# Phylogenetics and biogeography of the endemic Madagascan millipede assassin bugs (Hemiptera: Reduviidae: Ectrichodiinae) 

Michael Forthman*, Christiane Weirauch<br>University of California, Department of Entomology, 900 University Avenue, Riverside, CA 92521, USA

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#### Abstract

For at least the past 80 my , Madagascar, a major biodiversity hotspot, has been isolated from all other landmasses. This long-term isolation, along with geologic and climatic factors within Madagascar and throughout the Indian Ocean, has undoubtedly influenced the evolution of the island's biota. However, few systematic analyses incorporating modern divergence dating and biogeographic analyses have focused on Madagascan insects. The diverse Madagascan millipede assassin bugs (Heteroptera: Reduviidae: Ectrichodiinae) offer an opportunity to contribute to a limited body of insect-related research that explores Madagascar's historical biogeography. A molecular dataset (COI mtDNA and 18S, 28S D2 and D3-D5 rDNAs) for 56 taxa ( 39 ingroup) and a combined morphological ( 145 characters) and molecular dataset for 110 taxa ( 93 ingroup) are analyzed with maximum likelihood (ML) and parsimony approaches. Based on the molecular ML phylogeny, divergence times were estimated using fossil and secondary calibrations and biogeographic analyses performed using DIVA, DEC, and DEC +j models to determine the role and patterns of vicariance and dispersal in the origin of Madagascan Ectrichodiinae. Results indicate that Ectrichodiinae in Madagascar do not form a monophyletic group, different clades are closely related to Afrotropical and Oriental lineages, and have colonized the island via transoceanic dispersal at least twice from the Oriental region and once from the Afrotropical region in the last $\sim 68 \mathrm{my}$. Additionally, the DEC +j and DIVA models infer a single out-of-Madagascar dispersal event to the Afrotropical region. Oceanic and geologic factors that may have facilitated dispersal between these three regions are discussed. Results of the combined analyses are used to explore character support for Madagascan taxa and inform taxonomic diagnoses. Our results are congruent with the small but growing body of biogeographic research supporting Cenozoic transoceanic dispersal for Madagascan invertebrates to and from Oriental and Afrotropical regions.


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## 1. Introduction

Madagascar, the fourth largest island on Earth, is one of the world's biodiversity hotspots with more than 13,000 plant, 900 vertebrate, and 5800 invertebrate species known (Goodman and Benstead, 2005; Phillipson et al., 2006). For non-invasive species, $\sim 86 \%$ of invertebrates, $\sim 50 \%$ of birds, more than $95 \%$ of other vertebrates, and more than $90 \%$ of vascular plants are endemic to the island (Goodman and Benstead, 2005; Yoder and Nowak, 2006; Phillipson et al., 2006; Buerki et al., 2013). Numerous phylogenetic studies have provided evidence that a large proportion of Madagascar's biota is closely related to African lineages ( $\sim 37 \%$ of plants, $\sim 30 \%$ of vertebrates, $\sim 58 \%$ of invertebrates [see Yoder and Nowak, 2006]). However, a significant proportion of the biota is closely

[^0]related to Oriental taxa (see Vences, 2004; Yoder and Nowak, 2006; Warren et al., 2010); about $32 \%$ of plants, $32 \%$ of vertebrates, and $17 \%$ of invertebrates are sister to Oriental lineages (Yoder and Nowak, 2006). This extant, unique biodiversity is, in the words of Ganzhorn et al. (2014), "rooted in the plate tectonics of the Mesozoic" (i.e., long isolation period), as well as the island's geography, climate, and late colonization by humans (Goodman and Benstead, 2003; Scales, 2014).

Madagascar was once part of Gondwana, but $\sim 160$ mya, the Gondwanan landmass began to split with Indo-Madagascar separating from Africa (Plummer and Belle, 1995; Gnos et al., 1997). By at least 80 mya, Madagascar separated from India and has been isolated $\sim 400 \mathrm{~km}$ from Africa's southeastern coast since (Storey et al., 1995; Torsvik et al., 2000; Seward et al., 2004; Ali and Aitchison, 2008). Many plant and vertebrate studies have investigated historical biogeographic patterns for various Madagascan taxa. Although some systematic studies support influences of

Gondwanan vicariance for the presence of some Madagascan lineages (e.g., ranid frogs [Bossuyt et al., 2006], boid snakes [Noonan and Chippindale, 2006], chameleons [Okajima and Kumazawa, 2010]), many recent studies based on divergence dating analyses support post-Gondwanan transoceanic dispersal from Afrotropical and Oriental regions (e.g., endemic mammalian lineages [Poux et al., 2005], Chrysophylloideae [Bartish et al., 2011], gerrhosaurid lizards [Raselimanana et al., 2009]). Comparatively fewer studies have investigated biogeographic histories of Madagascan insects. Several Madagascan insects have been hypothesized to be relicts of Gondwanan vicariance, e.g., Diplatyidae (Dermaptera) (Popham, 2000), Notonemourinae (Plecoptera) (Illies, 1965; Paulian and Viette, 2003), some Blephariceridae (Diptera) (Paulian and Viette, 2003), and Sialidae (Liu et al., 2015), but there is an increasing number of systematic studies that support Cenozoic transoceanic dispersal from the Afrotropical and Oriental regions. For example, allodapine bees (Fuller et al., 2005; Schwarz et al., 2006; Chenoweth and Schwarz, 2011) and scarabid beetles (Wirta et al., 2008, 2010; Sole et al., 2011) colonized Madagascar multiple times in the last 65 my. Fungus-growing termites in Madagascar originated from a single colonization event $\sim 7-11$ mya (Nobre et al., 2010). Other studies on carpenter bees (Rehan et al., 2010), mayflies (Monaghan et al., 2005), pierid butterflies (Nazari et al., 2011), and dytiscid beetles (Bukontaite et al., 2015) have also supported Cenozoic colonization.

Millipede assassin bugs (Heteroptera: Reduviidae: Ectrichodiinae) are a diverse group of specialized millipede predators with 736 species in 121 genera that are distributed in circumtropical and some temperate regions (Maldonado, 1990; Dougherty, 1995; Carpintero and Maldonado, 1996; Forthman and Weirauch, 2012; Forthman et al., 2016). Recently, Forthman et al. (2016) published a monograph of Madagascan millipede assassin bugs (excluding the genus Distirogaster Horváth) in which 63 new species and three new genera were described, increasing the known Madagascan millipede assassin bug fauna by six-fold. All species and six of the eight genera found on Madagascar are endemic; of the 37 species of Glymmatophora Stål and 39 species of Maraenaspis Karsch, two and one, respectively, are endemic to Madagascar with the remainder distributed in Africa. This diverse Madagascan millipede assassin bug fauna thus presents an opportunity to investigate and contribute to a limited yet rapidly growing body of research exploring historical influences that have shaped Madagascar's terrestrial insect fauna. However, such an evolutionary investigation has been hindered by a lack of phylogenetic hypotheses; relationships between Ectrichodiinae genera have not been tested cladistically beyond a genus-level phylogeny of the New World fauna based on morphological data (Dougherty, 1995). A recent systematic study of the Reduviidae indicates that Ectrichodiinae originated $57-80$ mya and, therefore, after the Madagascar-India split (Hwang and Weirauch, 2012). Thus, dispersal is likely responsible for the current distribution of millipede assassin bugs worldwide, but it is unknown what factors (e.g., ocean currents, land bridges, and stepping-stone islands) may have facilitated dispersal between Madagascar and other biogeographic regions.

Given the recent taxonomic treatment, Upper Cretaceous age, and worldwide distribution of the subfamily, as well as the general restriction of genera to particular biogeographic regions, millipede assassin bugs are an excellent model for biogeographic studies of the Madagascan fauna. Here, we present molecular phylogenetic results that include representatives of six of the eight Madagascan Ectrichodiinae genera and provide a framework for testing the number, timing, and origin of Madagascan lineages. Based on these results, we perform divergence dating and biogeographic analyses to investigate the geographic and oceanic influences that could have facilitated long-distance dispersal between Madagascar
and other biogeographic areas. We also present results of a combined morphological and molecular phylogenetic analysis on a large sample of Madagascan Ectrichodiinae species that is used to formalize taxonomic decisions and inform diagnoses in Forthman et al.'s (2016) Madagascan Ectrichodiinae monograph.

## 2. Material and methods

### 2.1. Taxon sampling, vouchering, and databasing

A total of 110 terminal taxa were examined, comprising 93 ingroup (Ectrichodiinae) and 17 outgroup taxa (seven Reduviidae subfamilies). Ingroup sampling comprised all eight genera and 67 species of Madagascan Ectrichodiinae. Due to the lack of DNA quality material, only 56 terminal taxa were sequenced ( 39 ingroups, including 14 Madagascan species in six genera; sequence data for the Madagascan genus Toliarus Forthman, Chłond, and Weirauch and the species Maraenaspis bidens [Reuter] are unavailable). Each taxon is represented by a mounted primary specimen voucher (molecular vouchers are listed in Table 1). For specimens sampled for molecular data, a hind leg was removed for DNA extraction; once extraction was completed, the leg was card mounted and associated with the pinned specimen. Each molecular voucher is associated with a unique specimen identifier (USI) label to connect it with specimen information and, where possible, images in the Planetary Biodiversity Inventory database (http://www.research. amnh.org/pbi/locality/index.php) and Heteroptera Species Pages (http://research.amnh.org/pbi/heteropteraspeciespage/) maintained by the American Museum of Natural History. All USI labels are comprised of the prefix AMNH_PBI or UCR_ENT followed by an 8 -digit number. Sequenced specimens are also associated with an RCW number, which is the ethanol specimen collection number.

Specimens examined for this study are deposited in the following institutions: AMNH, American Museum of Natural History, New York, USA; BMNH, Natural History Museum, London, United Kingdom; CAS, California Academy of Sciences, California, USA; FCAP, Universidade Federal do Pará, Pará, Brazil; MNHN, Muséum National d'Histoire Naturelle, Paris, France; MRAC, Musée Royal de l'Afrique Centrale, Tervuren, Belgium; MTEC, Montana State University, Montana, USA; SU, Department of Zoology, University of Silesia, Poland; UCR, University of California, Riverside Entomological Research Museum, California, USA; USNM, National Museum of Natural History, Washington, D.C., USA.

### 2.2. Morphological methods, terminology, and abbreviations

Extreme sexual dimorphism (Fig. 1) is common among millipede assassin bugs and poses a significant problem when analyzing evolutionary relationships. As such, male ectrichodiine specimens were targeted for morphological character coding. However, two terminal taxa, Maraenaspis coccinea Horváth and Racelda sp., are represented by apterous females. The male of Maraenaspis coccinea has, to our knowledge, yet to be discovered and available specimens of Racelda were restricted to the one female at the time of this project. Despite the possibility of extreme dimorphism, some morphological features (e.g., antennal segmentation, relative lengths of labial segments, scutellar apical processes, etc.) remain similar among males and females within ectrichodiine species and genera. As such, these sexually static morphological features are inferred and coded in the analysis for taxa only represented by females.

External morphology and genitalic characters were examined using Nikon SMZ1000 and SMZ1500 dissecting microscopes. For males, genitalia (abdominal segment 8 , pygophore, and phallus) were dissected, cleared in heated $10 \%$ potassium hydroxide

Table 1
 sequences retrieved from GenBank.

| Subfamily | Taxon | USI | RCW | Depository | GenBank Accession No. |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  |  |  |  | COI | 18S | 28S D2 | 28S D3-D5 |
| Ectrichodiinae | Bannania n. sp. | UCR_ENT 00119027 | 3064 | UCR | KT221890 | KT221910 | KT221939 | KT221968 |
|  | nr Bannania sp. | UCR_ENT 00004465 | 609 | UCR | KT221908 | KT221937 | KT221966 | KT221996 |
|  | Brontostoma colossus | UCR_ENT 00119017 | 3049 | UCR | KT221891 | KT221911 | KT221940 | KT221969 |
|  | Brontostoma sanguinosum | UCR_ENT 00002617 | 1243 | UCR | KT221892 | KT221912 |  | KT221970 |
|  | Caecina sp. | UCR_ENT 00004440 | 2636 | UCR | KT221893 | KT221913 | KT221941 | KT221971 |
|  | Centraspis ducalis | UCR_ENT 00119021 | 3014 | UCR | KT221894 | KT221914 | KT221942 | KT221972 |
|  | Cleptria corallina | AMNH_PBI 00218770 | 14 | UCR |  | FJ230462* | FJ230543* | FJ230621*, FJ230700* |
|  | Cryptonannus n. sp. | UCR_ENT 00002757 | 1433 | UCR | KT221895 | KT221915 |  | KT221973 |
|  | nr Daraxa sp. | UCR_ENT 00119016 | 3076 | UCR | KT221909 | KT221938 | KT221967 | KT221997 |
|  | Distirogaster tarsalis | UCR_ENT 00006366 | 2898 | CAS | KR606396 | KT221918 | KT221945 | KT221976 |
|  | Distirogaster n. sp. 1 | UCR_ENT 00007158 | 2881 | UCR | KR606393 | KT221916 | KT221943 | KT221974 |
|  | Distirogaster n. sp. 2 | UCR_ENT 00088090 | 3018 | UCR | KR606395 | KT221917 | KT221944 | KT221975 |
|  | Ectrichodia crux | UCR_ENT 00119022 | 3026 | UCR | KT221896 | KT221919 | KT221946 | KT221977 |
|  | Ectrichodia lucida | AMNH_PBI 00218769 | 13 | UCR |  | FJ230461* | FJ230542* | FJ230620*, FJ230699* |
|  | Ectrychotes serdangensis | AMNH_PBI 00218830 | 76 | UCR |  | FJ230479* | FJ230560* | FJ230638*, FJ230717* |
|  | Ectrychotes sp. 1 | UCR_ENT 00119028 | 571 | UCR | KT221897 | KT221920 | KT221947 | KT221978 |
|  | Ectrychotes sp. 2 | AMNH_PBI 00218932 | 188 | UCR | JQ942322* | FJ230503* | FJ230584* | FJ230661*, FJ230740* |
|  | Gibbosella quadocris | UCR_ENT 00044860 | 2938 | UCR | KR606406 |  |  |  |
|  | Glymmatophora crassipes | UCR_ENT 00088087 | 3020 | UCR | KR606415 | KT221921 | KT221948 | KT221979 |
|  | Maraenaspis coccinea | AMNH_PBI 00218772 | 16 | UCR |  | FJ230463* | FJ230544* |  |
|  | Marojejycoris brevifrons | UCR_ENT 00006480 | 2923 | CAS | KR606417 | KT221922 |  | KT221980 |
|  | Mendis apicimaculata | UCR_ENT 00119026 | 2647 | UCR | KT221898 | KT221923 | KT221949 | KT221981 |
|  | Microstemmatoides atrocyanea | UCR_ENT 00119029 | 3029 | UCR | KT221899 | KT221924 | KT221950 | KT221982 |
|  | Neolibavius n. sp. | UCR_ENT 00003965 | 1830 | UCR | KT221900 | KT221925 | KT221951 | KT221983 |
|  | Pothea lugens | UCR_ENT 00119018 | 3304 | UCR | KT221902 | KT221927 | KT221953 |  |
| Ectrichodiinae | Racelda sp. | AMNH_PBI 00218801 | 41 | UCR |  | FJ230472* | FJ230553* | FJ230631*, FJ230710* |
|  | Rhiginia aimara | UCR_ENT 00119019 | 3077 | UCR | KT221903 | KT221928 | KT221954 |  |
|  | Rhiginia cinctiventris | AMNH_PBI 00218891 | 139 | UCR | JQ942333* | FJ230490* | FJ230571* | FJ230648*, FJ230727* |
|  | Rhiginia sp. | UCR_ENT 00119020 | 3047 | UCR | KT221904 | KT221929 | KT221955 | KT221985 |
|  | Santosia sp. | UCR_ENT 00004138 | 2046 | UCR | KT221905 | KT221930 | KT221956 | KT221986 |
|  | Tanindrazanus marginatus | UCR_ENT 00006887 | 2902 | CAS | KR606423 | KT221931 | KT221957 | KT221987 |
|  | Tanindrazanus tenebricus | UCR_ENT 00006723 | 2931 | CAS | KR606430 | KT221932 | KT221958 | KT221988 |
|  | Tanindrazanus varicolor | UCR_ENT 00006482 | 2949 | UCR | KR606433 | KT221933 | KT221959 | KT221989 |
|  | Toxopus brucei | UCR_ENT 00045338 | 2901 | UCR | KR606438 |  | KT221960 | KT221990 |
|  | Toxopus fisheri | UCR_ENT 00045431 | 2910 | CAS | KR606446 |  | KT221961 | KT221991 |
|  | Toxopus griswoldi | UCR_ENT 00006435 | 2916 | CAS | KR606448 | KT221934 | KT221962 | KT221992 |
|  | Toxopus toamasina | UCR_ENT 00007056 | 2951 | SU | KR606457 |  | KT221963 |  |
|  | Toxopus vazimba | UCR_ENT 00006472 | 2953 | CAS | KR606462 | KT221935 | KT221964 | KT221993 |
|  | Vilius macrops | UCR_ENT 00119025 | 3068 | UCR | KT221907 | KT221936 |  | KT221995 |
| Emesinae | Emesaya incisa | AMNH_PBI 00219017 | 282 | UCR | JQ942323* | FJ230515* | FJ230598* | FJ230672*, FJ230751* |
| Harpactorinae | Apiomerus lanipes | AMNH_PBI 00219016 | 281 | UCR | JQ942298* | FJ230514* | FJ230597* | FJ230671*, FJ230750* |
|  | Apiomerus ochropterus | AMNH_PBI 00218777 | 22 | UCR |  | FJ230466* | FJ230548* | FJ230625*, FJ230704* |
|  | Micrauchenus lineola | AMNH_PBI 00218790 | 35 | UCR | JQ942329* | FJ230471* | FJ230552* | FJ230630*, FJ230709* |
| Peiratinae | Peirates punctorius | AMNH_PBI 00218960 | 216 | UCR |  | FJ230508* | FJ230590* | FJ230666*, FJ230745* |
| Saicinae | Kiskeyana palassaina | AMNH_PBI 00190561 | 10 | USNM |  | FJ230460* | FJ230541* | FJ230619*, FJ230698* |
|  | Saica sp. | AMNH_PBI 00218796 | 42 | UCR |  | FJ230473* | FJ230554* | FJ230632*, FJ230711* |
| Stenopodainae | Oncocephalus sp. | UCR_ENT 00000182 | 79 | UCR |  | FJ230481* | FJ230562* | FJ230640*, FJ230719* |
|  | Stenopoda sp. | AMNH_PBI 00218904 | 154 | UCR |  | FJ230493* | FJ230574* | FJ230651*, FJ230730* |
|  | Stenopodessa sp. | UCR_ENT 00000078 | 398 | FCAP |  | FJ230532* | FJ230611* | FJ230688*, FJ230767* |
| Triatominae |  |  |  |  |  |  |  |  |
|  | Panstrongylus lignarius | UCR_ENT 00052166 | 1813 | UCR |  | JQ897584* | JQ897656* | JQ897741* |
|  | Triatoma recurva | AMNH_PBI 00218913 | 170 | UCR |  | FJ230496* | FJ230577* | FJ230654*, FJ230733* |
| Tribelocephalinae | Opistoplatys sp. 1 | UCR_ENT 00119024 | 3056 | UCR | KT221901 | KT221926 | KT221952 | KT221984 |
|  | Opistoplatys sp. 2 | UCR_ENT 00052187 | 1592 | UCR |  | JQ897612* | JQ897682* | JQ897767* |
|  | Tribelocephala peyrierasi | AMNH_PBI 00219033 | $287$ | CAS |  | FJ230521* | FJ230601* |  |
|  | Tribelocephala sp. | UCR_ENT 00119023 | 3048 | UCR | KT221906 |  | KT221965 | KT221994 |

( KOH ) for 3-8 min, washed with distilled water and subsequently with $100 \%$ ethanol (EtOH), stained with Chlorazol Black E in $70 \%$ EtOH solution to give contrast to membranous areas, examined in glycerol, and permanently stored in genitalic capsules pinned to the specimen. A total of 145 morphological characters were coded in the Descriptive Language for Taxonomy (DELTA) program (Dallwitz, 1980; Dallwitz et al., 1999) using a modified morphological matrix from Weirauch (2008) and Weirauch (2010). In general,
terminology follows a subset of terms used by Dougherty (1995), Weirauch (2008), and Forero and Weirauch (2012). Terminology for wing venation follows Hill (2014) and Weirauch (2008), although homology concepts are currently being reviewed across Paraneoptera (Dávid Rédei, pers. comm.). The morphological characters and character states used in this study are given in Table 2. The morphological matrix is provided in Supplementary Material (SM) 1 in nexus format.


Fig. 1. Examples and criteria of limited (top) and extreme (bottom) sexual dimorphism in Ectrichodiinae. Arrows in top panel indicate distal margin of wings.

### 2.3. Molecular markers, $P C R$, sequencing, and alignment

One mitochondrial (COI) and three nuclear (18S, 28S D2, and 28S D3-D5 rDNAs) gene regions were sampled. Sequences from Weirauch and Munro (2009) and Hwang and Weirauch (2012) were used for outgroup terminals and some Ectrichodiinae taxa (Table 1). To sequence remaining ectrichodiine taxa, primer sequences for 18 S (18SF, 18SR) and 28S D3-D5 (D3Fa, D5Ra) were obtained from Weirauch and Munro (2009); 28S D2 (D2Fa, D2Ra) from Forero et al. (2013); and COI (C1-J-2183F) from Simon et al. (1994) and (C1-N-2609R) from Damgaard et al. (2000). DNA was extracted from a hind leg for each specimen using QIAGEN DNeasy Blood and Tissue Kit protocols. PCR was performed using either GE Healthcare Life Sciences PuReTaq-Ready-To-Go-PCR-Beads or EmeraldAmp GT PCR Master Mix and Fisher Scientific or BioRad T100 Thermal Cyclers. Gene regions were amplified following Weirauch and Munro (2009), except the initial denaturation was set at 5 min , and for the 28 S gene regions, the annealing temperature was set to $50^{\circ} \mathrm{C}$. Gel electrophoresis with SyberSafe gel stain and a UV illuminator were used to check amplification results. PCR products were cleaned using Bioline SureClean and sequenced on an Applied Biosystems 3730xl DNA Sequencer at UCR's Institute for Integrative Genome Biology. Sequences were assembled and edited using Sequencher v4.8 and are available on GenBank (Table 1). Each gene region was treated as a separate partition and aligned in MAFFT (Katoh and Standley, 2013) using the G-INS-i algorithm. Gene regions were concatenated in SequenceMatrix v1.7.8 (Vaidya et al., 2011) and exported with external gaps coded as question marks. The concatenated, aligned molecular matrix is provided in nexus format in SM2.

### 2.4. Phylogenetic analyses

### 2.4.1. Molecular phylogenetic analyses

A maximum likelihood (ML) analysis was performed using the molecular dataset and RAxML-HPC2 on XSEDE v8.0.24 (Stamatakis, 2014) on the Cyberinfrastructure for Phylogenetic

Research Science Gateway v3.1 (CIPRES; http://www.phylo. org/sub_sections/portal/). Each gene partition was analyzed under a GTR + $\Gamma+$ I model, which is the best fit model for each partition as determined by MEGA v6.06 (Tamura et al., 2013). Rapid bootstrap analysis (BS) was performed for 1000 iterations and followed by a ML best tree search. Aside from estimating the proportion of invariable sites for each partition, default settings were used.

The molecular matrix was also subjected to parsimony analyses with New Technology search and equal (EW) and implied weights (IW) in TNT v.1.1 (Goloboff et al., 2008). Uninformative characters (1990) were inactivated prior to analysis. For IW, three concavity constant values ( $k$ ) were used: 3,6 , and 10 . Internal gaps were treated as a fifth state, while external gaps were treated as missing. Default settings were used for sectorial search, drift, and tree fusing. The initial driven search level was set at 100 with level checked every 3 hits, initial addition sequences $=14$, find minimum length 100 times, and random seed $=4325$. Standard BS resampling with absolute frequencies was performed using 500 replicates and New Technology search (initial driven search $=38$, check level every 3 hits, initial addition sequences $=7$, find minimum length 10 times).

### 2.4.2. Combined morphological and molecular phylogenetic analyses

Morphological and molecular datasets were concatenated using Mesquite v2.75 (Maddison and Maddison, 2011) and subjected to ML (RAxML-HPC v.8.1.15; Stamatakis, 2014) and EW parsimony analyses (New Technology search in TNT). For the ML analysis, each gene partition was analyzed under a GTR $+\Gamma$ model, while the morphological partition was analyzed with a Mkv model (Lewis, 2001). Rapid BS was performed for 1000 iterations and followed by a ML best tree search. Our equal weights parsimony analysis used the same search and bootstrap approaches applied to our molecular dataset (see Section 2.4.1). Unambiguous character optimizations were examined on the strict consensus tree in WinClada v1.00.08 (Nixon, 2002) (SM3).

### 2.5. Divergence dating estimation

Divergence dating estimates were generated on the molecular ML best tree using the CIPRES web server's BEAST2 on XSEDE (Bouckaert et al., 2014). Each gene region was assigned an unlinked site model (GTR $+\Gamma+\mathrm{I}$ ) and linked to the same tree model (Yule). The number of gamma rate categories for each site model was set to 4 and the proportion of invariant sites set to 0.5 , with the option to estimate the value of both parameters and the gamma shape. All other site model parameters and estimation options remained at default settings. Following Hwang and Weirauch (2012), a linked clock model was assigned to 28S D2 and 28S D3-D5 to reflect their single identity. As such, three unlinked relaxed lognormal clock models were assigned for $18 \mathrm{~S}, 28 \mathrm{~S}$, and COI. Initially, all noncalibration priors were not modified from default settings, however low posterior, prior, and mutation rate effective sample size (ESS) values were extremely low ( $<50$ ) after performing four independent runs in BEAST. To improve ESS values for these parameters, the default Jeffrey's prior was replaced with a $\log$ normal prior for the mutation rates (A. Rambaut \& A. Drummond, https://groups.google.com/forum/\#!forum/beast-users).

Given that there are no described Ectrichodiinae fossils, two outgroup fossils (Triatoma dominicana Poinar and Apicrenus fossilis Maldonado, Santiago-Blay, and Poinar) and two secondary calibrations (root node and Ectrichodiinae + Tribelocephalinae node) were used from a recent cladistic analysis of Reduviidae (Hwang and Weirauch, 2012). Fossils were assigned to nodes following Hwang and Weirauch (2012), using an apomorphy-based approach (Parham et al., 2011). For fossil-based node calibrations, a prior lognormal distribution was assigned with the minimum age

Table 2
Morphological characters and character state codings.

| Character no. | Character | Character states |
| :---: | :---: | :---: |
| General |  |  |
| 0 | Wing form | (0) Macropterous; (1) Brachypterous; (2) Apterous; (3) Micropterous |
| 1 | Body length | (0) Small; (1) Medium; (2) Large |
| 2 | General coloration | (0) Dull, ranging from pale to dark brown/black; (1) Metallic; (2) Red and black, sometimes with yellow or white markings; (3) Red and dull; (4) Orange and black; (5) Red and metallic |
| 3 | Bulbous hairs on integument | (0) Present; (1) Absent |
| 4 | Vestiture | (0) Very dense; (1) Moderately dense; (2) Sparse; (3) Glabrous |
| Head |  |  |
| 5 | Relative head length in dorsal view | (0) As long as wide; (1) Longer than wide; (2) Wider than long |
| 6 | Ventral surface of head | (0) Flat; (1) Anteromedially depressed; (2) Medially depressed; (3) Paramedially depressed |
| 7 | Depth of ventral head depression | (0) Shallow; (1) Deep |
| 8 | Ventral tubercles on head | (0) Absent; (1) Present |
| 9 | Ventrolateral tubercle on gula | (0) Absent; (1) Present |
| 10 | Anteocular length | (0) Shorter than postocular; (1) As long as postocular; (2) Longer than postocular |
| 11 | Postocular-neck constriction | (0) Absent, gradually transition; (1) Present, distinctly constricted |
| 12 | Gula shape in lateral view | (0) Flat, conforming to rounded head shape; (1) Moderately swollen ventrolaterally, not produced beyond ventral head margin; (2) Greatly swollen ventrolaterally, produced beyond ventral head margin |
| 13 | Postclypeal depression | (0) Flat; (1) Depressed |
| 14 | Width of postclypeal depression | (0) Narrow; (1) Broad |
| 15 | Depth of postclypeal depression | (0) Shallow; (1) Deep |
| 16 | Extension of postclypeal depression | (0) Posterior margin of clypeus to middle of synthlipsis; (1) Posterior margin of clypeus to interocular sulcus |
| 17 | Maxillary plate in lateral view | (0) Reaching or nearly reaching dorsal clypeal margin; (1) Not reaching dorsal clypeal margin |
| 18 | Clypeal process | (0) Absent; (1) Present |
| 19 | Shape of clypeus | (0) Without dorsal projection; (1) With dorsal projection |
| 20 | Clypeal apex relative to labral base | (0) Not elevated; (1) Elevated |
| 21 | Antennifer armature | (0) Unarmed; (1) With dorsolateral tubercle |
| 22 | Antennal shield | (0) Absent, not expanded; (1) Present, expanded |
| 23 | Antennal insertion site in lateral view | (0) Not concealed by antennal shield; (1) Concealed by antennal shield |
| 24 | Point of antennal insertion | (0) Sublateral or lateral; (1) Dorsal |
| 25 | Presence of ocellar lens | (0) Present; (1) Absent |
| 26 | Orientation of ocellar lens | (0) Medially; (1) Laterally |
| 27 | Ocellar lens size | (0) Large; (1) Small |
| 28 | Eye height in lateral view | (0) Less than or half of head height; (1) More than half of head height |
| 29 | Eye shape | (0) Adpressed; (1) Globbose |
| 30 | Anterior eye margin | (0) Convex; (1) Concave |
| 31 | Posterior eye margin | (0) Concave; (1) Straight |
| 32 | Dorsal eye margin | (0) Not attaining dorsal head surface; (1) Attaining dorsal head surface |
| 33 | Ventral eye margin | (0) Not attaining ventral head surface; (1) Attaining ventral head surface |
| 34 | Synthlipsis width | (0) About width of eye; (1) About 1.5 times width of eye; (2) About 2 times width of eye; (3) About 2.5 times width of eye; (4) About 3 times or more width of eye |
| 35 | Scapus length | (0) Not surpassing clypeal apex; (1) Surpassing clypeal apex |
| 36 | Pedicel curvature | (0) Straight; (1) Curved |
| 37 | Scapo-pedicellar articulation | (0) Slightly bent; (1) Strongly bent, pedicel and flagellomeres point posteriad |
| 38 | Antennal segmentation | (0) 4-segmented; (1) 6-segmented; (2) 7-segmented; (3) 8 -segmented; (4) $>8$ segments |
| 39 | Labrum structure | (0) Subdivided by transverse membrane; (1) Completely sclerotized |
| 40 | Ventral margin of labial segment III | (0) Straight; (1) Convex; (2) Concave |
| 41 | Labial segment III length | (0) Longer than labial segment II; (1) Shorter than labial segment II; (2) Subequal to labial segment II |
| Thorax |  |  |
| 42 | Pronotal length | (0) Wider than long; (1) As long as wide; (2) Longer than wide |
| 43 | Pronotal collar | (0) Distinct in dorsal view; (1) Indistinct in dorsal view |
| 44 | Anterior pronotal margin shape | (0) Medially concaved; (1) Straight or nearly straight |
| 45 | Anterior pronotal lobe shape | (0) Flat; (1) Dorsally elevated, conical |
| 46 | Anterior pronotal lobe structure | (0) Rugose; (1) Smooth; (2) Tuberculate |
| 47 | Anterolateral armature of pronotum | (0) Absent; (1) Small tubercle; (2) Strongly projecting process |
| 48 | Lateral carinae on anterior pronotal lobe | (0) Absent; (1) Present |
| 49 | Anterior pronotal lobe length | (0) Longer than posterior lobe; (1) Shorter than posterior lobe; (2) As long as posterior lobe |
| 50 | Anterior pronotal lobe width | (0) More than half as wide but not as wide as posterior lobe; (1) As wide as posterior lobe |
| 51 | Posterior pronotal lobe structure | (0) Striated; (1) Smooth; (2) Punctate; (3) Tuberculate; (4) Longitudinal ridges |
| 52 | Posterior pronotal lobe lateral depressions | (0) Present; (1) Absent |
| 53 | Posterior pronotal lobe lateral depression structure | (0) Not foveate; (1) Foveate |
| 54 | Extension of pronotal longitudinal depression on anterior lobe | (0) Reaching or nearly reaching anterior margin; (1) Not reaching anterior margin; (2) Absent |
| 55 | Presence of pronotal longitudinal depression on posterior lobe | (0) Absent; (1) Present |
| 56 | Structure of pronotal longitudinal depression on posterior lobe | (0) Not foveate; (1) Foveate |
| 57 | Pronotal transverse suture | (0) Incomplete, divided by paramedian ridges; (1) Complete, not divided by paramedian ridges |
| 58 | Pronotal transverse suture structure | (0) Not foveate; (1) Foveate |
| 59 | Scutellar processes | (0) None, scutellum triangular; (1) Two paramedian apical processes; (2) Two paramedian and one medial apical processes; (3) Two paramedian and two lateral processes |

Table 2 (continued)

| Character no. | Character | Character states |
| :---: | :---: | :---: |
| 60 | Distance between paramedian scutellar processes | (0) Narrow; (1) Broad |
| 61 | Length of paramedian scutellar processes | (0) Short; (1) Long |
| 62 | Orientation of paramedian scutellar processes | (0) Horizontally directed; (1) Dorsally directed |
| 63 | Scutellar disc surface | (0) Medially depressed; (1) Flat |
| 64 | Prosternal stridulatory process length | (0) Short; (1) Long, surpassing posterior margin of fore coxal cavity |
| 65 | Shape of apex of prosternal stridulatory process | (0) Acute; (1) Rounded |
| 66 | Transverse suture between meso- and metasterna | (0) Complete; (1) Incomplete, at least partially |
| 67 | Mesosternal surface | (0) Medially and laterally depressed; (1) Medially depressed; (2) Flat |
| 68 | Metasternal surface | (0) Medially depressed; (1) Flat or convex |
| 69 | Presence of metathoracic gland | (0) Absent; (1) Present |
| 70 | Presence of metathoracic gland evaporatorium | (0) Present; (1) Absent |
| 71 | Metathoracic gland evaporatorium size | (0) Small, not visible in lateral view; (1) Large, visible in lateral view |
| Leg |  |  |
| 72 | Shape of fore coxa | (0) Globular; (1) Elongate |
| 73 | Shape of fore femur | (0) Slender; (1) Incrassate |
| 74 | Presence of papillae on fore trochanter | (0) Absent; (1) Present |
| 75 | Presence of papillae on mid trochanter | (0) Absent; (1) Present |
| 76 | Presence of papillae on hind trochanter | (0) Absent; (1) Present |
| 77 | Presence of anteroventral row of spines/tubercles on fore femur | (0) Absent; (1) Present |
| 78 | Presence of posteroventral row of spines/tubercles on fore femur | (0) Absent; (1) Present |
| 79 | Presence of anterior subapical tubercle on fore femur | (0) Absent; (1) Present |
| 80 | Presence of posterior subapical tubercle on fore femur | (0) Absent; (1) Present |
| 81 | Presence of medial tubercle on fore femur | (0) Absent; (1) Present |
| 82 | Presence of carina on fore femur | (0) Absent; (1) Ventrally entirely carinate; (2) Ventrally carinate basally |
| 83 | Presence of papillae on fore femur | (0) Absent; (1) Present |
| 84 | Presence of anteroventral row of spines/tubercles on mid femur | (0) Absent; (1) Present |
| 85 | Presence of posteroventral row of spines/tubercles on mid femur | (0) Absent; (1) Present |
| 86 | Presence of anterior subapical tubercle on mid femur | (0) Absent; (1) Present |
| 87 | Presence of posterior subapical tubercle on mid femur | (0) Absent; (1) Present |
| 88 | Presence of medial tubercle on mid femur | (0) Absent; (1) Present |
| 89 | Presence of papillae on mid femur | (0) Absent; (1) Present |
| 90 | Presence of anteroventral row of spines/tubercles on hind femur | (0) Absent; (1) Present |
| 91 | Presence of anterior subapical tubercle on hind femur | (0) Absent; (1) Present |
| 92 | Presence of posterior subapical tubercle on hind femur | (0) Absent; (1) Present |
| 93 | Presence of subapical medial tubercle on hind femur | (0) Absent; (1) Present |
| 94 | Presence of medial tubercle on hind femur | (0) Absent; (1) Present |
| 95 | Presence of fossula spongiosa on fore tibia | (0) Present; (1) Absent |
| 96 | Presence of fossula spongiosa on mid tibia | (0) Present; (1) Absent |
| 97 | Protibial groove | (0) Absent; (1) Present |
| Fore wing |  |  |
| 98 | Extension of corium on fore wing | (0) Restricted to areas adjacent to basal wing veins and with pterostigma-like appearance; (1) Welldeveloped corium |
| 99 | Vestiture of corium on fore wing | (0) Long, simple setae; (1) Glabrous |
| 100 | Basal part of M and Cu on fore wing | (0) Separate veins; (1) Forming one vein, at least partially |
| 101 | Presence of cubital cell on fore wing | (0) Absent; (1) Present |
| 102 | Shape of cubital cell on fore wing | (0) Quadrate; (1) Hexagonal |
| 103 | Distal part of M and Cu on fore wing | (0) Separate veins; (1) Fused basally |
| 104 | Extension of distal part of M on fore wing | (0) Extends beyond distal $\mathrm{M}+\mathrm{Cu}$ junction; (1) No extension beyond distal $\mathrm{M}+\mathrm{Cu}$ junction |
| Abdomen |  |  |
| 105 | Color pattern of dorsal laterotergites | (0) Concolor; (1) Transversely bicolor; (2) Longitudinally bicolor |
| 106 | Expansion of dorsal laterotergite II | (0) Not expanded; (1) Laterally expanded; (2) Posterior tubercle |
| 107 | Dorsal laterotergite III armature | (0) Unarmed; (1) Posterior tubercle |
| 108 | Dorsal laterotergite IV armature | (0) Unarmed; (1) Posterior tubercle |
| 109 | Dorsal laterotergite V armature | (0) Unarmed; (1) Posterior tubercle |
| 110 | Dorsal laterotergite VI armature | (0) Unarmed; (1) Posterior tubercle |
| 111 | Connection of dorsal laterotergite and mediotergite | (0) Separated by membrane; (1) Sclerotized |
| 112 | Connection of ventral laterotergites and mediotergites III-VII | (0) Separated by membrane; (1) Sclerotized |

Table 2 (continued)

| Character no. | Character | Character states |
| :---: | :---: | :---: |
| 113 | Ventral connexival suture on sternite II | (0) Absent; (1) Present |
| 114 | Carinulation of intersegmental suture between abdominal sternites II and III | (0) Not carinulate; (1) Entirely carinulate |
| 115 | Carinulation of intersegmental suture between abdominal sternites III and IV | (0) Not carinulate; (1) Entirely carinulate; (2) Laterally carinulate |
| 116 | Carinulation of intersegmental suture between abdominal sternites IV and V | (0) Not carinulate; (1) Entirely carinulate; (2) Laterally carinulate |
| 117 | Carinulation of intersegmental suture between abdominal sternites V and VI | (0) Not carinulate; (1) Entirely carinulate; (2) Laterally carinulate |
| 118 | Carinulation of intersegmental suture between abdominal sternites VI and VII | (0) Not carinulate; (1) Entirely carinulate; (2) Laterally carinulate |
| 119 | Presence of paramedian carinae on abdominal sternites | (0) Absent; (1) Present |
| 120 | Abdominal sternite surface | (0) Flat; (1) Medially depressed; (2) Convex or keeled |
| 121 | Spiracle shape | (0) Ovoid or elliptical; (1) Circular |
| Male genitalia |  |  |
| 122 | Anteroposterior thickness of median pygophore process | (0) Flat; (1) Thickened, at least partially |
| 123 | Lateral thickness of median pygophore process | (0) Flat; (1) Thickened, at least partially |
| 124 | Lateral shape of median pygophore process | (0) Subtriangular; (1) Blade-like; (2) Hook-like; (3) Knob-like; (4) Rod-like |
| 125 | Shape of median pygophore process in caudal view | (0) Subtriangular; (1) Subquadrate; (2) Rod-like; (3) Spade-like |
| 126 | Apex of median pygophore process | (0) Entire; (1) Medially notched, bifid |
| 127 | Process on ponticulus basilaris | (0) Absent; (1) Present |
| 128 | Median process on ductifer | (0) Absent; (1) Present |
| 129 | Length of basal plate extension | (0) Shorter than basal plate; (1) As long as basal plate; (2) Longer than basal plate |
| 130 | Posterior margin of dorsal phallothecal sclerite | (0) Rounded; (1) Medially notched |
| 131 | Armature of dorsal phallothecal sclerite | (0) Unarmed; (1) Armed with denticle-like processes |
| 132 | Presence of vesica on endosoma | (0) Absent; (1) Present |
| 133 | Sclerotization of posterior endosomal margin | (0) Membranous; (1) Entirely sclerotized; (2) Paramedian sclerites; (3) Slender sclerotized processes; (4) Sclerotized lateral margin; (5) Sclerotized lateral hooks adjacent to fan-like lobs and with a medial dorsally projecting spine |
| 134 | Medial sclerotization of posterior endosomal region | (0) Membranous; (1) Slightly sclerotized; (2) Distinctly sclerite |
| 135 | Lateral sclerotization of posterior endosomal region | (0) Membranous; (1) Sclerotized |
| 136 | Medial sclerotization of medial endosomal region | (0) Membranous; (1) Slightly sclerotized; (2) Paramedial sclerites |
| 137 | Lateral sclerotization of medial endosomal region | (0) Membranous; (1) Sclerotized |
| 138 | Medial sclerotization of anterior endosomal region | (0) Membranous; (1) Sclerotized |
| 139 | Lateral sclerotization of anterior endosomal region | (0) Membranous; (1) Sclerotized |
| Glands |  |  |
| 140 | Presence of Brindley's gland | (0) Present; (1) Absent |
| 141 | Dorsal abdominal scent gland (DAG) I in adults | (0) Absent; (1) Present |
| 142 | DAG II in adults | (0) Absent; (1) Present |
| 143 | DAG III in adults | (0) Absent; (1) Present |
| 144 | Size of DAG III relative to DAGs I and II, external view | (0) Similar size; (1) Larger |

corresponding to the minimum age of the fossil and with the $95 \%$ confidence interval encompassing the range of estimated dates recovered by Hwang and Weirauch (2012). For secondary calibrations, a prior normal distribution was assigned with the $95 \%$ confidence interval corresponding to the estimated $95 \%$ highest posterior density (HPD) credibility interval from Hwang and Weirauch (2012). Calibration nodes, means, standard deviations or sigma values, and offsets (in real space) are given in SM4. Four Markov Chain Monte Carlo (MCMC) chains were ran for 200 million generations, with parameters and trees logged every 20,000 generations. Each chain was assessed for convergence in Tracer v1.6 and combined after discarding 5\% burn-in. Combined ESS values for each parameter were $\geqslant 200$. LogCombiner v2.1.3 was used to discard $5 \%$ of trees as burn-in and combine the remaining trees from each run. Median heights were annotated using TreeAnnotator v.2.1.2.

### 2.6. Biogeographic analyses

Ancestral ranges were reconstructed on the maximum clade credibility tree from the BEAST analysis using DIVA (DispersalVicariance Analysis) in RASP v3.02 (Ronquist, 1997; Yu et al., 2010, 2015) and DEC (dispersal-extinction-cladogenesis) (Ree et al., 2005; Ree and Smith, 2008) and DEC + j (Matzke, 2014) models in the BioGeoBEARS v0.2.1 package (Matzke, 2013) in R v3.2.0 ( R Core Team, 2015). Distribution ranges of Ectrichodiinae were divided into four areas: Neotropical, Afrotropical, Madagascar, and Oriental. Outgroup taxa were trimmed due to limited sampling of other assassin bug subfamilies.

The parsimony, event-based DIVA method was developed by Ronquist (1997) and reconstructs ancestral distributions based on a simple biogeographic model and a three-dimensional cost matrix. No penalty is allotted when speciation is the result of
vicariance, but dispersal and extinction have a penalty of one per unit area added to or deleted from a distribution (Ronquist, 1997). To reduce the acquisition of multiple areas at nodes near the root, the maximum number of areas for each node was two.

The DEC models geographic range evolution by stochastic dispersal and local extinction events along branches and estimates the likelihood of ancestral ranges at cladogenesis (Ree et al., 2005; Ree and Smith, 2008). This model has two free parameters specifying the dispersal rate ( $d$; range expansion) and extinction rate ( $e$; range contraction) along branches (Ree et al., 2005; Ree and Smith, 2008; Matzke, 2014). The DEC model also has a fixed cladogenesis model that assumes that one daughter lineage will always inherit one subarea of a widespread ancestral range (Matzke, 2014). The DEC + j model has an additional free parameter $j$ that enables one daughter lineage to disperse to a new range outside the ancestral range, thus modeling founder-event speciation events (Matzke, 2014). For both DEC and DEC + j models, the range constraint matrix was set at default with the maximum number of areas for each node set to two. Five time scales were constructed in the dispersal matrix to reflect changes in ocean currents in the Mozambique channel and the emergence of Indian Ocean islands: (1) 0-15 mya, (2) 15-23 mya, (3) 23-34 mya, (4) $34-56$ mya, and (5) 56-66 mya. Dispersal rates between areas were assigned as 0.1 (very low; oppositely directed ocean circulation, lack of islands and land bridges, and areas not connected), 0.25 (low; ocean circulation in dispersal direction or presence of islands or land bridges), 0.5 (moderate; ocean circulation in dispersal direction and presence of islands or land bridges), 0.75 (high; areas closely adjacent or directly connected), and 1.0 (same area) based on paleoceanographic and paleogeographic reconstructions (Sanmartín et al., 2001; von der Heydt and Dijkstra, 2006; Ali and Huber, 2010; Lomolino et al., 2010; Pyron, 2014). Dispersal rates assigned for each time scale are given in SM5. The DEC model is nested within the DEC +j model, and, as a result, we compare the two models using a Likelihood Ratio Test (LRT).

## 3. Results

### 3.1. Molecular phylogenetic analyses

The first published molecular ML best tree (Ln score $=-25426.19528)$ of millipede assassin bugs is shown in Fig. 2. Many nodes received moderate ( $B S=70-89$ ) to high support ( $\mathrm{BS}=90-100$ ), but several deeper nodes were poorly supported ( $\mathrm{BS}<70$ ). Ectrichodiinae is monophyletic with high support and recovered as the sister group to Tribelocephalinae, which is congruent with previous morphological and molecular cladistic analyses (Weirauch, 2008; Weirauch and Munro, 2009; Hwang and Weirauch, 2012). Two major ectrichodiine clades are recovered with low support: a predominantly Neotropical clade (node 1), which includes the Afrotropical genus Santosia Stål, and an Old World clade (node 2) that includes Afrotropical, Oriental, and Madagascan taxa.

Four Madagascan Ectrichodiinae lineages are recovered with low to high support. A representative of the Madagascan genus Gibbosella Chłond is nested within a clade of Oriental taxa with moderate support, but relationships within this clade, especially the Gibbosella and Caecina Stål sister group relationship, are poorly supported. The genera Tanindrazanus Forthman, Chłond, and Weirauch, Toxopus Bergroth, and Marojejycoris Forthman, Chłond, and Weirauch is a poorly supported clade. Relationships within this clade are largely weakly supported. Tanindrazanus is recovered as the sister group of Marojejycoris, and together are closely related to Toxopus. Distirogaster also forms a clade, but is weakly supported as the sister group to a clade comprising Afrotropical, Madagascan,
and Oriental taxa. The Madagascan species Glymmatophora crassipes Horváth is highly supported as the sister to the Afrotropical genus Ectrichodia Lepeletier and Serville.

A number of unresolved relationships are recovered throughout the EW parsimony phylogeny (SM6), e.g., near the root of the phylogeny (i.e., most outgroups and Ectrichodiinae + Tribelocephalinae), between Tanindrazanus + Marojejycoris and two other clades (Toxopus and Toxopus + Distirogaster), among others. Ectrichodiinae is also rendered paraphyletic with respect to Tribelocephalinae. The ectrichodiine genus Vilius Stål is sister to Tribelocephalinae genera, which is a questionable result. Due to the lack of resolution in our EW phylogeny, we used IW under three concavity constant values that resulted in one ( $k=6$ or 10 ) or three ( $k=3$ ) most parsimonious trees (SM6). Some of the relationships recovered from these IW analyses are similar with the molecular ML phylogeny. However, differences are observed (e.g., non-monophyly of Toxopus and Ectrichodiinae), some of which are questionable and unlikely based on morphology (e.g., Vilius nested with Tribelocephalinae, Maraenaspis coccinea sister to Caecina sp.). Some IW results consistently differed between weighting schemes, e.g., the phylogenetic positions of Marojejycoris, Toxopus toamasina, and Toxopus griswoldi. Despite topological differences between EW and IW parsimony trees, these results provide evidence for the non-monophyly of Madagascan Ectrichodiinae.

### 3.2. Combined morphological and molecular phylogenetic analyses

The TNT consensus tree of 938 parsimonious trees is shown in Fig. 3 (outgroups not shown but same as in Fig. 2) and was used to inform taxonomic decisions in Forthman et al. (2016). As in the molecular (SM2) and combined ML analyses, Ectrichodiinae are monophyletic and recovered as the sister to Tribelocephalinae with high support ( $B S=99$ ). A predominantly Neotropical clade (node 1) is also recovered with low support, but unlike the ML analysis, this clade includes the Oriental genus Vilius along with Santosia. An Old World clade (node 2) is recovered with several topological differences from the ML tree. Mendis Stål + Bannania Hsiao + Neolibavius Miller is weakly supported as the sister to the remaining taxa, followed by a weakly supported Microstemmatoides Putschkov + Synavecoris Villiers + nr Bannania clade. A clade (node 3) comprised of Ectrichodia, Glymmatophora, Cleptria Stål, Maraenaspis, and Ectrychotes Burmeister is recovered as sister to a predominantly Madagascan clade (node 4) with low support. Relationships within the latter clade are supported with BS < 50, with Marojejycoris sister to Gibbosella + Caecina, Marojejycoris + Gibbosella + Caecina sister to Distirogaster + Toliarus + Tanindrazanus + Toxopus (node 6), Distirogaster sister to Toliarus + Tanindrazanus + Toxopus (node 7), and Toliarus sister to Tanindrazanus + Toxopus. Character optimization results for the parsimony tree are given in SM3.

Our combined ML result (SM7) is largely congruent with the combined parsimony topology with the following exceptions: (1) Maraenaspis bidens is sister to Racelda sp., (2) Gibbosella is the sister to the Caecina + Mendis + Neolibavius + Bannania + nr Bannania clade, and (3) Distirogaster is the sister group to the Glymmatophora + Ectrichodia + Centraspis + Ectrychotes + Microstemmatoides + Cleptria + Maraenaspis coccinea clade. The ML result supports the taxonomic decisions of Forthman et al. (2016) and produces minor character optimization differences in comparison to the parsimony tree for taxa and clades of interest (data not shown).

### 3.3. Divergence dating estimation

Results of the BEAST molecular divergence dating analysis are shown in Fig. 4, with HPD intervals shown in SM8. Based on the


Fig. 2. Best tree based on ML analysis of 56 taxa, four G-INS-I aligned gene partitions (COI, 18S, 28S D2, 28S D3-D5), and GTR $+\Gamma+I$ model of sequence evolution (Final $\mathrm{Ln}=-25426.19528$ ). Terminal branches are colored by occurrence of a taxon in a given biogeographic region, which corresponds to the map legend. Numbers in gray circles refer to nodes discussed in the text. Bootstrap (BS) values $\geqslant 50$ are reported below branches, with the exception that BS values are reported next to the node of Microstemmatoides atrocyanea and Centraspis ducalis, as well as the node of the clade containing Microstemmatoides atrocyanea and Ectrichodia crux.
analysis, Ectrichodiinae diverged from Tribelocephalinae approximately 68.53 mya ( $95 \%$ HPD $=57.61-79.39$ mya), which is congruent with Hwang and Weirauch (2012). Within Ectrichodiinae, the Neotropical Ectrichodiinae + Santosia clade diverged from the Old World clade about 63.51 mya ( $95 \%$ HPD $=52.39-74.44$ mya). Within the Old World clade, all Madagascan Ectrichodiinae lineages and genera diverged from their respective sister groups within the last 46 my : (1) Gibbosella quadocris Forthman, Chłond, and Weirauch (median = 27.33 mya, $95 \% \quad H P D=12.22-42.12$ mya), (2) Marojejycoris + Tanindrazanus + Toxopus (median $=35.55$ mya, $95 \%$ HPD $=25.34-45.60 \mathrm{mya}$ ), (3) Distirogaster (median = 32.86 mya, $95 \%$ HPD $=23.48-42.59$ mya), and (4) Glymmatophora crassipes (median = 19.24 mya, $95 \%$ HPD $=11.46-27.85$ mya).

### 3.4. Biogeographic analyses

Ancestral range reconstruction results based on the DEC and DEC +j models are reported and shown in Fig. 4. Results of the ancestral area reconstruction using the DIVA model are shown in

SM9 and are most similar to the highest probability ranges reconstructed by DEC +j . The likelihood, $d, e$, and $j$ parameter values for the DEC and DEC +j models are as follows: (1) DEC: $\operatorname{Ln} L=-39.09$, $d=0.0056, e=0.0006, j=0$ and (2) DEC $+\mathrm{j}: \operatorname{Ln} L=-27.10, d=1 \mathrm{e}^{-12}$, $e=1 \mathrm{e}^{-12}, j=0.1238$. The DEC +j model performed significantly better than the DEC (LRT $X^{2}=23.97, \mathrm{df}=1, p=9.80 \mathrm{e}^{-7}$ ), with the $j$ parameter (or founder speciation effect) by far the most significant contributor to the current distribution.

In analyses with all three biogeographic models, a Neotropical and Oriental distribution is recovered for the ancestral node of Ectrichodiinae. The ancestral areas for the Neotropical Ectrichodiinae + Santosia clade and the Old World clade are inferred as Neotropical and Oriental, respectively, in all analyses. In the DEC +j analysis, the ancestral range of Gibbosella quadocris and Caecina sp. is recovered as Oriental with high probability, indicating the ancestor of Gibbosella dispersed to Madagascar from this region. An Oriental distribution is also reconstructed for the node that includes Gibbosella and Ectrychotes with subsequent dispersal to Madagascar for the node containing Marojejycoris and


Fig. 3. Strict consensus tree from 938 parsimonious trees from a TNT analysis of 110 taxa (outgroups removed from figure) and a combined morphological and molecular dataset (length $=7250$ steps; $\mathrm{RI}=0.587 ; \mathrm{CI}=0.356$ ). Terminal branches are colored by taxon distribution, which corresponds to the map legend. Numbers in gray circles refer to nodes discussed in the text and SM10. Bootstrap values $\geqslant 50$ are reported below branches. Examples of morphological characters that are discussed in the text are given: A.-D. Dorsal (A., B.) and lateral (C., D.) head morphology: A. Glymmatophora crassipes, B. Toxopus tibialis, C. Maraenaspis bidens, D. Tanindrazanus kathrynae; E. Lateral antennal morphology of Toxopus insignis; Dorsal (F.-H.), lateral (I.), and ventral (J.): F. Maraenaspis bidens, G. Toxopus italaviana, H. Marojejycoris notadichroa, I. Gibbosella conisimilis, J. Tanindrazanus irwini; K. Metathoracic gland evaporatorium of Tanindrazanus varicolor in lateral view; L. Ventral mid leg morphology of Glymmatophora crassipes. M. Hemelytral morphology of Gibbosella planiscutum; N. Ventral abdominal morphology of Distirogaster tarsalis. A., C.-G., and I.-M. modified from Forthman et al. (2016). Character numbers and character state codings are listed in Table 2. Abbreviations used in figures: 1A, first anal vein; aa, antennal articulation; ain, antennal insertion site; ap, antennal pseudoarticulation; apl, anterior pronotal lobe; app, anterolateral pronotal projection; asp, anterior femoral subapical protuberance; bf, basiflagellomere; cl, clypeus; co, corium; cp, corial pterostigma; Cu, cubitus; df, distiflagellomere; e, compound eye; exM, extension of $M$ beyond $M+C u d i s t a l$ junction; fmp, femoral medial protuberance; $\mathbf{g}$, gula; is, interocular sulcus; L2, labial segment II (first visible segment); L3, labial segment III (second visible segment); M, media; mc, metacoxa; mf, mid femur; mge, metathoracic gland evaporatorium; mms, transverse suture between meso- and metasterna; mss, mesosternum; mt, mid trochanter; mts, metasternum; $\mathbf{p}$, pedicel; pa, papillae; pc, sternal paramedian carinae; pcd, postclypeal depression; ppl, posterior pronotal lobe; psp, posterior femoral subapical protuberance; pts, pronotal transverse suture; R, radius; s, scape; s2, sternite II (fused sternites I and II); s3, sternite III; sc, scutellum; sis, sternal intersegmental suture; sl, synthlipsis; sld, sternal medial longitudinal depression.

# $\square$ Neotropical $\square$ Afrotropical $\square$ Madagascar $\square$ Oriental $\square$ Neotropical+Afrotropical $\square$ Neotropical+Oriental $\square$ Neotropical+Madagascar $\square$ Madagascar+Oriental $\square$ Afrotropical+Oriental $\square$ Afrotropical+Madagascar 



Fig. 4. Ancestral range reconstructions from DEC (left) and DEC +j (right) models for select nodes. Pie charts indicate the relative likelihoods of each reconstructed distribution, with geographical distributions color coded according to the legend at the top of the figure. The DEC + j model performed significantly better than the DEC: DEC $+\mathrm{j} \operatorname{Ln} L=-27.10$; DEC $\operatorname{Ln} L=-39.09$; LRT $p$-value $=9.80 \mathrm{e}^{-7}$. Select dispersal events contributing to Madagascar's Ectrichodiinae diversity is shown for each model. Dispersal events unique to each model are indicated by dashed arrows.

Ectrychotes. The ancestor of the clade containing Centraspis Schaum and Ectrychotes is reconstructed to have dispersed to the Afrotropical region from Madagascar, with a re-colonization from Africa to Madagascar by Glymmatophora crassipes. Results from DIVA are similar to DEC +j highest probability results, but differ in the
following with respect to the Madagascan fauna: (1) the node containing Gibbosella and Ectrychotes is inferred as Madagascar + Oriental, (2) the ancestral range of Gibbosella quadocris and Caecina sp. is Madagascar + Oriental, (3) an Afrotropical + Madagascar distribution is recovered for the clade containing Distirogaster
and Ectrychotes, and (4) the ancestral range of Glymmatophora crassipes + Ectrichodia is Afrotropical + Madagascar.

Reconstructions from the DEC analyses differ from DEC +j results for some nodes and by the presence of more widespread ancestral ranges. In the DEC analysis, a Madagascar + Oriental ancestral distribution is recovered for Gibbosella quadocris + Caecina sp. with high probability, as well as for the node containing Marojejycoris and Ectrychotes and the node containing Distirogaster and Ectrychotes. An Afrotropical + Oriental ancestral range is recovered for the clade including Centraspis and Ectrychotes, with the ancestral node of Glymmatophora crassipes + Ectrichodia having an Afrotropical + Madagascar distribution.

## 4. Discussion

### 4.1. Phylogeny of Madagascan Ectrichodiinae

Results of our molecular ML phylogeny are used to investigate the number of Madagascan Ectrichodiinae lineages, as well as to estimate temporal divergences and biogeographic history. Our combined morphological and molecular phylogeny was used by Forthman et al. (2016) to formalize taxonomic decisions and inform diagnostic features for Madagascan millipede assassin bugs. Both molecular ML and combined parsimony phylogenetic analyses recovered some similar relationships. Both phylogenetic hypotheses support a paraphyletic New World fauna, with respect to Santosia in the molecular ML phylogeny or Santosia + Vilius in the combined parsimony result. This predominately New World clade is recovered as sister to a larger clade of Afrotropical, Oriental, and Madagascan Ectrichodiinae with relatively weak support. Madagascan lineages are recovered with close relationships to Oriental and Afrotropical taxa in both analyses, which is congruent with Vences' (2004) and Yoder and Nowak's (2006) conclusions that a large proportion of the Madagascan biota exhibits relationships with Afrotropical and Oriental taxa. Species of Glymmatophora share a close relationship with Ectrichodia species. Gibbosella species are recovered as sister to Caecina, albeit with low support in both phylogenies. Although the molecular and combined phylogenies differ in their phylogenetic placement of Marojejycoris, the genera Tanindrazanus and Toxopus retain a close relationship in both analyses.

Despite recovering similar relationships, the two phylogenies show some degree of discordance in higher-level relationships within Ectrichodiinae, a result that may be due to the increase in missing molecular data in the combined analysis. Unlike the molecular phylogeny, most Madagascan taxa form a clade in the combined phylogeny (node 4, including Caecina) with low support. The phylogenetic placement of Marojejycoris and Distirogaster differ; in the ML phylogeny, Marojejycoris is recovered as sister to Tanindrazanus and Distirogaster as sister to a clade containing Ectrichodia and Ectrychotes, whereas the two genera are included within node 4 of the combined analysis. In general, more nodes are recovered with $\mathrm{BS} \geqslant 50$ in the molecular phylogeny compared to the combined phylogeny. Regardless of the discordance between the two phylogenetic hypotheses, the combined phylogeny has permitted exploration of potential character support for taxa and been useful in determining diagnostic features for Forthman et al.'s (2016) taxonomic monograph.

### 4.2. Temporal divergence and biogeography

Based on our temporal divergence estimates and ancestral range reconstructions, millipede assassin bugs colonized Madagascar via transoceanic dispersal more than once within the last $\sim 68 \mathrm{my}$. The DEC +j model outperformed the DEC model based
on LRT and infers two colonization events from the Oriental region to Madagascar, once between 25 and 57 mya and once within the last 42 my. Subsequently, a single colonization event from Madagascar to the Afrotropical region is inferred to have occurred around the Eocene-Oligocene boundary (21-43 mya) with a back-colonization event (ancestral node of Glymmatophora + Ectrichodia) from the Afrotropical region to Madagascar within the last $\sim 28 \mathrm{my}$. These results are congruent with the DIVA results (SM9). Although the DEC results differ from the DEC +j in terms of the ancestral ranges reconstructed for several nodes, in general we find similar patterns: two dispersal events into Madagascar from the Oriental region and one dispersal from Africa to Madagascar. However, an out-of-Madagascar dispersal event to the Afrotropical region is not recovered in the DEC analysis; upon diverging from Distirogaster, the branch leading to the node containing Centraspis and Ectrychotes has an Oriental distribution, with a range expansion into the Afrotropical region at that node.

Several hypotheses have been proposed for dispersal events from the Oriental region to Madagascar and may explain the two colonization events reconstructed from the DEC +j model. While many researchers reconstruct an isolated India in the Late Cretaceous (~65 mya) (see Yoder and Nowak, 2006; Ali and Aitchison, 2008), Ali and Aitchison (2008) proposed a paleogeographic model in which India became progressively isolated but remained connected to Madagascar via the Seychelles-Mascarene Plateau. This connection was extended in the Palaeogene by the development of volcanic islands (Ali and Aitchison, 2008; Zhou et al., 2012) and would have facilitated dispersal as India moved north. Warren et al. (2010) reviewed geologic evidence and also proposed that the rise and fall of sea levels exposed islands between India and Madagascar over the last 65 my, thus reducing the distance of open ocean to traverse for potential colonizers. Warren et al. (2010) further proposed climatic influences that would facilitate long-distance dispersal (e.g., winter monsoon winds blowing from India toward Madagascar facilitating aerial dispersal).

Several studies have supported "out-of-Madagascar" dispersal events to surrounding islands and continents for plants and vertebrates (e.g., Jansa et al., 1999; Wikström et al., 2010; Harmon et al., 2008), as well as for several groups of insects. Torres et al. (2001) concluded that satyrine butterflies in the subtribe Mycalesina have a complex biogeographical history that involves dispersal from Madagascar to Africa. The mayfly genus Cloeodes Traver is inferred to have a Madagascan origin followed by dispersal into Africa (Monaghan et al., 2005). Zakharov et al. (2004) inferred out-ofMadagascar dispersal for species of the butterfly genus Papilio Linnaeus to Oriental and Afrotropical regions within the last 10 my. Recently, the out-of-Madagascar biogeographical hypothesis has been supported by systematic analyses of Madagascan diving beetles (Dytiscidae), with results indicating dispersal to Oriental and Afrotropical regions around the Oligocene and Miocene (Bukontaite et al., 2015).

Our DEC +j results support an out-of-Madagascar dispersal event to Afrotropical regions about 21-43 mya. Westward dispersal could not have been facilitated by ocean currents as these currents had an eastward movement during the Eocene and reversed direction only $\sim 15$ mya (Ali and Huber, 2010). McCall (1997) hypothesized that a land bridge known as the Davie Ridge once connected Africa and Madagascar during the mid-Eocene to early Miocene (i.e., 45-26 mya). However, this land bridge has not been supported by some studies (e.g., Leclaire et al., 1989; Bassias, 1992; Rogers et al., 2000), although some suggest that isolated islands may have been present (Rogers et al., 2000; de Wit, 2003). Thus, it is possible that islands in the Mozambique Channel have facilitated westward aerial dispersal during this time. Female specimens for extant Madagascan species of Distirogaster ( 5 spp .), Toxopus (2 spp.), and Glymmatophora ( 1 sp. ) are apterous, which
makes present-day aerial dispersal unlikely. However, it is unknown if the ancestors of these taxa were apterous or capable of flight, thus enabling long-distance aerial dispersal. Female specimens for the majority of Madagascan species, as well as many Afrotropical and Oriental taxa, remain unknown and hinder our ability to reconstruct ancestral states. Future discoveries of female specimens will allow us to investigate the evolution of winglessness for each sex and to subsequently refine hypotheses on how ancestral species may have dispersed in and out of Madagascar.

Based on our DEC +j analysis, the presence of islands and the eastward ocean currents of the Mozambique Channel within the last $\sim 28$ my may have facilitated the re-colonization of Madagascar from Africa. The divergence time estimate of Glymmatophora crassipes ( $95 \%$ HPD $=11.46-27.85$ mya) is consistent with this hypothesis. However, aerial dispersal cannot be ruled out as ocean currents reversed direction $\sim 15$ mya, which would hinder eastward dispersal via rafting. Glymmatophora crassipes is apterous in both sexes, but some African species in this genus are macropterous, and it is uncertain when and where the ancestor of $G$. crassipes has lost the capability of flight. Apterous and macropterous Glymmatophora species will need to be targeted for future analyses to determine if ocean currents or aerial dispersal are responsible for extant Madagascan Glymmatophora. Ancestral state reconstructions on the evolution of winglessness across Glymmatophora may provide further insight into possible avenues for the colonization of Madagascar.

Five Ectrichodiinae species in three genera - Mascaregnasa Distant, Quinssyana Distant, and Rochonia Distant - are known from another Indian Ocean island, the Seychelles (Maldonado, 1990). Each species is only known from the male holotype and, thus, were not included in our molecular or morphological analyses. We hypothesize, based on morphological similarities examined in habitus images of the holotypes, that Quinssyana is closely related to the Madagascan genus Gibbosella and the African genus Synavecoris, while Toxopus is closely related to Rochonia. Mascaregnasa is very different from other millipede assassin bug genera, and we are unable to hypothesize affinities with other taxa. Future inclusion of these taxa in systematic analyses will be critical for testing the role of the Seychelles as a stepping-stone island, which has been postulated for other taxa (e.g., Tachycnemis frogs [Vences et al., 2003a,b], Nephilengys hermit spiders [Kuntner and Agnarsson, 2011], and baetid mayflies [Monaghan et al., 2005]). Millipede assassin bugs from other Indian Ocean islands are currently unknown, but future taxonomic surveys on these islands may result in material that would also benefit future biogeographic investigations for Madagascan Ectrichodiinae.

### 4.3. Combined parsimony analysis to inform taxonomic diagnoses of Madagascan Ectrichodiinae

Character optimizations of the combined parsimony analysis were used as the basis for diagnoses in the taxonomic monograph of Madagascan Ectrichodiinae (Forthman et al., 2016). A detailed discussion of important morphological characters for selected nodes shown in Fig. 3 is provided in SM10. Here, we only discuss some examples of male diagnostic features for endemic Madagascan genera that are also included in Forthman et al. (2016). Species of Marojejycoris are distinguished by features such as a complete pronotal transverse suture (57-1; Fig. 3H), the laterally visible MGE (71-1; Fig. 3K), and the fore wing lacking the distal part of M beyond the $\mathrm{M}+\mathrm{Cu}$ junction (104-1; Fig. 3M). Diagnostic features for Gibbosella include the 8 -segmented antennae (38-3; except 6 -segmented in G. pallidalata Forthman, Chłond, and Weirauch), dorsally directed paramedian scutellar processes (62-1; except in G. planiscutum Forthman, Chłond, and Weirauch) (Fig. 3I), and transversely bicolored dorsal laterotergites (105-1)
with posterior protuberances (107-1, 108-1, 109-1, 110-1; shared with Caecina). A number of features are diagnostic for Distirogaster, e.g., the metallic coloration (2-1), 8 -segmented antennae (38-3), posterior tubercles on dorsal laterotergites III-VI (107-1, 108-1, 109-1, 110-1), and paramedian carinae on abdominal sternites (120-1; Fig. 3N). The small (1-0) red and black (2-2) body and a punctate posterior pronotal lobe (51-2) distinguish Toliarus. Although Tanindrazanus is not supported by any synapomorphic characters in our analysis, it is consistently recovered as a clade in molecular and combined analyses. An incomplete transverse suture between the meso- and metasterna (66-1), a laterally expanded dorsal laterotergite II (106-1), and the transversely bicolor dorsal laterotergites (105-1) are treated as diagnostic characters for Toxopus.

## 5. Conclusion

Madagascar's unique biodiversity is, at least partially, the result of long-term geologic isolation, transoceanic dispersal events followed by speciation, local geography, and climate change. The recently revised and diverse millipede assassin bug fauna of Madagascar have presented an opportunity to contribute to this growing knowledge of Madagascan invertebrates by investigating its historical biogeography. Phylogenetic hypotheses generated from molecular and combined morphological and molecular cladistic analyses are similar in some respects but discordant at higher-level relationships. Overall, the molecular phylogeny received higher BS support compared to the combined phylogeny. Regardless, results from the combined phylogeny have been important for informing taxonomic diagnoses in Forthman et al. (2016).

Based on our molecular dataset, millipede assassin bugs are shown to have colonized Madagascar within the last ~68 my. Given this relatively recent age, transoceanic dispersal rather than vicariance is responsible for the Madagascan Ectrichodiinae fauna we see today. Millipede assassin bugs colonized Madagascar twice from the Oriental region and once from the Afrotropical region. However, DEC +j and DIVA models reconstruct an out-ofMadagascan dispersal event to the Afrotropical region, whereas the DEC model does not. Temporal divergence estimates and biogeographic results indicate that dispersal from the Oriental region to Madagascar may have been facilitated by the SeychellesMascarene Plateau and volcanic islands in the Indian Ocean over the last $\sim 65 \mathrm{my}$. Factors facilitating dispersal from the Afrotropical region to Madagascar are more difficult to determine, but aerial dispersal via stepping-stone islands in the Mozambique Channel is the most probable hypothesis when accounting for ancient oceanographic reconstructions (i.e., ocean currents had an eastward direction). Further testing of the geologic and oceanic factors facilitating dispersal will require a larger sample of Afrotropical millipede assassin bugs, as well as Seychellois species. Furthermore, apterous females are known for several Madagascan species, making aerial dispersal impossible. However, it remains to be investigated if the ancestors of these taxa possessed an apterous condition or were capable of flight. Our results are congruent with a small, yet, largely growing body of biogeographic studies for Madagascan invertebrates; Madagascan millipede assassin bugs have a complex biogeographic history, with Cenozoic transoceanic dispersal between Oriental, Afrotropical, and Madagascan regions solely responsible for the endemic fauna we currently find present on the island.

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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.2016.03. 011.

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[^0]:    * Corresponding author.

    E-mail address: mfort001@ucr.edu (M. Forthman).

