Distinguishing the parasitic wasp, *Peristenus howardi*, from some of its congeners using polymerase chain reaction and restriction endonuclease digestion

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Abstract

A molecular procedure incorporating polymerase chain reaction (PCR) of the COI gene and restriction endonuclease digestion of PCR products was used to distinguish *Peristenus howardi* (Hymenoptera: Braconidae) from four other *Peristenus* species. Non-solvent extraction of parasite DNA using a commercially available kit proved to be very effective in producing amplifiable template. Use of SfiI endonuclease produced restriction fragments with banding patterns in agarose gel electrophoresis that readily separated *Peristenus* spp.. However, while the restriction fragment banding patterns of both *P. pallipes* and *P. pseudopallipes* were easily distinguishable from the other *Peristenus* species, they could not be reliably separated from one another. This molecular procedure can be used in applied and ecological research to better understand the role of *P. howardi* in the *Peristenus-Lygus* parasite-host system within the Pacific Northwest. Consensus sequences of our amplimers for all five *Peristenus* spp. are deposited in GenBank under accession numbers AY626370, AY626371, AY626372, AY626373, and AY626374.

Introduction

*Lygus* spp., particularly *L. hesperus* Knight and *L. elisus* Van Duzee, (Heteroptera: Miridae) are the most serious pests of alfalfa, *Medicago sativa*, grown for seed in the Pacific Northwest and California. In addition to direct yield reductions caused by feeding on alfalfa flowers and seeds (Sorenson 1939), insecticides used to manage *Lygus* spp. can indirectly cause further reductions by negatively impacting the activity of the alfalfa leafcutting bee, *Megachile rotundata* (F.) (Hymenoptera: Megachilidae), the principal pollinator of alfalfa seed in the Pacific Northwest (Peterson et al. 1992). Moreover, insecticide-resistant *Lygus* populations have been reported in the Pacific Northwest (Xu and Brindley 1994). Establishment of an effective biological control program for *Lygus* spp. would benefit alfalfa seed production by reducing direct damage to alfalfa seed, minimizing the disruption of pollinators through reduced insecticide use, and delaying or preventing the development of insecticide resistance.

Native and introduced *Peristenus* spp. (Hymenoptera: Braconidae: Euphorinae) are known to parasitize *Lygus* spp. in the northeastern USA (Day 1996) and the Canadian prairies (Braun et al. 2001). Until recently, little information was available concerning parasitism of *Lygus* spp. in the Pacific Northwest (Mayer et al. 1998). Earlier surveys reported that *Lygus* spp. collected in Idaho and Utah were parasitized by *P. pallipes* (Curtis) (Clancy and Pierce 1966; Musebeck et al. 1951). More recently, a braconid wasp was found to be parasitizing a high percentage of *L. hesperus* nymphs collected in Idaho. Although this parasite is morphologically similar to *P. pallipes* and *P. pseudopallipes* (Loan), which are common in the northeastern USA, it was determined to be a new species, *P. howardi* Shaw, apparently native to the Pacific Northwest (Mayer et al. 1998; Day et al. 1999). Because *P. howardi* has been found to parasitize a high percentage of both the first and second generations of *L. hesperus* in some Idaho and Washington locations, it may be a potentially important biological control agent for *Lygus* spp. in alfalfa seed and other seed, vegetable, fruit and forage crops in the Pacific Northwest (Mayer et al. 1998). Little is known about the biology, distribution, and extent of *P. howardi* parasitism of *Lygus* spp. in alfalfa seed fields and nothing is known about the effects of crop and pest management practices on its biological control potential. Research efforts have been hindered in that the reduced morphology of euphorine parasites renders *P. howardi* larvae indistinguishable from other parasites of mirids in the genera *Peristenus* and *Leiophron* Nees (Day and Saunders 1990). Furthermore, although *P. howardi* is apparently multi-voltine, only a small percentage of the larvae do not diapause prior to pupation and adult emergence. This diapause results in a 9-10 month delay in the recovery of data related to percentage parasitism and species composition from field research programs. Additionally, mortality of parasites during the rearing process for species identification can be 40% or higher resulting in a significant and unavoidable loss of data important to understanding the within-season impact of *P. howardi* (Day 1994). A reliable method for detecting *Peristenus* parasitism of *Lygus* spp. occurring...
in the Pacific Northwest that incorporates positive species identification, particularly that for *P. howardi*, would benefit research aimed at studying the ecology and biology of the *Lygus-Peristenus* host-parasite interactions and may prove useful for monitoring some of the biological control components of future IPM programs designed to control *Lygus* damage in alfalfa seed and other Pacific Northwest crops.

Tilmon et al. (2000) developed a two-step molecular method that uses the polymerase chain reaction (PCR) followed by restriction endonuclease digestion of amplimers to detect and identify several *Peristenus* spp. parasitizing *L. lineolaris* (Palisot de Beauvois) nymphs. Using a slightly different approach, Erlandson et al. (2003) developed species-specific PCR primers that allowed for the identification of *Peristenus* and *Lygus* spp., but found that their procedure was less sensitive in detecting *Peristenus* DNA than that of Tilmon et al. (2000). Neither of these studies included *P. howardi*, considered the most important *Peristenus* parasite of *Lygus* spp. in the Pacific Northwest (Mayer et al. 1998; Day et al. 1999). Therefore, we decided to modify the methods of Tilmon et al. (2000) to allow for the definitive separation of *P. howardi* from several other *Peristenus* spp. based on restriction endonuclease digestion of PCR amplimers. The specific purpose of this modification was to provide same-season identification of *P. howardi* from parasitized *Lygus* spp.

**Materials and Methods**

Specimens of adult *Hesperus, P. howardi, P. digoneutis* Loan, *P. conradi* Marsh, *P. pallipes*, and *P. pseudopallipes* were autoritatively identified by and obtained from W.H. Day (Beneficial Insects Research Laboratory, USDA-ARS, 501 S. Chapel Street, Newark, DE 19713). They were preserved in 95% ethanol and held in the Insects Research Laboratory, USDA-ARS, 501 S. Chapel Street, Newark, DE 19713). They were preserved in 95% ethanol and held until used for DNA extraction. DNA was extracted from these specimens using the Qiagen DNeasy® Purification System kit (Qiagen Inc., www.qiagen.com) and a modification of the Qiagen procedure was less sensitive in detecting DNA than that of Tilmon et al. (2000). Neither of these studies included *P. howardi*, considered the most important *Peristenus* parasite of *Lygus* spp. in the Pacific Northwest (Mayer et al. 1998; Day et al. 1999). Therefore, we decided to modify the methods of Tilmon et al. (2000) to allow for the definitive separation of *P. howardi* from several other *Peristenus* spp. based on restriction endonuclease digestion of PCR amplimers. The specific purpose of this modification was to provide same-season identification of *P. howardi* from parasitized *Lygus* spp.

To estimate the DNA yield from the above extraction procedure, we devised a microplate procedure based on quantification of DNA by spot testing using ethidium bromide (Sambrook and Russell 2001). Ten µl of 2 mg/ml ethidium bromide in nuclease-free water were added to the appropriate wells of a conical-bottom, polystyrene ELISA plate followed by addition of 5 µl of extracted DNA. Standards were prepared from *E. coli* genomic DNA (Product No. D2001, Sigma-Aldrich, Inc.) in nuclease-free water at concentrations of 0, 1.25, 2.5, 5, 10, and 20 µg/ml and included in every assay. The plate was placed on a transilluminator emitting UV light at 312 nm and the wells of interest were photographed using a digital camera (CoolPix® 990, Nikon, Inc., www.nikon-coolpix.com) with the room lights off. The image was transferred to a computer and converted to gray scale using Paint Shop Pro® version 7.04 (Jasc Software, Inc., www.jasc.com). The gray scale image was opened in SigmaScan Pro® version 5.0 image analysis software (SPSS, Inc., www.systat.com) and the density in the center of each well representing samples and standards was measured using the built-in point intensity tools of the software. The densities of the standards were adjusted by subtracting the density for the 0 µg/ml concentration from those for all concentrations and the results were fitted to an exponential function for estimating the DNA concentrations of the extracted samples.

PCR reactions were carried out in a PowerBlock® 1 thermocycler (Ericomp, Inc., San Diego, CA) equipped with a heated lid using a protocol similar to that described by Tilmon et al. (2000), but adjusted to increase the volumes of all components included in each PCR reaction mixture. A portion of all DNA extracts was diluted 1:5 in nuclease-free water for use as the template in PCR reactions. The primers C1-J-2252 (Tilmon et al. 2000) and TL2-N-3014 (Simon et al. 1994) were synthesized by Integrated DNA Technologies, Inc. (www.idtdna.com), diluted to 6.4 µM in nuclease-free water, and stored in 50 µl aliquots at -25°C. Each 50 µl PCR reaction contained 5 µl of each 6.4 µM primer, 10 µl of 5 mM MgCl₂, 25 µl of Promega PCR Master Mix (Promega Corporation, www.promega.com), and 5 µl of diluted template DNA. Amplification was carried out in 35 cycles of 94°C for 60 seconds, 52°C for 60 seconds, and 72°C for 90 seconds. PCR products were electrophoresed in 10 cm, 1% gels of 1:1 agarose:Synergene [FMC Corporation, Rockland, ME]) in 1x TBE buffer at 57 volts for 3 hours using a Sigma Model E0638 horizontal submarine electrophoresis unit (Sigma-Aldrich, www.sigmaaldrich.com).

PCR products from amplification of *P. digoneutis, P. howardi, P. pallipes*, and *P. pseudopallipes* DNA were sequenced in a LI-COR (www.licor.com) 4000L automated sequencing machine available through the University of Idaho Automated DNA Sequencing Facility. Amplified DNA from *P. conradi* was sequenced commercially by SeqWright DNA Technology Services, Houston, TX. Sequences were verified in both directions to maximize accuracy. Consensus sequences were derived from the chromatograms using PHRED (Ewing et al. 1998) for base calling, PHRAP (Gordon et al. 1998) for sequence assembly, and CONSED (Gordon et al. 1998) for sequence finishing and the resulting sequences were aligned using ClustalX v. 1.83 (Chenna et al. 2003). A phylogenetic tree based on the Hasegawa-Kishino-Yano substitution model (Hasegawa
et al. 1985) was generated using PAUP 4.0b10 (Swofford 1998) with the inclusion of the sequence from Apis mellifera L. (Crozier and Crozier 1993) as a reference and the GenBank sequence for L. lineolaris (Palisot) (AF189240) as an outgroup.

Restriction sites were mapped in these five sequences to identify a restriction endonuclease that would produce species-specific restriction fragment-length polymorphisms (RFLPs) in the respective PCR products. We chose SfcI (restriction site: 5′...CTRYAG...3′) because the predicted fragment lengths provided the clearest separation of P. howardi from the other Peristenus species. PCR products obtained from amplification of all five Peristenus species were digested with SfcI following the protocol supplied with the enzyme (New England Biolabs, Inc., www.neb.com). All 20 µl digestion reactions were carried out in 0.6 ml PCR tubes containing 9 µl of PCR product, 2 µl of NE buffer 4 (50 mM K-acetate, 20 mM Tris-acetate, 10 mM Mg-acetate, 1 mM dithiothreitol, pH 7.9), 8 µl of 0.25 mg/ml BSA, and 1 µl of SfcI. Reactions were incubated for 1 hour at 25°C and the digestion products electrophoresed in 3% Low Range Ultra Agarose (Bio-Rad Laboratories, www.bio-rad.com) gels in 1x TBE buffer at 57 volts for 4 hours.

Results

The Qiagen DNeasy Purification System proved to be very efficient in extracting DNA suitable for PCR amplification from adult Peristenus spp. Microplate quantification revealed that the extraction procedure produced 65-190 ng DNA in the 50 µl of final eluate. We subsequently found that this yield range remained consistent when extracting DNA from Peristenus larvae dissected from parasitized Lygus spp (data not shown). Consequently, the PCR amplifications in these experiments were seeded with approximately 1-4 ng of Peristenus template DNA.

The approximately 760 base pair PCR products from P. conradi, P. digoneutis, and P. pallipes were identical to those produced by Tilmon et al. (2000) using our DNA extracts with the same primers and amplification protocol (Figure 1). Similar PCR products were obtained using template DNA from P. howardi and P. pseudopallipes, indicating that the same region of the cytochrome oxidase I gene in these species was amplified. No PCR products were detected for L. hesperus or the negative extraction control. Consensus sequences of our amplifiers for all five Peristenus spp. are deposited in GenBank under accession numbers AY626370, AY626371, AY626372, AY626373, and AY626374. ClustalX alignment of our sequences for P. conradi, P. digoneutis, and P. pallipes with the approximately 820 base pair sequences published by Tilmon et al. (2000) (AF189243, AF189241, AF189242) resulted in 99.5, 98.3, and 98.3% homology, respectively.

Nucleotide sequencing of the PCR products for P. conradi, P. digoneutis, P. howardi, P. pallipes, and P. pseudopallipes revealed fragment sizes of 766, 757, 758, 762, and 759 base pairs, respectively (Figure 2). For these sequences, we found the phylogenetic relationships presented in Figure 3. The same tree topology was obtained by substituting the sequences published by Tilmon et al. (2000) for P. conradi, P. digoneutis, and P. pallipes.

Based on the SfcI recognition sequence, the predicted restriction-fragment lengths are: 96, 165, and 505 for P. conradi, 39, 60, 102, 117, 181, and 219 for P. digoneutis, 102, 155, 165, and 336 for P. howardi; 165, 237, and 360 for P. pallipes; 163, 237, and 359 for P. pseudopallipes. Electrophoretic banding patterns of SfcI restriction fragments produced by digestion of the PCR products were consistent with these predicted fragment lengths (Figure 4), although fragments less than 150 base pairs were very diffuse and difficult to resolve. The choice of SfcI for digestion resulted in the inability to separate P. pallipes from P. pseudopallipes, but P. howardi was easily distinguishable from all other Peristenus spp. tested.

Discussion

We have successfully extended the PCR protocol of Tilmon et al. (2000) to the amplification of DNA from P. howardi and P. pseudopallipes. Adoption and modification of a commercially available DNA extraction procedure (Qiagen DNeasy Purification System) did not impact the PCR results, but significantly improved our ability to more easily process large numbers of field-collected samples. Moreover, replacement of AluI with SfcI permitted the precise identification of P. howardi, the predominant Peristenus species parasitizing Lygus spp. in the Pacific Northwest. The predicted AluI restriction fragments indicated that P. howardi (76, 108, 574) could not be reliably separated from P. pseudopallipes (42, 66, 79, 572) and P. pallipes (42, 66, 80, 574) with agarose electrophoresis. In addition, we found that AluI did not completely digest P. howardi PCR products, further complicating the definitive identification of this species through RFLP analysis with AluI. This supports the speculation of Tilmon et al. (2000) that restriction site variation might result in the inability to identify new parasitoid species.
Figure 2. ClustalX alignment of the DNA sequences obtained from PCR amplification of the cytochrome oxidase I gene of *Peristenus* spp. with primers C1-J-2252 and TL2-N-3014. SfcI cleavage sites are highlighted in red. (Continued on page 5).
within the genus *Peristenus*. The restriction fragments produced by digestion with either *Alu*I or *Sfc*I did not allow for the separation of *P. pallipes* and *P. pseudopallipes*, but *Sfc*I digestion accurately separated *P. howardi* from all other *Peristenus* species tested, fulfilling the primary objective of this research.

Our PCR protocol for *Peristenus* DNA amplification calls for relatively large volumes of all reagents in the reaction mixture. This was an intentional goal to reduce potential pipetting errors when the procedure is applied to routine analysis of large numbers of field-collected samples. This was accomplished by preparing five-fold dilutions of the template DNA and diluting the primers to 6.4 µM. Using 5 µl of undiluted template DNA proved to be excessive as the PCR reaction with this amount of template often produced little or no amplimer. Additional MgCl₂ was necessary as the concentration present in the Promega PCR Master resulted in 1.5 mM MgCl₂ in the final reaction mixture. We found that MgCl₂ concentrations less than 2.5 mM produced inconsistent amplification results.

**Figure 2.** (Continued from page 4).
Mowry TM, Barbour JD. 2004. Distinguishing the parasitic wasp, *Peristenus howardi*, from some of its congeners using polymerase chain reaction and restriction endonuclease digestion. 7pp. *Journal of Insect Science*, 4:25, Available online: insectscience.org/4.25

Peristenus howardi was first detected and described as a new species in 1997 from parasitized *L. hesperus* collected in Idaho and parasitism rates can reach 80-100% in untreated alfalfa seed fields (Day et al. 1999; JDB, personal observation). However, the host and geographic ranges of *P. howardi* are largely unknown, as is the potential for use of this native species for biological control of *Lygus* spp. in seed, fruit, vegetable, and forage crops in the Pacific Northwest. Prior to this report, identification of *P. howardi* through molecular techniques had not been accomplished. This extension of a species-specific PCR protocol coupled to SfcI endonuclease digestion provides a useful tool for studying *Peristenus* incidence and distribution within *Lygus* spp. populations. In addition, it will greatly reduce the time required for sample identification and the loss of data resulting from mortality incurred during the rearing of larval parasites.

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