Argentina, and a clade of mostly Nearctic species that we here refer to as the erosa species group. In the present analysis, the erosa group comprises all included Nearctic species of *Phymata* except *P. luxa* (i.e., *P. fasciata*, *P. pacifica*, *P. rossi* Evans, *P. borica* Evans, *P. saileri* Kormilev, *P. arctostaphylae* Van Duzee, *P. americana*, and *P. pennsylvanica*; BS = 90%; PP = 100%). *Phymata americana* is rendered paraphyletic by the eastern Nearctic *P. pennsylvanica* and the western Nearctic *P. arctostaphylae* in the ML tree, with the two eastern subspecies of *P. americana* (*P. americana americana* Melin and *P. americana coloradensis* Melin) forming a clade with *P. pennsylvanica*, and *P. arctostaphylae* being recovered as sister to the two western subspecies (*P. americana metcalfi* Evans and *P. americana obscura* Kormilev). "*Phymata americana*", *P. arctostaphylae*, and *P. pennsylvanica* together are strongly supported in both analyses (BS = 100%; PP = 100%). *Phymata fasciata* with five described subspecies, three of which here included, is polyphyletic. While the nominate subspecies *Phymata fasciata fasciata* (Gray) and *Phymata fasciata mexicana* Melin are monophyletic in both analyses (BS = 100%; PP = 98%), *Phymata fasciata mystica* (Evans) is recovered as sister taxon to the "*P. americana*", *P. pennsylvanica*, and *P. arctostaphylae* clade (BS = 100%; PP = 100%). *Phymata borica* is rendered polyphyletic. *Phymata pacifica* (two of the three subspecies included) is monophyletic in the ML tree and sister to *P. f. fasciata* + *P. f. mexicana*, but rendered paraphyletic by *P. f. fasciata* + *P. f. mexicana* in the Bayesian tree; the *P. pacifica* and *P. f. fasciata* + *P. f. mexicana* clade is weakly (BS = 54%) or well (PP = 100%) supported in the two analyses.

3.2. Divergence times

Age estimates (median age values and 95% Highest Posterior Density [HPD]) from the BEAST 2 analysis are depicted in Fig. 3 and reported in Table S5a. Themonocorini diverged from the MRCA of the other tribes early on in ambush bug evolution (77.6 mya; 95% HPD [57.68, 100.26]). Phymatini likely diverged from "Macrocephalini" + Carcinocorini during the Late Cretaceous (71.18 mya; 95% HPD [52.91, 90.92]) and Carcinocorini from Old World "Macrocephalini" during the Eocene (44.31 mya; 95% HPD [26.35, 63.74]). The divergence between the ambush bug subgenera *P.* (*Phymatispa*) and *P.* (*Phymata*) also occurred during the Eocene (47.77 mya; 95% HPD [31.42, 67.13]). *Phymata interjecta* Dudich, a species from Hispaniola, diverged from the main land taxa during the Oligocene (29.86 mya; 95% HPD [17.69, 43.81]). *Phymata crassipes* likely diverged from the New World *Phymata* no earlier than around the Oligocene–Miocene boundary (23.93 mya; 95% HPD [14.38, 36.34]).

3.3. Ancestral distributions

The ancestral ranges inferred from the DEC and S-DIVA analyses are summarized in Table S5b. Major results from the DEC analysis are illustrated on the maximum clade credibility chronogram (Fig. 3). While somewhat ambiguous, our results indicate that the MRCA of Phymatinae occurred in the Neotropics + Oriental + Afrotropics, potentially suggesting an origin in the tropics of Gondwana during the early stages of its break up. Themonocorini then became confined to Africa, while DEC recovered the ancestral range of Phymatini + (“Macrocephalini” + Carcinocorini) as the Neotropical and Oriental regions. “Macrocephalini” gave rise to Carcinocorini in the Oriental region. Phymatini, i.e., the genus *Phymata* and the subgenera *P.* (*Phymatispa*) and *P.* (*Phymata*) originated in the Neotropics. Together, DEC and S-DIVA estimated multiple colonization events of the Nearctic from the Neotropics by Phymatini with an initial dispersal occurring sometime between 29.86 and 23.93 mya. S-DIVA estimated a second dispersal from the Neotropics to the Nearctic region in the Miocene (13.71–11.44 mya). Both analyses also estimated dispersal of *Phymata* into the Palearctic from the New World occurring after 29.86 mya, and then subsequent vicariance roughly following the Oligocene-Miocene boundary at 23.03 mya (Cohen et al., 2013).

4. Discussion

4.1. Phylogeny of Phymatinae

Our phylogenetic analyses confirm the monophyly of Phymatinae and reconstruct Holoptilinae + Centrocnemidinae as its sister group, corroborating the hypothesis proposed by Weirauch et al. (2011). The phallus of male Holoptilinae and Elasmodeminae are very similar (Carayon et al., 1958), as well as that of Centrocnemidinae (Rédei and Tsai, 2011), in being elongate, membranous, without sclerotization, and with whip-like extensions of the basal plate struts. A combined analysis incorporating molecular and morphological data, particularly of the male phallus, is needed to further test subfamily-level relationships in the Phymatine Complex.

Reconstruction of tribal-level relationships of Phymatinae revealed paraphyly among “Macrocephalini” with respect to Carcinocorini. This result is not entirely surprising given the cosmopolitan distribution of “Macrocephalini”, their diverse morphology, and observations associating the two tribes made by early taxonomists. Handlirsch (1897) speculated that Carcinocorini may be closely related to certain Oriental “Macrocephalini” based on similar wing venation, especially the genera *Glossopelta* Handlirsch, *Amblythyreus* Westwood, *Cnizocoris* Handlirsch, and *Agreuocoris* Handlirsch. Later, Maa and Lin (1956) suggested that “Macrocephalini” could be divided into two groups, one containing the putatively more plesiomorphic New World genera (*Extraneza* Barber + (*Macrocephalus* + *Lophoscutus*)) and the other all Old World genera (*Cnizocoris* + (*Amblythyreus* complex + *Agreuocoris* complex). Maa and Lin (1956) further postulated that *Cnizocoris* may represent the earliest diverging lineage of the Old World clade. They also suggested that *Amblythyreus* and several Afrotropical genera such as *Narina* Distant, and *Oxythyreus* Westwood form a clade sister to the remaining Oriental “Macrocephalini” (e.g., *Glossopelta*, *Agreuocoris*, and *Diurocoris* Maa & Lin). Among the suspected New World clade, *Extraneza* and *Kormilevida* van Doesburg, two monotypic Caribbean genera, are the only known “Macrocephalini” that possess foretarsi, while the foretarsus is completely reduced in all remaining “Macrocephalini” and the Carcinocorini. *Kormilevida* shares antennal characteristics with *Agdistocoris* Kormilev, an Oriental genus of “Macrocephalini”, and possesses ultraconnexiva, a unique trait for “Macrocephalini” although also documented in several species of Phymatini (Kormilev and van Doesburg, 1991). To further examine relationships of “Macrocephalini”, future studies will need to include additional genera from the Oriental region, as well as Afrotropical taxa, and the two obscure genera from the Greater Antilles.

Our phylogenetic hypothesis indicates that several currently recognized species of *Phymata* are either paraphyletic or polyphyletic, especially within the largely Nearctic erosa group. Some *Phymata* taxa are based primarily on coloration (e.g., *P. pallida* and *P. luteomarginata* Kormilev), a trait that can be highly variable across populations and one that is unreliable for species delimitation. Morphological characters such as the shape of the pronotal margin, however, may be more useful in delineating species. We observe congruence between pronotal morphology and relationships reconstructed with molecular data. For instance, the pronotal margin of *P. f. mystica* is quite different from that of other subspecies of polyphyletic *P. fasciata* and shares more in common with “*P. americana*” and its kin, taxa that it is closely related to genetically. *Phymata fasciata mystica* bears spiniform lateral and posterior angles on the pronotal hind lobe, while the remaining *P. fasciata* subspecies have angles that are generally more obtuse. *Phymata pennsylvanica* also shares many morphological characters with “*P. americana*” and a recent study has demonstrated that the two “species” lack prezygotic barriers and can successfully hybridize (Punzalan and Rowe, 2017).

Kormilev (1962) used scutellar morphology to divide *P.* (*Phymata*) into two main groups. One consists of taxa with a linear or sublinear median carina on the scutellum (a trait shared across all other *Phymata* subgenera), while the other is comprised of taxa that possess a cruciform or subcruciform carina. All of the erosa group taxa sampled for our study form a clade and possess cruciform or subcruciform carinae. Neotropical and Palearctic *Phymata* more distantly related to this clade tend to exhibit linear or sublinear carinae. Since all species of the subgenera *Phymata* (*Euryphymata*) Kormilev, *Phymata* (*Neophymata*) Kormilev, and *P.* (*Phymatispa*) possess linear median carinae, cruciform or subcruciform median carinae appear to be a relatively recently derived characteristic exhibited by only some Neotropical and Nearctic *P.* (*Phymata*).

Our study also indicates that Palearctic *P. crassipes* is derived from a New World ancestor. This finding supports earlier hypotheses by Handlirsch (1897) and Kormilev (1957, 1962), who believed Old World *Phymata* to be closely related with *P. vicina* and its kin from the Nearctic. The two subgenera *P.* (*Phymata*) and *P.* (*Phymatispa*) diverged during the Eocene (Fig. 3), a relatively ancient divergence for reduviid subgenera. This result was not unsuspected given the distinct morphology between the two groups. In particular, male *P.* (*Phymatispa*) are notable for being easily distinguished from taxa of other *Phymata* subgenera by their forked parameres (Kormilev, 1962). A majority of erosa group taxa, on the other hand, share relatively recent ancestors having split within the past 10 million years. Additional taxon sampling, including rare species, Neotropical taxa, and male and female conspecifics, will be required to fully resolve species-level relationships. Considering the para- and polyphyly observed in this phylogenetic reconstruction, it is evident that Nearctic *Phymata* will need to undergo extensive reclassification following more detailed molecular, morphological, and ecological investigations including more comprehensive taxonomic and geographic sampling.

4.2. Biogeography of the ambush bugs

Our biogeographic analyses generally substantiate previously held views regarding the origins and distributions of ambush bugs, but some ancestral biogeographic patterns remain ambiguous. We incorporated an additional tribe of Phymatinae (Themonocorini) that is endemic to sub-Saharan Africa and whose biogeographic history in relation to other ambush bugs was never hypothesized. Given a Cretaceous origin (Hwang and Weirauch, 2012), it is plausible that the ancestor of Phymatinae was subjected to circumtropical vicariance (sans Australia) as the southern landmasses comprising Gondwana drifted apart.

Based on our analyses, we suspect that Phymatini originated in the Neotropics, colonized the Nearctic multiple times, and dispersed into the Palearctic. The Greater and Lesser Antilles support substantial ambush bug diversity, harboring taxa from three of the five genera of Phymatini (∼11 spp.) and three genera of “Macrocephalini” (∼28 spp.), but how these tribes colonized and diversified on this archipelago has remained unclear. Long before the closure of the Isthmus of Panama (∼3.5 mya) (Coates et al., 1992), Reduviidae may have colonized the Caribbean Islands by way of GAARlandia (Justi et al., 2016), which reached maximum land exposure around 35–33 mya (Iturralde-Vinent and MacPhee, 1999). The ancestor of *P. interjecta* that today is restricted to Hispaniola may have colonized the archipelago during that time.

Phymata could have colonized the Nearctic through the Caribbean archipelago (29.86–23.93) or the Panama Island Arc (∼15 mya) prior to the closure of the Isthmus of Panama (Sanmartín and Ronquist, 2004). However, given their small size and association with plants and the prevalence of tropical storms in this area, we cannot rule out the possibility that ambush bugs may also have dispersed throughout the Caribbean over water. The relationships between and among Caribbean and Nearctic taxa remain unclear, and additional sampling of Caribbean taxa is necessary to fully test our biogeographic hypotheses.

Based on our analyses, *Phymata* colonized the Palearctic from the New World during the last 30 million years and *P. crassipes*, the only Palearctic species sampled, diverged from New World taxa roughly around the Oligocene-Miocene boundary. DEC recovered highest support for a dispersal event from the Nearctic + Neotropical regions to the Palearctic, while S-DIVA recovered the highest support for a long distance dispersal into the Palearctic from either South America or the Caribbean. This S-DIVA result could be an artifact due to the fact that no Nearctic taxa putatively closely related to *P. crassipes* (i.e., *P. vicina*, *P. maculata*, *P. pallida*) were included in our dataset. The addition of any of these Nearctic taxa may greatly reduce the estimated divergence time for *P. crassipes* (currently estimated at ∼23.9 mya) from New World taxa.

Phymata crassipes (or its ancestor) may have dispersed to the Old World via a high latitude land connection that linked North America with the Palearctic or via a chance transoceanic dispersal event. The latter hypothesis is difficult to test since the sustained dispersal capabilities of ambush bugs are not well understood, but seems highly unlikely given that *Phymata* do not appear to be apt long distance fliers (personal observation) and typically spend most of their time on blooming vegetation (Balduf, 1941).

Holarctic land bridges facilitated the intercontinental dispersal of many terrestrial species during the Cenozoic (Sanmartín et al., 2001). The Bering land bridge was a terrestrial isthmus that linked eastern Asia with western North America and allowed for the overland exchange of taxa from the Early Cenozoic to as recently as the Late Pliocene (3.5 mya) (Sanmartín et al., 2001). Mean annual temperatures of Beringia are estimated to have stayed relatively warm (∼11 to 12 °C) through the Middle Miocene (Wolfe, 1994). As a result, many temperate insects are believed to have dispersed between North America and Asia over the Bering land bridge during the Miocene, including bumble bees (Hines, 2008), colletid bees (Kuhlmann et al., 2009), and various groups of butterflies (Pena et al., 2010; Vila et al., 2011; Wu et al., 2015). The Thulean and DeGeer land bridges are thought to have enabled faunal exchange between North America and Eurasia across the North Atlantic. The Thulean route, in particular, is believed to have served as a major avenue that allowed the exchange of both temperate and tropical taxa during its exposure (Tiffney, 1985). However, while the Bering land bridge was emergent throughout most of the Cenozoic, the Thulean and DeGeer bridges were exposed for shorter periods and disappeared approximately 50 and 39 mya, respectively (Sanmartín et al., 2001).

The time periods estimated for the major trans-Atlantic bridges, however, do not coincide with the more recent estimated dates for *Phymata* dispersal into the Palearctic (no earlier than ∼30 mya), so we speculate, that like many other temperate insects, *Phymata* may have crossed the Bering land bridge and spread across Eurasia following the dispersal of temperate flowering plants (Milne, 2006) and their pollinators. Considering the relatively few species of *Phymata* present in the Palearctic, dispersal into Eurasia may have occurred relatively recently or Old World *Phymata* may have faced one or several major extinction events. Of all the ambush bugs, *Phymata* show the greatest tolerance towards seasonal variability and mild climate, exhibiting a genus-wide distribution that spans from the equator to relatively high temperate latitudes (∼50°N and ∼40°S) (AEC database). Their ability to persist in cool temperate habitats leads us to believe that they could have crossed Beringia possibly up until the Late Miocene, similar to other temperate insects.

Our divergence dating and biogeographic hypotheses can be more rigorously tested in the future by including additional *Phymata* taxa from the Palearctic and Caribbean, and several rare Nearctic taxa that share similarities with those from the Palearctic such as *P. vicina* and *P. maculata*. Inclusion of “Macrocephalini” from the Afrotropical region and Caribbean (i.e., *Extraneza* and *Kormilevida*) may also help resolve poorly supported early diverging nodes of Phymatini + (“Macrocephalini” + Carcinocorini) and New World “Macrocephalini” + (Old World “Macrocephalini” + Carcinocorini). Future analyses will also benefit from the inclusion of representatives of Elasmodeminae, a rare reduviid subfamily that is believed to be sister to Holoptilinae based on morphological similarities (Carayon et al., 1958) and Phimophorinae (Davis, 1961).

5. Conclusions and future directions

The first comprehensive phylogeny of Phymatinae offers an improved understanding of phymatine tribal-level relationships. Given support for a New World origin of Phymatini and barring any long distance transoceanic dispersal events, we hypothesize that *Phymata* dispersed across the Caribbean region via GAARlandia, underwent multiple colonizations of the Nearctic, and dispersed into the Palearctic by way of the Bering land bridge. This study also provides a framework to further test species-level relationships within Nearctic *Phymata*. Of special interest are the relationships within the erosa species complex, a group of species that are commonly used in studies on the evolution of sexual selection and predator-prey systems, and general ecology.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.06.010.

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