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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 *Sfc*I endonuclease produced restriction fragments with banding patterns in agarose gel electrophoresis that readily separated *P. howardi*, *P. digoneutis*, *P. conradi*, *P. pallipes*, and *P. pseudopallipes*.. 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 *L. Hesperus*, *P. howardi*, *P. digoneutis* Loan, *P. conradi* Marsh, *P. pallipes*, and *P. pseudopallipes* were authoritatively 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 at -25°C until used for DNA extraction. DNA was extracted from all insect species using the Qiagen DNeasy® Purification System kit (Qiagen Inc., www.qiagen.com) and a modification of the Qiagen protocol for isolation of genomic DNA from insects. A whole, adult insect was placed in an autoclaved 1.5 ml microcentrifuge tube containing 180 µl of buffer ATL (from kit), 20 µl of 20 mg/ml proteinase K (from kit), and 40 µl of 10 mg/ml RNase A (Product No. R6513, Sigma-Aldrich, Inc., www.sigmaaldrich.com) and homogenized by hand with disposable microtube pestles. The homogenate was incubated in water bath at 55°C for 4 hours, after which 200 µl of buffer AL (from kit) was added and the tube vortexed. The homogenate was incubated at 70°C for 10 minutes after which 200 µl of 100% ethanol was added and the tube vortexed. The entire mixture was transferred to a DNeasy® spin column in a 2 ml collection tube and centrifuged at 6000 x *g* for 1 minute. The spin column was transferred to a new collection tube, 500 µl of buffer AW1 (from kit) added, and again centrifuged at 6000 x *g* for 1 minute. The spin column was again transferred to a new collection tube, 500 µl of buffer AW2 (from kit) added, and the centrifugation repeated. Finally, the spin column was transferred to an autoclaved 1.5 ml microcentrifuge tube (with lid removed), 50 µl of nuclease-free water added, incubated for 30 minutes at room temperature, and centrifuged at 6000 x *g* for 1 minute. The eluate containing extracted DNA was stored at 4°C until used as a PCR template. Negative controls were prepared in parallel with all extractions by performing the above procedure without insect material included.

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® I 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 $MgCl_2$, 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 (1:1 agarose:Synergel [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 *Sfc*I (restriction site: 5'…C$^{\nabla}$TRYAG…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 *Sfc*I 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 *Sfc*I. 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.

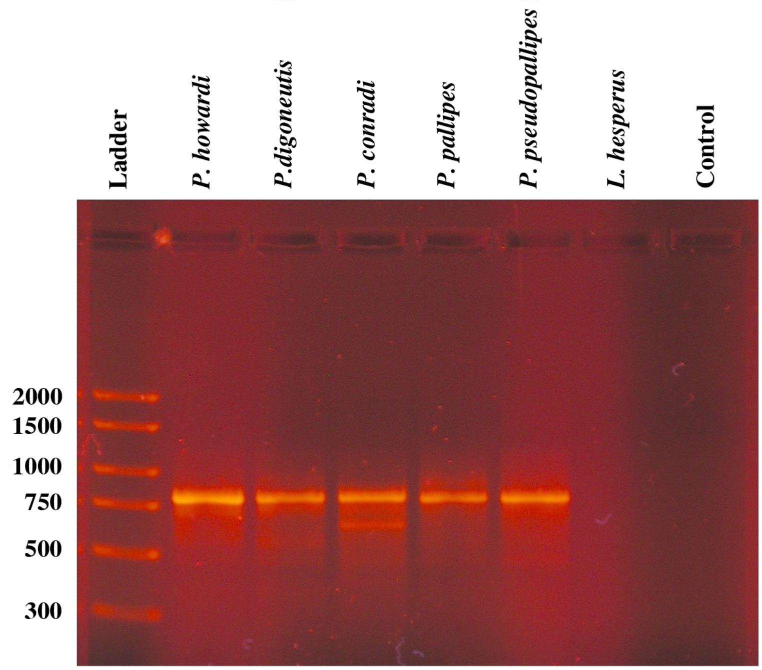

Figure 1. Banding patterns of PCR products obtained from amplification of the cytochrome oxidase I gene of *Peristenus* spp. with primers C1-J-2252 and TL2-N-3014.

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 amplimers 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 *Sfc*I 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 *Sfc*I 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 *Sfc*I 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 *Alu*I with *Sfc*I permitted the precise identification of *P. howardi*, the predominant *Peristenus* species parasitizing *Lygus* spp. in the Pacific Northwest. The predicted *Alu*I 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 *Alu*I did not completely digest *P. howardi* PCR products, further complicating the definitive identification of this species through RFLP analysis with *Alu*I. This supports the speculation of Tilmon *et al.* (2000) that restriction site variation might result in the inability to identify new parasitoid species

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P. howardi          ---CGCGCAAAGGACGTTAAGGTGTTTCTCC-TAATTTATGCTATAATAA 46
P. pseudopallipes   -CCGGCACTTCAGCAGGTGAGGTGTTTGTCCATAATTTATGCTATAATAA 49
P. pallipes         AGAGAAAAAAAGAACATTTGGTTGTATGGGCCTAATTTATGCTATAATAA 50
P. digoneutis       ---GGTAAAAGAAACATTCGGTTGTATAGGTATAATTTATGCTATAGTAA 47
P. conradi          GGGGAAAAAAGAGACATTTGGATGTTTAGGAATAATTTATGCTATAATAA 50

P. howardi          CAATTGGCATTTTAGGATTTATTGTTTGAGCTCATCATATATTTACAGTT 96
P. pseudopallipes   CAATTGGAATTTTAGGATTTATTGTTTGAGCTCATCATATATTTACAGTT 99
P. pallipes         CAATTGGAATTTTAGGGTTTATTGTTTGAGCTCATCATATATTTACAGTT 100
P. digonuetis       CTATTGGTATTTTAGGATTTATTGTTTGAGCACATCATATATTTACAGTT 97
P. conradi          CAATTGGAATCTTAGGATTTATTGTTTGAGCTCATCACATATTTACTGTA 100

P. howardi          GGAATGGATATTGATACACGAGCCTATTTTACTTCTGCTACTATAATTAT 146
P. pseudopallipes   GGTATGGATATTGATACACGAGCTTATTTTACTTCTGCTACTATAATTAT 149
P. pallipes         GGTATGGATATTGATACACGAGCTTATTTTACTTCTGCTACTATAATTAT 150
P. digonuetis       GGGATAGATATTGATACTCGGGCTTATTTTACTTCTGCTACAATAATTAT 147
P. conradi          GGGATAGATATTGATACACGAGCTTATTTTACTTCTGCTACAATAATTAT 150

P. howardi          TGCCGTTCCTACAGGGATTAAAATTTTTAGTTGGTTAGCTACATTTAGTG 196
P. pseudopallipes   TGCTGTTCCTACGGGGATTAAAATTTTTAGTTGGTTAGCTACATTTAGTG 199
P. pallipes         TGCTGTTCCTACGGGGATTAAAATTTTTAGTTGGTTAGCTACATTTAGTG 200
P. digonuetis       TGCTGTTCCTACAGGAATTAAAATTTTTAGTTGATTAGCTACATTTAGAG 197
P. conradi          TGCGGTACCTACTGGAATTAAAATTTTTAGTTGATTAGCGACATTTAGGG 200

P. howardi          GAGTAAAAATAAAATATAATTTAAGAATTTTGTGATCAATAGGTTTTATT 246
P. pseudopallipes   GAGTAAAAATAAAATATAATTTAAGAATTTTGTGATCGATAGGTTTTATT 249
P. pallipes         GAGTAAAAATAAAATATAATTTAAGAATTTTGTGATCGATAGGTTTTATT 250
P. digonuetis       GTGTAAAAATAAAATATAATTTAAGAATTTTATGAGCAATAGGATTTATT 247
P. conradi          GTATAAAAATAAAATATAATTTAAGAATTTTATGATCGATAGGATTTATT 250

P. howardi          TTTTTATTTACTATAGGGGGATTAACAGGAGTAGTATTATCAAATTCTTC 296
P. pseudopallipes   TTTTTATTTACCATAGGGGGATTAACAGGAGTAGTATTATCAAATTCTTC 299
P. pallipes         TTTTTATTTACCATAGGTGGTTTAACAGGGGTAGTATTATCAAATTCTTC 300
P. digonuetis       TTTTTATTTACTATAGGGGGTTTAACAGGAGTTGTTTTATCAAATTCTTC 297
P. conradi          TTTTTATTTACTATAGGAGGTCTAACAGGAGTAGTTTTATCTAATTCTTC 300

P. howardi          TGTTGATTTACTTTTACATGATACATATTATGTTGTTGCTCATTTTCATT 346
P. pseudopallipes   TGTTGACTTACTTTTACATGATACATATTATGTTGTTGCTCATTTTCATT 349
P. pallipes         TGTTGACTTACTTTTACATGATACATATTATGTTGTTGCTCATTTTCATT 350
P. digonuetis       TGTAGATTTACTTTTACATGATACTTATTATGTTGTCGCTCATTTTCATT 347
P. conradi          TGTTGACTTGCTTTTACATGATACTTATTATGTTGTTGCTCATTTTCATT 350

P. howardi          ATGTTCTTTCTATGGGGGCTGTATTTTCAATTATTGGTGGATTAATTTTT 396
P. pseudopallipes   ATGTTCTTTCTATAGGGGCTGTATTTTCAATTATTGGTGGATTAATTTTT 399
P. pallipes         ATGTTCTTTCTATAGGGGCTGTATTTTCAATTATTGGTGGATTAATTTTT 400
P. digonuetis       ATGTTCTTTCTATAGGAGCTGTATTTTCAATTATTGGTGGATTAATTTTT 397
P. conradi          ATGTTTTATCTATGGGTGCAGTATTTTCTATTATTGGGGGCTTAATTTTT 350

P. howardi          TGATATCCTTTATTTACAGGGGTATCCTTAAATGAAAAATGATTAAAAAT 446
P. pseudopallipes   TGATATCCCTTATTTACAGGGGTATCTTTAAATGAAAAATGATTAAAAAT 449
P. pallipes         TGATATCCCTTATTTACAGGGGTATCTTTAAATGAAAAATGATTAAAAAT 450
P. digonuetis       TGATATCCCCTATTTACAGGTTTATCATTAAATGATAAATGATTAAAAAT 447
P. conradi          TGATATTCGTTATTTACAGGGGTATCATTAAATAAAAAATGATTAAAAGT 450
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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*).

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P. howardi          TCATTTTTATTTAATTTTTATTGGTGTAAATATAACTTTTTTCCCCAAC 496
P. pseudopallipes   TCATTTTTATTTAATTTTTATTGGTGTAAATATAACTTTTTTCCCCCAAC 499
P. pallipes         TCATTTTTATTTAATTTTTATTGGTGTAAATATAACTTTTTTCCCCCAAC 500
P. digonuetis       TCATTTTTATTTAATTTTTATTGGTGTAAATATAACTTTTTTCCCTCAAC 497
P. conradi          TCATTTTTATTTAATTTTTATTGGGGTAAATATAACTTTTTTCCCACAAC 500

P. howardi          ATTTTTTAGGTTTAAGAGGGATACCTCGACGATATAGGGATTATCCTGAT 546
P. pseudopallipes   ATTTTTTAGGTTTAAGAGGGATACCTCGACGATATAGGGATTATCCTGAT 549
P. pallipes         ATTTTTTAGGTTTAAGAGGGATACCTCGACGATATAGAGATTATCCTGAT 550
P. digonuetis       ATTTTTTAGGATTAAGAGGGATACCTCGGCGGTATAGGGATTATCCTGAT 547
P. conradi          ATTTTTTAGGGTTGAGAGGGATACCTCGTCGGTATAGAGATTATCCGGAT 550

P. howardi          ATATATATAAATTGAAATATTTTATCTTCTATTGGGTCAATTATTTCTAT 596
P. pseudopallipes   ATATATATAAATTGAAATATTTTATCTTCTATTGGGTCAATTATTTCTAT 599
P. pallipes         ATATATATAAATTGAAATATTTTATCTTCTATTGGGTCAATTATTTCTAT 600
P. digonuetis       ATATATATAAATTGAAATTTATTATCTTCTATAGGATCAATTATTTCTAT 597
P. conradi          ATGTATATAATATGAAATATTTTATCATCAATAGGTTCAATTATTTCTAT 600

P. howardi          AGTTGGAATATTGTATTTTATTTATATTATTTGAGAAAGATTAGTTAGTA 646
P. pseudopallipes   AGTTGGTATATTGTATTTTATTTATATTATTTGAGAAAGGTTAGTTAGTA 649
P. pallipes         AGTTGGTATATTGTATTTTATTTATATTATTTGAGAAAGGTTAGTTAGTA 650
P. digonuetis       AATTAGAATATTATATTTTATTTATATTATTTGAGAGAGGTTGGTCAGAA 647
P. conradi          AATTGGTATATTATATTTTATTTATATTATTTGAGAAAGTTTAGTGAGGA 650

P. howardi          AGCGGTATATAATTTATAATAAGTATATAAATACTTCTATTGGATGGTTA 696
P. pseudopallipes   AGCGGCATATAATTTATAATAAGTATATAAATACTTCTATTGAATGGTTA 699
P. pallipes         AGCGGCATATAATTTATAATAAGTATATAAATACTTCTATTGAATGGTTA 700
P. digonuetis       AGCGATATGTAATTTTTAATAAATATATAAATACTTCAATTGAATGAGTA 697
P. conradi          AACGTTATATAATTTTTAATAAATATATGAATTCTTCTATTGAATGATTT 700

P. howardi          CAAATGTATCCTCCTCAATTTCACAGGTATAATCAATTACCTCTAATATT 746
P. pseudopallipes   CAAAGGTATCCCCCTCAATTTCACAGATATATTAGAGTACCTCTAAT-TT 748
P. pallipes         CAAATGTATCCCCCTCAATTTCATAGATATAATCACTTACCTTTAAGTTG 750
P. digonuetis       CAAATATATCCTCCTCAATTTCACAGGTATATTCAA-TACCCATTAA-TT 745
P. conradi          CAGATATATCCTCCCCAATATCATAGTTATAATCAATTACCATTAATTTT 750

P. howardi          AAAAAATAATAG---- 758
P. pseudopallipes   TAAAAAAACCC----- 759
P. pallipes         AAAAAATACTTC---- 762
P. digonuetis       CATAGGCACTTC---- 757
P. conradi          TAAAAATATTAAATTT 766
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Figure 2. (*Continued from page 4*).

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 $MgCl_2$ was necessary as the concentration present in the Promega PCR Master resulted in 1.5 mM $MgCl_2$ in the final reaction mixture. We found that $MgCl_2$ concentrations less than 2.5 mM produced inconsistent amplification results.

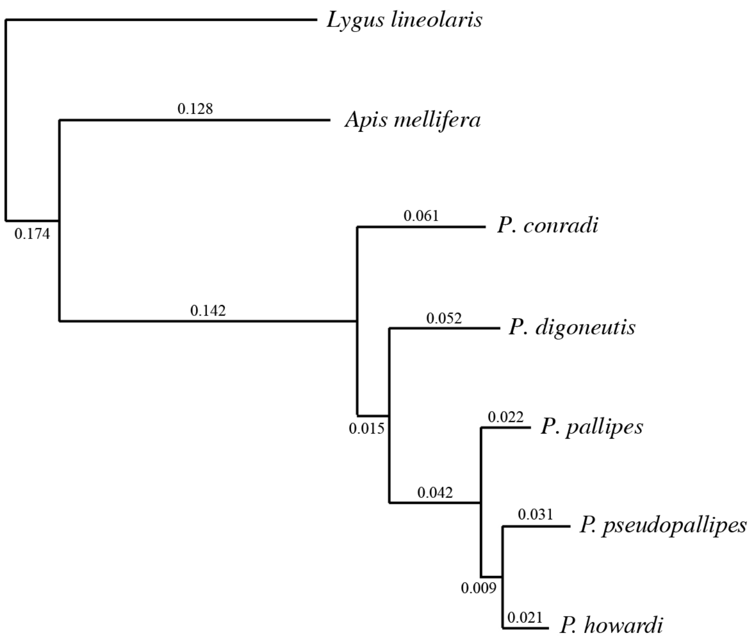

Figure 3. Phylogenetic tree of *Peristenus* spp. derived from PCR amplification of the cytochrome oxidase I gene with primers C1-J-2252 and TL2-N-3014. Distances are based on the Hasegawa-Kishino-Yano substitution model. The same tree topology was obtained using the sequences published by Tilmon *et al.* (2000).

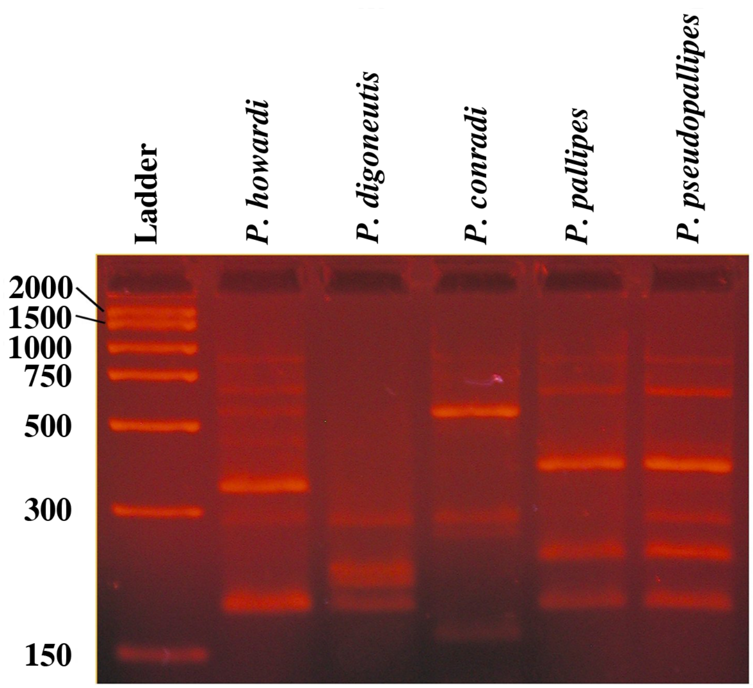

Figure 4. Banding patterns of the *Sfc*I restriction fragments produced by digestion of PCR products obtained from amplification of the cytochrome oxidase I gene of *Peristenus* spp. with primers C1-J-2252 and TL2-N-3014.

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 *Sfc*I 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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References

Braun L, Erlandson M, Baldwin D, Soroka J, Mason P, Footit R, Hegedus D. 2001. Seasonal occurrence, species composition, and parasitism of *Lygus* spp. (Hemiptera: Miridae) in alfalfa, canola, and mustard in Saskatchewan. *The Canadian Entomologist*, 133:565-577.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research*, 31:3497-3500.

Clancy DW, Pierce HD. 1966. Natural enemies of some lygus bugs. *Journal of Economic Entomology*, 59: 853-858.

Crozier RH, Crozier YC. 1993. The mitochondrial genome of the honey bee *Apis mellifera*: Complete sequence and genome organization. *Genetics*, 133:97-117.

Day WH. 1994. Estimating mortality caused by parasites and diseases of insects: comparisons of the dissection and rearing methods. *Environmental Entomology*, 23: 543-550.

Day WH. 1996. Evaluation of biological control of the tarnished plant bug (Hemiptera: Miridae) in alfalfa by the introduced parasite *Peristenus digoneutis* (Hymenoptera: Braconidae). *Environmental Entomology*, 25: 512-518.

Day WH, Baird CR, Shaw SR. 1999. New, native species of *Peristenus* (Hymenoptera: Braconidae) parasitizing *Lygus hesperus* (Hemiptera: Miridae) in Idaho: biology, importance, and description. *Annals of the Entomological Society of America*, 92:370-375.

Day WH, Saunders LB. 1990. Abundance of the garden fleahopper (Hemiptera: Miridae) on alfalfa and parasitism by *Leiophron uniformis* (Gahan) (Hymenoptera: Braconidae). *Journal of Economic Entomology*, 83: 101-106.

Erlandson M, Braun L, Baldwin J, Soroka M, Ashfaq M, Hegedus

D. 2003. Molecular markers for *Peristenus* spp. (Hymenoptera: Braconidae) parasitoids associated with *Lygus* spp. (Hemiptera: Miridae). *The Canadian Entomologist*, 135: 71-83.

Ewing B, Hillier L, Wendl M, Green P. 1998. Base-calling of automated sequencer traces using *Phred*. I. Accuracy Assessment. *Genome Research*, 8:175-185.

Gordon D, Abajian C, Green P. 1998. *Consed*: A graphical tool for sequence finishing. *Genome Research*, 8:195-202.

Hasegawa M, Kishino H, Yano T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, 21:160-174.

Mayer DF, Baird CR, Simko B. 1998. Parasitism of *Lygus* spp. (Hemiptera: Miridae) by *Peristenus* in the Pacific Northwest. *Journal of the Entomological Society of British Columbia*, 95: 53-57.

Musebeck CFW, Krombein KV, Townes HK. 1951. Hymenoptera of America north of Mexico. *Synoptic catalogue United States Department of Agriculture*. *Agricultural Monographs* 2: 1

Peterson SS, Baird CR, Bitner RM. 1992. Current status of the alfalfa leafcutting bee, *Megachile rotundata*, as a pollinator of alfalfa seed. *Bee Science* 2: 135-142.

Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual. Volume 3. Cold Spring harbor Laboratory Press. Cold Spring Harbor, NY.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America,* 87: 651-701.

Sorenson CJ. 1939. *Lygus hesperus* Knight and *Lygus elisus* Van Duzee in relation to alfalfa seed production. *Utah Agricultural Experiment Station Bulletin* 284.

Swofford DL. 1998. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, MA.

Tilmon KJ, Danforth BN, Day WH, Hoffmann MP. 2000. Determining parasitoid species composition in a host population: a molecular approach. *Annals of the Entomological Society of America*, 93: 640-647.

Xu G, Brindley WA. 1994. Esterase isozymes in *Lygus hesperus*: characterization and relationship with organophosphate resistance. *Pesticide Science*, 42: 273-289.