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Determining Parasitoid Species Composition in a Host Population: A Molecular Approach

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ABSTRACT Larvae of closely related parasitoid taxa often lack morphological differences that can be used for species level identification. Determining the parasitoid species present in a host population may require rearing, often a time-consuming process. To monitor field parasitism rates by several species of *Peristenus* wasps (Hymenoptera: Braconidae) that are natural enemies of *Lygus* (Heteroptera: Miridae), we have developed a two-step molecular approach. Polymerase chain reaction (PCR) of the COI gene with wasp-targeting primers is performed on DNA extracted from a *Lygus* nymph and the parasitoid larva (if any) therein. A positive reaction indicates parasitoid presence. A restriction digest of the PCR product then indicates which parasitoid species is present among known alternatives, and a diagnosis is achieved in days rather than weeks or months.

KEY WORDS *Lygus lineolaris*, *Peristenus*, parasitoid, DNA identification technique, PCR-RFLP

DETERMINING THE IDENTITY of parasitoids attacking a host species in different habitats and locations is relevant to understanding both ecological and evolutionary relationships between hosts and parasitoids, and to assessing biological control of pestiferous hosts. The host range of a parasitoid species is similarly important. Predictions about the host range and impact of an introduced parasitoid in a new region, with new potential hosts, are difficult to make with accuracy. Postintroduction data for already-established introduced parasitoids could prove valuable for developing criteria for release programs, and shed light on natural processes of geographic dispersion and host range expansion.

The parasitoid–host associations between *Lygus* Hahn (Heteroptera: Miridae: Mirini) and *Peristenus* Foerster (Hymenoptera: Braconidae: Euphorinae) are good models for studying the pattern and impact of parasitoid introduction events. In North America, there are both native and introduced *Peristenus* species with overlapping geographic and host ranges. This system can provide important data on the abundance and impact of a new parasitoid on target and nontarget hosts, and on the interaction between native and introduced natural enemies of a common host (Kuhlmann et al. 1998).

Because the period of interaction between the adult parasitoid and its individual target is quite brief, field studies must often focus on already-parasitized hosts and the immature parasitoids within them. However, a frequent obstacle to obtaining parasitoid–host data is the issue of identity. The reduced morphology of parasitic larvae such as *Peristenus* immatures can preclude species and even generic determination (Loan and Shaw 1987). A careful study of larval head sclerites by Carignan et al. (1995) failed to reveal characters that separate three geographically overlapping nymphal parasitoids of *L. lineolaris* (Palisot), *P. digoneutis* Loan, *P. pallipes* (Curtis), and *P. pseudopallipes* Loan. Also, new *Peristenus* species such as *P. conradi* Marsh (Day et al. 1992) and *P. stygicus* Loan continue to be introduced from abroad. Larval morphology also fails to distinguish *Peristenus* from *Leiophron* Nees, another euphorine genus parasitic on *Lygus* and other mirids (Loan and Shaw 1987, Day and Saunders 1990), further complicating the study of this system.

Studies of parasitoid populations in *Lygus* have relied on a combination of dissection and rearing (Day 1994). The more easily identified wasp adults are ephemeral and difficult to sample. Dissection of a *Lygus* subsample gives the best estimate of percent parasitism, and rearing of a paired subsample gives the identity of the parasitoid species present (Day 1994). However, a postemergence diapause of up to 11 mo, depending on the species, precedes adult emergence. With species or generations that have long diapause, the wasp cocoons must be held in summer and then winter conditions for several months. With parasitoids of *Lygus*, it is not uncommon to wait 8 mo or more for information on the parasitoid species composition in a host population.

Studies on the ecological interaction of *Lygus* parasitoids, as well as programs to monitor the spread of introduced *Peristenus* species, could benefit from a simple, rapid, and reliable laboratory assay to detect and distinguish among known parasitoid species. Though related species may be morphologically indistinct as immatures, their molecular differences can provide useful characters for classification. Molecular

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variation at the protein and DNA levels has been used for insect species diagnostics (Black et al. 1992; Sperling et al. 1995; Antolin et al. 1996; Taylor et al. 1996, 1997). Furthermore, in parasitological studies, the molecular genetic differences between parasite and host may be used for parasite detection. Serological and electrophoretic techniques have proven useful for detecting pathogens in insect vectors (Higgins and Azad 1995) as well as parasitism in herbivorous pests (Höller and Braune 1988, Stuart and Greenstone 1997). The development of the PCR technique has further simplified the detection of insect endobionts (Higgins and Azad 1995, Zhu and Greenstone 1999).

The purpose of this study was to identify some of the genetic variation between *Lygus* and *Peristenus*, and genetic variation among select species of *Peristenus*, and use this information to develop a diagnostic tool for the detection and identification of parasitism in *L. lineolaris*.

Host and Parasitoid Species. The host species in this study, *Lygus lineolaris*, is native to and occurs throughout North America (Kelton 1975). One of the broadest herbivore generalists, it has been documented on 328 plant species of 55 plant families in 30 orders (Young 1986). In addition to its food plants, *Lygus* has been documented to feed opportunistically on numerous live and dead insects (Wheeler 1976), and on the blood of faculty (Myers 1929) and graduate students (K.J.T., unpublished data).

Peristenus digoneutis, a European species parasitic on *L. rugulipennis* Poppius, was successfully introduced in northern New Jersey by the USDA in the late 1970s and early 1980s for biological control of *L. lineolaris* in alfalfa (Day et al. 1990). It has since spread into New York, and five other New England states (Fig. 1), achieving levels of parasitism well above those of native euphorine parasitoids of *L. lineolaris* (Day 1996, Day et al. 1998). Adults emerge from cocoons and are active for 10–14 d in the field, parasitizing early instar *L. lineolaris* nymphs. Usually only one egg is oviposited per host, and the immature parasitoid develops over a 17-d period (Carignan et al. 1995). Upon emergence from the dying mirid nymph, the larva burrows into the ground and spins a cocoon. *P. digoneutis* has two generations per year, some of which go into overwintering diapause. Research on the spread and impact of *P. digoneutis* in the northeast has been conducted since its introduction (Day et al. 1990, Day 1996). Because *P. digoneutis* has been the focus of a monitoring program, it is a prime candidate for inclusion in a diagnostic assay.

Peristenus pallipes is a parasitoid of *L. lineolaris* and other mirids in the northeast and other parts of the country as well (Day et al. 1990, Snodgrass and Fayad 1991, Day 1996). Its life cycle is similar to that of *P. digoneutis* except that it has only one generation per year, in the spring/early summer. Its geographic range includes the range of *P. digoneutis*, making it important to distinguish between the two. A closely allied native species, *P. pseudopallipes*, whose single generation follows that of *P. pallipes*, was not included in this study because specimens were not available at the time. The third *Peristenus* species included in this initial study is *P. conradi*. *P. conradi* prefers the introduced *Adelphocoris lineolatis* (Goeze), but parasitizes *L. lineolaris* at a low frequency (Day et al. 1992). A fourth native species parasitic on *L. lineolaris* in the northeast is *Leiophron uniformis* (Gahan); our initial efforts have focused on the genus *Peristenus* and *L. uniformis* was not included in this study.

Materials and Methods

Characterization of Genetic Variability. The basis of this laboratory technique to detect and distinguish parasitoid species is that portions of the parasitoid DNA will differ from the host as well as from each other. We extracted DNA from adult specimens of *L. lineolaris*, *P. digoneutis*, *P. pallipes*, and *P. conradi*. Wasps were collected from several locations in the northeastern United States and authoritatively identified by W.H.D., and preserved at −80°C. *L. lineolaris* specimens were field collected from alfalfa in Tompkins County, NY, and preserved in 95% ethanol at −20°C. We removed the abdomen from adult *L. lineolaris* specimens before DNA extraction to ensure against parasitoid contamination (though parasitoid larvae are seldom found in adult *Lygus*). We followed standard protocols for DNA extraction (Doyle and Doyle 1987, 1990) as outlined below. Specimens were ground in individual 1.5-ml Eppendorf tubes in the presence of 2× CTAB extraction buffer and 100 μg of proteinase K. Tubes were incubated for 2 h at 55°C, homogenates were extracted with chloroform-isoamylalcohol, digested for 30 min in the presence of 10 μg RNase, and then extracted again with phenol-chloroform-isoamylalcohol, and then chloroform-isoamylalcohol. The DNA was precipitated with 2.5 volumes of ice cold ethanol (100%) and 1/10 volume 3 M sodium acetate, washed once in 80% ethanol, and resuspended in 50 μl Tris-EDTA (pH 7.6) buffer.

We selected the mitochondrial protein-coding gene cytochrome oxidase I (COI) because this gene exhibits interspecific variability in other insect genera, but low intraspecific variability (Vogler et al. 1993). The mitochondrial genome has the added advantage of high copy number per cell (Crozier and Crozier 1992, Simon et al. 1994).

We determined the DNA sequence for an ≈820 bp region in the COI gene for *L. lineolaris* (five individuals), and for *P. digoneutis*, *P. pallipes*, and *P. conradi* (two individuals each). Initial PCR reactions were performed using primers C1-J-2183 and TL2-N-3014 (Simon et al. 1994). PCR reactions were carried out in a Biometra Uno I machine (Biometra, Tampa, FL) using the following cycle conditions: 94°C for 60 s; 52°C, 60 s; 72°C, 90 s (×35 cycles). To avoid contamination of PCR reactions: (1) all glassware and pipettors were cleaned with a dilute solution of sodium hypochlorite on a regular basis (Prince and Andrus 1992); (2) separate areas of the laboratory and separate pipettors were used for DNA extractions, DNA amplifications, and PCR product purification; (3) negative controls were included in all sets of reactions.

Fig. 1. Range expansion of *P. digoneutis* in the northeastern United States, 1984–1996. Numbers within counties represent the year of first county record. Lines represent inferred edges of distribution for 1993 and 1996. Adapted from Day et al. 1998.

All PCR products destined for sequencing were gel-purified overnight at 4°C in low-melting point agarose gels (FMC, Rockland, ME). DNA was recovered from ≈400 mg gel slices using the Promega Wizard PCR Preps DNA Purification kit (Promega, Madison, WI). PCR products purified this way provided consistently good sequence. Automated sequencing was done on an Applied Biosystems 377A automated sequencing machine available through the Cornell Oligonucleotide Facility. All sequences were verified in both directions.

Peristenus and *L. lineolaris* sequences were aligned using MegAlign in the Lasergene software package (DNAStar, Madison, WI). *Apis mellifera* L. sequence (Crozier and Crozier 1993) was included as a reference to determine the reading frame of the sequences. To verify that samples were not identical, we generated a distance tree using the Hasegawa-Kishino-Yano model of base substitution in PAUP* 4.0 d64 (Hasegawa et al. 1985, Swofford et al. 1996). Based on these alignments, we identified a region that was fairly conserved among *P. digoneutis*, *P. pallipes*, and *P. conradi*,

but highly variable between *Peristenus* and *L. lineolaris*. This region served as a basis for designing a new primer, C1-J-2252 (after nomenclature in Simon et al. 1994), [5′-ATTTCYCATATAATTTWTAATGAAAG-3′], which we refer to as 'Peristenus Ia'. This primer was designed to selectively amplify *Peristenus* over *Lygus* for this region.

Polymerase Chain Reaction of Parasitoid Species Using Peristenus Ia Primer. Using the Peristenus Ia primer in conjunction with the primer TL2-N-3014 we tested the ability of this primer pair to selectively amplify *Peristenus* over *L. lineolaris* DNA, and the primer sensitivity to low concentrations of *Peristenus* DNA. It is necessary for the primers to amplify only parasitoid DNA because they are to be used on whole-bug extractions to indicate which *L. lineolaris* are parasitized. First, we tested the Peristenus Ia and TL2-N-3014 primers in a PCR reaction with *P. digoneutis*, *P. pallipes*, *P. conradi*, and *L. lineolaris* DNA, with a negative control of water in place of DNA template. Next, to test primer sensitivity, we mixed *P. digoneutis* DNA with *L. lineolaris* DNA in concentrations of 50, 9, 1, 0.01, and 0.001% *P. digoneutis*. Aliquots of these solutions, and a pure *L. lineolaris* control, were used in PCR with the Peristenus Ia and TL2-N-3014 primers using cycle conditions described above.

Selection and Use of Restriction Enzyme. The aligned *Peristenus* sequences were used to locate sites that varied among parasitoid species. By comparing the sequence of these variable regions with the recognition sites of various restriction enzymes, we identified enzymes that would provide species-specific restriction fragment patterns when used to digest Peristenus Ia to TL2-N-3014 parasitoid PCR products. Restriction fragment-length polymorphism (RFLP) in PCR products (PCR-RFLP) has been used to detect variation within and between populations and species (Deng 1988, Chen et al. 1992, Ota et al. 1992, Aquadro et al. 1998). We chose *Alu*I (recognition site: 5′..AG$^{\triangledown}$CT..3′) for subsequent digests.

Peristenus Ia to TL2-N-3014 PCR products obtained from amplifications of adult *P. digoneutis*, *P. pallipes*, and *P. conradi* were digested with *Alu*I following standard protocols (Sambrook et al. 1989) and those provided by the manufacturer (Promega, Madison, WI). For each 15-μl reaction we used 0.5 U of *Alu*I and ≈0.2 ng of PCR product. Reactions were incubated for 4 h at 37°C under mineral oil and run out on 2% gels [1:1.28 SeaKem LE agarose: Synergel (FMC, Rockland, ME; Diversified Biotech, Boston, MA)] in 1× TBE buffer at 57v for 3 h.

Verification of PCR-Digest Identifications. To confirm that the DNA protocol described above yields parasitoid identification results consistent with traditional dissection/rearing methods, we collected *L. lineolaris* nymphs (28 and 31 July, 1997) from an alfalfa field in Wallkill (Ulster County), NY. We randomly assigned *L. lineolaris* nymphs to two groups: (1) 60 to be analyzed using the DNA protocol, and (2) a group to be divided and dissected (40) or reared (181) at the USDA Beneficial Insect Research Laboratory in Newark, DE, using protocols of Day (1994). Samples for DNA analysis were stored at −20°C before extraction. We individually extracted the DNA from 60 *L. lineolaris* nymphs using the protocol described above. PCR was performed as described above, using the Peristenus Ia and TL2-N-3014 primers. Positive reactions were counted, and the DNA templates from those specimens were reamplified to provide material for the *Alu*I restriction digests, which were then run on gels as described above. The resulting banding patterns were compared with positive controls (digests of identified *Peristenus* spp.). Parasitism rates obtained from the two methods were compared using a comparison of binomial proportions with normal distribution (Ott 1993).

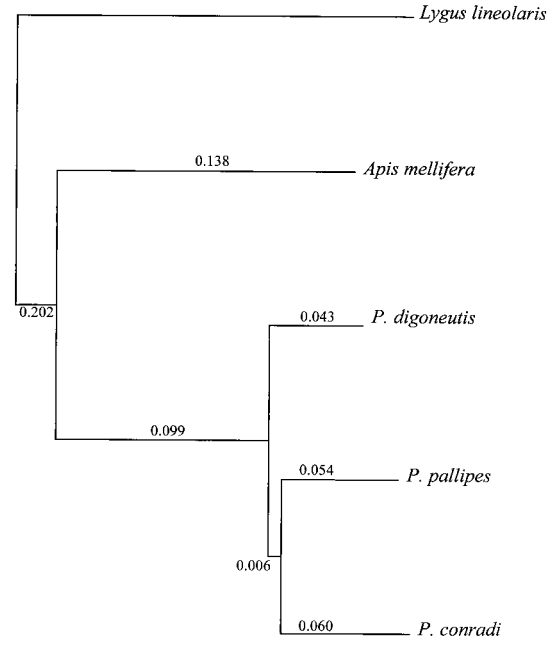

Fig. 2. Distance tree based on HKY85 substitution model. The same tree topology is obtained irrespective of the substitution model selected. Numbers along branches indicate branch lengths.

Results

Characterization of Genetic Variability. Alignments were unambiguous and no insertions/deletions were observed (alignments are available from the authors). Sequences are deposited in GenBank (accession numbers AF189240, AF189241, AF189242, AF189243). We chose a region 26 bp long as a basis for the Peristenus Ia primer (above).

For the 820 bp region sequenced for *L. lineolaris*, *P. digoneutis*, *P. pallipes*, and *P. conradi*, we found species sequence divergence as presented in Fig. 2. This confirms that the *Peristenus* sequences are more similar to each other than to either *A. mellifera* or *L. lineolaris*, and that sequences were not contaminates of each other.

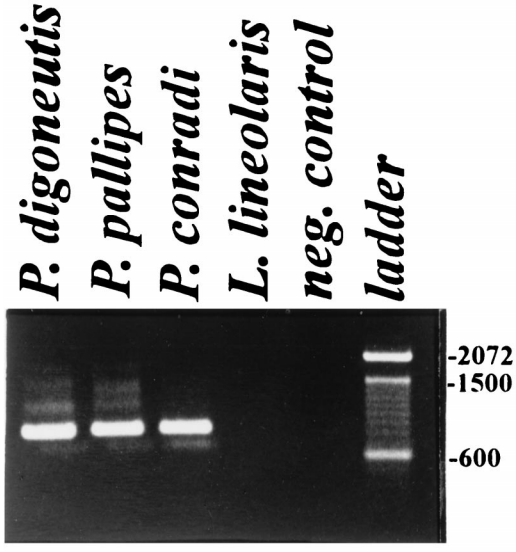

Fig. 3. Bands produced by PCR using Peristenus Ia and TL2-N-3014 primers.

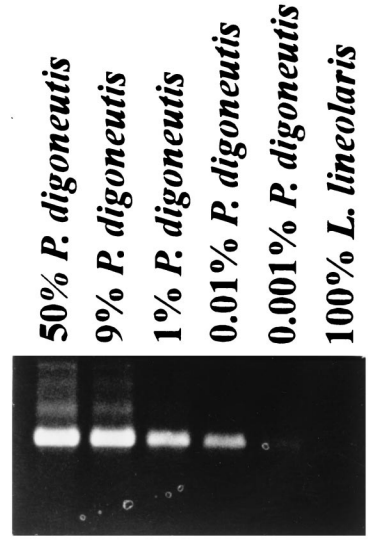

Fig. 4. Bands produced by PCR with Peristenus Ia and TL2-N-3014 primers on varying concentrations of *P. digoneutis* DNA relative to *L. lineolaris* DNA.

Polymerase Chain Reaction of Parasitoid Species Using Peristenus Ia Primer. The primer designed in this study is intended for use with DNA extractions of *L. lineolaris* nymphs to determine *Peristenus* parasitoid presence through positive PCR (with subsequent identification). For such, it is important that the primer pair (1) amplifies only parasitoid, and not host, DNA, and (2) detects a very low concentration of parasitoid DNA so that the earliest stages of parasitism (e.g., parasitoid eggs) are not overlooked.

Using the Peristenus Ia primer in conjunction with TL2-N-3014 we obtained positive PCR for each *Peristenus* species, but not for *L. lineolaris* (Fig. 3). PCR on descending concentration of *P. digoneutis* (relative to *L. lineolaris*) DNA was positive at 50, 9, 1, and 0.01% *P. digoneutis*, and the 0.001% *P. digoneutis* and the *L. lineolaris* control DNA did not amplify (Fig. 4). We calculated that even a newly laid parasitoid egg comprises at least 0.07% of the host, a level well above the minimum threshold (0.01%) of primer sensitivity found here.

Use of Restriction Enzyme. Based on the *Alu*I recognition sequence, predicted band lengths for restriction digest on Peristenus Ia to TL2-N-3014 PCR products are *P. digoneutis*, 180, 188, and 393 bp; *P. pallipes*, 42, 66, 80, and 574 bp; *P. conradi*, 42, 80, and 644 bp. When we used the restriction enzyme *Alu*I to digest the PCR product from the Peristenus Ia to TL2-N-3014 reaction, observable bands on the gel (Fig. 5) ranged from 184 to 644 bp. Predicted bands smaller than ≈150 bp are not resolvable under these conditions. However, bands >150 bp provide a species specific pattern that varied among the 3 *Peristenus* species in this study.

Verification of PCR-Digest Identifications. Of the *L. lineolaris* nymphs processed using the DNA protocols described in this article, we found that 33.3% (20/60) were parasitized, all by *P. digoneutis.* Dissection revealed a 32.5% (13/40) parasitism rate. The two parasitism rates were not significantly different (SE = 0.096; $P < 0.05$). Rearing revealed a 13.8% (25/181) parasitism rate: 23 *P. digoneutis*, 1 hyperparasite (probably *Mesochorus curvulus*), and 1 dead (and therefore unidentifiable) parasitoid larva.

Discussion

The technique developed in this study can be used to identify and quantify the known parasitoid species in a host population. Although this technique was developed to detect *Peristenus* parasitoids of *L. lineolaris*, it can be expanded to include additional taxa, or can be adapted to different parasitoid–host systems, giving it broad applicability. The two main steps in the procedure are as follows: (1) DNA extraction of individual potential hosts and PCR with parasitoid-targeting primers, and (2) restriction digest of positive PCR products. The technique reduces identification time in the *L. lineolaris-Peristenus* system from months to days; further refinement may reduce this time even further. It eliminates the need to laboratory-rear samples, and also allows for the storage of samples until it is convenient to analyze them.

The parasitism rates revealed by the DNA and dissection protocols are consistent with each other, suggesting that the two methods are equally accurate. Rearing and identification of adult parasitoids confirmed that the species identifications obtained using the DNA protocols were also accurate. The discrepancy between parasitism rates revealed by rearing (13.8%) and DNA or dissection (33.3 and 32.5%) is not unexpected, because a higher mortality of parasitized individuals is not unusual in the rearing process [a fact that has previously necessitated paired dissections (Day 1994)].

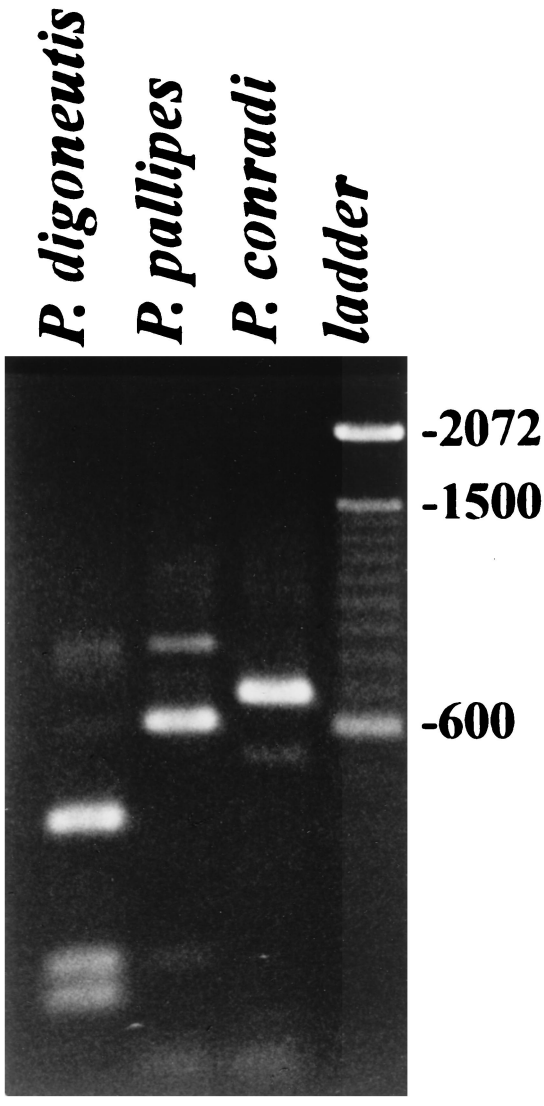

Fig. 5. Banding pattern produced by *Alu*I restriction digest of PCR products from Peristenus Ia and TL2-N-3014 reactions.

A potential pitfall of this technique is that restriction site variation could lead to false readings for new or unexpected parasitoid species or conspecific variants (Sperling and Hickey 1995, Sperling et al. 1996). Furthermore, primer mismatch for new or variable species could prevent the initial amplification that indicates parasitoid presence. To reduce the possibility that new, unexpected, or variable species could escape detection through nonspecificity in the restriction digest, it would be good practice to routinely select a random subsample of PCR 'positives' for direct sequencing to verify identity and screen for variation. The danger of encountering the latter problem of primer mismatch can be reduced by using primer pairs effective at the family level for both braconids and ichneumonids. The Peristenus Ia and TL2-N-3014 primer combination does in fact amplify a variety of Hymenoptera including bees, the 7 *Peristenus* species tested thus far, other braconids, and ichneumonids including *M. curvulus* (a hyperparasite that will ultimately be incorporated into the assay). However, the primers do not work universally within the Ichneumonoidea. We plan to design a new primer specifically for the Ichneumonoidea to replace TL2-N-3014, which could help ensure universal amplification of hymenopterous parasitoids. The presence of dipterous parasitoids, if suspected, would be revealed best through dissection; however, none have been found in mirid nymphs to date (Day 1995).

The *Lygus lineolaris–Peristenus digoneutis* system is useful for careful study because its successful introduction to the United States 15 yr ago can help answer questions about what has happened in the years after the introduction. The ease of detecting particular parasitoid species makes the technique outlined here useful for monitoring geographic range expansion of an introduced parasitoid like *P. digoneutis*, and for assessing parasitoid activity in previously unstudied habitats or crops. It can aid in assessing parasitoid impact on target and nontarget hosts and also in studying the interactions of native and introduced parasitoid species. The technique is particularly useful in situations where multiple hosts are under study (as with assessing impact on nontargets), because the effort required to rear different hosts and parasitoids must certainly limit the array of different host species and samples which can be maintained in the lab; instead, potential hosts and other samples can be collected, identified, and stored for laboratory analysis.

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