BARCODING ARTICLE

Vouchering DNA-barcoded specimens: test of a nondestructive extraction protocol for terrestrial arthropods

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Abstract

Morphology-based keys support accurate identification of many taxa. However, identification can be difficult for taxa that are either not well studied, very small, members of cryptic species complexes, or represented by 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 nondestructive extraction of DNA from terrestrial arthropods, using as examples members of the orders Acarina, Araneae, Coleoptera, Diptera, and Hymenoptera chosen to represent the ranges in size, overall sclerotization, and delicacy of key morphological characters in the group. 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. We provide links from the carcasses and DNA vouchers to image (MorphBank) and sequence (GenBank) databases.

Keywords: Acarina, Arachnida, barcode, cytochrome oxidase I, Insecta, mitochondrial DNA, voucher

Received 6 January 2007; revision accepted 11 June 2007

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, and for eggs and other 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 (Hebert *et al.* 2002), may provide a new source of characters for species-level identification.

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; Anderson *et al.* 2000; Gleeson *et al.* 2000; Wells & Sperling 2001; Brunner *et al.* 2002; Chen *et al.* 2002, 2006; Jarman *et al.* 2002; Besansky *et al.* 2003; Ball *et al.* 2005; Barrett & Hebert 2005; Greenstone *et al.* 2005, 2007; Hogg & Hebert 2005; Mitchell *et al.* 2005; Monoghan *et al.* 2005; Ball & Armstrong 2006; Barber & Boyce 2006; Greenstone 2006; Hajibabael *et al.* 2006; Kaila & Ståhls 2006). An important

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part of this enterprise is the deposition of sequences into publicly accessible sequence databases, the most comprehensive of which is GenBank (Benson *et al.* 2007). However, GenBank contains numerous errors, some of which are due to misidentification of the species whose DNA has been sequenced (Harris 2003; Vigalys 2003). Ecologists and evolutionary biologists routinely 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). This would make it possible to determine whether the organism from which the DNA was extracted had been correctly identified in the first place.

Unfortunately, many protocols for DNA extraction, especially for small specimens, require crushing of the entire sample, precluding deposition of the carcass as a museum voucher (Whitfield & Cameron 1994). One suggested approach is to take multiple images of the specimen before crushing (De Ley *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 rest of the specimen useless as a voucher. Alternatively, the removed appendage, if essentially intact, could be curated with the rest of the specimen, but this is tedious and introduces opportunities for mix-ups.

Here we present and evaluate a protocol for the nondestructive extraction of mitochondrial 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 diverse group. 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.

Materials and methods

Taxa

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 colouration and stationary behaviour, 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 coconuts, bananas, and several ornamental plants

(Flechtmann & Etienne 2004). *Raoiella* contains several species that are not well known or are poorly described. The genus is defined by the shape of the dorsal setae; *R. indica* is distinguished from its congeners by the *size* 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. The limits between *Coleomegilla* and the related genera *Naemia*, *Paranaemia* and *Eumegilla* are in need of clarification and the subject of a forthcoming revision (N.J.V., J. Obrycki, J.A. Giorgi, and W. Steiner, in progress). Genitalia and colour 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* (see Fig. 3C). These look-alikes are most easily separated by the form of the tarsal claw, simple and scythe-like in *Naemia* (Fig. 3F) but bearing a large quadrate tooth in *Coleomegilla* (Fig. 3D, E).

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 colour 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 (Hymenoptera), 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 (US Department of Agriculture 1986; Aguiar & 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 selected 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 collectors, localities, and geopositioning coordinates for all specimens 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% NaClO. Depending upon the size of the specimen, 100, 200, or 400 µL of a GuSCN-based extraction buffer, originally developed to extract DNA from mammalian teeth and bones (Rohland *et al*. 2004), was added to the tube, which was then placed in a 60 °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 a volume of isopropanol equal to the volume of the extraction buffer in which the specimen had been incubated. The sample was stored overnight at –20 °C and centrifuged at 4 °C for 20 min at 13 000 *g*. After a single rinse in 70% EtOH, the DNA was vacuum-dried, resuspended in a volume of 0.1× TE pH 8.0 equal to that of the extraction buffer, and stored at –20 °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 additional DNA samples of each species, extracted by conventional means from whole-body homogenates (Greenstone *et al*. 2005), were provided for reference.

Following extraction, the extracted arthropod carcasses and unextracted controls were again immersed in 80% EtOH and stored at 4 °C until prepared for imaging.

PCR and sequencing

Polymerase chain reaction (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 cytochrome oxidase I (COI) primers C1-J-1751and C1-N-2191, as well as C1-J-2195 and C1-N-2568 (Simon *et al*. 1994), with expected amplicon sizes of 488 and 421 bp, respectively. *Mecynogea* DNA was additionally amplified with COI 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. The *Raoiella* samples required a preliminary round of amplification utilizing a GenomiPhi kit (GE Healthcare) in order to obtain sufficient amplification product for sequencing.

Amplified DNA was visualized by electrophoresis of 6 µL of the PCR/stop reaction (12 µL for *Raioella* and *Eurytoma* because 6 µL did not image well) in 1.5% agarose. The remainder of the reaction mixture was loaded, electro-phoresed in 1.5% NuSieve agarose (Cambrex Bio Science) in 1× TAE modified to have a final EDTA concentration of 0.1 mM, and the fragments excised for sequencing by BigDye terminator version 3.1 kits on an ABI 3100 sequencer (Applied Biosystems). Editing was performed with Lasergene (DNAStar).

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. Carcasses 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. *D. 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 micro-scopy. 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 × 3.2 mm Leica/Cambridge aluminium SEM stubs with carbon adhesive tabs (Electron Microscopy Sciences no. 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 (~20–30 nm coating). SEM images were taken with an Amray 1810 with LaB_6 source (Amray).

Deposition of morphological vouchers, specimen images, DNA vouchers, 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 (NMNHENTM) in Washington, D.C.; the companion DNA samples were deposited in the NMNHENTM Tissue Collection. Accession numbers for both carcasses and DNA are provided in Table 1. DNA sequences were deposited in GenBank and images in MorphBank; their accession numbers are also given in Table 1.

Results and discussion

When the GuSCN-based buffer we used was employed to extract DNA from vertebrate teeth and bones, it did not affect the appearance of the specimens, other than to cause them to 'appear a little cleaner' (Rohland *et al*. 2004). Its

Table 1 Data for animals used in the study. *Delphinia picta* were collected in fruit fly traps and *Eurytoma rhois* by sweeping; all other species were collected by hand. All localities are in the USA unless noted otherwise

| Taxon	DNA extraction treatment	MorphBank no.	NMNH-ENTM no. Carcass	NMNH-ENTM no. DNA	GenBank no.	Locality, habitat, collection dates, collectors, and GPS coordinates
Raoiella indica Hirst (ACARI: Tenuipalpidae)	4-h GuSCN	137781, 137782	00530938	00530920	EF185151	Saint Lucia, West Indies, Castries
	Conventional			00530918	EF185151	On coconut palm (*Cocos nucifera*)
	Conventional			00530919	EF185151	10 September 2005; Coll.: R. Ochoa &
	None	137780	00530939			G. Mathurian 13.900000; 60.833333
Mecynogea lemniscata (Walckenaer) Araneae: Araneidae	2-h GuSCN		00447378	00530922	EF185153	Maryland, Howard CO, Ellicott City
	2-h GuSCN		00447379	00530923	EF577426	Understorey of deciduous forest remnant
	4-h GuSCN	137705	00447380	00530924	DQ029239	22 July 2006; Coll.: A. Greenstone &
	4-h GuSCN		00447381	00530925	EF185152	M. Greenstone
	Conventional			00530910	DQ029238	39.230236; 76.823228
	Conventional			00530911	DQ029239	
	None	137703	00447382			
Delphinia picta (Fabricius) Diptera: Ulidiidae	2-h GuSCN	137793	00529373	00530921	EF185150	Georgia, Dekalb CO, Atlanta,
	1-h GuSCN	137788	00530935	00530940	EF185150	3310 Buford Highway
	4-h GuSCN	137789	00530936	00530941	EF185150	May 2005; Coll.: J. Orr
	None	137791	00530937			33.834931; 84.318761
	Conventional			00530912	EF185150	
	Conventional			00530913	EF185150	
	NONE	137795	00529374			
Coleomegilla maculata (De Geer) Coleoptera: Coccinellidae	1-h GuSCN		00529370	00530926	EF192134	Maryland, Prince Georges CO, Beltsville
	1-h GuSCN		00529371	00530927	EF192134	Laboratory colony, originally from potato fields
	1-h GuSCN		00529376	00530928	EF192134	20 June 2003; Coll.: M. Greenstone
	2-h GuSCN	137837	00529372	00530929	EF192134	39.033333; 76.927500
	1-h GuSCN	137868	00530976			
	None	137869	00529369			
	None	137834	00530968			
	Conventional			00530934	EF192134	Maryland, Prince Georges CO, Beltsville
	Conventional			00530914	EF192134	Potato fields
						3 July 2005; Coll.: D. Rowley & M. Greenstone
						39.033333; 76.927500
Naemia sp. Coleoptera: Coccinellidae	None	137827	00529379			Nova Scotia, Canada, Granville Beach On *Spartina* sp. 6 August 2006; Coll.: C.Majka & B. Maybank. 44.752031; 65.520858
Naemia seriata (Melsheimer) Coleoptera: Coccinellidae	None	137860	00529380			Maryland, Talbot Georges CO, Wittman On *Spartina* sp. 24 July 2005; Coll.: W. E. Steiner 38.800000; 76.283333
Eurytoma rhois Crosby Hymenoptera: Eurytomidae	2-h GuSCN	135296–135299	00529377	00530930	EF185155	Rhode Island, Kent CO, Warwick
	4-h GuSCN			00530931	EF185156	Powerline right-of-way at junction
	4-h GuSCN			00530932	EF185157	of Route 117 and Toll Gate Road
	4-h GuSCN			00530933	EF185157	On blooming *Rhus copallina*
	Conventional			00530916	EF185154	25 July, 2006; Coll.: C. Gates
	Conventional			00530917	EF577427	41.701919; 71.487636
	None	135292–135295	00529378			
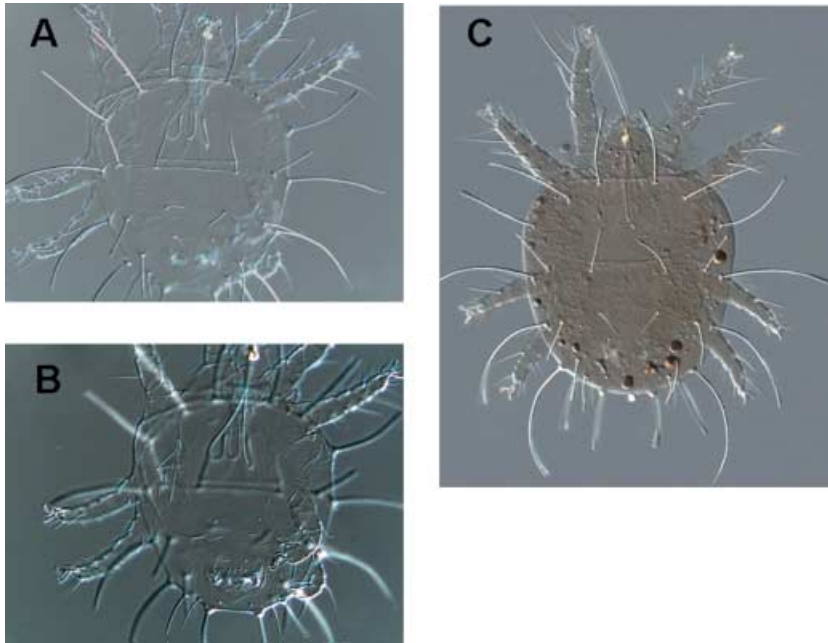
Fig. 1 *Raioella indica* morphological vouchers: (A) extracted for 4 h, dorsal view; (B) same specimen, ventral view; (C) unextracted control.

effects on the terrestrial arthropods studied here ranged from slight discoloration to slight-to-moderate distortion of surface features.

The diagnostic shapes of the dorsal setae and other characteristics of *Raoiella 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 *Mecynogea 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 *Coleomegilla maculata*, the cuticle became more translucent, lost the saturated red or pink tones, and tended to brown after extraction (Fig. 3A, B). However, the black pigment that forms the dorsal maculae seems to be quite stable, so that the colour pattern could be easily assessed even in the specimens that underwent 4 h of extraction (not shown), and distinguished from those of *Naemia* sp. (Fig. 3B, C). After DNA extraction, the claw of *C. maculata* was sometimes more flaccid, but still clearly exhibited the diagnostic large quadrate tooth (Fig. 3D, E). 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 tem-
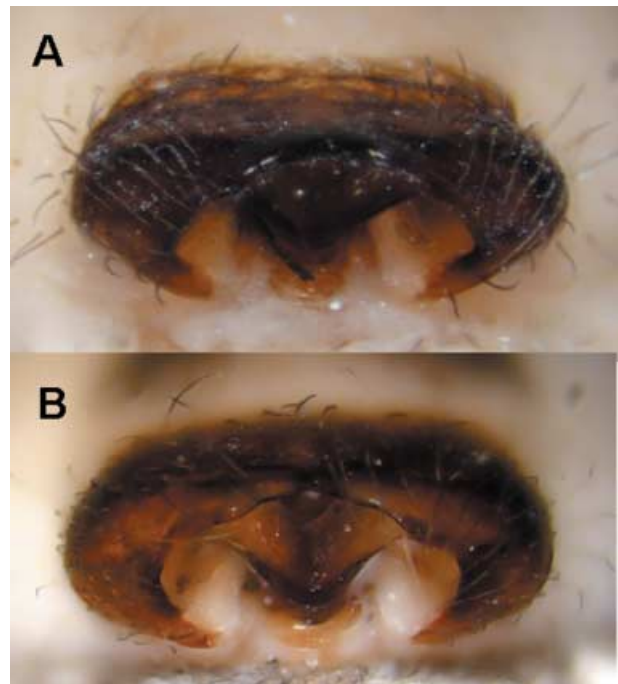

Fig. 2 *Mecynogea lemniscata* morphological vouchers: (A) epigynum extracted 4 h; (B) unextracted control epigynum.

porary support until dry. Limiting extraction time to 1 h decreased the severity of this effect.

There was no visible effect of the extraction on colour pattern or cuticular structures of *Delphinia picta* (Fig. 4); genitalic morphology was also not affected (not shown). Specimens that were dried after extraction using critical
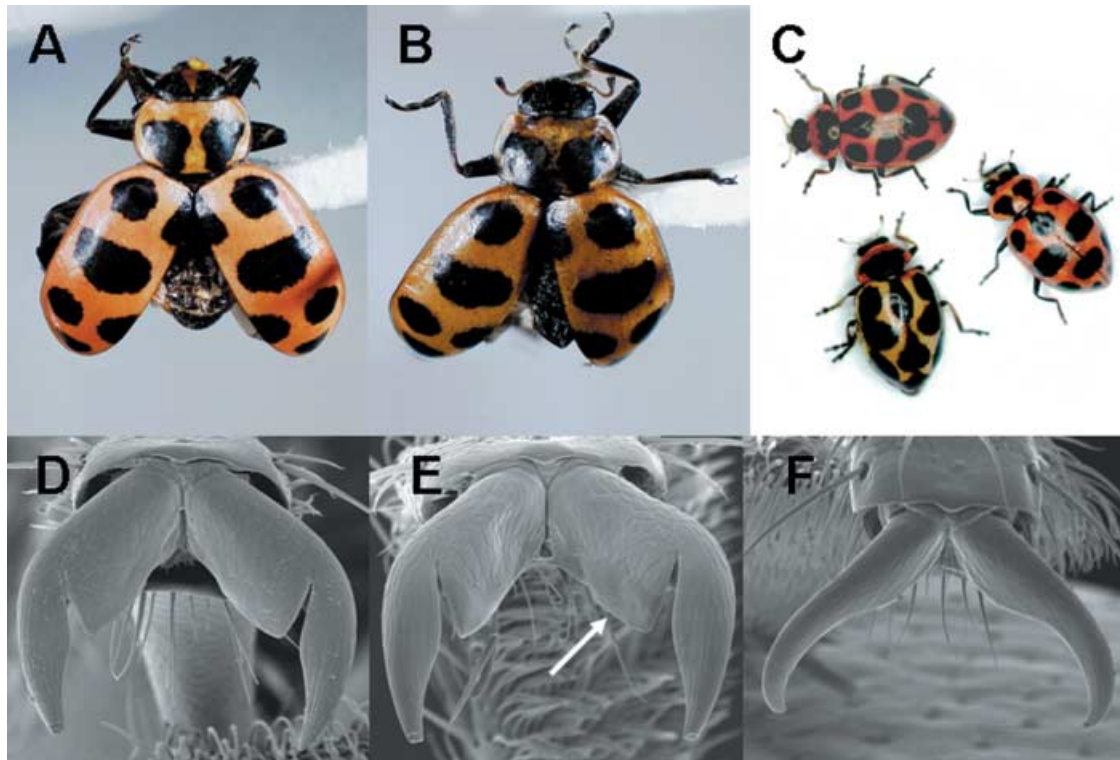
Fig. 3 *Coleomegilla maculata* morphological vouchers and related taxa. Top, pinned and live specimens; bottom, scanning electron micrographs of anterior tarsomere showing shape of claw. (A) pointed unextracted *C. maculata* control; (B) pointed *C. maculata* extracted for 2 h; (C) group of three live beetles: *Naemia seriata* from Maryland (bottom), *C. maculata* from Maryland (middle 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.

point drying or after transfer from alcohol to ethyl acetate shrivelled to varying degrees (Fig. 4A, B), somewhat less in the critical-point-dried material. In some cases, this limited the study of chaetotaxy and colour 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 (Fig. 4D–F).

Eurytoma rhois exhibited setae that were slightly lighter in colour 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. 5B). Setation on extracted material generally appeared to be abraded more easily (compare controls vs. extracted mesosoma and head, Fig. 5C, D, G, H). We do not know whether these setation artefacts 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). 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, while *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-moderate distortion of surface features that did not prevent identification as to species and effective presentation as morphological vouchers. The flocculent precipitate noted on the *Mecynogea* and *Delphinia* specimens might be removed by more assiduous rinsing of the specimens following extraction. Other so-called nondestructive 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). Although these
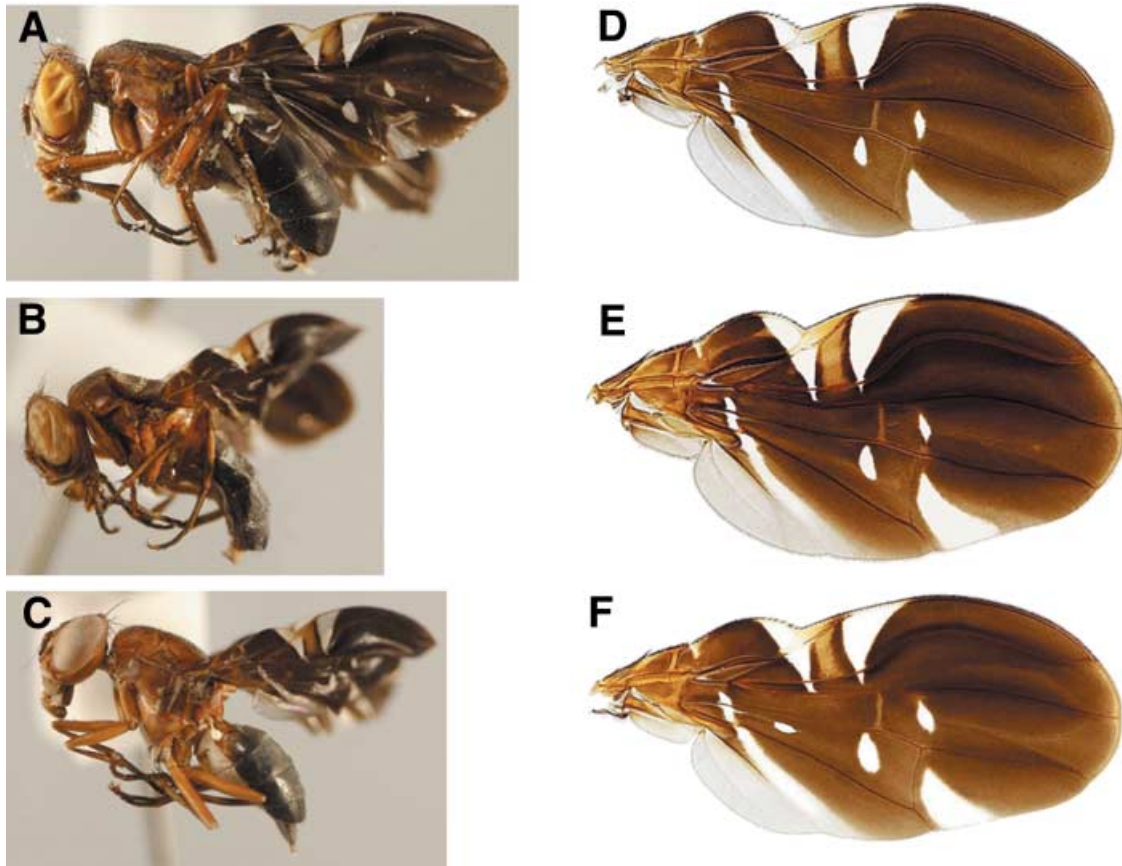
Fig. 4 *Delphinia picta* morphological vouchers: (A) extracted for 2 h and critical-point-dried; (B) extracted for 2 h and ethyl acetate-dried; (C) unextracted control; (D) wing extracted for 1 h; (E) wing extracted for 4 h; (F) wing of unextracted control; (E) and (F) were air-dried after dehydration in ethyl acetate.

protocols may provide useable vouchers, they are more tedious and labour-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.

Cytochrome oxidase I is an effective barcoding sequence for arachnids and insects (Barrett & Hebert 2005; Greenstone *et al*. 2005). However, other genomic regions, including nuclear as well as mitochondrial sequences, have also proven useful for separating closely related arthropod taxa (Besansky *et al*. 1997; Chen *et al*. 2000, 2006; Gleeson *et al*. 2000; Bull *et al*. 2006; Greenstone 2006) and may therefore be used, by definition, as barcodes. Although we examined only mitochondrial sequences, there is no a priori reason not to expect our DNA extraction technique to be effective in extracting nuclear sequences as well.

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 meantime, use of our protocol to extract a series of specimens from 1 to 4 h should provide a number of individuals for which sequenceable DNA can be linked 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. This, coupled with hot-linked databases like MorphBank and GenBank, will improve the reliability and utility of barcoding by making it possible to view all pertinent attributes of a specimen — morphology, taxonomy, DNA sequence, voucher identity, and collection data — available simultaneously.
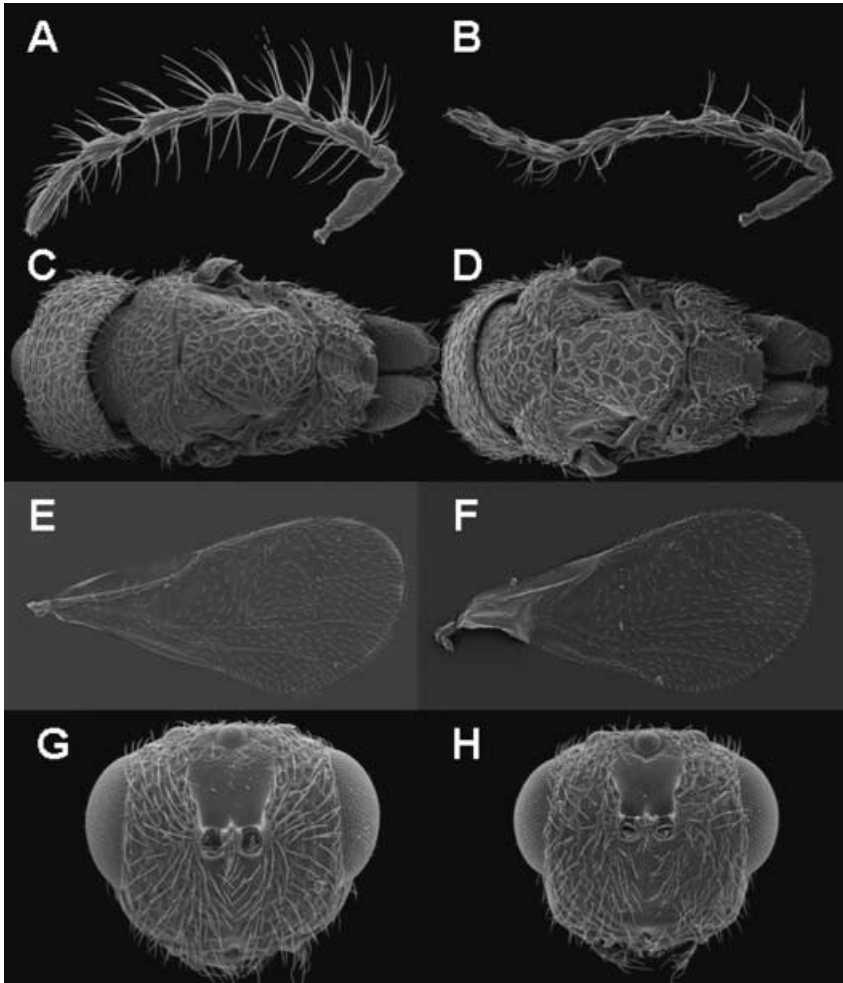
Fig. 5 *Eurytoma rhois* morphological voucher. Unextracted control, left; extracted for 4 h, right. A and B, antenna; C and D, mesosoma; E and F, forewing; G and H, head.
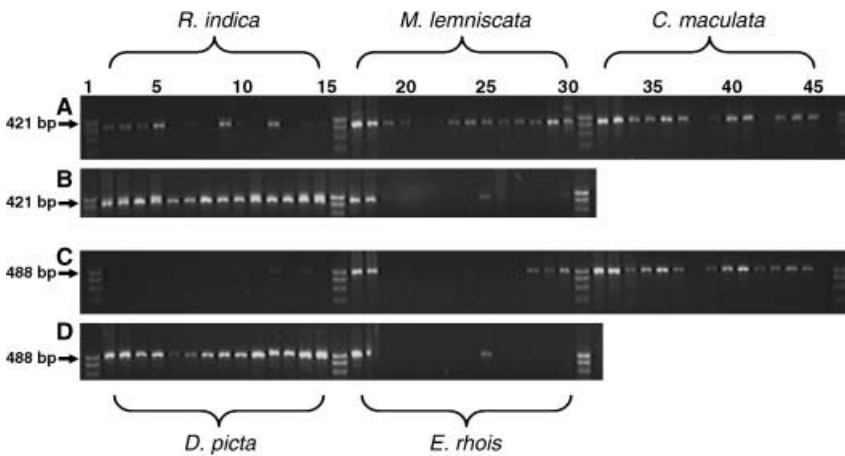

Fig. 6 421 bp (A and B) and 488 bp (C and D) gels. Lanes 1, 16, 31, and 47, 100 bp ladder. Lane 46, no-DNA control. For A and C, lanes 2–15, *Raioella indica*; lanes 17–30, *Mecynogea lemniscata*; lanes 32–45, *Coleomegilla maculata*. For (B and D), lanes 2–15, *Delphinia picta*; lanes 17–30, *Eurytoma rhois*. For each species, the first two lanes are conventionally extracted DNA, the next four wells are 1-h extractions, the following four wells are 2-h extractions, and the last four wells are 4-h extractions.

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