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Taxon-specific multiplex-PCR for quick, easy, and accurate identification of encyrtid and aphelinid parasitoid species attacking soft scale insects in California citrus groves

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Abstract Citricola scale, *Coccus pseudomagnoliarum* Kuwana (Hemiptera: Coccidae), is a serious pest of citrus in California's San Joaquin Valley, but not in southern California where a complex of *Metaphycus* spp. Mercet (Hymenoptera: Encyrtidae) suppress it. This has created interest in using these (and other *Metaphycus*) species for biological control in the San Joaquin Valley. A critical step in assessing an organism's potential for biological control is the ability to accurately identify it. For *Metaphycus* spp., this currently requires slide mounted adult specimens and expert taxonomic knowledge. We present a simple, quick and accurate method to identify any life stage of the ten major parasitoids of soft scales in California citrus, based on amplification of ribosomal DNA, using the polymerase chain reaction (PCR). Three multiplex-PCR protocols amplify products of taxon-specific sizes, allowing direct diagnosis of taxa accommodated by the PCR, and reducing identification time to a fraction of that of existing methods.

Keywords Hymenoptera · *Metaphycus* · *Coccophagus* · *Microterys* · Citricola scale · rDNA

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Introduction

In recent years, citricola scale, *Coccus pseudomagnoliarum* Kuwana (Hemiptera: Coccidae), has re-emerged as a serious pest of citrus in central California's San Joaquin Valley. Having once been a major pest of California citrus (Quayle 1938), citricola scale was incidentally reduced to a minor pest following the advent of broad-spectrum insecticide use, in the late 1940s, for the control of armored scales (Kennet 1988). However, the re-emergence of citricola scale as a pest in the San Joaquin Valley coincided with the evolution of resistance by the economically important armored scales *Aonidiella aurantii* Maskell and *A. citrina* Coquillet to broad-spectrum organophosphate and carbamate insecticides (Grafton-Cardwell 1994), and a subsequent shift to biological control methods to suppress armored scale (Luck et al. 1997; UCIPM 2009a). In contrast, citricola scale remains extremely rare in southern California citrus groves (Morse et al. 2006; Kapranas et al. 2007; UCIPM 2009b), following its fortuitous demise in the 1930s, when several parasitoids were introduced to combat black scale, *Saissetia oleae* Olivier (Bartlett 1953, 1978).

The stark difference in the occurrence of citricola scale between the two regions has been largely credited to a relative paucity in the diversity and abundance of encyrtid species (Hymenoptera: Encyrtidae) in the San Joaquin Valley, particularly those in the genus *Metaphycus* Mercet (Lampson and Morse 1992; Bernal et al. 2001; Kapranas et al. 2007). As primary endoparasitoids, mainly of soft or armored scales, species of *Metaphycus* play a crucial role in the natural regulation of their hosts (Guerrieri and Noyes 2000). In the San Joaquin Valley, only two *Metaphycus* species are commonly associated with citricola scale: *M. helvolus* (Compere) and *M. luteolus* (Timberlake). However, together they account for <14% of total scale parasitism and the overwhelming majority of scale parasitism in that region (83.9%) is attributed to the aphelinid autoparasitoid *Coccophagus lycimnia* (Walker) (Bernal et al. 2001). In contrast, in southern California, a recent survey of the parasitoid complex associated with brown soft scale, *Coccus hesperidum* L. (an alternative host for parasitoids of citricola scale), identified four *Metaphycus* species, *M. helvolus*, *M. luteolus*, *M. stanleyi* (Compere), and *M. angustifrons* (Compere), which together accounted for 75% of scale parasitism (Kapranas et al. 2007). Another encyrtid, *Microterys nietneri* (Motschulsky), accounted for a further 7% of scale parasitism, but *Coccophagus* spp. (abundance of individual species was not determined) accounted for only 11% of total scale parasitism (Kapranas et al. 2007). It is thought that the complex of *Metaphycus* spp. exercises complete control over citricola scale in southern California (UCIPM 2009b) creating interest in the possibility of using the additional *Metaphycus* species in an augmentative release program in the San Joaquin Valley. Interestingly, although *M. angustifrons* was found to be by far the most abundant parasitoid of brown soft scale in southern California, this was actually a new record of establishment in California for this species. However, *M. angustifrons* and *M. stanleyi* are morphologically very similar (indeed males of the two species are acknowledged as inseparable; Kapranas et al. 2007) and it seems highly likely that *M. angustifrons* has previously been misidentified.

A further species of *Metaphycus* that has attracted the attention of citrus researchers in California is *M. flavus* (Howard) (Bernal et al. 1999; Schweizer et al. 2002, 2003). This species was collected in 1996 from citricola scale at Kozan, Turkey, and has since been introduced to central California's San Joaquin Valley for the biological control of citricola scale (Bernal et al. 1999; Schweizer et al. 2002), and to southern California orchards for the control of black scale (Schweizer et al. 2003). However, it is unknown whether this species has established in either region, at least in part because it is morphologically indistinguishable from the native *M. luteolus*. In their authoritative revision of European species of *Metaphycus*, Guerrieri and Noyes (2000) concede that the two species may actually be synonymous.

A critical step in assessing an organism's potential for biological control is the ability to accurately identify it (Bigler et al. 2005). This can be problematic if definitive morphological characters are elusive or difficult to interpret. The identification of scale parasitoids is hampered by their small size (typically less than 2 mm in length), and whilst several diagnostic keys based on morphological characters are in circulation, in most cases, high quality, expertly prepared, slide-mounted specimens are requisite for reliable identification. For example, accurate identification of some *Metaphycus* spp. requires slide preparations that include a dissection of the specimen's mouthparts (see Guerrieri and Noyes 2000). Thus, traditional methods of identifying soft scale parasitoids are time consuming and require specialized knowledge. Furthermore, morphological keys rely on characters of adult specimens and cannot be used to reliably identify immature life stages.

Over the last decade, the use of molecular techniques to identify species has increasingly been advocated in genera that lack definitive morphological characters. Typically, this has involved the use of the polymerase chain reaction (PCR) to amplify short stretches of DNA, which are then characterized by secondary experimental methods such as sequencing or digestion with restriction endonucleases (e.g., Stouthamer et al. 1999; Rugman-Jones et al. 2006, 2009a). However, such secondary methods require further investment of time and money. Thus, methods that allow direct interpretation of the product of the PCR reaction are favorable. In situations where a specimen is likely to be only one of a relatively small number of species, taxon-specific multiplex-PCR offers one solution. This technique involves the use of one PCR primer located in a region shared by several taxa (a universal primer) alongside several

opposing primers, each of which lies in a region that is specific to only one of the taxa under consideration, and which when it amplifies, produces a PCR product of a size that is unique to that taxon. As a result, taxa can be identified directly following gel electrophoresis of the PCR product (e.g., Cornel et al. 1996; Fritz et al. 2004; Gariepy et al. 2005; Rugman-Jones et al. 2009b).

Here we present a molecular identification method based on multiplex-PCR, enabling rapid and reliable identification of ten parasitoid species (from three genera), which are (Kapranas et al. 2007), or through augmentative releases may become (Bernal et al. 1999; Schweizer et al. 2002, 2003), part of the complex of parasitoids attacking soft scales in California citrus groves: *Metaphycus luteolus*, *M. stanleyi (M. swirskii)*, *M angustifrons*, *M. helvolus*, *M. flavus*, *Microterys nietneri*, *Coccophagus cowperi* (Girault), *C. lycimnia*, *C. rusti* (Compere), and *C. semicircularis* (Forster). Of these, *M. luteolus* is the only California native, the others having been originally imported into California for release against various soft scale species (Bartlett 1978; Daane et al. 1991; Lampson and Morse 1992; Bernal et al. 1999). This work seeks to provide a quick and accurate means to identify the complex of parasitoid species currently attacking soft scales in California citrus, and to monitor the success in the San Joaquin Valley of future augmentative releases of parasitoids introduced from southern California against citricola scale.

Materials and methods

Origin of parasitoids

Four of the five *Metaphycus* species used in this study were taken from separate cultures maintained on brown soft scale at the University of California, Riverside (UCR), USA. Bernal et al. (1999) gave original collection details for the cultures of *M. luteolus*, *M. stanleyi,* and *M. flavus*. The *M. angustifrons* culture originated from wasps collected at the Citrus Heritage Park, Riverside, Riverside Co., California, USA (N3°53.534 W117°25.278) on 4 March 2004. The fifth *Metaphycus* species, *M. helvolus,* was identified from field-collected material (Kapranas et al. 2007) during testing of our multiplex-PCR methods (see below). The four species of *Coccophagus* were identified from this same field-collected material. Specimens of *Microterys nietneri* were taken from a culture maintained on brown soft scale at UCR, which originated from wasps collected at Rancho Pauma Granite, Pauma Vista, San Diego Co., California, USA (N33°20.105 W116°58.483) on 10 May 2004.

DNA extraction, amplification and sequencing

DNA was extracted from intact individual wasps using the EDNA HiSpEx™ Tissue Kit (Fisher Biotech, Wembley, Australia) according to the manufacturer's guidelines for extracting from 1 mm^3 tissue. For each species, the polymerase chain reaction (PCR) was used to amplify separately two stretches of nuclear ribosomal DNA (rDNA): a section of the D2 expansion region of 28S, and the internal transcribed spacer 2 (ITS2). PCR was performed in 25 µl reactions containing 1× ThermoPol PCR buffer (New England BioLabs, Ipswich, MA, USA), 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.4 mM dUTP, 2 µl BSA (NEB), 1 U *Taq* polymerase (NEB), and 0.2 µM each of the primers: ITS2-forward (5′-TGTGAACTGCAGGACACATG-3′; Campbell et al. 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White et al. 1990) for ITS2; and D2-CF (5′-CGTGTTGCTTGATAGTGCAGC-3′) and D2-CR (5′-TTGGTCCGTGTTTCAAGACGGG-3′; Campbell et al. 1993, 2000) for 28S. Amplifications were performed in a Mastercycler® ep gradient S thermocycler (Eppendorf North America Inc., New York, NY, USA) with an initial denaturing step at 95°C for 3 min, followed by 38 cycles of 94°C for 45 s, 55°C for 30 s, 72°C for 1 min 30 s, and a final extension of 3 min at 72°C. The presence of amplified products was confirmed by gel electrophoresis and PCR products were cleaned using the Wizard® PCR Preps DNA purification kit (Promega, Madison, WI, USA) and direct-sequenced in both directions at the University of California Riverside Genomics Institute, Core Instrumentation Facility using an Applied Biosystems 3730 DNA analyzer with a Big-Dye V3.1® kit (Applied Biosystems, Foster City, CA, USA). For most species, sequences were obtained for at least eight specimens, including three specimens from each of the cultures held at UCR (see above) and at least five specimens from field-collected material (see below). Exceptions to this were *M. flavus* and *Microterys nietneri*, which

were not detected in the field and therefore were represented only by the three culture specimens. Sequences were aligned manually in BioEdit version 7.0.5.3 (Hall 1999) and representative sequences were deposited in GenBank® (Benson et al. 2008). For each 'genetic' species, several intact specimens (from the field-collected 'test' material; see below) were retrieved from the DNA extractions and species identity was confirmed using available keys (Compere 1931, 1940; Annecke and Mynhardt 1971, 1972, 1981; Guerrieri and Noyes 2000) and by comparing the specimens with the types (where available), or with authoritatively identified specimens housed in the collections of the Natural History Museum (NHM, London) and of the Department of Agricultural Entomology and Zoology (DEZA), University of Naples (Portici, Italy). Voucher specimens are deposited in the Entomology Museum at UCR, in the NHM and in DEZA.

Multiplex-PCR design

Taxon-specific PCR primers were designed for three separate multiplex-PCRs (one genus-specific [based on 28S sequences], and two species-specific [based on ITS2 sequences]) with the aid of Primer3 v.0.4.0 (Rozen and Skaletsky 2000) using the following criteria: (1) reverse primers should work with a common forward primer; (2) at least one nucleotide at the 3′ end of the primer must be unique to one target taxon in the alignment; (3) complementarity between primers should be minimal; (4) they should have similar melting temperatures (can be manipulated by substituting nucleotides at the 5′ end); and (5) they should produce a PCR product of a unique size for each included taxon, which can be readily distinguished using standard agarose gel electrophoresis. The specificity of several primers for each group of taxa was tested individually and in multiplex-PCR against all included taxa, identifying a set of primers which gave the best combination of the desired characteristics (Tables 1, 2). The specificity of each of the two species-specific multiplex-PCRs was also tested against all the species included in the other to determine if false positives were produced (i.e., the *Metaphycus* spp. multiplex-PCR was tested against specimens of all four *Coccophagus* species, and vice versa).

Multiplex-PCR conditions

In each multiplex-PCR, DNA from individual wasps was amplified in 25 µl reactions containing 1× ThermoPol buffer (NEB), 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.4 mM dUTP, 2 µl BSA (NEB), 0.2 µM each primer (Tables 1, 2), 1 U *Taq* polymerase (NEB), and 2 µl template DNA (concentration undetermined). Amplification was performed in a Mastercycler® ep gradient S thermocycler (Eppendorf North America, Inc.). The thermocycler profile for the three multiplex-PCRs was identical: an initial denaturing at 95°C for 3 min followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, and a final extension of 3 min at 72°C. Following amplification, 5 µl of each PCR product was visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Gels were run for 1.5 h at a voltage of 4.5 V cm^{-1} to ensure ample separation of the different sized bands.

Confirmation of morphological identifications of adult specimens using the species-specific multiplex-PCRs

The respective species-specific multiplex-PCR was used to identify specimens collected and preserved in 95% ethanol between February 2004 and March 2006 from several southern California citrus groves. These

Table 1 Multiplex-PCR primers for identification of parasitoids to genus based on amplification of 28S rDNA

Taxon	Primer name	Primer sequence	PCR product (bp)
	MMC uni-F	5′-CCGTGAGGGAAAGTTGAAAA-3′	
Coccophagus	Coc-R	5′-GAAGTGCACGTCGACCAC-3′	206
Metaphycus	Met-R	5′-TCCTGAAAGTACCCAAAGCAAA-3′	~530
Microterys nietneri	Mic-R	5′-GTCTACCGTACGAGCCTTGG-3′	277

Table 2 Species-specific multiplex-PCR primers for identification of species of (a) *Metaphycus* and (b) *Coccophagus* based on amplification of ITS2 rDNA

Taxon	Primer name	Primer sequence	PCR product (bp)
(a) *Metaphycus* spp.			
	M.uni-F	5′-GGCTGAGGGTCGTTCSATAA-3′	
M. angustifrons	ang-R1	5′-GCGTTCGAACACGAATTGTA-3′	184
M. luteolus	lut-R3	5′-GTGCCATGAAACAACCAGAG-3′	638
M. flavus	fla-R2	5′-CACAACAACACAGCACAGCA-3′	531
M. stanleyi (*M. swirskii*)	sta-R1	5′-ACCACAAATTGGACGAGAGG-3′	323
M. helvolus	hel-R1	5′-GCTCAGGCTTTCGCTGTC-3′	75
Microterys nietneri[a]	Mic-R1	5′-TTCGAGCGAGTGAGTGAGTG-3′	671
(b) *Coccophagus* spp.			
	C.uni-F	5′-GACATTTCGAACGCACATTG-3′	
C. cowperi	cow-R1	5′-AGCCAATTCGCTCGTTAGAC-3′	109
C. lycimnia	lyc-R2	5′-GGCGTTTTAAAATAAACGCAAG-3′	388
C. rusti	rus-R2	5′-CGCGTTGTTACCTTCCTTCT-3′	183
C. semicircularis	sem-R2	5′-ACGAGCGTACTCAACTGCTG-3′	495

[a] Inclusion of the primer for *Microterys nietneri* is optional

specimens were collected as part of a survey of parasitoids of brown soft scale carried out by Kapranas et al. (2007). The material included 347 batch-reared *Metaphycus* individuals, omitted from their abundance data, but tentatively identified to species based on morphology, as well as 77 specimens of *Coccophagus* spp. identified only to genus (see Kapranas et al. 2007). The quality of the DNA extraction of any specimen that failed to produce a multiplex-PCR product was assessed by attempting to amplify the 28S and ITS2 regions as above.

Parasitoid species diagnosis from parasitized host scales

Current morphology-based identification methods only work with adult wasps, and thus require "rearing out" of the parasitoids from their hosts prior to identification. Thus, if time and space is of the essence, it would be beneficial if parasitoids could be identified from the host scale material (i.e. prior to their emergence). Molecular-based identification offers this potential. A colony of brown soft scales on a single *Yucca recurvifolia* Salisbury leaf was isolated with a *M. luteolus* population allowing four days for the females to parasitize the scales. Immediately following that period, the *Yucca* leaf was isolated, preventing further parasitism, and five scales were collected at random. Two days later, another five scales were collected. At such an early stage of parasitoid development in these two collections (i.e., 1–6 days), it was impossible to definitively tell if a scale was parasitized or not. However, a further two days later (eight days after initial exposure to the parasitoids), larvae could be seen within the host scales under a dissecting microscope, and a further sample of five parasitized scales was collected. A final collection of five scales containing visible pupae was made on day 10. DNA was extracted from intact individual scales as described for the parasitoids, and tested using the *Metaphycus* species-specific multiplex-PCR. A second test was performed with scales exposed separately to *M. angustifrons* and *M. stanleyi*, but parasitized scales were collected only once (after eight days, when larvae were visible).

Results

As expected, 28S rDNA sequences (GenBank accessions HM856874-856887) were highly conserved within species. Only *C. rusti*, showed any intraspecific variation in this region. Sequences of ITS2 were slightly more variable, but variation was almost exclusively restricted to microsatellite repeats

(GenBank accessions HM856888-856901). The one exception to this was *C. semicircularis* in which we found two different ITS2 sequences differing from each other by one insertion (nucleotide position 412) and two substitutions (nucleotide position 141–142). The DNA sequences obtained for the 28S and ITS2 regions of each species led to the design of three sets of taxon-specific multiplex-PCR primers: one for identification of specimens to genus (based on 28S; Table 1), and one set each for identification of species of *Metaphycus* and *Coccophagus,* respectively (based on ITS2; Table 2). Authoritative morphological identification confirmed the specific identity of specimens of nine of the ten parasitoid species. However, both the UCR colony, and specimens from the field that were initially thought to be *Metaphycus stanleyi,* actually keyed out as *M. swirskii* Annecke & Mynhardt. Each multiplex-PCR successfully amplified the DNA of each included taxon, yielding PCR products of characteristic (and hence diagnostic) taxon-specific sizes (Figs. 1, 2, 3). Furthermore, no false positive bands were produced when the *Coccophagus* spp. were tested with the *Metaphycus* spp. multiplex-PCR, and vice versa (results not shown).

Use of the *Metaphycus* spp. multiplex-PCR to diagnose specimens previously identified using morphological methods found that many specimens were incorrectly identified using those morphological methods (Table 3). While molecular diagnoses concurred that females of *M. luteolus* and *M. stanleyi* had consistently been correctly identified using morphology (although *M. stanleyi* actually appears to be *M. swirskii*, see above), the morphological identifications of males of these two species were not so reliable, with 16% and 63% of specimens identified as male *M. luteolus* and *M. stanleyi* respectively, actually being found to be *M. angustifrons* (Table 3). A further 13% of specimens identified morphologically as *M. helvolus* (male or female) were also found to be *M. angustifrons*, and 5% of specimens identified morphologically as female *M. angustifrons* were found to be *M. helvolus* (Table 3). The *Coccophagus* spp. had not previously been identified to species, but all four of the expected species were present, with almost half (48%) identified as *C. semicircularis*

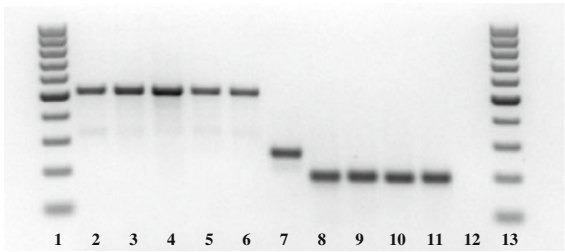

Fig. 1 Visualization of the 28S multiplex-PCR products of the complex of ten parasitoid species attacking soft scales (Hemiptera: Coccidae) in California citrus groves. PCR products were electrophoresed on a 1.5% agarose gel. Lane 1, 100 bp DNA size standard (Fermentas, Inc., Glen Burnie, MD); lane 2, *Metaphycus angustifrons*; lane 3, *M. luteolus*; lane 4, *M. stanleyi*; lane 5, *M. helvolus*; lane 6, *M. flavus*; lane 7, *Microterys nietneri*; lane 8, *Coccophagus semicircularis*; lane 9, *C. lycymnia*; lane 10, *C. rusti*; lane 11, *C. cowperi*; lane 12, no template control; lane 13, 100 bp DNA size standard

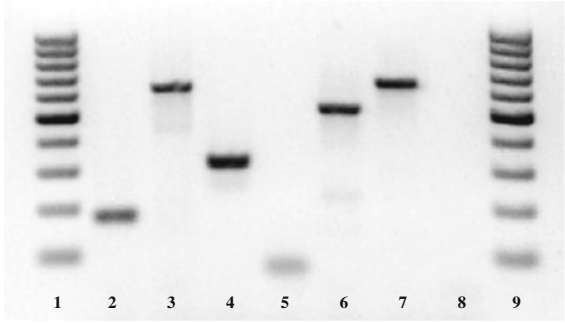

Fig. 2 Visualization of the *Metaphycus* spp. multiplex-PCR products following electrophoresis on a 1.5% agarose gel. Lane 1, 100 bp DNA size standard (Fermentas); lane 2, *M. angustifrons*; lane 3, *M. luteolus*; lane 4, *M. stanleyi*; lane 5, *M. helvolus*; lane 6, *M. flavus*; lane 7, *Microterys nietneri*; lane 8, no template control; lane 9, 100 bp DNA size standard

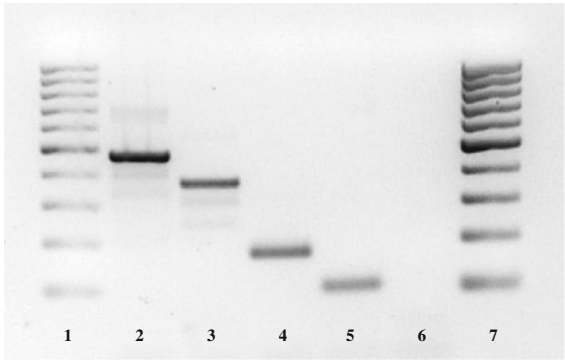

Fig. 3 Visualization of the *Coccophagus* spp. multiplex-PCR following electrophoresis on a 1.5% agarose gel. Lane 1, 100 bp DNA size standard (Fermentas); lane 2, *C. semicircularis*; lane 3, *C. lycymnia*; lane 4, *C. rusti*; lane 5, *C. cowperi*; lane 6, no template control; lane 7, 100 bp DNA size standard

Table 3 Molecular diagnosis of morphologically identified, alcohol preserved, adult parasitoid specimens using multiplex-PCR

Morphological ID	Molecular ID				
	M. helvolus	*M. angustifrons*	*M. luteolus*	*M. stanleyi* (*swirskii*)	No amplification[a]
M. helvolus female ($n = 15$)	13	2	–	–	–
M. helvolus male ($n = 60$)	47	5	–	–	8
M. stanleyi female ($n = 46$)	–	–	–	45	1
M. stanleyi male ($n = 46$)	–	29	1	16	–
M. luteolus female ($n = 42$)	–	–	41	–	1
M. luteolus male ($n = 19$)	–	3	16	–	–
M. angustifrons female ($n = 119$)	6	109	–	1	3
	C. cowperi	*C. lycimnia*	*C. rusti*	*C. semicircularis*	No amplification
Coccophagus spp. ($n = 77$)	11	11	18	37	–

[a] Quality of the DNA extractions was subsequently tested using general 28S and ITS2 PCR primers (see text)

(Table 3). Of the 424 specimens tested using multiplex-PCR, only 13 failed to yield a PCR product. Of those, 12 also yielded nothing in conjunction with the universal 28S and ITS2 primers and it is assumed that these represent failed DNA extractions. The 28S and ITS2 regions of the remaining specimen (previously identified as a *M. helvolus* male) were sequenced (GenBank accessions HM856886 and HM856900, respectively), and the carcass was subsequently slide-mounted and identified as *Metaphycus alberti* (Howard), an unexpected species that was not originally incorporated into the multiplex assays due to its lack of occurrence in previous field surveys.

The *Metaphycus* spp. multiplex-PCR detected *M. luteolus* in a total of seven of the ten brown soft scales from which DNA was extracted immediately or two days after exposure to the *M. luteolus* colony (i.e., before parasitism could be definitively assessed under a light microscope). All brown soft scales in which larvae or pupae could be seen prior to DNA extraction yielded a multiplex-PCR product of the expected size for the parasitoid species to which it had been exposed: *M. luteolus*, *M. angustifrons* or *M. stanleyi* (*M. swirskii*).

Discussion

Accurate identification of insect parasitoids used (or being considered for use) in biological control programs is a fundamental step in assessing their suitability and performance (Bigler et al. 2005; Greenstone 2006). The taxon-specific multiplex-PCRs developed in the present study provide means by which any life stage of the major members of the complex of parasitoids attacking soft scales in California citrus groves (Lampson and Morse 1992; Bernal et al. 2001; Kapranas et al. 2007) can be accurately and rapidly identified. When coupled with a universal forward PCR primer, the genus-specific reverse primers yield a PCR product of a characteristic size that allows diagnosis of a specimen as *Metaphycus* spp., *Coccophagus* spp. or *Microterys nietneri*. Specimens identified as a *Metaphycus* spp. or *Coccophagus* spp. can then be identified to species using the respective species-specific multiplex-PCR. Since adults of these two genera are superficially easy to discriminate from one another, it may be possible to jump straight to one or other of the species-specific multiplex-PCRs for their identification. However, the two-step system potentially allows identification of immature life stages (eggs, larvae and pupae), even when extracted along with host scale DNA. In principal, we have demonstrated this using three *Metaphycus* spp. parasitizing brown soft scale. However, we cannot be certain this holds true for the remaining species, and it is also possible that a different host scale species may disrupt the PCR.

Tests of our multiplex-PCR protocols on field-collected material highlight the value of attaching a genetic signature to morphologically similar species (see also Rugman-Jones et al. 2009a, b). In the past, reliable identification of *Metaphycus* spp. has required well-prepared adult specimens that were

normally sent to one of a small number of taxonomic experts familiar with these parasitoids (Guerrieri and Noyes 2000). Even in studies where an individual was expected to be one of only a small number of species, reliable species diagnosis involved rearing out adult parasitoids from individual scales in isolation, and subsequent identification based on differences in the color of the mummified host scale and/or association with females emerging from the same host (Kapranas et al. 2007). The present method provides a genetic profile for each species of interest, thereby removing the ambiguity from species identification, and allowing identification of any parasitoid life stage. Our entire protocol, from DNA extraction through to the completion of two consecutive rounds of multiplex-PCR and gel electrophoresis, can be performed in approximately 10 h. We estimate that using our method, a single laboratory technician could readily diagnose specimens at a rate of 100 per day, and at a cost (in laboratory reagents and consumables) of around $1 per specimen.

Multiplex-PCR can be an extremely efficient identification tool if a specimen is expected to be one of only a handful of species (Gariepy et al. 2008). However, herein lies a limitation of the multiplex-PCR method: it is context-specific and its initial design is dependent on having specimens to test that are representative of the spectrum of species one might later expect to encounter. For the purpose of our objectives (i.e., diagnosing species of *Metaphycus* and *Coccophagus* on California citrus), all of the major species identified in earlier survey work were included (Lampson and Morse 1992; Bernal et al. 2001; Kapranas et al. 2007). However, the occurrence of rare, unexpected, or recently introduced species may be overlooked, as was the case here in a single incidence of the unanticipated detection of *M. alberti* (discussed in more detail below). In some cases this is easily resolved; for example, with the deliberate introduction of new species (e.g., *M. flavus*), preemptive design and incorporation of additional species-specific primer(s) into the multiplex assay is possible and would allow one to monitor their establishment and success. In other instances, such as accidental introduction of additional species, it is difficult to predict how our multiplex assay would "respond" to the presence of an unknown species. It is likely that for such a specimen, the multiplex assay would fail to yield PCR product (or product of an unexpected size). In such cases, subsequent amplification and sequencing of the ITS2 and/or 28S genes would at the very least provide a DNA sequence that could then be compared to known species. In addition, the use of the non-destructive EDNA HiSpEx^{TM} Tissue Kit would allow for the recovery, mounting, and morphological identification of the voucher specimen associated with the genetic profile produced by DNA sequencing. As previously mentioned, this was the case for a single specimen of *M. alberti* among the 424 specimens used to challenge the validity of our multiplex assay. This not only demonstrates the specificity of our assay, but also provides guidelines for detecting rare or unexpected species that may occasionally occur in field-collected samples. In the case of *M. alberti*, this species was originally introduced to California from Australia in 1898, for the control of brown soft scale, and initially appeared to become established in the Riverside area. However, despite extensive sampling of brown soft scale in southern California, *M. alberti* was not detected after 1922, until it was "rediscovered" in 1997, attacking extremely low population densities of brown soft scale on English ivy *Hedera helix* L. (Stauffer and Rose 1997). It appears to be a very rare parasitoid of brown soft scale on citrus in California and therefore, not worthy of inclusion in our multiplex assay.

Application of our methodology to other geographic regions should be undertaken with care. Each multiplex-PCR has been designed to work within the constraints of a known degree of inter- and intraspecific genetic variation. In their survey of parasitoids attacking black scale on citrus and olives in Spain, Tena et al. (2008) were unable to differentiate between the larval stages of *M. flavus* and *M. helvolus*, and between those of *C. lycimnia* and *C. semicircularis*. Our method provides an accurate means of achieving this in California citrus groves, but it may be unwise to use it elsewhere without first surveying and sequencing the resident parasitoid fauna. Populations outside the geographic range of our study may differ sufficiently in their ITS2 sequence (on which the two species-specific multiplex-PCRs are based) such that they prevent annealing of the "correct" primer and/or facilitate annealing of an "incorrect" primer. This may result in the production of spurious PCR products and the potential for false positive identifications.

The discovery that what has long been regarded as *M. stanleyi* in California citrus (Bernal et al. 1999, 2001; Kapranas et al. 2007), in fact appears to be *M. swirskii,* further highlights the difficulties that can be associated with interpreting morphological characters for the purpose of identification. No details are given about how species identity was determined when the UCR colony was originally collected (Bernal et al. 1999) but Kapranas et al. (2007) used morphological keys prepared by Annecke and Mynhardt (1971, 1972, 1981) to diagnose field-collected specimens as *M. stanleyi* (though their interpretation of these keys may have been influenced by what had been reported previously). In their revision of the European species of *Metaphycus*, Guerrieri and Noyes (2000) noted in their description of *M. swirskii* that it is very similar to *M. stanleyi* in general coloration and structure of the antennae, but "…can be separated from *stanleyi* on the basis of the colour of the genae, scape and wings". In their description of *M. stanleyi* they also suggested that "males of *stanleyi* are very similar to those of *swirskii* but can be separated by the less expanded scape. In *stanleyi* the male scape is about 2.5× as long as broad, whereas in *swirskii* it is slightly less than 2× as long as broad". Use of these characters to diagnose these species seems particularly surprising because, in the 'Introduction' to their revision (p. 151), Guerrieri and Noyes (2000) question the reliability of identification keys that rely on "the use of variable characters in order to separate species or groups of species, e.g., general body colouration, or very small differences in relative width of scape…", and note that several species "vary quite extensively in these characters (e.g. *stagnarum*), possibly as a result of ambient temperature experienced during development or some influence of the age or species of host or host's food plant". However, the same authors also indicated a strong stability in the color of the scape and of the gena following the examination of long series of the most common species, and as a consequence, these characters were used in their key to species. This raises some doubt over the validity of *M. stanleyi* and *M. swirskii*, which is exacerbated by the lack of a type specimen for *M. stanleyi* (Guerrieri and Noyes 2000), and by the fact that in at least one instance, Guerrieri and Noyes (2000) appear to include material from a single collection [ex star scale on coffee from Mchana, Kenya, x.1984 (A17534)] in their description of both species. To solve this problem, specimens that match the morphological description of *M. stanleyi* given by Guerrieri and Noyes (2000) will need to be collected and their DNA sequences compared with those of our *M. swirskii* specimens. It would also be interesting to determine whether the "*M. stanleyi*" that parasitizes black scale in California (Daane et al. 1991; Lampson and Morse 1992) is the same species.

While raising questions about the possible synonymy of *M. stanleyi* and *M. swirskii*, the sequence data provided herein also supports the validity of two other 'questionable' species, *M. luteolus* and *M. flavus* (Guerrieri and Noyes 2000). This will prove invaluable for accurately assessing the latter species as a potential biological control agent for the suppression of citricola scale and black scale in California citrus (Bernal et al. 1999, 2001; Schweizer et al. 2003). As reported by Guerrieri and Noyes (2000), it should be noted here that *M. flavus* and *M. luteolus* (both originally described from the USA) may still actually be synonymous, and that the species collected in Turkey and referred to herein as *M. flavus*, may represent a valid, undescribed species. Indeed, the true identity of these species may only be solved by the molecular and morphological determination of specimens of both sexes from a large number of localities, including the type locality of *M. flavus* in Florida.

The objective of this study was to create a quick, easy and above all, accurate, alternative to traditional morphology-based methods used to identify the major parasitoid species attacking soft scales in California citrus. The protocols developed herein provide significant reductions in the time, costs and expertise required to produce reliable species diagnoses. Furthermore, our multiplex-PCR methods allow the identification of both sexes and the immature life stages of each parasitoid species, even eggs inside a host scale. As such, our methodology could greatly improve efforts to monitor the success of augmentative releases of parasitoids (introduced from southern California) against citricola scale in the San Joaquin Valley.

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