Entomologische Blätter und Coleoptera

Are *Miquihuana rhadiniformis* Barr, 1982 and *Pseudamara arenaria* (LeConte, 1847) (Coleoptera, Carabidae) sphodrines?
Phylogenetic analysis of data from next-generation sequencing of museum specimens resolves the tribal-group relationships of these enigmatic taxa

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Abstract

Miquihuana rhadiniformis Barr, 1982 and *Pseudamara arenaria* (LeConte, 1847) are enigmatic North American carabid beetles whose tribal-group placement has been in doubt. Both are currently placed in Sphodrini, although *Pseudamara* has typically been placed in Zabrini by most authors. As these species are relatively rarely collected, neither *Miquihuana* Barr, 1982 nor *Pseudamara* Lindroth, 1968 have been sampled in previous phylogenetic studies and attempts to obtain DNA sequences through standard PCR of available specimens failed. We used Illumina sequencing to recover nuclear gene fragments (18S rDNA, 28S rDNA, *wingless*, and CAD) from pinned *M. rhadiniformis* and 70%-ethanol-preserved *P. arenaria*. Combining these data with data from exemplar taxa from Sphodrini, Zabrini, Pterostichini, Platynini, and other, putatively related tribes, we examine the tribal relationships of *Miquihuana* and *Pseudamara*. The phylogenetic reconstructions consistently and with high support place *M. rhadiniformis* with other sphodrines excluding Atranopsina. There is evidence that *Miquihuana* is a distinct lineage from Sphodrina, but its placement varies among the single gene analyses. *Pseudamara arenaria* is not a sphodrine, as it nests within Zabrini, supporting the traditional placement. We transfer *Pseudamara* back to Zabrini based on these molecular data and we present additional morphological insights corroborating these results.

Zusammenfassung

Sind *Miquihuana rhadiniformis* Barr, 1982 und *Pseudamara arenaria* (LeConte, 1847) (Coleoptera, Carabidae) Sphodrinen? Die phylogenetische Datenanalyse nach Next-Generation-Sequenzierung von Museumsexemplaren klärt die Zugehörigkeit dieser rätselhaften Taxa zu den Tribus-Gruppen. *Miquihuana rhadiniformis* Barr, 1982 und *Pseudamara arenaria* (LeConte, 1847) sind rätselhafte Laufkäfer Nordamerikas, bei denen Uneinigkeit über die Zugehörigkeit in eine der Tribus besteht. Beide werden derzeit zu den Sphodrini gestellt, obwohl *Pseudamara* von den meisten Autoren den Zabrini zugeordnet wurde. Da beide Arten relativ selten gesammelt werden, wurden bisher weder *Miquihuana* Barr, 1982 noch *Pseudamara* Lindroth, 1968 in phylogenetische Studien einbezogen. Versuche, DNA-Sequenzen mittels Standard-PCR aus verfügbaren Exemplaren zu erhalten, blieben erfolglos. Wir verwendeten Illumina-Sequenzierung, um nukleare Genfragmente (18S rDNA, 28S rDNA, „wingless" und CAD) aus genadelten Exemplaren von *M. rhadiniformis* sowie in 70%igem Alkohol konservierten Exemplaren von *P. arenaria* zu gewinnen. Basierend auf der Kombination dieser Daten mit solchen von exemplarischen Taxa der Sphodrini, Zabrini, Pterostichini, Platynini und anderen, vermutlich verwandten Tribus untersuchten wir die Verwandtschaftsverhältnisse von *Miquihuana* und *Pseudamara*. Übereinstimmend und mit starker Stützung stellen die phylogenetischen Rekonstruktionen *M. rhadiniformis* zwischen andere Sphodrini abgesondert von Atranopsina. Es gibt Anzeichen dafür, dass *Miquihuana* eine von den Sphodrina verschiedene Linie darstellt, aber die Platzierung des Taxon variiert im Resultat der Analysen verschiedener einzelner Gene. *Pseudamara arenaria* ist keine Sphodrini; das Taxon platziert sich innerhalb der Zabrini, was die traditionelle Einordnung bestätigt. Basierend auf molekularen Daten transferieren wir *Pseudamara* zurück in die Tribus Zabrini, und wir präsentieren weitere morphologische Erkenntnisse, welche diese Resultate bekräftigen.

Key words: Harpalinae, Pterostichitae, Sphodrini, Zabrini, degraded DNA, nuclear DNA, Illumina sequencing

Introduction

Among the more fascinating biogeographic mysteries are isolated species found on continents far from their nearest relatives. The carabid beetles *Miquihuana rhadiniformis* Barr, 1982 and *Pseudamara arenaria* (LeConte, 1847) are two little-known Nearctic species whose nearest relatives are thought to be many thousands of kilometers away, in Europe and Asia (Barr 1982, Hieke 2010). Both species have been classified in the tribe Sphodrini, each having the special status of being the only North American representative of an otherwise Old World subtribe of Sphodrini: *Miquihuana* Barr, 1982 with Sphodrina and *Pseudamara* Lindroth, 1968 with

Atranopsina (Bousquet 2012, Hieke 2010).

The tribe Sphodrini (Fig. 1) includes approximately 825 species in 40 genera (Lorenz 2005), which are grouped into six subtribes: Atranopsina (100 species), Calathina (185 species), Dolichina (17 species), Pristosiina (65 species), Sphodrina (360 species), and Synuchina (100 species). Sphodrini is most diverse in the Palearctic with few species occurring in northern Africa and eastern Asia (Lorenz 2005). New World Sphodrini are notably less diverse than their Old World counterparts and include four genera: *Calathus* Bonelli, 1810 (22 species), *Synuchus* Gyllenhal, 1810 (three species), *Miquihuana* (one species), and *Pseudamara* (one species).

Miquihuana rhadiniformis is a true cave specialist or troglobite (Fig. 1H). It is one of the most morphologically aberrant and distinctive species of Sphodrini in North America, in part because it is the only known troglobitic sphodrine in the New World (Barr 1982, Casale et al. 1998). Many carabid beetles live in or spend part of their lives in caves or subsurface habitats the world over (Barr 1960, 1982, Casale et al. 1998, Jeannel 1943, Tian et al. 2014), although the distribution of cave species across Carabidae is non-randomly distributed phylogenetically and geographically (Casale et al. 1998). Thus far most known cave carabids are members of Trechini, Platynini, or Sphodrini (Casale et al. 1998). The similarity in form of these beetles to each other and to other cave beetles (e.g., some Staphylinidae, Peck & Thayer 2003, and Leiodidae, Fresneda 1998) is striking. The widespread convergence in external features of subterranean species makes morphology-based phylogenetic placement difficult (Wiens et al. 2003). For this reason, DNA data has proved especially useful to infer the phylogenetic history of various groups with numerous subterranean lineages (Faille et al. 2010, Gomez et al. 2016, Page et al. 2008, Ribera et al. 2010, Wiens et al. 2003).

Pseudamara arenaria is a small, stout beetle that is unusual for its combination of morphological features typical of various Harpalinae tribes, particularly Harpalini, Pterostichini, Sphodrini, and Zabrini (Fig. 2, Hieke 2010). Unlike the other genera classified in Sphodrini that occur in North America, *Pseudamara* has historically been listed within Zabrini (e.g., Lindroth 1968). It was recently transferred to Sphodrini by Hieke (2010) who hypothesized that it was closely related to the Atranopsina, particularly the Canary Island endemic, *Amaroschema* Jeannel, 1943. He further speculated that *Pseudamara* is a critical missing link between Harpalini and Zabrini (Hieke 2010).

Neither *M. rhadiniformis* nor *P. arenaria* has been sampled in a phylogenetic analysis to date. Understanding their connections to the World fauna is essential for developing a picture of the origin and diversification of these carabid lineages within North America. Given the parallel hypotheses that both *M. rhadiniformis* and *P. arenaria* are isolated species with Old World connections, and that evaluating Hieke's (2010) placement of *Pseudamara* necessitated sampling members of both Sphodrini and Zabrini, an analysis including exemplars from across these tribes was pursued to address their placement and consider the biogeographic implications in light of our reconstruction. The known specimens of *M. rhadiniformis* and the specimens of *P. arenaria* available to us were not preserved explicitly for DNA applications. The degraded DNA of museum specimens often precludes their use in PCRs of longer fragments or requires the use of targeted, custom short primers and many additional PCRs to recover a full-length genomic region. Because many current next-generation sequencing (NGS) platforms produce short (<500 base) reads, the library preparations for these sequencing platforms are built using short fragments of DNA. The highly fragmented DNA of museum specimens is thus suitable for many NGS library preparations (Heintzman et al. 2014, Kanda et al. 2015, Staats et al., 2013). We attempted to obtain sequences for *M. rhadiniformis* and *P. arenaria* using PCR, but most of these reactions failed. We then used NGS for low-depth genomic sequencing of these species in order to retrieve nuclear genes from our museum specimens, allowing us to include these species in our phylogenetic analyses.
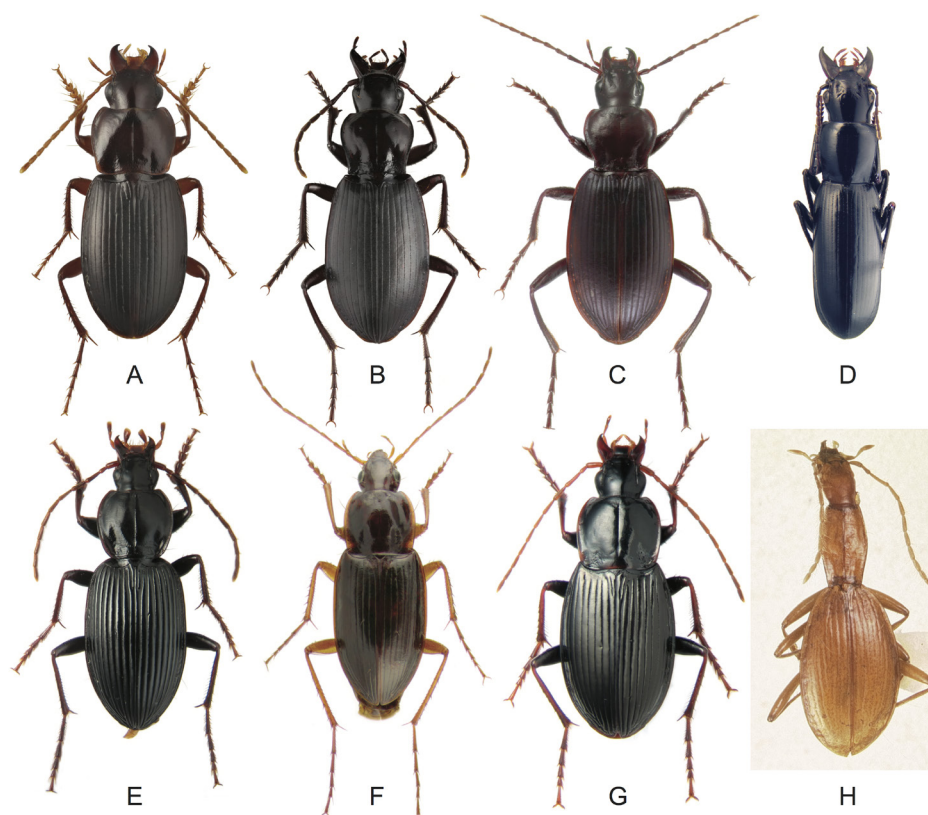
We dedicate this paper to Dr. Fritz Hieke, whose exemplary work on zabrines serves as a model to those who study carabid diversity, and whose studies of *Pseudamara* provided inspiration for the current study.
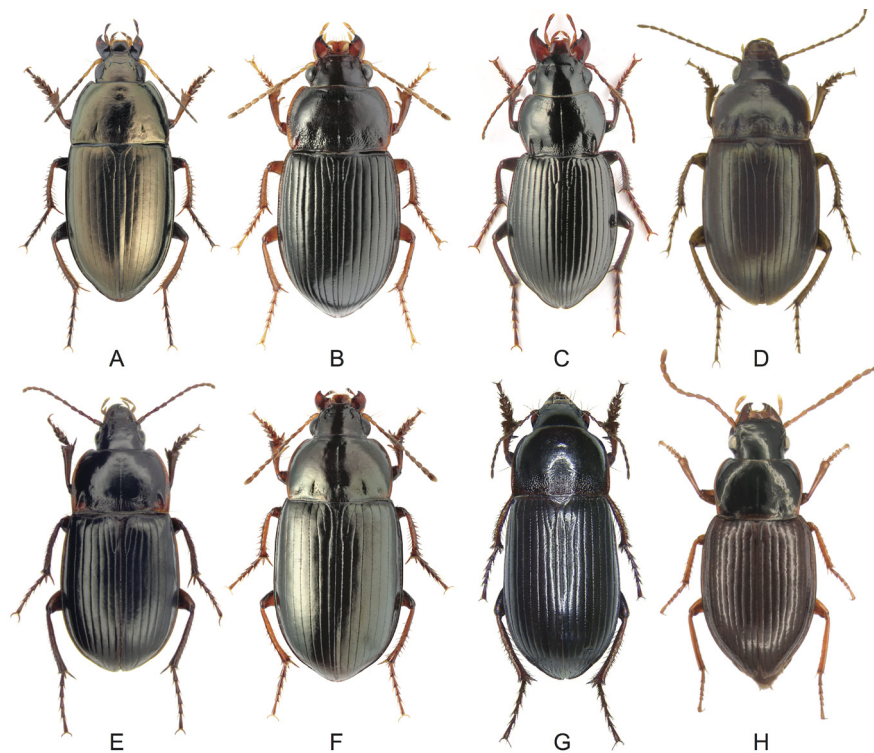
Materials & methods

Morphological methods. Specimens used for morphological studies are deposited in the following collections: California Academy of Science, San Francisco, California, USA (CAS); Carnegie Museum of Natural History, Pittsburgh, Pennsylvania, USA (CMNH); Cornell University Insect Collection, Ithaca, New York, USA (CUIC); Essig Museum of Entomology, Berkeley, California, USA (EMEC); Oregon State Arthropod Collection, Corvallis, Oregon, USA (OSAC); EH Strickland Entomological Museum, Edmonton, Alberta, Canada (UASM).

Gross morphological examination and male and female genitalia preparation used the same methods as Will (2002, 2011). Images were taken using either a modified Microptics XLT digital or a Leica Z6 lens with a JVC KY-F75U camera and followed the methods of Maddison & Cooper (2014). The images were then edited to enhance clarity using standard image editing software. The majority of the dorsal habitus images shown in Figures 1 and 2 were generously provided to us, and the photo credits are provided in the figure captions.

Taxon sampling for molecular phylogenetics. In order to place *P. arenaria* and *M. rhadiniformis* in tribal-groups, 32 Harpalinae species were selected as a representative sampling from across Sphodrini (11 species) and Zabrini (nine species), and exemplars from tribes thought to be potential near-relatives of sphodrines and

Fig. 1. Dorsal habitus images of representative species of Sphodrini. **A** – *Platyderus caucasicus* Kryzhanovskij, 1968; **B** – *Laemostenus piceus* (Dejean, 1828); **C** – *Laemostenus terricola* (Herbst, 1784); **D** – *Stenolepta cylindrica* Semenov, 1889; **E** – *Synuchus nitidus* (Motschulsky, 1861); **F** – *Calathus gregarius* (Say, 1823); **G** – *Pristosia proxima* (A. Morawitz, 1862); **H** – *Miquihuana rhadiniformis* Barr, 1982. Photo credits: A, B, D, E, G (Kirill Makarov), C, F (Henri Goulet).

Fig. 2. Dorsal habitus images of representative species of Zabrini. **A** – *Amara (Amara) aenea* (DeGeer,1774); **B** – *Amara (Bradytus) simplicidens* Morawitz, 1863; **C** – *Amara (Curtonotus) gebleri* Dejean, 1831; **D** – *Amara (Paracelia) quenseli* (Schoenherr, 1806); **E** – *Amara (Percosia) obesa* (Say, 1823); **F** – *Amara (Xenocelia) ambulans* C. Zimmerman, 1832; **G** – *Zabrus tenebrionoides* Goeze, 1777; **H** – *Pseudamara arenaria* (LeConte, 1847). Photo credits: A, B, C, F (Kirill Makarov), D, E, H (Henri Goulet), G (Udo Schmidt, Creative Commons CC-BY-SA 2.0).

zabrines: Platynini (five species), Pterostichini (three species), and a single representative each of Abacetini, Morionini, Cnemalobini, and Harpalini. *Patrobus longicornis* (Say, 1823), *Bembidion perspicuum* (LeConte, 1848), and *Sitaphe parallelipennis* Baehr, 2003, were included as outgroup taxa (Table 1). Within Sphodrini, we sampled five of six recognized subtribes, only lacking Pristosiina, and our sampling focused primarily on New World species. Within Zabrini, we included five of the 47 subgenera of *Amara* Bonelli, 1810 (Hieke 2007) and three species of *Zabrus* Clairville, 1806 (Table 1).

Unlike most specimens from which we extracted genomic DNA, the specimens of *P. arenaria* and *M. rhadiniformis* we examined were not deliberately preserved for DNA sequencing. The specimen of *P. arenaria* was collected in a pitfall trap and stored in 70% EtOH at ambient temperature for 17 years. *Miquihuana rhadiniformis* was described in 1982 and is only known from a single collecting event from 1981. The type series consists of four pinned adults, and we chose one of the three female paratypes for sequencing.

Gene sampling & DNA extraction. The methods for collecting molecular sequence data closely follow those employed by Kanda et al. (2015) for next generation sequencing (NGS) and PCR or Maddison (2012) for PCR.

Genomic DNA was extracted from muscle tissue using the standard protocol for Qiagen's Blood & Tissue DNEasy kit (Carlsbad, CA). DNA from *P. arenaria* and *M. rhadiniformis* specimens was extracted using the Qiagen kit but to minimize the risk of cross-sample contamination was done in a separate room intended solely for extraction of samples we predict to have degraded DNA.

Fragments of the following nuclear genes were chosen for analysis: the ribosomal genes, 18S and 28S, and the nuclear protein-coding genes, CAD (the carbamoyl phosphate synthetase domain of the *rudimentary* gene) and *wingless*. The molecular data chosen for this study include newly generated sequences as well as sequences from GenBank. Of the 37 species sampled for this study, 11 include only newly generated sequences, 20 include some combination of previously generated sequences from GenBank, and six species are represented entirely by sequences from GenBank (Table 1).

PCR, capillary sequencing, and contig assembly of PCR data. For all taxa other than *M. rhadiniformis* and *P. arenaria*, sequence data was obtained through PCR and Sanger sequencing. PCR products were obtained using the primers and protocols described in Kanda et al. (2015) and Maddison (2012) with TaKaRa Ex Taq polymerase and the manufacturer's recommendations on an Eppendorf Mastercycler ProS Thermal Cycler. The PCR products were then purified and quantified prior to sequencing at the University of Arizona's Genetic Core on either a 3730 or 3730 XL Applied Biosystems automatic sequencer.

The resulting chromatograms were processed using Phred (Green & Ewing 2002) and Phrap (Green 1999) as orchestrated by Mesquite's Chromaseq package (Maddison & Maddison 2014, Maddison & Maddison 2015b) with subsequent modifications by Chromaseq and manual inspection. The contigs were manually inspected, and final base calling was performed using Chromaseq. Sites with more than one peak in both chromatograms files were coded using IUPAC ambiguity codes.

For *Miquihuana* and *Pseudamara*, we attempted to acquire data using PCR, both using the standard protocols, as well as primers designed to amplify smaller pieces of 28S (approximately 300 bases), but the reactions consistently failed for these species aside from PCR of *wg* from *P. arenaria*. Given these PCR failures, *P. arenaria* and *M. rhadiniformis* were sequenced using high-throughput sequencing on the Illumina platform (described below).

Library preparation for high throughput sequencing. The concentration and fragment sizes of the DNA extractions of *Miquihuana* and *Pseudamara* were quantified in order to inform the library preparation process. DNA sample concentrations were measured using fluorometry and Qubit dsDNA high sensitivity assays. The fragment size distribution of the DNA extractions of *M. rhadiniformis* and *P. arenaria* were analyzed using a high sensitivity DNA chip on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, California). The fragment size distribution of the total DNA of *M. rhadiniformis* included a broad peak between roughly 35 and 500 bases. The Bioanalyzer trace of the DNA fragments of *P. arenaria* displayed a gentle incline near the high standard of approximately 10.3 kb. Because of the large fragments present in the DNA sample of *P. arenaria*, we sheared an aliquot of the extraction using a Bioruptor Pico Sonication System (Diagenode) with ten cycles of one minute each (30 seconds on, 30 seconds off).

The library preparation of *M. rhadiniformis* was made for Illumina sequencing using the NEBNext Ultra DNA kit and protocols, NEB adapters for Illumina, and a single index NEB barcode. We followed the basic protocol for size selection with an average insert size of 200 bases.

Prior to library preparation of our extraction of *P. arenaria*, we purified the DNA using AMPure beads followed by a repair step using NEB's double-stranded repair enzymes. The library preparation of *P. arenaria* was similar to that of *M. rhadiniformis* aside from the following details: the preparation was made using the NEBNext Ultra DNA II kit and protocols, we did not size select prior to enrichment, and we used dual-index NEB barcodes.

The DNA concentrations of the resulting libraries were initially quantified using Qubit, and the mean insert size was determined on an Agilent Bioanalyzer 2100. The concentration of adapter-ligated DNA was quantified using qPCR with KAPA library quantification kits for Illumina on an ABI PRISM 7500 FAST Sequence

Table 1. Taxa and gene fragments sampled for this study. The five columns on the right refer to separate gene fragments. Entries in these columns correspond to GenBank records or newly sequenced data. Entries that begin with only two letters refer to GenBank records, those that begin with 'DRM' refer to D.R. Maddison extraction codes, and those that begin with 'kww' refer to K.W. Will extraction codes. For additional details regarding locality data of sequenced vouchers and specimen accession data see Table S1.

Species	28S	18S	*wg*	CAD2	CAD4
Platynini					
Agonum extensicolle (Say, 1823)	AF398643	AF002775	AF398564	KR604890	DRM2409
Metacolpodes buchannani (Hope, 1831)	KR604891	kww426	kww426	KR604889	kww426
Atranus pubescens (Dejean, 1828)	AF438026	DRM0947	AF437911	KR604899	DRM0947
Platynus brunneomarginatus (Mannerheim, 1843)	KR604895	kww420	kww420	KR604909	kww1003
Rhadine cf. *perlevis* Casey, 1913	AF438128		AF437990	DRM0656	DRM0656
Sphodrini: Atranopsina					
Amaroschema gaudini Jeannel, 1943	kww684	kww684	kww684	kww684	
Platyderus varians Schaufuss, 1862	kww683	FJ173122	kww683	kww683	
Sphodrini: Calathina					
Calathus (*Neocalathus*) *aztec* Ball & Nègre, 1972	GU254425		kww1005		
Calathus (*Neocalathus*) *marmoreus* Ball & Nègre, 1972	GU254413		kww1008	kww1008	
Calathus (*Acalathus*) *advena* (LeConte, 1846)	GU254408	kww1007	kww1007	kww1007	
Sphodrini: Sphodrina					
Laemostenus complanatus (Dejean, 1828)	kww40	kww40	kww40	kww726	kww726
Laemostenus terricola (Herbst, 1784)	JF778812	JF778829			
Sphodrini: Synuchina					
Synuchus dubius (LeConte, 1854)	AF398674	DRM0353	AF398629	DRM0353	DRM0353
Synuchus vivalis (Illiger, 1798)	FJ173083	FJ173120			
Synuchus impunctatus (Say, 1823)	kww1004		kww1004	kww1004	kww1004
Sphodrini: Dolichina					
Dolichus halensis (Schaller, 1783)	FJ173105	FJ173129	kww1002	kww1002	kww1002
Sphodrini incertae sedis					
Miquihuana rhadiniformis Barr, 1982	kww0999	kww0999	kww0999		kww0999
Zabrini					
Amara (*Amara*) *aenea* (DeGeer, 1774)	FJ173093	FJ173123	kww1001	kww1001	kww1001
Amara (*Amara*) *chalcites* Dejean, 1828	AB243496		AB243550		
Amara (*Curtonotus*) *lacustris* LeConte, 1855	DRM0315	DRM0315	DRM0315	DRM0315	DRM0315
Amara (*Bradytus*) *apricaria* (Paykull, 1790)	DRM0314	AF002774	AF398565	DRM0314	DRM0314
Amara (*Paracelia*) *quenseli* (Schoenherr, 1806)	kww1000	kww1000	kww1000	kww1000	kww1000
Amara (*Percosia*) *obesa* (Say, 1823)	kww1006		kww1006		kww1006
Zabrus ignavus Csiki, 1907	FJ173096	FJ173125			
Zabrus vasconicus Uhagon, 1904	kww724	kww724	kww724	kww724	kww724
Zabrus seidlitzi Schaum, 1864	DRM0911	DRM0911	DRM0911	DRM0911	
Pseudamara arenaria (LeConte, 1847)	kww998	kww998	kww998	kww998	kww998
Pterostichini					
Pterostichus melanarius (Illiger, 1798)	KP419605	KP419252	KP813551	KP812985	KP812985
Poecilus lucublandus (Say, 1823)	EU142440	EU142291	EU142321	EU142306	EU142306
Hybothecus flohri (Bates, 1882)	kww18	kww18	kww18	kww18	DRM1053
Abacetini					
Abacetus sp.	AF398681	DRM0688	AF398635	DRM0688	DRM0688
Cnemalobini					
Cnemalobus sulciferus Philippi, 1864	AF398706	AF012474	AF398580	DRM0455	DRM0455
Harpalini					
Pelmatellus sp.	AF398690	AF398720	AF398615	DRM0621	DRM0621
Morionini					
Morion aridus Allen, 1968	AF398698	AF002783	AF398606	DRM0136	
Moriomorphini					
Sitaphe parallelipennis Baehr, 2003	GU556118	GU556146	GU556061	DRM2247	DRM2247
Patrobini					
Patrobus longicornis (Say, 1823)	AF398700	AF002786	AF398613	DRM0114	DRM0114
Bembidiini					
Bembidion perspicuum (LeConte, 1848)	GU454740	JN170213	JN171477	DRM1120	JN170877

Detection System. The qPCR concentration values and the mean fragment sizes were used to make two to three mM aliquots of the libraries for accurate pooling.

High throughput sequencing and demultiplexing. Library aliquots were submitted to the Center for Genome Research and Biocomputing at Oregon State University for pooling and sequencing. The initial multiplexing scheme chosen for the libraries of *M. rhadiniformis* and *P. arenaria* were a sixteenth and a tenth of an Illumina HiSeq 2000 100-base paired-end lane and an Illumina HiSeq 3000 150-base paired-end lane respectively. The reads were demultiplexed using CASAVA v. 1.7 (Illumina), and FastQC reports were created from the fastq files.

Assembly of short read data. Following the methods employed by Kanda et al. (2015) we made *de novo* assemblies from our Illumina read data in addition to making reference-based assemblies of the gene fragments chosen for tree inference. The paired-end reads were imported into CLC Genomics Workbench v. 8.5.1 (CLC Bio, Qiagen, Aarhus, Denmark), specifying the observed range of insert sizes. The reads were quality trimmed in CLC using the default parameters. The *de novo* assembly from these trimmed reads was made using CLC with the default settings.

Reference-based assemblies were made in CLC using sequences for 18S, 28S, *wg*, and CAD from *Pterostichus melanarius* (Illiger, 1798) as a reference. The trimmed reads from *M. rhadiniformis* and *P. arenaria* were mapped to the reference, and the contigs were extracted from the mappings while excluding the reference sequence in the consensus.

Loci identification and contig selection. We first queried the *de novo* assemblies for contigs that are highly similar to the focal gene fragments chosen for this study by using BLASTn against a database of *P. melanarius* sequences in Geneious v. 6.1.4 (Altschul et al. 1990; Kearse et al. 2012). Generally 28S and 18S searches included more than one best hit. We selected a single potential orthologous sequence for further study following the methods of Kanda et al. (2015). If the hits were non-overlapping, we examined the alignments and created a single sequence from the highest scoring contigs with the most query cover.

To inform our selection of a single potential orthologous sequence for phylogenetic analysis we compared sequences identified using our BLAST approach with sequences from our reference-based assemblies. We compared sequences for the percentage of the reference fragment length that was recovered and the impact of the reference on contig assembly. We favored more complete sequences over less complete sequences and sequences that were not missing nucleotides that are absent in the reference over sequences with these biases.

Based on our results, we chose 18S, 28S, and CAD sequences from the *de novo* assembly for *M. rhadiniformis*, and we selected the *wingless* sequence from the reference-based assembly. For *P. arenaria*, we selected

contigs for 18S and CAD from the *de novo* assembly. The 28S sequence was generated using reference-based assembly, and we chose to retain our PCR data for *wingless*.

Data availability. The sequences of 18S, 28S, CAD, and *wingless* resulting from both PCR/Sanger sequencing and Illumina sequencing are deposited in GenBank with accession numbers KX091904, KX091905, KX091907-KX091977, KX091979-KX091999. The raw Illumina reads for *M. rhadiniformis* and *P. arenaria* are available through NCBI Sequence Read Archive with the accession number SRP073745. Data matrices and phylogenetic trees are deposited with Dryad and are accessible from the Dryad Digital Repository.

Multiple sequence alignment. Alignment of our sampled genes varied between fragments depending on the need to propose indels. Our rDNA and *wg* sequence data have regions that require indels for alignment, while the CAD fragments do not. Alignment of CAD was trivial and was performed manually in Mesquite. The aligned CAD sequences were colored by amino acid to inspect the reading frame for accuracy and absence of stop codons. Our rDNA sequences were aligned using MAFFT Q-INS-I (Katoh & Standley 2013) and visually inspected for accuracy. Obvious misaligned blocks were corrected manually. *Wingless* nucleotide sequences were translated into their amino acid sequences, and the nucleotide data were then matched to the protein alignment. The AA matrix was aligned using MAFFT L-INS-I (Katoh et al. 2005) as implemented through Mesquite, and obvious misaligned blocks were corrected manually prior to matching back the nucleotide data.

Putatively ambiguously aligned regions in 18S and 28S were selected with the extended Gblocks algorithm (Talavera & Castresana 2007) as implemented in Mesquite, and excluded from the analysis. The following Gblocks-like analysis parameters in Mesquite were used: minimum fraction of identical residues for conserved positions = 0.2, minimum fraction of identical residues for highly conserved positions = 0.4, counting fraction within only those taxa that have non-gaps at that position, maximum length of non-conserved blocks = 4, minimum length of a block = 4, and fraction of gaps allowed in a character = 0.5.

Phylogenetic reconstruction. Aligned single gene matrices and the concatenated matrix of the five gene fragments were used for inference of a species tree. Optimal model and partitioning schemes were sought using Partitionfinder v. 1.1.1 (Lanfear et al. 2012), searching for schemes based on initial partitioning of the data by codon for *wg* and CAD.

Phylogenetic trees were inferred using RAxML v. 8.1.4 (Stamatakis 2014), as orchestrated by the Zephyr package of Mesquite (Maddison & Maddison 2015a), with the optimal partitioning scheme for RAxML found by Partitionfinder with the GTR+G model of nucleotide substitution. The optimal partitioning scheme for the

concatenated matrix includes three partitions: subset 1 = 28S, subset 2 = 18S, position 1 and 2 of *wg*, position 1 and 2 of CAD, subset 3 = position 3 of *wg*, position 3 of CAD. The optimal schemes for the single gene analyses for CAD and *wg* also include partitioning between the first and second codon positions and the third codon position. Maximum likelihood tree searches based on single gene matrices included 500 search replicates. Tree searches based on the concatenated data were repeated twice from different starting seeds to explore the variance between runs with 500 replicates.

Bootstrap replicates for single gene matrices and the concatenated matrix were based on 250 and 500 replicates respectively.

Results

Recovery of focal nuclear genes from *M. rhadiniformis* and *P. arenaria*. Illumina sequencing of our library preparations of *M. rhadiniformis* and of *P. arenaria* yielded approximately 25 million 100-base and 67 million 150-base paired-end raw reads respectively. Default trimming and *de novo* assembly using CLC produced assemblies with L50 values (using 'L' to designate a length not a number, cf. Bushnell (2015)) of 287 and 552 bases. Based on the BLAST-based approach of Kanda et al. (2015), we identified all 5 focal gene fragments in *P. arenaria*, all of which included sequence data for 95% or more of the length of our reference *Pterostichus melanarius* PCR data. The *de novo* assembly of *M. rhadiniformis* captures less gene space than that of *P. arenaria*. Using this approach with the *de novo M. rhadiniformis* genome assembly, we identified 28S, 18S, and a shorter fragment of 484 bases of CAD corresponding to CAD4 (Table 1).

The reference-based assemblies of our focal gene set were more successful for identifying putative orthologs. We identified all five genes in *P. arenaria* and recovered a short region of approximately 30 bases of only Ns in the *de novo* 28S contig. Reference-based assembly of our focal genes using *Pterostichus melanarius* reference sequences and our reads for *M. rhadiniformis* produced a similarly sized fragment of CAD, 28S, and 18S as well as a short, 191 base contig for *wingless*. Similar to the results of Kanda et al. (2015), the rDNA contigs from our reference-based assemblies differ from our *de novo* assemblies with respect to indels. However, length and sequence identity differences in 28S and 18S were only observed in *M. rhadiniformis*, and because of this, the *de novo M. rhadiniformis* rDNA contigs were chosen for phylogenetic inference.

Phylogenetic reconstruction. The highest likelihood tree from the concatenated matrix is shown in Figure 3, and the majority rule consensus tree of this same dataset is presented in Figure 4. Our results refer to these trees unless specified otherwise. Individual gene trees inferred using maximum likelihood are shown separately (Figs. 5, S1). The nuclear gene fragments sequenced for this study

place *Miquihuana* with other sphodrines and resolve *Pseudamara* within Zabrini. The following tribal-level clades were recovered with good to strong support (Table 2): Platynini, Zabrini (including *Pseudamara*), and Sphodrini (including *Miquihuana*). Harpalinae was recovered as monophyletic, but most deeper-level relationships within this clade are not well supported.

We examined the monophyly of four subtribes of Sphodrini: Atranopsina, Calathina, Synuchina, and Sphodrina. Atranospina is recovered as monophyletic with modest support and is placed as the sister group of all remaining Sphodrini. We resolved Calathina and Synuchina as monophyletic with very high support. Sphodrina monophyly was not recovered because of the placement of *Miquihuana*. The placement of *Miquihuana* varies between individual gene analyses, but the ML trees of 28S, *wg*, and CAD place *Miquihuana* with other sphodrines, excluding Atranopsina (Fig. 5). The ML tree of our concatenated dataset places *Miquihuana* as sister to all other sphodrines, excluding Atranopsina, and *Miquihuana* is not inferred to be more closely related to Sphodrina. The Sphodrini clade is strongly supported in the majority-rule bootstrap tree of the concatenated data, but the deeper relationships among subtribal lineages of Sphodrini and *Miquihuana* are ambiguous.
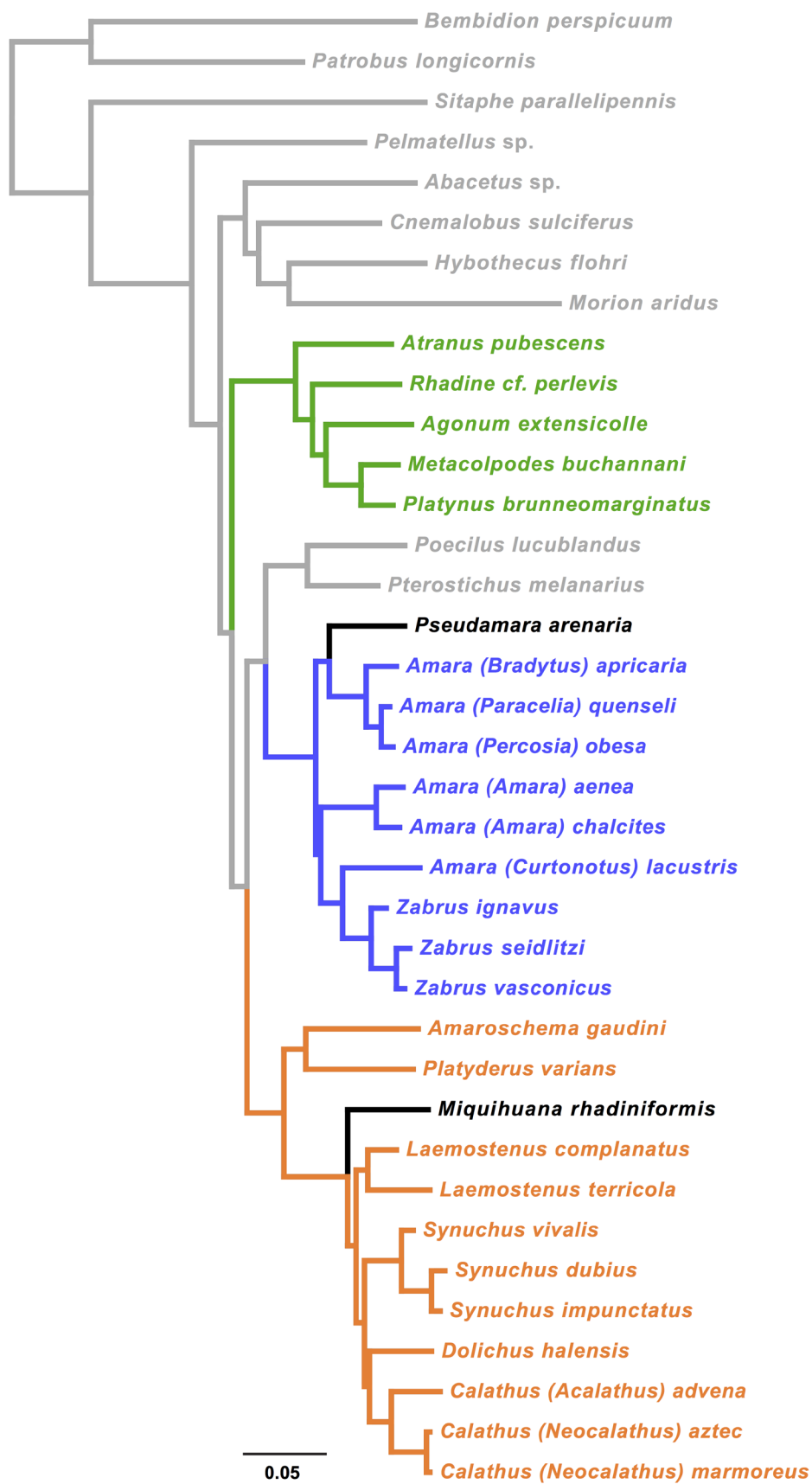
Among zabrines, the cosmopolitan genus *Amara* is not resolved as monophyletic in our analyses. *Pseudamara* is recovered as sister to a clade with three species of *Amara* in our concatenated dataset. Zabrini monophyly and the nesting of *Pseudamara* within Zabrini are strongly supported, and *Pseudamara* is never resolved with *Amaroschema* or within or even sister to Sphodrini. A clade including *Pterostichus melanarius* and *Poecilus lucublandus* (Say, 1823) is resolved as sister to Zabrini in our bootstrap analysis of the concatenated matrix and the CAD gene tree.

Discussion

For many rare species, such as *Pseudamara*, or those in habitats that are difficult to visit or sample, such as *Miquihuana*, it may not be possible to acquire specimens preserved specifically for DNA studies. Such species have traditionally been omitted from molecular phylogenetic studies. However, the rise of NGS, and its ability to sequence the degraded DNA of old specimens in museums (e.g., Heintzman et al. 2014, Kanda et al. 2015, Staats et al. 2013), has enabled full inclusion of those species in modern systematic works. As available specimens of *M. rhadiniformis* and *P. arenaria* were not preserved explicitly for DNA applications, and as most PCRs of our genomic DNA extractions of *M. rhadiniformis* and *P. arenaria* failed, these taxa were good candidates for the use of NGS.
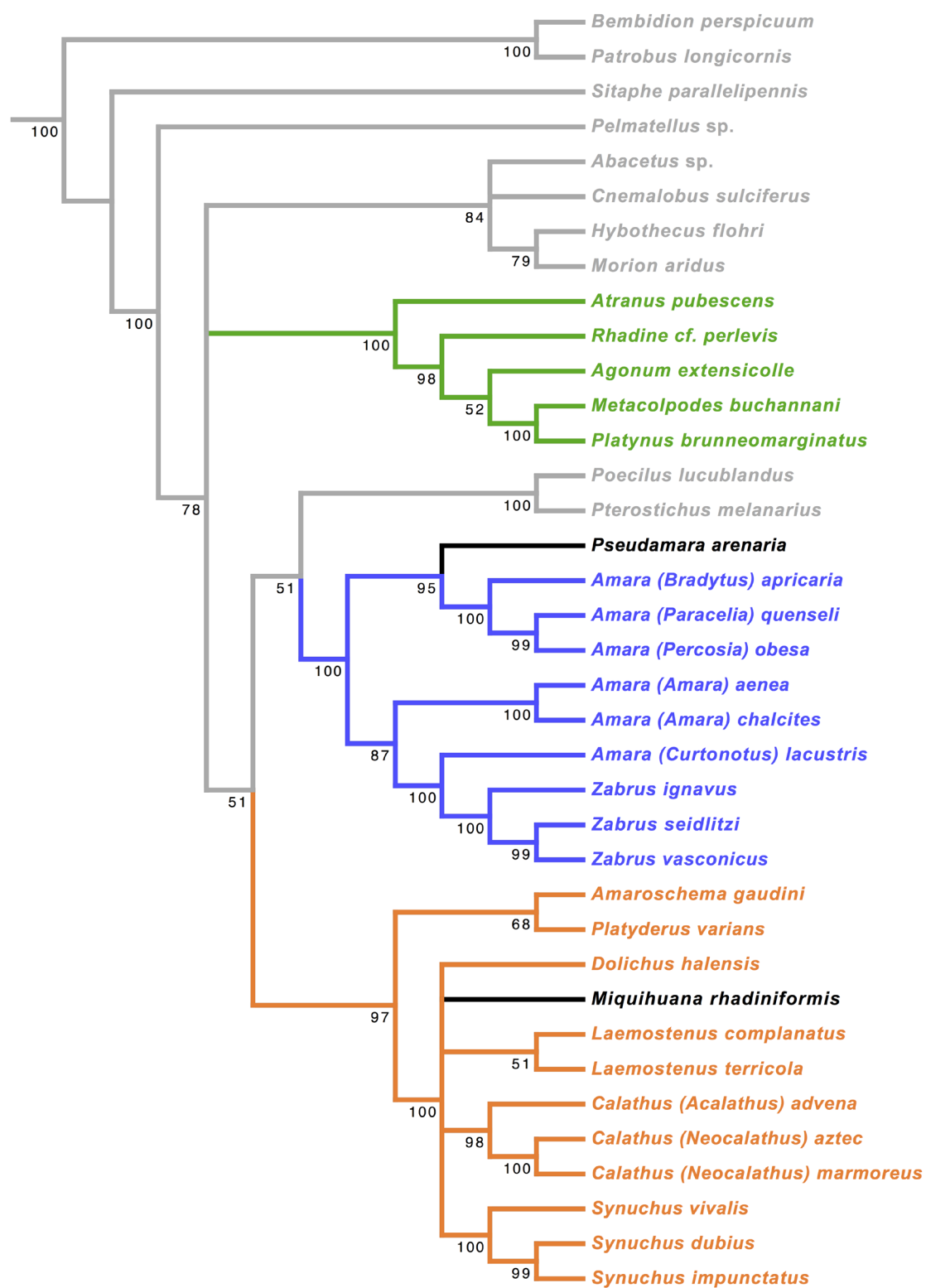
Our use of NGS on extractions of old museum specimens of *M. rhadiniformis* and *P. arenaria* was successful, and overall less costly than attempting to collect fresh

Fig. 3. Highest likelihood tree inferred from the concatenated matrix. Scale bar = 0.05 expected substitutions per site as estimated by RAxML. The color scheme for Platynini, Sphodrini, and Zabrini is consistent throughout all our trees.

Fig. 4. Majority rule consensus tree from maximum likelihood bootstrap analysis of the five gene fragment concatenated matrix (18S, 28S, *wg*, and the two fragments of CAD) partitioned by gene and codon.

Fig. 5. Highest likelihood trees inferred from individual gene matrices. CAD included analysis of a concatenated matrix of both gene fragments. Analyses based on CAD and wingless fragments were partitioned by codon. Scale bars = 0.05 expected substitutions per site as estimated by RAxML.

Table 2. Support for or against particular clades across individual gene matrices based on clade frequencies across 250 bootstrap replicates.

Clade	28S	18S	*wg*	CAD
Platynini	77	-32	-3	74
Sphodrini including *Miquihuana*	27	-10	-2	40
Atranopsina	-13	-17	15	46
Miquihuana + all other Sphodrini excluding Atranopsina	100	-17	2	98
Zabrini including *Pseudamara*	37	-12	-27	95
Pseudamara + *Amara (Bradytus)* + *A. (Percosia)* + *A. (Paracelia)*	63	-12	-10	57

specimens. The cost of collecting these beetles would not be trivial, especially *M. rhadiniformis*, which is only known from a cave in northeastern Mexico. For addressing the goals of our study, the time and cost of a collecting expedition to the type locality of *M. rhadiniformis* far exceed the costs we incurred to sequence a paratype. For less than 300 USD per specimen, we assembled large pieces of the ribosomal gene complex and low-expression, low-copy nuclear protein coding genes that have been shown to be very valuable sources of phylogenetic signal in carabids (KANDA et al. 2015, MADDISON 2012, WILD & MADDISON 2008). The data we collected allowed us to include *M. rhadiniformis* and *P. arenaria* in phylogenetic analyses based on nuclear loci, and to resolve their tribal-level placement with confidence.

Miquihuana BARR, 1982

Miquihuana rhadiniformis is a true cave specialist (troglobite), which previous workers considered biogeographically enigmatic (CASALE 1988) or relictual (BARR 1982). The species is only known from Sótano de Riachuelo, a cave in Nuevo León, Mexico, with the type series consisting of three female and one male adult specimens found during a single collecting event. It is no surprise that very little is known about *M. rhadiniformis*. BARR (1982) described the single species, giving it the specific epithet *rhadiniformis*, thereby drawing attention to its resemblance to the platynine genus *Rhadine* LECONTE, 1846*,* which includes numerous troglobitic species in North America (BARR 1974, GOMEZ et al. 2016). However, he did not classify the genus near *Rhadine*, but instead within the Sphodrini, a group with many forest dwelling and cave species that has only recently been consistently separated from Platynini. Many authors still include Sphodrini within Platynini (LINDROTH 1956 as Agonini, LORENZ 2005 as Platyninae), but recent evidence indicates that these groups may not be particularly closely related (MADDISON et al. 1999, OBER & MADDISON 2008, RUIZ et al. 2009). BARR (1982) suggested that *Miquihuana* is a true sphodrine and fit the *Sphodrus* CLAIRVILLE, 1806 group of JEANNEL's (1937) Sphodrina. CASALE (1988) further promoted this view of the Old World affinities of
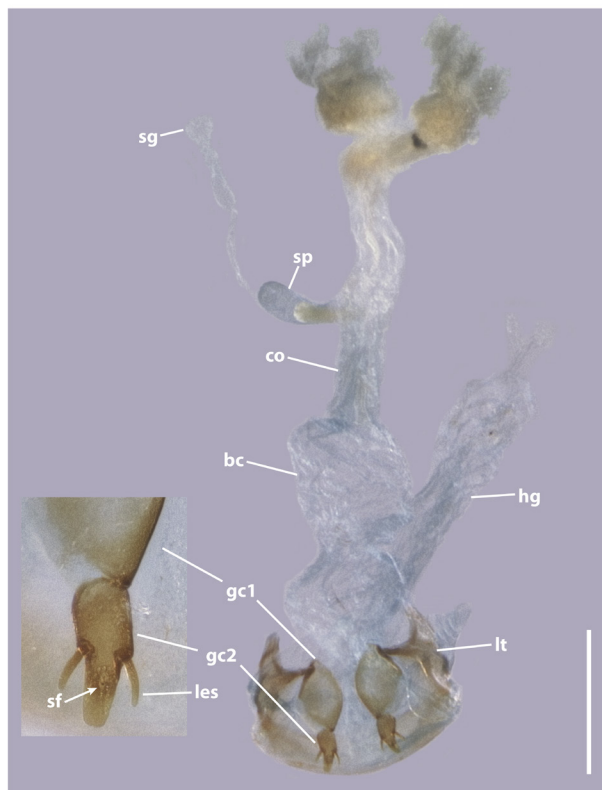
Miquihuana by treating it within his newly reclassified Sphodrina, thereby making *Miquihuana* the only native North American Sphodrina.

Based on the morphological similarity between distantly related subterranean lineages of beetles, it is likely that many features in these lineages are under the strong selective influence of their habitat (CULVER et al. 1990). Many characteristics of *Miquihuana* are likely adaptations to its cave-dwelling habit: small compound eyes, elongate pronotum, absence of flight wings, well-developed antennal pubescence, and elongate umbilical setae (Fig. 1H). BARR (1982) was well aware of this adaptive syndrome in cave insects, and for this reason he did not rely on many external morphological features for classifying *Miquihuana*. The two characters that he discussed in this context are the absence of a longitudinal ridge on the protibia and the shape of the prosternal process (BARR 1982). The presence of an external ridge on the protibia is common in Platynini, including the deceptively named *Mexisphodrus* BARR, 1965, and *Rhadine,* both of which are known from caves in Mexico (BARR 1982). The shape of the prosternal process is important for distinguishing sphodrines from platynines as sphodrines possess a posteriorly carinate prosternal process (CASALE 1988). The prosternal process of *M. rhadiniformis* is laterally compressed and apically truncate (BARR 1982). Based on our examination of a paratype of *M. rhadiniformis*, the posterior edge of the prosternum is perpendicular in lateral aspect, but it lacks a well-delimited carina along its midline, best seen in ventral or ventroposterior aspect. In rare instances platynines are known to have a posteriorly carinate prosternal edge (e.g., *Dalatagonum* FEDORENKO, 2011, *Aparupa* ANDREWES, 1930), and various Sphodrini exhibit some variation in this feature (JEANNEL 1937, *Calathus* spp. Gomez personal observation), such that the character state observed in *M. rhadiniformis* does not preclude the inclusion of *Miquihuana* within Sphodrini. These structures alone do not provide decisive evidence for a relationship in the tribe.

BARR (1982) proposed classifying *Miquihuana* within Sphodrini primarily based on characters from the male and female genitalia. Characters of the female genitalia (Fig. 6), particularly setation, and the number and lo-

cation of spines of the gonocoxites, have been crucial for distinguishing sphodrines from platynines (Habu 1978). All sphodrines lack the posterior fringe of setae along gonocoxite-1, whereas most carabids have a series of setae along this margin, notably well-developed in platynines (Liebherr & Will 1998). The gonocoxite-1 of *Miquihuana* is glabrous (Fig. 6), thus matching the state found in sphodrines.

In the male genitalia, *Miquihuana,* unlike *Rhadine* and most other platynines, possesses distinctly asymmetrical male parameres, a broad, short left paramere and a slender, elongate right paramere with a right-angled base. The parameres of Platynini are short and broad, conchoid, and although the right paramere is typically more twisted than the left, overall the parameres are nearly equal in size and shape (Liebherr 1986). The param-

Fig. 6. Female genitalia of *Miquihuana rhadinformis* Barr, 1982. bc = bursa copulatrix, co = common oviduct, gc1 = gonocoxite-1, gc2 = gonocoxite-2, hg = hind gut, les = lateral ensiform seta, lt = laterotergite, sf = sensory furrow, sg = spermathecal gland, sp = spermatheca. Scale bar = 1mm.

eres of sphodrines vary between subtribe with regard to overall shape, presence or absence of an apical hook or acuminate tip, and degree of asymmetry between right and left paramere (Casale 1988). Synuchina and Atranopsina possess short parameres, a broad left paramere and a much smaller and narrower right paramere with an angle between the base and apex. Dolichina, Sphodrina, and Calathina possess distinctly asymmetrical parameres, a broad left paramere, often with an acuminate tip, and a styloid right paramere with an angle between the

base and apex that also bears an apical hook in Calathina. Pristosiina is unusual in that the male genitalia are inverted in repose relative to the plesiomorphic condition, with the right side superior (Lindroth 1956), and the right paramere is apically narrowed to a slender point (Casale 1988). *Miquihuana* thus has parameres that are more similar to those found in sphodrines than those in platynines.

Although morphological evidence suggests that *Miquihuana* is a Sphodrini, its relationships within the tribe are less clear. Casale (1988) used both male and female genital structures to classify the Sphodrini into six subtribes, which have been recovered in a recent molecular phylogenetic study of the tribe (Ruiz et al. 2009). The right paramere of *Miquihuana* lacks the apical hook as found in Calathina, and is similar to the state found in Sphodrina. Barr (1982) and Casale (1988) used this feature as evidence for a close relationship of *Miquihuana* to the otherwise Old World Sphodrina (e.g., *Laemostenus* Bonelli, 1810). The most prominent female genital character used by Casale (1988) for characterizing the subtribes of Sphodrini was the presence or absence of an apical sensory furrow or fovea on gonocoxite-2. The secondary loss of a sensory furrow on gonocoxite-2 united Dolichina with Synuchina in Casale's (1988) classification of Sphodrini. Unlike Synuchina and Dolichina, *Miquihuana* possesses a sensory furrow on gonocoxite-2, and a small nematiform seta arises from it (Fig. 6). Because this sensory structure is present in *Miquihuana* and is rarely absent in carabids (e.g., *Nototylus* Banniger, 1927, Liebherr & Will 1998), it appears unlikely that *Miquihuana* nests within Synuchina or Dolichina. The morphological evidence for placing it with subtribe Sphodrina is not decisive. The right paramere is indeed styloid (without a hook at the tip), but a styloid right paramere may be convergent within the tribe (Ruiz et al. 2009), and thus may not by shared by *Miquihuana* and Sphodrina because of common ancestry. Other features that define Casale's (1988) Sphodrina (e.g., setation of the dorsal surface of the tarsomeres, and extent of pectination of claws) are variable enough within subtribes as to provide at best weak evidence of placement.

In summary, the morphological evidence, particularly from the genitalia, is consistent with a placement of *Miquihuana* within Sphodrini, but the exact relationship of *Miquihuana* to other sphodrines is not evident based upon examined morphological data.

Despite limited taxon sampling across the tribe, our nuclear gene dataset strongly supports the inclusion of *Miquihuana* within Sphodrini. The individual gene trees each support this conclusion, and *Miquihuana* does not nest within either of the other New World sphodrine genera, *Calathus* or *Synuchus* (i.e., *Miquihuana* is not a lineage with highly modified features derived from within *Calathus* or *Synuchus*). However, the sister group of *Miquihuana* remains unclear. This is evident from conflicting relationships across gene trees (Fig. 5). The gene

trees do not conflict in one point: *M. rhadiniformis* does not group with Sphodrina in any of the trees. The strong performance of the sampled genes in recovering Calathina and Synuchina (Figs. 3, 4, 5, S1) provides some evidence that these genes are good phylogenetic markers within sphodrines, making the consistent placement of *Miquihuana* with non-Sphodrina species somewhat more compelling. Given its lack of evident placement within any sampled subtribe, *Miquihuana rhadiniformis* may represent a previously unrecognized major lineage of Sphodrini or at least a significantly divergent taxon within an existing lineage.

Though there are several troglobitic sphodrines in Europe and Asia, *M. rhadiniformis* is currently the only known troglobitic Sphodrini in North America (Casale et al. 1998). The bulk of the diversity in Sphodrini is Palearctic, and the tribe is largely missing from the tropics and the Southern Hemisphere. The only other known native New World sphodrines are all part of *Calathus* and *Synuchus*, both of which are more diverse in the Palearctic than the Nearctic (Lorenz 2005). North American species of *Calathus* and *Synuchus* are also exclusively surface dwelling species, and there are many montane endemics in *Calathus* in Mexico (Ball & Nègre 1972). This biogeographic pattern led Barr (1982) to hypothesize that *M. rhadiniformis* was a relictual species, and that all other members of the radiation of Sphodrina that once existed in North America have become extinct. Casale (1988) also noted the disparity in Sphodrini diversity in North America and regarded it as a question without an easy answer. Casale (1988, p. 948) writes [translation from Italian] "[What are] the causes of the massive subtraction that has resulted in the apparent total loss of the subtribe Sphodrina from Alaska to Mexico, in an area where the Synuchina and Calathina are, to the contrary, well represented [?]".

Highly disjunct distributions are known from many groups of animals with cave species (see Juberthie & Decu 1998 for a general review of the distribution of cave animals). A commonly used hypothesis for the mechanism responsible for these disjunct distributions often entails range expansion into an area followed by extinction of the above-ground elements (Assmann et al. 2010); the species that remain in the region are considered relicts of their former distribution. This expansion-extinction hypothesis has been proposed as a mechanism behind the present day distribution of other animal groups with similar disjunct distributions such as cave salamanders of the family Proteidae (e.g., *Speleomantes* Dubois, 1984 and *Atylodes* Gistel, 1868, Durand 1998, Weisrock et al. 2005) and the European cave carabid beetle *Dalyat mirabilis* Mateu, 2002 (Mateu & Belles 2003). *Dalyat* Mateu, 2002 in particular is striking: a genus found only in three caves in Spain, whose nearest living relatives (based upon a molecular phylogenetic analysis) are in South Africa and the west coast of North America (Ribera et al. 2005).

Although our study does unequivocally resolve the tribal-level placement of *Miquihuana* within Sphodrini, the general biogeographic pattern of North American sphodrines remains unchanged. The available evidence indicates that there are likely three distinct lineages of Sphodrini in North America, possibly corresponding to at least three separate invasions of North America.

Pseudamara Lindroth, 1968

Pseudamara arenaria has a checkered taxonomic history (Hieke 2010). In general habitus (Fig. 2H) it resembles a stout harpaline or sphodrine leading early authors to describe it in *Geobaenus* Dejean, 1829 (itself a problematic genus, KWW unpublished) or place it near *Bradycellus* Erichson, 1837. As compared to *Amara*, *P. arenaria* was noted by Lindroth (1968) as being unusual, specifically "not very closely related to *Amara*." Nevertheless, most classifications maintained *Pseudamara* in Zabrini (or Amarini). Fritz Hieke was unparalleled in his knowledge of Zabrini, and in the late 1990s and early 2000s, in a series of correspondence with one of us (KWW), Hieke discussed a number of possible placements for *Pseudamara,* including treating it as separate tribe between Harpalini and Zabrini or placing it among Sphodrini. Lindroth (1968), Bousquet (2012), and Hieke (2010) thoroughly review the taxonomic and nomenclatural history of *Pseudamara.* Hieke (2010) then made the decisive step to place *Pseudamara* among Sphodrini based on the parameres of the aedeagus, gonocoxites, palp setation and elytral plica. Variation within *P. arenaria* in the palp setae and elytral plica form are absent from discussions of *Pseudamara* by Hieke and Lindroth (1968) before him, which seems to indicate those studies are founded on limited material. Based on a total of about 80 specimens, 10 of which we studied in detail including dissections, the morphological characters above and other characters that may be pertinent to tribal placement of the genus are discussed below.

The head of *P. arenaria* is relatively broad, has very shallow, slightly transverse frontal impressions and short, heavy mandibles. The relative proportions of the head of *Pseudamara* are much more typical of Harpalini, and thus differ from the large majority of *Amara* species. However, the generally harpaline-like head shape (i.e., relatively broad between and anterior to the eyes) is found in many different lineages of carabids, presumably due to convergent evolution of a feeding strategy that includes seed feeding (Acorn & Ball 1991, Forsythe, D.J. 1982, Forsythe, T.G. 1983). Some notable and diverse examples of potential convergence in head form include *Simodontus* Chaudoir, 1843 (Pterostichini), *Badister* Clairville, 1806 (Licinini), *Amaroschema* (Sphodrini), and *Geobaenus* (Platynini). The evolution of the general form of the head is probably functionally driven. Given this, it does not appear to provide compelling evidence for tribal-level placement of *Pseudamara. Pseudamara*

constantly has two supraorbital setae above each eye, in contrast to the single seta of members of Harpalini. It is not uncommon for groups with nearly all members having two supraorbital setae to exhibit loss in some taxa, e.g., in *Gastrellarius honestus* (Say, 1823) (Bousquet 1999) or more rarely, taxa are polymorphic for supraorbital setal number having both one and two setae states, e.g., *Cuneipectus* Sloane, 1907 (Will 2015). There is no case known to us in which a clade that ancestrally had a single supraorbital seta has regained a second seta. The presence of two supraorbital setae in *Pseudamara* is thus consistent with a placement in either Zabrini or Sphodrini, but not within Harpalini.

The palps of *P. arenaria* have been a key feature for identification of the genus. The terminal palpomeres are clearly setose, a character state not known in *Amara*, and this remains a feature distinguishing *P. arenaria* from all zabrines. The penultimate labial palpomere was noted by Hieke (2010) and Lindroth (1968) as being bisetose in contrast to the plurisetose state in Zabrini and many Harpalini. However, we found that *P. arenaria* specimens exhibit a range of setal states. While most specimens have two large setae and no or only one or two inconspicuous additional setae, some individuals have two or more additional setae that approach the size of the two primary setae, blurring the line between bisetose and plurisetose. The bisetose state is common to many groups of Harpalinae, and many carabids outside of Harpalinae, and so may be the plesiomorphic condition in Harpalinae from which the plurisetose state is derived.

The elytra and flight wings show marked variation in *P. arenaria*. The flight wing may be full, with a large fold apically, or reduced to a small pad. The flight wing dimorphism does not have a clear geographic pattern, nor does it appear to be correlated with sex. The elytral striae range from impunctate to clearly crenulate, and the plica of the epipleuron, a key feature in Hieke's (2010) argument for placement in Sphodrini, varies from fully crossed and distinct as in *Amara*, to only represented by a small ridge that does not cross the epipleuron externally or only very shallowly crosses the epipleuron, a state approached by some Platynini and Sphodrini. The polymorphic form of the elytral plica (Fig. 7) is ambiguous evidence for placement of *P. arenaria* at best and may be the result of independent reduction as has been hypothesized to have occurred in Abacetini (Straneo 1991, Will 2011) and Pterostichini (Will 2007).

The female reproductive tract and gonocoxites in *P. arenaria* are typical of *Amara* and species of *Zabrus* with an evident spermatheca (Ortuño et al. 2003). Gonocoxite-2 is triangular and narrower than many zabrine species, but is not unique in its form. Gonocoxite-2 has two, long, subapical nematiform setae and a large ensiform seta laterally and medially. Gonocoxite-1 has scattered, fine setae apicomedially. In some specimens there may be only four to six setae, and they are difficult to see. This may have led Hieke (2010) to assume that gono-
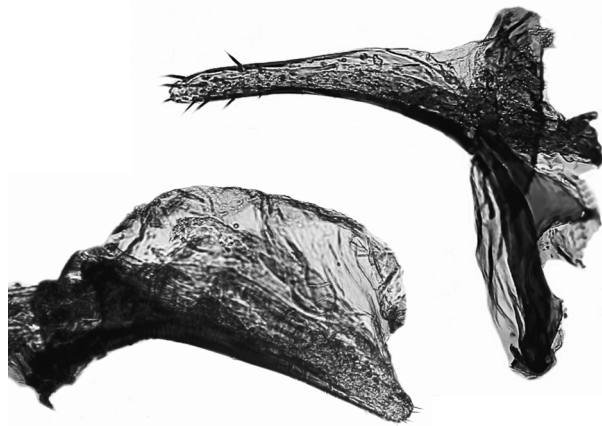

Fig. 7. Left lateral aspect of the apex of the elytron showin the range of variation in the development of the plica. Edges have been enhanced for clarity.

coxite-1 was glabrous. The female tract has a well-developed spermatheca broadly joined to the common oviduct at its base, an appended spermathecal gland and a villous canal extended from the base of the spermatheca, up the common oviduct.

The male aedeagus of *P. arenaria* is relatively very small with a simple median lobe and endophallus that lacks any ostensible sclerotized plates or spines. The key feature of the aedeagus that Hieke (2010) felt excluded it from Zabrini was the form of the parameres. In Zabrini the left paramere is modestly sized and broad or more or less conchoid, while the right is narrow, elongate and usually hooked at the tip. The length of the right is much greater than the left, reaching or nearly reaching the apex of the median lobe. This general pattern is similar to that of the male parameres of *Calathus*. And, as far as known, all *Amara* and *Zabrus* have glabrous parameres. *Pseudamara arenaria* males have parameres that are nearly the same length, the left being broad and longer than in a typical *Amara* and the right narrow, but shorter than a typical *Amara*. The right paramere in *P. arenaria* is not hooked at the tip. Additionally, the apices of both parameres in *P. arenaria* are distinctly setose (Fig. 8). While the parameres are not typically zabrine in form, they only bear a superficial resemblance to *Amaroschema* and other sphodrines that have more or less equal length parameres.

The pygidial gland reservoirs of sphodrines, platynines and harpalines have a distinct dorsal lobe (Forsythe 1972, Will & Liebherr 2002). *Pseudamara arenaria* lacks the dorsal lobe, and the reservoir is gener-

ally cordiform, similar to the shape found in many taxa, including *Amara*. The pygidial gland reservoir of *Amaroschema* has not been examined, but is predicted to have a dorsal lobe like other sphodrines. Harpalini species have only a single testis, a derived feature that is inferred to the ancestral state for the Harpalini clade (WILL et al. 2005). A single *P. arenaria* male that was preserved such

Fig. 8. Photographs of the male parameres of *Pseudamara arenaria* (LECONTE, 1847), indicating the minute hairs present at the apices of both right and left lobes.

that the dry internal organs were still relatively intact had distinct sinuate accessory glands and what appeared to be the remnants of a pair of testes.

In summary, morphological evidence for the placement of *Pseudamara* is limited due to variability (penultimate labial palp setae, elytral plica), apparently autapomorphic states (head shape modifications, setose terminal palpomeres, setose parameres), or likely plesiomorphic states (two supraorbital setae, cordiform pygidial gland reservoirs, two testes, generalized Harpalinae female reproductive tract). *Pseudamara arenaria* is an enigmatic and problematic taxon for which it appears there is limited phylogenetic evidence borne by morphological features. The trouble this caused FRITZ HIEKE for several decades was well-founded.

In this case, however, the DNA sequence data is decisive. Though we cannot speak on the sister-group relationships within Zabrini given our limited exemplar sampling, it is clear that *P. arenaria* is a member of the tribe and not closely related to any Sphodrini nor any of the other taxa in our analysis. Based on its phylogenetic position it is here returned to Zabrini.

Acknowledgements

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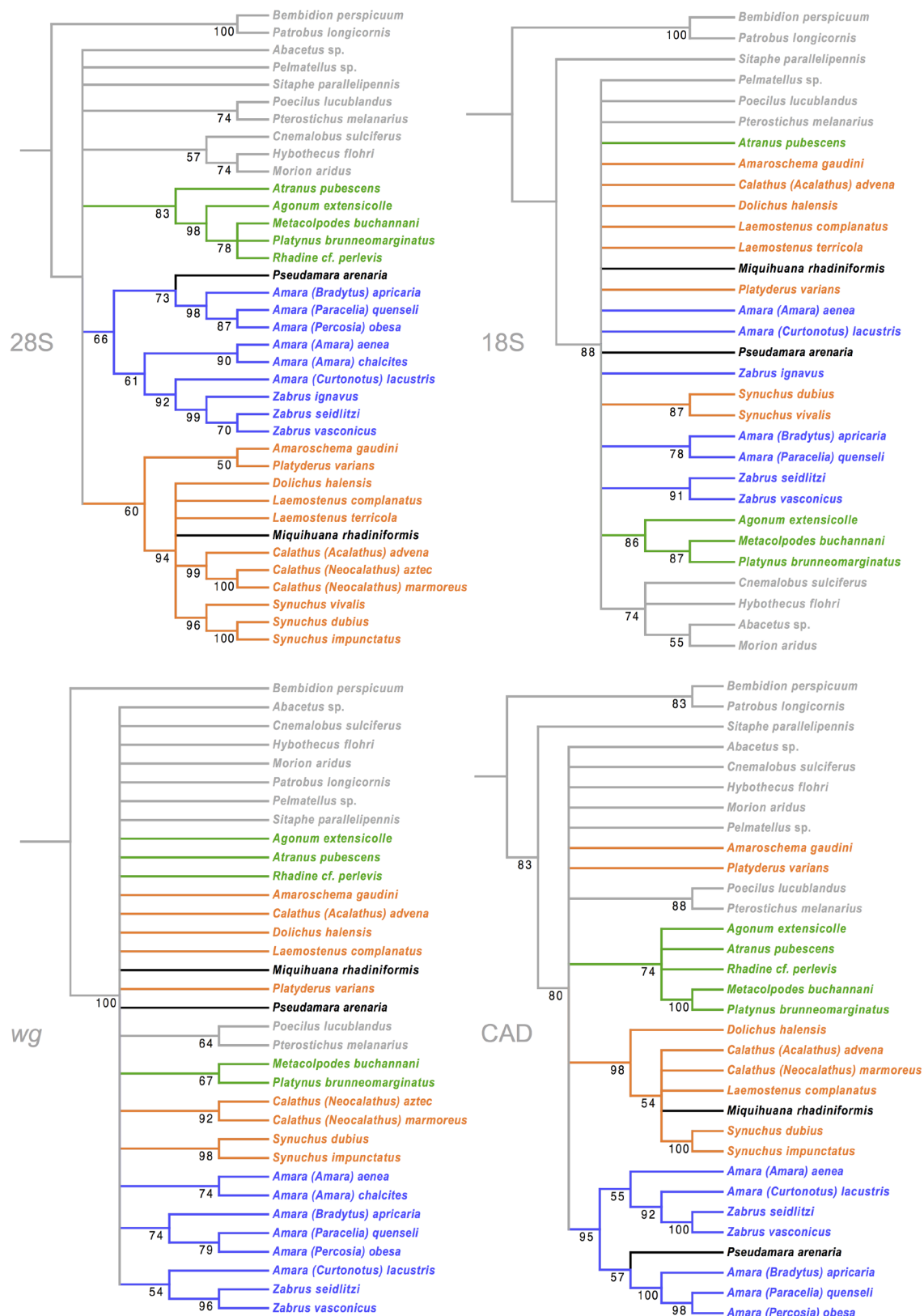
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Table S1. Locality data for newly sequenced voucher specimens.

Species	Code	Locality
Abacetus sp.	DRM0688	India: 15-60 km W Belgaum, 3.iii.-4.iv.1997
Agonum extensicolle	DRM2409	USA: Arizona: Santa Cruz River, Tumacocori, 31.5622°N 111.0452°W, 995m, 15.xi.1998.
Amara aenea	kww1001	USA: Washington: Pierce Co. North Tacoma, 17.v.2004
Amara apricaria	DRM0314	Canada: Alberta: Edmonton, 8.vi.1993
Amara lacustris	DRM0315	Canada: Alberta: Kenilworth Lake, 53.341°N 110.487°W, 10.vi.1993
Amara obesa	kww1006	USA: Arizona, Gila Co. Sierra Ancha, Tonto National Forest, Workman Creek, 33.8146°N 110.9256°W, 2060m, 27.vii.2001
Amara quenseli	kww1000	USA: California: Mono Co. White Mts., N. Fork Crooked Creek, 37.5063°N 118.1631°W, 2960m. 11.vii.2010
Amaroschema gaudini	kww684	Spain: Tenerife: carretera de Bailadero a Chamoraga, 10.i.2007
Atranus pubescens	DRM0947	USA: New York: Tompkins Co., Ithaca
Bembidion perspicuum	DRM1120	USA: Arizona: Cochise Co., San Pedro Riparian Area at Charleston Bridge, 31.6300°N 110.1774°W, 1200m, 17.xi.2001
Calathus advena	kww1007	USA: Utah: Toele Co. Stansbury Mountains, Loop Campground, 2265m, 40.4838°N 112.6061°W, 5.vi.2000
Calathus aztec	kww1005	Mexico: Jalisco: Nevado de Colima, 19.6119°N 103.5700°W, 2560m, 28.vii.2007
Calathus marmoreus	kww1008	Mexico: Hidalgo: Parque Nacional El Chico, 20.1754°N 98.7181°W, 2946m, 19.viii.2015
Cnemalobus sulciferus	DRM0455	Chile: Talca Province: Area de Protección Vilches Visitor Center area, 35.6058°S 71.0725°W, 1200m, 17-19.xii.1996
Dolichus halensis	kww1002	South Korea, Jeju Province, Jeomhual Resort, 19.x.2005
Hybothecus flohri	kww18	USA: Arizona: Pima Co., Box Canyon
Hybothecus flohri	DRM1053	USA: Arizona: Pima Co., Box Canyon
Laemostenus complanatus	kww40	USA: California: Contra Costa Co. Concord, 37.9475°N 121.9719°W, 88m. 14.iii.2010
Laemostenus complanatus	kww726	USA: California: Contra Costa Co. Concord, 88m. 30.iv.2004
Metacolpodes buchannani	kww426	USA: California: Del Norte Co. Rock Creek Ranch, 41.7306°N 123.9756°W, 100m, 12.vi.2008
Miquihuana rhadiniformis	kww999	Mexico: Tamaulipas: 6.5 km and 2 km E Miquihuana, Sótano de Riachuelo
Morion aridus	DRM0136	USA: Arizona: Pima Co., Tucson Mountains, 32.244°N 111.167°W, 885m, November 1993
Patrobus longicornis	DRM0114	Canada: Alberta: Edmonton, 53.530°N 113.513°W, 15-20.vii.1992
Pelmatellus sp.	DRM0621	Costa Rica: Cerro de la Muerte: Pan American Highway marker 89.3km, 5.7 km from La Jorgina
Platyderus varians	kww683	Spain: Madrid: Pto de Navacerrada. Fte de los geólogos. 1700m, 08.v.2004
Platynus brunneomarginatus	kww420	USA: California: Santa Barbara Co. Cachuma Camp, 34.6978°N 119.9133°W, 635m, 24.v.2006
Platynus brunneomarginatus	kww1003	USA: California: Fresno Co. San Joaquin River Gorge, 37.0961°N 119.5350°W, 345m. 12.v.2012
Pseudamara arenaria	kww998	USA: New York: Franklin Co., Adirondack Mountains, 1999
Rhadine cf. perlevis	DRM0656	USA: Arizona: Pima Co., Santa Catalina Mountains, Mount Lemmon, 32.4465°N 110.7740°W, 2550m, 2.ix.1992
Sitaphe parallelipennis	DRM2247	Australia: Queensland: Upper Whitehall Gully, 1290m, 26.vi.1997
Synuchus dubius	DRM0353	USA: Arizona: Pima Co., Santa Catalina Mountains, Mt Lemmon, 32.447°N 110.781°W
Synuchus impunctatus	kww1004	USA: Vermont: Washington Co. Camel's Hump, 44.3064°N 72.8589°W, 550m, 14.vi.2010
Zabrus seidlitzi	DRM0911	Spain: Madrid: Navacerrada, 14.ix.1996
Zabrus vasconicus	kww724	Spain: Navarra: Pto. De Lizarraga

Fig. S1. Majority rule consensus trees from analysis of individual gene matrices and 250 bootstrap replicates. CAD included analysis of a concatenated matrix of both gene fragments. Analyses based on CAD and *wingless* fragments were partitioned by codon.