Molecular EcologyNotes

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Vouchering DNA-barcoded specimens: Test of a non-destructive extraction protocol for arthropods

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Running head: VOUCHERING DNA-BARCODED SPECIMENS

Abstract

The correct identification of species is essential to the performance of ecological and evolutionary research. Morphology-based keys support accurate identification of many taxa. However, for taxa that are not well studied, or for which distinguishing morphological characters have not been discerned, identification can be difficult. Accurate identification is especially problematic for very small organisms, for members of cryptic species complexes, for eggs, and for immature stages. For such cases, DNA barcodes may provide diagnostic characters. Ecologists and evolutionary biologists deposit museum vouchers to document the species studied in their research. If DNA barcodes are to be used for identification, then both the DNA and the specimen from which it was extracted should be vouchered. We describe a protocol for the non-destructive extraction of DNA from terrestrial arthropods, using as examples members of the orders Acarina, Araneae, Coleoptera, Diptera, and Hymenoptera, which were chosen to represent the ranges in size, overall sclerotization, and delicacy of key morphological characters in terrestrial arthropods. We successfully extracted sequenceable DNA from all species after 1 – 4 h of immersion in extraction buffer. The extracted carcasses, processed and imaged using protocols standard for the taxon, were distinguishable from closely related species, and adequate as morphological vouchers.

Introduction

The correct identification of species is essential to the performance of ecological and evolutionary research. Morphology-based keys support accurate identification of many taxa. However, for taxa that are not well studied, or for which distinguishing morphological characters have not been discerned, identification can be difficult. Accurate identification is especially problematic for very small organisms, for members of cryptic species complexes, for eggs, and for immature stages (Toft 1983; Cockburn 1990; Sperling& Hickey 1994; Brunner *et al.* 2002; Chen *et al.* 2002; Armstrong & Ball 2005; Ball *et al.* 2005; Greenstone *et al.* 2005; Barber & Boyce 2006; Grosjean *et al*. 2006). For such situations, species-specific fragments of DNA, known as DNA barcodes (Ball *et al.* 2005), may provide a new source of characters for species level identification.

Ecologists and evolutionary biologists should deposit museum specimens, referred to as vouchers, to document the species studied in their research (Thomas 1994). If DNA barcodes are to be used for identification, then the DNA as well as the specimen from which it was extracted should be vouchered (Hafner 1994). Many protocols for DNA extraction, especially for small specimens, require maceration of the entire specimen, precluding deposition of the carcass as a museum voucher (Whitfield & Cameron 1994). One suggested approach is to take multiple images of the specimen before maceration (De Lay *et al.* 2005). Another approach, for sufficiently large, bilaterally symmetric animals, is to remove a single appendage for DNA extraction (Starks & Peters 2002). However, if there are appendage-specific characters essential for species identification, subsequent loss of the remaining appendage of the pair, during shipment or routine examination, would render the specimen useless as a voucher. Alternatively,

the removed appendage could be curated with the rest of the specimen, but this is tedious and introduces opportunities for mix-ups.

An on-line resource, MorphBank (http://morphbank.net), links specimen images directly to GeneBank sequences. However, GenBank contains numerous errors (Harris 2003; Vilgalys 2003). For this reason as well, it is important to be able to link a DNA sequence to the morphologically identifiable specimen from which the DNA was extracted.

Besides providing new characters for hitherto poorly known groups, the study of DNA sequence data has enabled new insights into the ecology and phylogenetic relationships of well studied taxa, including the largest phylum of organisms, Arthropoda (e.g., Paskewitz & Collins 1990; Brower 1999; Gleeson *et al.* 2000; Anderson *et al.* 2000; Wells & Sperling 2001; Chen *et al.* 2002; Brunner *et al.* 2002; Jarman *et al.* 2002; Besansky *et al.* 2003; Ball *et al.* 2005; Barrett & Hebert 2005; Greenstone *et al*. 2005; Hogg & Hebert 2005; Mitchell *et al* 2005; Monoghan *et al.* 2005; Greenstone 2006; Ball & Armstrong 2006; Barber & Boyce 2006; Hajibabael *et al.* 2006; Kaila & Ståhls 2006). Here we present and evaluate a protocol for the non-destructive extraction of DNA from terrestrial arthropods, using as our subjects a variety of animals chosen to be representative of the ranges in size, overall sclerotization, and delicacy of key morphological characters in this group.

Materials and methods

Choice of taxa

On the basis of our intimate knowledge of their systematics and taxonomy, we selected five species from two arachnid and three insect orders of great ecological significance in terrestrial ecosystems.

Tenuipalpidae is a cosmopolitan mite family comprising more than 800 phytophagous species in 32 genera. Because they are small (200-300 μm), slow moving, and exhibit cryptic coloration and stationary behavior, tenuipalpids are very difficult to recognize, collect, and identify in the field (Jeppson *et al*. 1975). Having another tool to help distinguish closely related species is therefore of utmost importance. The red palm mite, *Raoiella indica* Hirst (ACARI: Tenuipalpidae), is a new invasive pest in the Americas that infests coconut, bananas, and several ornamental plants (Flechtmann & Etienne 2004). *Raoiella* contains several species that are not well known or are poorly described; *R. indica* is distinguished from its congeners by the shape of the dorsal setae.

The basilica spider, *Mecynogea lemniscata* (Walckenaer) (ARANEAE: Araneidae), belongs to a New World genus comprising about a dozen species (Levi 1980; Platnick 2006); as with spiders generally, congeners are distinguished primarily on the basis of subtle morphological differences in the genitalia.

The pink ladybug, *Coleomegilla maculata* (De Geer) (COLEOPTERA: Coccinellidae), is a common denizen of row crops in eastern North America; the genus is restricted to the New World and is most diverse in the tropics and subtropics. Although numerous names have been recognized, most species have been incorrectly treated as subspecies or varieties of *C. maculata*. The limits between *Coleomegilla* and the related genera *Naemia, Paranaemia* and *Eumegilla* are in need of clarification and the subject of a forthcoming revision (NJV, J. Obrycki, and W. Steiner, in progress). Genitalia and color patterns in this group are conservative, and often appear

very similar among related taxa. *Naemia* can generally be recognized by the fact that the elytral spots are connected longitudinally, but some specimens from the northern and southern limits of the range have disconnected spots and may be mistaken for *Coleomegilla*. These look-alikes are most easily separated by the form of the tarsal claw, simple and scythelike in *Naemia* but bearing a large quadrate tooth in *Coleomegilla*. Other structural differences used to distinguish certain species of *Coleomegilla* and allied New World taxa include the length of the legs, the shape and size of the pronotum, and the exact shape of the basal lobe and aedeagus of the male genitalia. Some *Coleomegilla* species can also be distinguished on the basis of small differences in the shape of the black maculae on the elytra or pronotum.

Delphinia picta (Fabricius) (DIPTERA: Ulidiidae) is a ubiquitous saprophage in eastern North America with larvae that feed primarily on decaying vegetation. Ulidiids are closely related to fruit flies (Tephritidae), a group that includes numerous agricultural pests, and the key characters for distinguishing species within both families are similar. These include wing patterns, body color patterns, number and positions of setae (chaetotaxy), microtrichia patterns, and genitalic morphology.

Eurytoma rhois Crosby (HYMENOPTERA: Eurytomidae) belongs to the most commonly collected genus of the family Eurytomidae (Hymeoptera), a cosmopolitan family of phytophagous and entomophagous parasitic wasps. The key diagnostic features for *E. rhois* concern relative sizes of sclerites and the propodeal surface sculpture

Collection of arthropods

A variety of methods, some of them taxon-specific, are used to collect and preserve terrestrial arthropods (U.S. Department of Agriculture 1986; Aguiar and Sharkov 1997; Noyes 1998;

Triplehorn & Johnson 2004). Because all specimens were to be extracted in an aqueous buffer, all were collected into EtOH, regardless of customary methods of preservation for the taxon; we chose an EtOH concentration of 80% as sufficiently high to preserve DNA but not so high as to cause desiccation that would unduly distort the animal's external morphology.

The methods and localities of collection for all taxa are given in Table 1.

DNA extraction protocol

Each specimen was removed from the 80% EtOH, allowed to air dry, and placed in a 1.5 ml microfuge tube with forceps that had been soaked in 0.5 % NaCIO. A minimum 100 µl of a GuSCN-based extraction buffer (Rohland *et al.* 2004) was added to the tube, which was then placed in a 60 ^{0}C water bath for 1, 2, or 4 h. The extraction buffer was removed to a clean tube and the DNA was precipitated by addition of an equal volume of isopropanol. The sample was incubated over night at -20 ^{0}C and centrifuged for 20 min at 13,000 x g and 4 ^{0}C. After a single rinse in 70% EtOH, the DNA was vacuum-dried, resuspended in an equal volume of 0.1X TE pH 8.0, and stored at -20 ^{0}C. Four individuals of each taxon were subjected to each extraction interval, and two were used as controls that went through all procedures except extraction. Two DNA samples of each species, extracted by conventional means from whole-body homogenates (Greenstone *et al.* 2005), were provided for reference.

The extracted arthropod carcasses and unextracted controls were again immersed in 80% EtOH and stored at 4 ^{0}C until prepared for imaging.

PCR and sequencing

PCR conditions and components were as described in Greenstone *et al.* (2005), with 5-6 µl of the DNA extract used in the reaction. DNA of all species was amplified with primers C1-J-1751and C1-N-2191, and C1-J-2195-C1-N-2568 (Simon *et al.* 1994), with expected amplicon sizes of 488 and 421 bp, respectively. *Mecynogea* DNA was additionally amplified with cytochrome oxidase I primer pairs C1-J-1751 "SPID" (Hedin & Maddison 2001) and C1-N-2776 (Simon *et al.* 1994), with an expected amplicon size of 1070 bp, to ensure complete coverage of the sequence.

Amplified DNA was visualized by electrophoresis of 6 µl of the PCR/Stop reaction (12 µl for *Raioella* and *Eurytoma* because of their very small size) in 1.5 % agarose. The remainder of the reaction mixture was loaded, electrophoresed in 1.5% NuSieve agarose (Cambrex Bio Science Rockland Inc., Rockland, Maine, USA) in 1x TAE modified to have a final EDTA concentration of 0.1 mM, and the fragments excised for sequencing by BigDye terminator v3.1 kits on an ABI 3100 sequencer (Applied Biosystems, Foster City, California, USA). Editing was performed with Lasergene (DNAStar, Madison, Wisconsin, USA).

Deposition of morphological vouchers, DNA, and DNA sequences

Extracted arthropod carcasses were deposited as morphological vouchers, prepared according to standard museum practices, in the Insect and Mite National Collection of the Smithsonian Institution, National Museum of Natural History (NMNH) in Washington, D.C.; the companion DNA samples were deposited in the NMNH Tissue Collection. DNA sequences were deposited in GenBank (Accession Nos. EF185147-EF185157, and EF192134).

Imaging of extracted carcasses

Arthropod carcasses were processed and imaged after DNA extraction using protocols standard for the taxon. Carcasses of *R. indica* were slide-mounted and viewed by Nomarski interference. Specimens of *M. lemniscata* were photographed in EtOH under a dissecting microscope. Carcasses of *C. maculata* were removed from the EtOH and point-mounted; scanning electron microscope (SEM) images of the tarsi were made from coated specimens mounted on stubs. *Delphinia picta* carcasses were removed from EtOH and dried for 48 h in ethyl acetate, then air-dried or critical-point-dried, mounted on pins, and imaged by light microscopy. Wings of several specimens were removed and slide mounted in Euparal. Carcasses of *E. rhois* were removed from the EtOH and dehydrated using HMDS (Heraty & Hawks 1998); dried specimens were carefully disarticulated prior to imaging via SEM.

For SEM imaging, specimens were affixed to 12.7 x 3.2 mm Leica/Cambridge aluminum SEM stubs with carbon adhesive tabs (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA; #77825-12). Stub-mounted specimens were sputter-coated using a Cressington Scientific 108 Auto with a gold-palladium mixture from at least three different angles to ensure complete coverage (~20-30nm coating). SEM images were taken with an Amray 1810 with LaB_6 source (Amray, Inc., Bedford, Massachusettes, USA).

Results and discussion

The effects of our extraction protocol on the resultant morphological vouchers ranged from slight discoloration to slight-to-significant distortion of surface features that did not prevent identification to species.

The diagnostic shapes of the dorsal setae and other characteristics of *R. indica* were readily observable in the extracted specimens (Fig. 1). Spider genitalia are in most cases highly sclerotized and thus resistant to most chemical treatments: our DNA extraction protocol had no visible effect on the morphology of the female genitalia of *M. lemniscata*, (Fig. 2), but did leave a flocculent precipitate on most of the specimens (Fig. 2A). This precipitate could be removed manually, but might pose a problem for some kinds of morphological research, such as examination of spinneret spigots (which typically are not species-specific).

In *C. maculata*, the cuticle became more translucent, lost the saturated red or pink tones, and tended to brown after extraction (Figs. 3A and 3B). However, the black pigment that forms the dorsal maculae seems to be quite stable, so that the color pattern could be easily assessed even in the specimens that underwent 4 h of extraction (not shown), and distinguished from those of *N. seriata* (Figs. 3B and 3C). After DNA-extraction the claw of *C. maculata* was sometimes more flaccid, but still clearly exhibited the diagnostic large quadrate tooth (Figs. 3D and 3E). Generally the pronotal shape was not altered by extraction, with the exception of one of the 4-h specimens where the disk buckled (not shown). The thick portions of the legs retained their shape well, but the narrow last tarsal segment sometimes became droopy. Problems with structural integrity could be minimized by placing the specimen on its back to prevent the abdomen from folding down, and carefully positioning key structures and providing temporary support until dry. Limiting extraction time to 1 h decreased the severity of this effect.

There was no visible effect of the extraction on color pattern or cuticular structures of *D. picta* (Fig. 4); genitalic morphology was also not affected (not shown). Specimens that were dried after extraction using critical point drying or after transfer from alcohol to ethyl acetate shriveled to varying degrees (Figs. 4A and 4B), somewhat less in the critical-point-dried material. In some cases this limited the study of chaetotaxy and color and microtrichial patterns of various parts of

the body, particularly the thorax. Extracted specimens often were unevenly covered with a powdery whitish precipitate that sometimes partially obscured surface features. This sometimes made it difficult to observe microtrichia patterns or other surface characters. The wings of the *D. picta* specimens were virtually indistinguishable regardless of treatment (Figs. 4D – 4F).

E. rhois exhibited setae that were slightly lighter in color than the control after dehydration but prior to SEM. The key diagnostic features for *E. rhois,* relative sizes of sclerites and propodeal surface sculpture, were little affected by the DNA extraction process. The extracted specimen pictured, a male, had matted antennal setation (Fig. 4B). Setation on extracted material generally appeared to be abraded more easily (compare controls versus extracted mesosoma and head, Figs. 4C and 4D, 4G and 4H). We do not know whether these setation artifacts were a direct result of the extraction or might be due to the transferral of extraction buffer into and out of the extraction tube.

Gels containing the amplified 421 and 488 bp PCR products from all specimens used in the study are shown in Fig. 6 (the 1070 bp *M. lemniscata* amplicons, used for sequencing, are not shown but gave similar results). We were able to amplify DNA from all species, with the success of amplification roughly proportional to the size of the specimen and the length of time of extraction. Thus almost all *D. picta* and *C. maculata* specimens produced PCR products regardless of extraction time, *M. lemniscata* specimens were most effectively extracted after 2-4 h extraction, and both *R. indica* and *E. rhois* tended to require the full 4 h for effective extraction.

Overall, our extraction protocol yielded DNA suitable for sequencing, with effects on the extracted carcass ranging from slight discoloration to slight-to-significant distortion of surface features that did not prevent identification to species and effective presentation as morphological vouchers. The flocculent precipitate noted on the *Mecynogaea* and *Delphinia* specimens might

be removed by more assiduous rinsing of the specimens following extraction. Other so-called non-destructive DNA extraction protocols have required breaches of the cuticle ranging from pin-pricks (Phillips & Simon 1995; Favret 2005) to amputation (Stark & Peters 2002) and even decapitation (Johnson *et al.* 2001) or more extensive disarticulation, slicing, and injection (Knölke *et al.* 2005; Barr & McPheron 2006). Though these protocols may provide useable vouchers, they are more tedious and labor-intensive than our procedure, which requires only soaking the specimen in buffer. They also create opportunities for cross-contamination via the piercing or cutting instrument and, if structures that have been separated for extraction are rejoined in the voucher specimen, for creation of chimaeric morphological vouchers.

Ongoing research on different preservation methods (e.g., Paabo *et al.* 2004; Mulligan 2005) may lead to protocols that cause less distortion, leaving more photogenic morphological vouchers following DNA extraction. In the mean time, use of our protocol to extract a series of specimens from 1-4 h should provide a number of individuals for which sequenceable DNA can be matched to a presentable morphological voucher. Optimized protocols would make joint vouchering of morphological specimens and the DNA extracted from them a routine part of DNA barcoding.

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Author identification box

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441 taxonomy of the exemplary taxa: Acari (Ronald Ochoa); Araneae (Jonathan Coddington);

442 Coleoptera (Natalia Vandenberg); Diptera (Allen Norrbom); and Hymenoptera (Michael Gates).

Figure captions

Fig. 1. *Raioella indica* morphological vouchers. A, extracted for 4 h, dorsal view. B, same specimen, ventral view. C, unextracted control.

Fig. 2. *Mecynogea lemniscata* morphological vouchers. A, epigynum extracted 4 h. B, unextracted control epigynum.

Fig. 3. *Coleomegilla maculata* morphological vouchers and related taxa. Top, pinned and live specimens; bottom, scanning electron micrographs of anterior tarsomere showing shape of claw. A, pinned unextracted *C. maculata* control. B, pinned *C. maculata* extracted for 1 h. C, group of three live beetles: *Naemia seriata* from Maryland (bottom); *C. maculata* from Maryland (midle right); *Naemia* sp. from Nova Scotia (top). D, unextracted *C. maculata* control. E, *C. maculata* extracted for 1 h; arrow = quadrate tooth. F, unextracted *N. seriata* from Maryland.

Fig. 4. *Delphinia picta* morphological vouchers. A, extracted for 2 h and critical-point-dried. B, extracted for 2 h and ethyl acetate-dried. C, unextracted control. D, wing extracted for 1h. E, wing extracted for 4 h. F, wing of unextracted control. E and F were air-dried after dehydration in ethyl acetate.

Fig. 5. *Eurytoma rhois* morphological voucher. Unextracted control, left; extracted for 4 h, right. A and B, antenna; C and D, mesosoma; E and F, forewing; G and H, head.

Fig. 6. 421 bp (A and B) and 488 bp (C and D) gels. Lanes 1, 16, 31, and 46, 100 bp ladder. Lane 46, no-DNA control. For A and C, lanes 2-15, *Raioella indica*; lanes 17-30, *Mecynogea*

lemniscata; lanes 32-45, *Coleomegilla maculata.* For B and D, lanes 2-15, *Delphinia picta*; lanes 17-30, *Eurytoma rhois.* For each species, the first two-lanes are conventionally-extracted DNA, the next 4 wells are 1-h extractions, the following 4 wells are 2-h extractions, and the last 4 wells are 4-h extractions.

472 **Table 1.** Collecting localities for animals used in the study. *Delphinia picta* were collected in
473 fruit fly traps and *Eurytoma rhois* by sweeping; all other species were collected by hand.
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Taxon	**Locality and Habitat**
Raoiella indica Hirst (ACARI: Tenuipalpidae)	Saint Lucia, West Indies On coconut palm (*Cocos nucifera*)
Mecynogea lemniscata (Walckenaer) ARANEAE: Araneidae	Maryland, Howard CO, Ellicott City Understory of deciduous forest remnant
Delphinia picta (Fabricius) DIPTERA: Ulidiidae	Georgia, Fulton CO, Atlanta
Coleomegilla maculata (De Geer) COLEOPTERA: Coccinellidae	Maryland, Prince Georges CO, Beltsville Laboratory colony, originally from potato fields
Naemia seriata (Melsheimer) COLEOPTERA: Coccinellidae	Granville Beach, Nova Scotia, Canada On *Spartina* sp. Maryland, Talbot Georges CO, Wittman On *Spartina* sp.
Eurytoma rhois Crosby HYMENOPTERA: Eurytomidae	Rhode Island, Kent CO, Warwick Powerline right-of-way at junction of Route 117 and Toll Gate Road On blooming *Rhus copallina*

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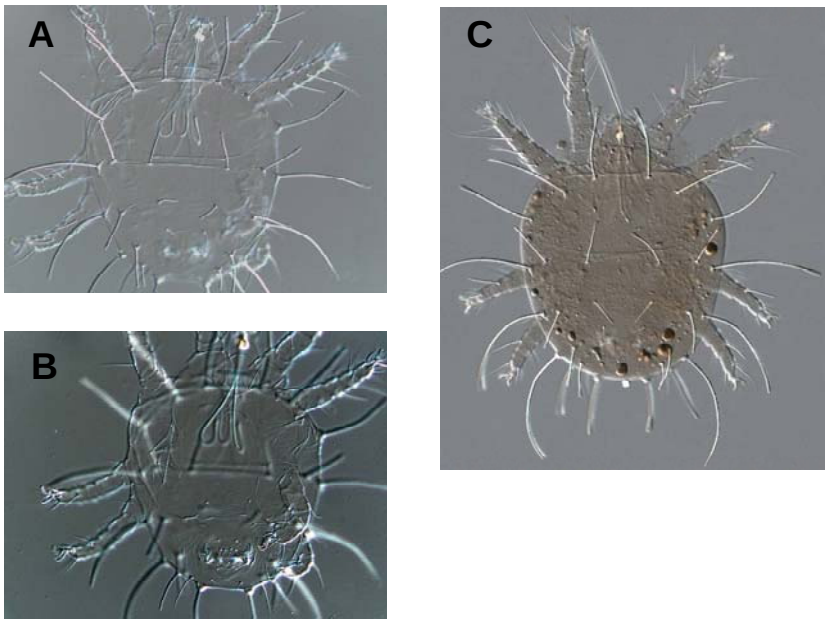

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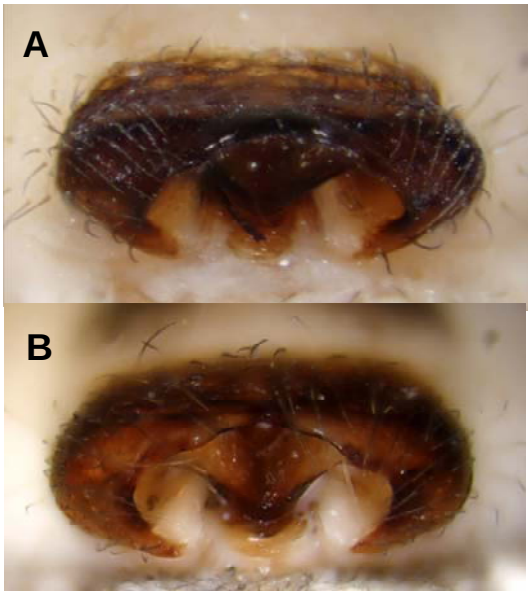

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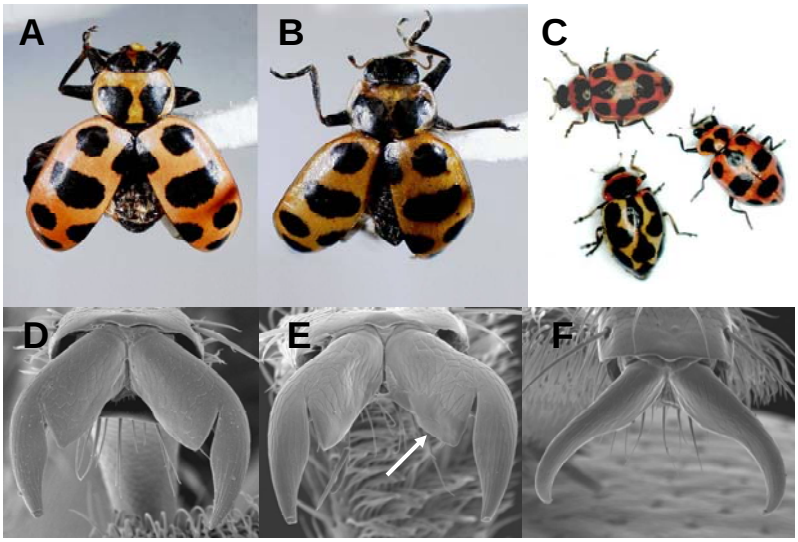

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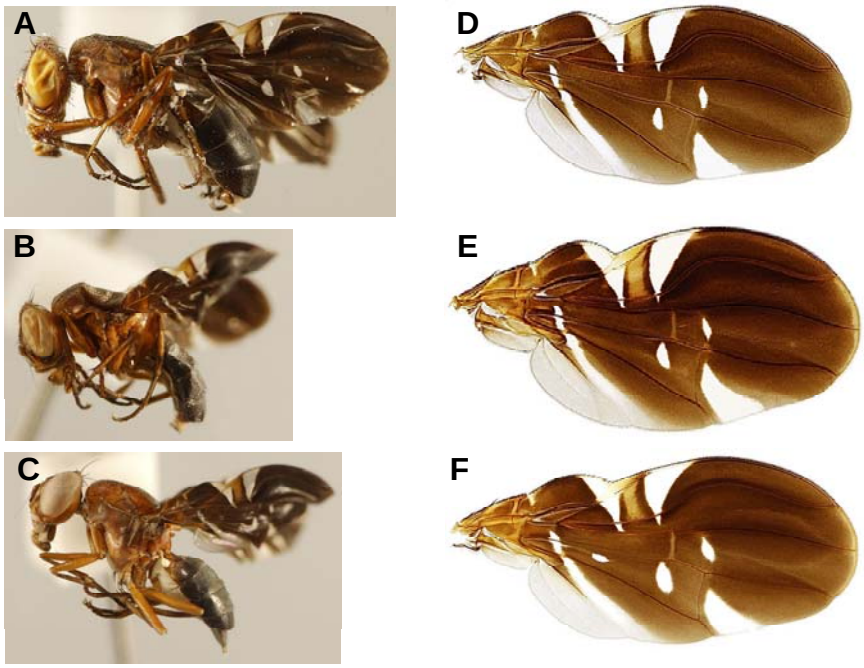

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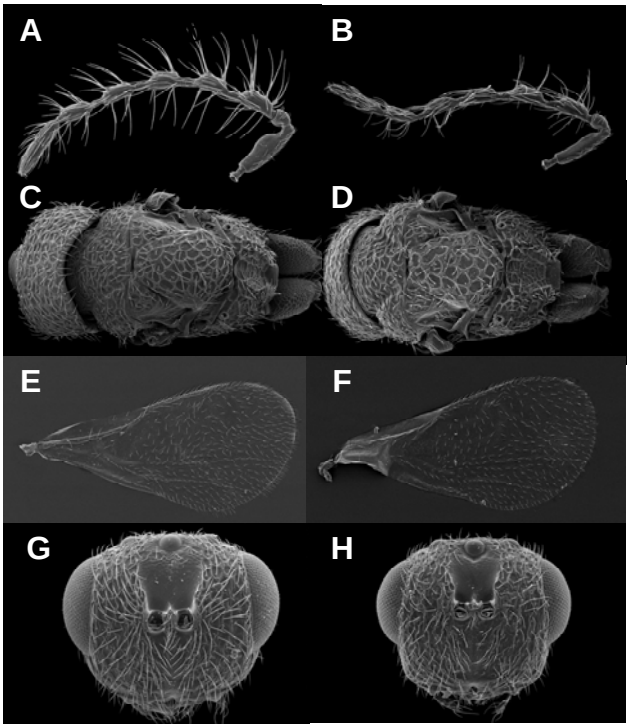

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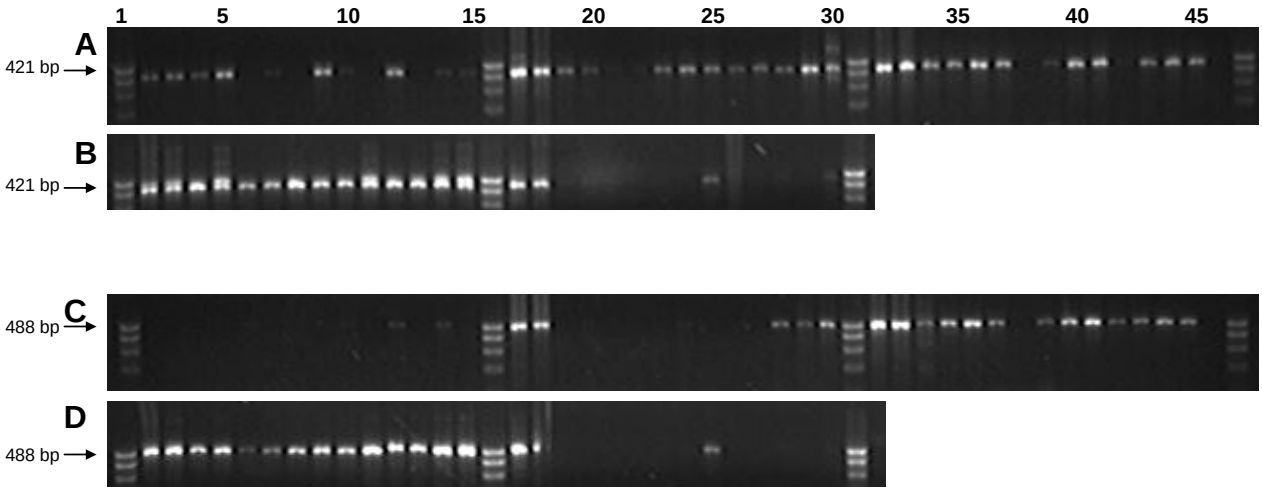

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