

DNA barcoding of rhopalosomatid larvae reveals a new host record and genetic evidence of a second species of *Rhopalosoma* Cresson (Hymenoptera, Rhopalosomatidae) in America north of Mexico

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Academic editor: Michael Ohl	Received 15 July 2019	Accepted 19 October 2019	Published 30 December 2019		
http://zoobank.org/9E2BBCDA-59CF-42E2-BAE4-907A2896AA6C					

Citation: Miller LA, Benefield TD, Lounsbury SA, Lohrmann V, Blaschke JD (2019) DNA barcoding of rhopalosomatid larvae reveals a new host record and genetic evidence of a second species of *Rhopalosoma* Cresson (Hymenoptera, Rhopalosomatidae) in America north of Mexico. Journal of Hymenoptera Research 74: 35–46. https://doi.org/10.3897/jhr.74.38276

Abstract

Rhopalosomatidae are unusual wasps whose larvae develop as ectoparasitoids on crickets. In America north of Mexico, three genera and six species are recognized. Host species are known only for *Rhopalosoma nearcticum* Brues and include *Hapithus agitator* Uhler, *H. brevipennis* (Saussure), and *H. saltator* (Uhler) (Gryllidae: Hapithinae). Here we report a new host species: the *Anaxipha exigua* (Say) species-group (Trigonidiidae: Trigonidiinae) discovered by barcoding nine rhopalosomatid larvae collected from Cypress Grove Nature Park, Jackson, Tennessee. *Rhopalosoma nearcticum* is currently the only documented species of *Rhopalosoma* Cresson in America north of Mexico, but our phylogenetic analyses recovered two genetically distinct clades of *Rhopalosoma* and thus reveal the presence of at least two species of *Rhopalosoma* in America north of Mexico.

Keywords

Parasitoid wasps, molecular phylogenetics, Vespoidea, Orthoptera

Introduction

Rhopalosomatidae (Hymenoptera) are unusual aculeate wasps that apparently develop as ectoparasitoids on crickets (Grylloidea) (Perkins 1908; Hood 1913; Gurney 1953; Lohrmann et al. 2014; Lohrmann and Engel 2017). Of the 72 extant species in the family (Aguiar et al. 2013), only six have been recorded in America north of Mexico. Whereas *Liosphex* Townes and *Olixon* Cameron are represented by two and three species respectively, *Rhopalosoma* Cresson (Fig. 1) is represented solely by the widespread species *Rhopalosoma nearcticum* Brues (Townes 1977; Lohrmann and Ohl 2010; Lohrmann et al. 2012).

Within the Rhopalosomatidae, *R. nearcticum* is the only species whose biology and life cycle has been investigated in detail (Lohrmann and Engel 2017). Hood (1913) was the first to document observations of a larval *R. nearcticum* (originally misidentified as *R. poeyi* Cresson) attached to a *Hapithus saltator* (Uhler) (Orthoptera: Gryllidae: Hapithinae) host. Later, Gurney (1953) provided detailed descriptions of the immature stages of *R. nearcticum* and added *Hapithus agitator* Uhler and *H. brevipennis* (Saussure) as hosts.

In contrast to other hymenopteran parasitoids of crickets, *R. nearcticum* oviposit without relocating the host (Melo et al. 2011). The egg is laid behind the coxa of the host's hind leg. With its mandibles imbedded in the abdomen of the host, the larva develops from first to fourth instar. As it grows, the hind leg of the cricket is forced outward at an unnatural angle (Gurney 1953). The fifth instar detaches from the host, usually killing it in the process (JDB, pers. obs. 2017), and borrows in the soil where it spins a cocoon and pupates (Gurney 1953; fig. 2). The following spring it emerges as an adult (Hood 1913; Gurney 1953).

Although rhopalosomatids are rarely collected, they can be locally abundant (e.g., Smith 2008), in particular in floodplain forests where hosts are numerous (Barrows 2013). Adults are most often collected passively in Malaise traps (e.g., Townes 1977; Smith 2008; Barrows 2013), especially in the summer and early fall (Freytag 1984). Gurney (1953) observed a group of ~10 adults flying over shrubbery at twilight "until no longer visible in the gathering dark", and this, coupled with their unusual large ocelli, their inconspicuous pale brown color, and their occasional appearance in light trap samples (e.g., Stange 1991), indicates that *R. nearcticum* is a crepuscular or nocturnal species.

Morphological phylogenies have been reconstructed for the closely related *Paniscomima* Enderlein (Guidotti 2007), the brachypterous *Olixon* Cameron (Krogmann et al. 2009), and the family as a whole (Guidotti 1999). However, no comprehensive molecular study has focused on the Rhopalosomatidae itself, nor thoroughly examined any individual genus within it. However, *Rhopalosoma nearcticum* has been included in several molecular analyses of Hymenoptera (Carpenter and Wheeler 1999; Hines et al. 2007; Pilgrim et al. 2008; Szafranski 2009*; Heraty et al. 2011; Klopfstein et al.

^{*} A crosscheck of Szafranski's sequence of *R. nearcticum* (GenBank: EU567206.1) against the data in BOLD and GenBank reveals the sequence belongs to *Drosophila melanogaster*.



Figure 1. Adult female *Rhopalosoma* cf. *nearcticum* attracted to a mercury-vapor lamp in Fairfax County, VA, USA on July 29, 2018. Photo by Ashley Bradford, initially posted on bugguide.net.

2013; Branstetter et al. 2017), and several rhopalosomatids have been sequenced for the Barcode of Life Database (BOLD) (Ratnasingham and Hebert 2007).

In September, 2016, a bush cricket of the *Anaxipha exigua* (Say) species-group (Trigonidiidae) was collected at Cypress Grove Nature Park (CGNP) in Jackson, TN. Attached to the abdomen directly behind the right hind leg was a dark brown sac-like protuberance that was identified as a potential rhopalosomatid larva. Given the rarity of documented rhopalosomatid larvae and the novelty of the host record, our objectives were to 1) collect additional larvae and hosts from CGNP, 2) attempt to rear larvae to adulthood, and 3) sequence the barcoding gene COI of each specimen for molecular identification. Here, we report the *Anaxipha exigua* species-group as a new host record for *Rhopalosoma* and identify two genetically distinct clades of *Rhopalosoma* in America north of Mexico.

Materials and methods

Collection and rearing attempts

Cricket specimens were collected from CGNP using sweep nets in 2017 and 2018 from July–October when *Rhopalosoma* seems to be at peak abundance (Barrows 2013).

Parasitized crickets were retained at the Blaschke Lab at Union University for observation and attempted rearing of parasitoids. Crickets were maintained at 26 °C with a 16:8 photoperiod and were supplied apple slivers and raisins for food. Soil from CGNP was provided for burrowing/cocoon-formation when the larva detached from its host. If the cricket expired before the larva was mature enough to detach, the parasitoid and its host were stored in a freezer at 4 °C for subsequent molecular analysis. Photographs of parasitized crickets and larvae were taken using a camera phone mounted on a Fisher Stereomaster microscope and the images were stacked and edited using Helicon Focus 6 (v.6.8.0) software. Voucher specimens are retained at Union University, Jackson, TN.

Barcoding

Genomic DNA was extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands). Due to their small size, rhopalosomatid specimens were extracted whole using whatever material was available (larvae, pupae, and/or exuviae). A BIO-RAD T100 Thermal Cycler was used to perform 50 µL PCR reactions modeled after Hebert et al. (2003). The following settings were used for the PCR reaction: 30 s denaturation at 98 °C followed by five cycles of 98 °C for 5 sec, 45 °C for 5 sec, and 72 °C for 15 sec; 35 cycles of 98 °C for 5 sec, 51 °C for 5 sec, and 72 °C for 15 sec; 35 cycles of 98 °C for 5 sec, 51 °C for 5 sec, and 72 °C for 15 sec; and a final extension at 72 °C for 1 min. The traditional invertebrate primers for COI were used for barcoding (LCO1490/HCO2198; Folmer et al. 1994). Difficult templates were amplified using a custom rhopalosomatid-specific forward primer (5'-CYATAT-GATCAGGAATAGTAGGWT-3'). Successful amplifications were confirmed via thin gel electrophoresis and the samples were sent to GeneWiz (South Plainfield, New Jersey) for post-PCR clean-up and sequencing.

Sequence quality was determined by the quality scores provided by GeneWiz and by examining the chromatograms visually using Geneious Prime (v.2019.0.4). The novel rhopalosomatid COI barcodes were uploaded to GenBank (Table 1). Sequences were aligned manually with a *R. nearcticum* reference sequence from GenBank (ID# GQ374638.1). Additional sequences from BOLD included barcodes from two unidentified *Rhopalosoma* specimens from Florida (initially assumed to be *R. nearcticum*), two unidentified *Rhopalosoma* specimens from Costa Rica, and an *Olixon* specimen as the outgroup (max = 657bp, min = 456bp, avg = 602.3pb) (Table 1).

Phylogenetic relationships were reconstructed using maximum likelihood (ML) and neighbor joining (NJ). ML trees were generated using RAxML (v.8.2.12) (Stamatakis 2014) through the CIPRES Science Gateway (Miller et al. 2010) with default parameters and analyzed statistically using 1000 bootstrap (BS) replicates. A strict consensus neighbor joining (NJ) tree was constructed using Geneious Prime with default parameters and one million bootstrap replicates. All phylogenies were visualized and clades compared in Geneious Prime. The Species Delimitation plug-in (Masters et al. 2011) was used to assess species boundaries and diversity using the Intra Dist, Inter Dist, P ID(Liberal) and P(Randomly Distinct) calculations.

Results

Collection and parasitoid rearing

In total, 12 parasitized crickets were collected and nine rhopalosomatid larvae were successfully barcoded (Table 1). Seven larvae were found on *H. agitator* hosts (five of these larvae were barcoded) (Fig. 2G), one on an *H. saltator* (larva barcoded) (Fig. 2H), and four were discovered attached to crickets from the *A. exigua* species-group (three larvae barcoded) (Fig. 2I).

Six parasitoid larvae detached from their host and four of these successfully spun cocoons to begin pupation. One specimen from an *H. agitator* host developed into an adult, but failed to eclose properly from the cocoon, resulting in the death of the wasp (Fig. 2C). Because so little is known about the phenology of these wasps, two cocoons from *A. exigua* hosts were dissected to observe the pupae *in situ* after attempting to activate the final molt to adulthood by incubating the pupae at 26 °C for >4 weeks. One larva had failed to develop into a pupa and the only identifiable tissue inside the cocoon was a disarticulated mandible (Fig. 2D). The other specimen had not pupated either, but was alive and appeared to be in a suspended animation "pre-pupa" state (Fig. 2E). The final cocoon is currently still in incubation (Fig. 2F).

Barcoding and phylogenetics

The topologies from the ML and NJ analyses were identical and statistically robust (Figs 3–4). Both trees recovered two distinct clades of Nearctic *Rhopalosoma*. Clade 1 contained the reference *R. nearcticum* sequence from GenBank along with seven of the

Tree ID	Species	Host	Sample	Accession #
Parasitoid_1_TN	R. ?nearcticum sp. 1	H. agitator	Larva	MK991300
Parasitoid_2_TN	R. ?nearcticum sp. 1	A. exigua s.g.	Pupa	MK991301
Parasitoid_3_TN	R. ?nearcticum sp. 1	A. exigua s.g.	Larva	MK991302
Parasitoid_4_TN	R. ?nearcticum sp. 1	H. agitator	Larva	MK991303
Parasitoid_5_TN	R. ?nearcticum sp. 2	H. agitator	Larva	MK991304
Parasitoid_6_TN	R. ?nearcticum sp. 1	H. agitator	Larva	MK991305
Parasitoid_7_TN	R. ?nearcticum sp. 1	A. exigua s.g.	Larva	MK991306
Parasitoid_8_TN	R. ?nearcticum sp. 1	H. saltator	Larva	MK991307
Parasitoid_9_TN	R. ?nearcticum sp. 2	H. agitator	Larva	MK991308
Rhopalosoma_BBHYA1357_FL	R. ?nearcticum sp. 2	_	Adult	BBHYA1357-12*
Rhopalosoma_BBHYA1345_FL	R. ?nearcticum sp. 2	_	Adult	BBHYA1345-12*
Rhopalosoma_JICAX021_CR	<i>Rhopalosoma</i> sp.	_	Adult	JICAX021-16*
Rhopalosoma_JIAAG042_CR	<i>Rhopalosoma</i> sp.	_	Adult	JIAAG042-16*
Olixon_BBHY2946_TX	Olixon sp.	_	Adult	BBHYA2946-12*
R. nearcticum_GQ374638.1	R. ?nearcticum sp. 1	_	Adult	GQ374638.1

Table 1. Specimens used in phylogenetic analyses of Rhopalosomatidae with GenBank accession numbers. 'ID numbers from BOLD.



Figure 2. Life stages and representative specimens of *Rhopalosoma* **A** 5th instar larva prior to burrowing (MK991305) **B** pupal case extracted from soil (MK991302) **C** adult after failing to emerge properly from cocoon (MK991303) **D** disarticulated mandible from pupal case (MK991302) **E** pupal case extracted from dirt showing still living pre-pupa (MK991301) **F** pupal case awaiting adult emergence (MK991300) **G–I** early instar larvae attached to: **G** *Hapithus agitator* adult (larva: MK991304) and **H** *H. saltator* nymph (larva: MK991307) **I** *Anaxipha exigua* species group (inset: detached larva: MK991302).



Figure 3. Maximum Likelihood phylogeny of *Rhopalosoma* with *Olixon* sp. as the outgroup. Bootstrap support shown for important nodes. Cricket photos by Carl Strang (*Hapithus agitator*), and Wil Hershberger/Lang Elliott (*H. saltator* and *Anaxipha exigua* s.g.).



Figure 4. Neighbor Joining phylogeny of *Rhopalosoma* with *Olixon* sp. as the outgroup. Consensus support shown for important nodes. Cricket photos by Carl Strang (*Hapithus agitator*), and Wil Hershberger/Lang Elliott (*H. saltator* and *Anaxipha exigua* s.g.).

new barcodes, representing three larvae from *H. agitator*, one larva from *H. saltator*, and three larvae from *A. exigua* (BS = 100). Clade 2 included the two specimens from Florida along with two new barcodes representing two larvae from CGNP, both from *H. agitator* hosts (BS = 96). These clades were not sister to each other. Instead, Clade 2 was recovered sister to a specimen from Costa Rica (BS = 68).

The intraspecific distance was low within each clade (Clade 1 = 0.002; Clade 2 = 0.006), while the interspecific distance between these clades was high (0.148). Similarly, the P ID(Liberal) calculations, which serve as predictions of the utility of the gene for species delimitation (Ross et al. 2008), were high (Clade 1 = 0.97; Clade 2 = 1.00). The P(Randomly Distinct) values were <0.05 indicating a high probability that these two clades represent two distinct lineages (Rodrigo et al. 2008).

Discussion

The 12 Nearctic *Rhopalosoma* specimens included in the analyses (nine novel, plus two from BOLD and one from GenBank) were recovered in two distinct clades with convincing statistical support (BS = 98 and BS = 100). Clade 1 establishes one species of *Rhopalosoma* to be a generalist parasitoid of Grylloidea by expanding the known hosts to include the *Anaxipha exigua* species group. Unfortunately, even after barcoding the crickets, the exact species of the new host *Anaxipha* was not possible to determine. Six nominal species are included in the *A. exigua* species-group, only reliably distinguishable by the cadence of their mating calls (Walker and Funk 2014). However, *A. exigua* is the only member of the group whose range would include CGNP, indicating the host is most likely *A. exigua*, but we leave confirmation of the new host beyond the species-group level to future researchers.

Clade 2 reveals a second distinct genetic lineage of *Rhopalosoma* in the Nearctic. This clade includes specimens from Tennessee and Florida, and was recovered sister to a specimen from Costa Rica. This implies that this species has a closer evolutionary relationship with at least one Neotropical species than with its sympatric species in North America, even while developing on the same host as its Tennessee relative (*H. agitator*).

Although a *R. nearcticum* reference sequence was recovered within Clade 1, it is not possible at this time to determine if the sequence actually belongs to true *R. nearcticum*. Now that it is apparent that two species of *Rhopalosoma* inhabit America north of Mexico, adults of each clade need to be compared with the type specimen of *R. nearcticum* and with other members of the genus, *Rhopalosoma simile* Brues and the Caribbean species in particular. Either clade could be *R. nearcticum*, or neither group could be. Morphologically, *Rhopalosoma simile* is quite similar to *R. nearcticum* and apart from the color of the scape, pedicel, and flagellomeres I–V there is no character known that distinguishes these two taxa (Townes 1977). Gauld (1995) interpreted *R. nearcticum* and *R. simile* as conspecific and reported the occurrence of *R. nearcticum* in Costa Rica. However, this assumption has never been tested and a more detailed study should address the question of whether the two clades revealed in the study might represent these two species. Unfortunately, no high quality adult specimens were obtained during this study to compare with type specimens of *Rhopalosoma* spp.

The relatively few barcodes of *Rhopalosoma* generated here reveal the desperate need for thorough revision of this genus and phylogenetic analysis of intrageneric relationships. Although a species-level identification key for *Rhopalosoma* exists (Townes 1977), accurate identification remains a challenge. Other genera within Rhopalosoma-tidae (i.e., *Liosphex, Olixon* and *Paniscomima*) have received major taxonomic updates since Townes' family revision in 1977 and many new species have been discovered (e.g., Guidotti 2007; Krogmann et al. 2009; Lohrmann and Ohl 2010; Lohrmann 2011; Lohrmann et al. 2012). *Rhopalosoma*, however, has remained almost untouched apart from minor, mostly faunistic notes (e.g., Freytag 1984; Coronado Blanco and Cancino 2002; Smith 2008) with the exception of the description of the first fossil species in the genus (Lohrmann et al. 2019).

The evidence that two non-sister clades of *Rhopalosoma* develop on *Hapithus* hosts indicates that other members of the genus may do so as well. There is a striking similarity in the overall distribution range of *Rhopalosoma* and *Hapithus* (Townes 1977; Cigliano et al. 2019), and future studies could explore the hypothesis that *Hapithus* served as the ancestral host for a larger clade within *Rhopalosoma* or even the genus as a whole.

Cypress Grove Nature Park has shown to be an excellent site for observing and collecting these unusual wasps. Future studies should focus on describing adult and larval morphologies of the rhopalosomatids of CGNP and correlating them with the two genetic clades discovered here.

Acknowledgements

This research was supported by an undergraduate research grant given to TB and JDB and by the biology department of Union University. We thank David Funk for assistance in identifying cricket specimens and Ashley Bradford (Alexandria, VA), Carl Strang (nature inquiries.wordpress.com), and Wil Hershberger/Lang Elliott (songsofinsects.com) for their permission to use the photos of the female *Rhopalosoma* (Fig. 1) and the crickets (Figs 3–4). Finally, we thank Denis J. Brothers and James P. Pitts for their valuable comments on the manuscript.

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