HIGH-FIDELITY PCR ASSAY DISCRIMINATES BETWEEN IMMATURE LIPOLEXIS OREGMAE AND LYSIPHLEBUS TESTACEIPES (HYMENOPTERA: APHIDIIDAE) WITHIN THEIR APHID HOSTS

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HIGH-FIDELITY PCR ASSAY DISCRIMINATES BETWEEN IMMATURE *LIPOLEXIS OREGMAE* AND *LYSIPHLEBUS TESTACEIPES* (HYMENOPTERA: APHIDIIDAE) WITHIN THEIR APHID HOSTS

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ABSTRACT

Species-specific molecular markers were developed to identify and distinguish between two parasitoids of the brown citrus aphid, *Toxoptera citricida* Kirkaldy, in Florida. PCR primers were developed for *Lysiphlebus testaceipes* Cresson and *Lipolexis oregmae* Gahan (= *scutellaris* Mackauer) with DNA sequences from the internal transcribed spacer (ITS) region between the 5.8S and 28S nuclear rRNA genes. With High-fidelity PCR, the *L. testaceipes*-specific primer produced a 520-bp band while that of *L. oregmae* resulted in a 270-bp band. Eggs of both parasitoids within their aphid hosts could be detected by 6 h after oviposition, but 100% detection rates only occurred after 24 h. A sensitivity analysis indicated that a parasitoid egg within a single aphid could be detected 100% of the time when combined with DNA from up to 36 unparasitized aphids. A single first instar parasitoid could be detected by High-fidelity PCR when the parasitized aphid was combined with up to 500 unparasitized aphids, indicating a high level of sensitivity. Species-specific primers detected both immature parasitoid species within aphids commonly found in citrus in Florida, including *Aphis craccivora* Koch, *Aphis gossypii* Glover, *Aphis spiraecola* Patch, *Toxoptera aurantii* Boyer and *T. citricida*. This High-fidelity PCR assay provides an efficient method to monitor establishment of *L. oregmae* in citrus groves in this classical biological control program in Florida.

Key Words: High-fidelity PCR, *Toxoptera citricida, Lysiphlebus testaceipes*, *Lipolexis oregmae*, citrus

RESUMEN

Los marcadores moleculares específicos de las especies fueron desarrollados para identificar y distinguir entre dos parasitoides del áfido pardo de los cítricos, *Toxoptera citricida* Kirkaldy, en la Florida. Se desarrollaron cebadores (= primers) de PCR para *Lysiphlebus testaceipes* Cresson y *Lipolexis oregmae* Gahan (= *scutellaris* Mackauer) usando secuencias de ADN de la región del separador transcrito interna (STI) entre los genes 5.8S y 28S del rARN nuclear. Usando PCR de Alta-fidelidad, el cebador específico de *L. testaceipes* produjo una banda de 520-bp (pares de bases) mientras que el de *L. oregmae* resultó en una banda de 270-bp. Los huevos de ambos parasitoides dentro de sus hospederos áfidos pudieron ser detectados a los 6 horas después de la oviposición, pero una tasa de 100% de detección solamente ocurrio después de 24 horas. Un analísis de sensibilidad indicó que un huevo del parasitoide dentro de un solo áfido podian ser detectado 100% de las veces cuando fué combinado con ADN de hasta con 36 áfidos no parasitados. Un solo parasitoide en la primera estadia podia ser detectado por el PCR de Alta-fidelidad cuando el áfido parasitado fué combinado con hasta 500 áfidos no parasitados, indicando un alto nivel de sensibilidad. Los cebadores específicos de las especies detectaron ambas especies de parasitoides inmaduros dentro los áfidos encontrados frecuentemente en los cítricos en la Florida, incluyendo *Aphis craccivora* Koch, *Aphis gossypii* Glover, *Aphis spiraecola* Patch, *Toxoptera aurantii* Boyer y *T. citricida*. Este ensayo de PCR de Alta-fidelidad provee un método eficaz para realizar un monitoreo del establecimiento de *L. oregmae* en los huertos de cítricos en este programa de control biológico clasico en la Florida.

The brown citrus aphid, *Toxoptera citricida* Kirkaldy (Homoptera: Aphididae), currently occurs throughout Florida and is a threat to citrus because it is the most efficient aphid vector of citrus tristeza virus. In an effort to control *T. citricida*, the parasitoid *Lipolexis oregmae* (Gahan) (= *scutellaris* Mackauer, Miller et al. [2002]) (Hymenoptera: Aphidiidae) was imported, mass reared and released in a classical biological control program (Hoy & Nuygen 2000). Another aphidiid, *Lysiphlebus testaceipes* Cresson, is abundant in citrus groves and also parasitizes *T. citricida*.

Sampling for *L. oregmae* is difficult because mummified *T. citricida* containing *L. oregmae* are found off the citrus plant (Hill & Hoy 2003). The majority of mummies of *T. citricida* containing *L. testaceipes* also may occur off citrus foliage (Persad & Hoy 2003a). Thus, collection of *T. citricida* on foliage before mummification has occurred is necessary to monitor for establishment and abundance of *L. oregmae*.

To determine if *L. oregmae* has established, aphids on foliage were collected in citrus groves and held in air-inflated plastic bags in the labora-

tory for 7-9 d so that adult parasitoids could emerge. With this technique, adults of *L. oregmae* and *L. testaceipes* emerged from field-collected samples taken from citrus groves throughout Florida. However, because mortality of immature parasitoids may occur under these conditions due to mold, it is likely that the abundance of *L. oregmae* is underestimated, resulting in loss of critical data.

Dissections and microscopic examinations of immature parasitoids of both species revealed that they are similar in appearance after the first instar (Persad & Hoy, unpublished data) and, thus, morphology is not adequate to resolve the identity of immature parasitoids within field-collected aphids.

To resolve these problems, we developed and evaluated a molecular assay to detect immature *L. testaceipes* and *L. oregmae* within *T. citricida* and other aphid hosts found on citrus in Florida.

Materials and Methods

Cultures

Cultures of *T. citricida* and *L. oregmae* were maintained on potted citrus in 63 × 63 × 63 cm mesh cages in the laboratory at 22-24°C and 55-65% RH and 16 L: 8 D as described by Hill & Hoy (2003) and Walker (2002). Cultures of *L. testaceipes* were initiated from field-collected populations of parasitoids on *T. citricida* in citrus groves throughout Florida (Persad & Hoy 2003a). Adult parasitoids that emerged were held in batches of 20-30 individuals in 2.5 × 6 cm plastic vials with moistened honey-saturated paper strips for 24 h. One batch of *L. testaceipes* was released onto nine potted citrus plants that were each infested with 250-300 aphids of mixed instars in a 63 × 63 × 63 cm mesh cage in the laboratory under similar conditions.

DNA Extractions

Genomic DNA from individual adults of *L. testaceipes*, *L. oregmae* and *T. citricida* was extracted with PUREGENE reagents by the method suggested by the manufacturer (Gentra Systems, Minneapolis, MN) and resuspended in 50 µl sterile water. For screening large populations of field-collected specimens, genomic DNA was extracted from batches of adults by grinding in 50µl Chelex (Bio-Rad, Hercules, CA) resin and treating the extracts for 1 h at 60°C and 5 min at 94°C (Edwards & Hoy 1993). One microliter of PUREGENE or Chelex preparations was used for High-fidelity PCR.

Primers

PCR primers for the amplification of four insect mitochondrial gene fragments (12S, 16S, COI and NADH) (Kambhampati & Smith 1995) and nuclear rRNA primers (5.8S-F and 28 S-R) (Porter & Collins 1991) (Table 1) were used to amplify extracted DNA.

High-fidelity PCR Protocol

High-fidelity PCR was performed in a 50-µl reaction volume containing 50 mM Tris, pH 9.2, 16 mM ammonium sulfate, 1.75 mM $MgCl_2$, 350 µM each of dATP, dGTP, dCTP, dTTP, 800 pmol of primers, 1 unit *Tgo* DNA polymerase and 5 units of *Taq* DNA polymerase (Roche Molecular Biochemicals) (Barnes 1994). Reactions were overlaid with 50 µl of mineral oil and High-fidelity PCR was conducted with three linked temperature profiles: (i) To eliminate possible template secondary structure, hot-start PCR at 94°C for 2 mins was used for 1 cycle followed by (ii) 10 cycles, each consisting of denaturation at 94°C for 10 s, annealing at 49°C for 30 s and 40°C for 30 s for

Table 1. Primers used for amplification of *Lysiphlebus testaceipes*, *Lipolexis oregmae* and *Toxoptera citricida* DNA.

Primer	Sequence	Gene segment
SR-J-14199	5'-TACTATGTTACGACTTAT- 3'	Mitochondrial 12S rRNA
SR-N-14594	5'-AAACTAGGATTAGATACCC-3'	"
LR-J-13017	5'-TTACGCTGTTATCCCTAA-3'	Mitochondrial 16S rRNA
LR-N-13398	5'-CACCTGTTTAACAAAAACAT-3'	"
CI-J-1632	5'-TGATCAAATTTATAAT-3'	Mitochondrial CO I
CI-N-2191	5'-GGTAAAATTAAAATATAAACTTC-3'	"
N5-J-7502	5'-CTAAAGTTGATGAATGAACTAAAG-3'	Mitochondrial NADH+ NADH5
N4-N-8925	5'-GCTCATGTTGAAGCTCC-3'	"
5.8 S-F	5'-GTGAATTCTGTGAACTGCAGGACACATGAAC-3'	Nuclear rRNA ITS-2
28 S-R	5'-ATGCTTAAATTTAGGGGGTA-3'	"
LO-ITSF	5'-GGCCAGTTGTCGAGTCC-3'	ITS-2
LT-ITSF	5'-CTAGCGATAAATGAATGTTC-3'	"

LO = *Lipolexis oregmae* and LT= *Lysiphlebus testaceipes*

ITS-2 and mitochondrial gene segments, respectively, and elongation at 68°C for 1 min 20 s and (iii) 20 cycles, each consisting of denaturation at 94°C for 10 s, annealing at 49°C for 30 s, and extension at 68°C for 1 min 20 s plus an additional 20s for each consecutive cycle. PCR products were separated on a 2% agarose gel, stained with ethidium bromide and photographed under UV light.

The PCR products were ligated into a PCR 2.1 TOPO vector and used to transform competent One Shot *E. coli* cells with subsequent ampicillin selection following the manufacturer's directions (Invitrogen, Carlsbad, CA). Clones were incubated overnight in Luria-Bertani (LB) medium on plates containing ampicillin, IPTG and X-gal. Sixteen clear colonies were randomly picked from each plate and separately cultured for 16 h in 5 ml of LB medium. Plasmid DNA was extracted with a QIAGEN Plasmid Mini-prep Kit (QIAGEN, Inc., Valencia, CA). All plasmids were incubated and digested with *Eco*R1 and visualized on a 1% agarose gel to verify that the inserts corresponded to the expected size of the PCR products. Three clear colonies containing plasmids with the inserts were recultured in 50 ml of LB medium with ampicillin for each species. Plasmids were extracted with QIAGEN Plasmid Midi-prep kits. DNA inserts were sequenced with a Perkin-Elmer Applied Biosystems ABI PRISM Automated DNA sequencer located at the University of Florida Interdisciplinary Center for Biotechnology, Research Core Facility.

Accuracy of Species-Specific Primers

Once primers were designed based on the sequences obtained, ten adults each of *L. testaceipes* and *L. oregmae* and each of 10 third instars of *T. citricida* were placed individually into 0.5-ml thick-walled eppendorf tubes each containing 50 µl of 5% Chelex resin suspension. A pestle was made by slowly heating a standard pipette tip which was then inserted into an empty 0.5-ml eppendorf tube so that the tip assumed the shape of the base of the tube to form a close-fitting pestle. New pestles were used to grind each adult specimen, a procedure which lasted 30 to 40 sec. After grinding, each tube was placed in a water bath at 60°C for one h. The tubes were collected and placed in a Perkin-Elmer DNA Thermal Cycler model 480 at 94°C for 5 mins after which samples were centrifuged for 30 sec.

High-fidelity PCR was used to evaluate the specificity of the assay by subjecting 10 replicates of DNA from *L. testaceipes*, *L. oregmae* and *T. citricida* to either primer. PCR products were separated on a 2% agarose gel, stained with ethidium bromide and photographed under UV light.

Detection of Parasitoid Eggs and First-instars

Third instars of *T. citricida* were exposed to single oviposition opportunities by each parasitoid in petri-dish arenas and returned to potted citrus plants in the laboratory. After periods of 6, 12, 18, 24 and 48 h during which aphids were exposed to parasitoids, individual aphids were ground in 50 µl of Chelex and incubated for 1 h to extract DNA from the parasitoid eggs. Under laboratory rearing conditions, eggs of *L. testaceipes* and *L. oregmae* hatch after 55 h and 75 h, respectively (Persad & Hoy 2003b). Ten aphids were exposed to each parasitoid species and evaluated by the High-fidelity assay for each of the five-time intervals. Sub-groups of exposed aphids were routinely dissected 4 d after assumed oviposition and, if parasitism was below the expected 98-100% (Persad & Hoy 2003b), then the group and the PCR results observed were rejected.

The experiment was repeated to detect parasitoid larvae 70 h after exposing aphid hosts to *L. testaceipes* or *L. oregmae*, respectively (first instars eclose at 55 and 61 h, respectively, Persad & Hoy 2003b). High-fidelity PCR was used to determine the presence of eggs or larvae of both parasitoid species in each of 10 trials with the aim of finding the earliest time after the oviposition opportunity when 100% of 10 trials resulted in detection of parasitoid eggs or larvae in *T. citricida.*

Other Hosts of *L. testaceipes* and *L. oregmae* and Sensitivity of the Assay

Because other aphids are found in citrus groves in Florida and both *L. testaceipes* and *L. oregmae* are known to parasitize aphids other than *T. citricida* (Fasulo & Halbert 1998; Hoy & Nguyen 2000), four additional aphid species were collected from the field and greenhouse cultures were initiated. *Aphis spiraecola* Patch, *A. gossypii* Glover, and *Toxoptera aurantii* Boyer were maintained on potted citrus, while *A. craccivora* Koch was cultured through several generations on potted eggplants.

In a preliminary experiment, 10 third instars of each aphid species were exposed individually to females of either *L. testaceipes* or *L. oregmae* in petri-dish arenas. After exposure for 24 h, High-fidelity PCR assays were conducted and these confirmed that parasitoid DNA was present in all exposed aphids.

Because field samples may involve hundreds or thousands of aphid individuals of different species, we wanted to determine whether aphids could be pooled, yet still yield qualitative data for presence/absence of *L. testaceipes* or *L. oregmae*. Ten replicates of High-fidelity PCR were conducted on samples containing ratios of one parasitized (24 h after oviposition opportunity) to nine unparasitized third instars of brown citrus

aphids. Incremental increases of nine unparasitized aphids (ratios of 1: 9, then 1: 18, followed by 1: 27, etc.) were evaluated with High-fidelity PCR until detection dropped from 100% to under 50%.

Interspecific Interactions of *L. testaceipes* and *L. oregmae*

Each of eight third instars of *T. citricida* was exposed individually to an *L. oregmae* female in a petri-dish arena, and immediately afterwards the same aphid was exposed to a *L. testaceipes* female in the method described by Persad & Hoy (2003b). After the oviposition opportunities, the aphid was returned to a potted citrus plant for 24 h. The experiment was repeated with the reverse oviposition sequence. The DNA from single aphids in all trials was extracted with Chelex and High-fideltiy PCR was performed on each to determine if the DNA from more than one parasitoid within a single aphid would affect the specificity of the *L. testaceipes* or *L. oregmae* primer.

Other Parasitoids

To determine whether the presence of DNA from other parasitoid species would give false positives we tested additional aphid parasitoids, some of which may occur in Florida; these included *Lysiphlebus japonica* Ashmead (obtained in 70% alcohol, from the Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville), *Aphelinus gossypi* Timberlake, *Aphidius colemani* Vierick, *Aphidius ervi* Haliday and *Aphidius matricariae* Haliday, (all obtained alive from BioBest International) and the hyperparasitoids *Alloxysta megourae* complex and *Pachyneuron aphidisi* Bouche (field-collected live specimens from Florida, identified by guidelines of Evans and Stange [1997]). Ten specimens of each parasitoid species were ground individually in 50 µl Chelex and the DNA was tested with the primers for both *L. testaceipes* and *L. oregmae* in High-fidelity PCR.

Results and Discussion

Primers

Mitochondrial 12S and NADH primers produced no discernible PCR products when used with DNA from *L. testaceipes* and *L. oregmae*. Only the 16S and COI primers produced discernible DNA bands of the expected size (data not shown). The PCR products obtained with the16S primers were cloned and sequenced (GenBank accession numbers AY498553 through and including AY498558) because sequence differences are expected to be higher than with the COI fragment (Simon et al. 1994). The entire 0.55 kb ITS-2 PCR products of *L. oregmae* were cloned and sequenced but only 398 bp of the 0.75 kb product from *L. testaceipes* could be sequenced because the 3' end was 'AT' rich and difficult to sequence. The cloned sequences of the PCR products from *T. citricida*, *L. testaceipes* and *L. oregmae* were aligned by CLUSTAL W. Unfortunately the 16 S rRNA sequences from *L. testaceipes* and *L. oregmae* displayed low sequence divergence (14.8%) and no species-specific primers could be designed. However, the sequence divergences of the nuclear ITS-2 region for *L. testaceipes* and *L. oregmae* and *T. citricida* were high, allowing species-specific forward primers to be designed (Table 1). An *L. oregmae*-specific forward primer (LO-ITSF 5'-GGCCAGTTGTCGAGTCC-3') and an *L. testaceipes*-specific forward primer (LT-ITSF 5'-CTAGCGATAAATGAATGTTC-3') were designed after obtaining the complete ITS-2 sequence from *L. oregmae* (Gen Bank accession no. AY498553) and a partial ITS-2 sequence from *L. testaceipes* (Gen Bank accession no. AY498554). PCR products from *L. testaceipes*-specific primers produced 520 bp bands while those of *L. oregmae* produced 270 bp bands (Fig. 1).

Accuracy of Species-Specific Primers

Both primers yielded bands specific to the respective adult parasitoid in each of the 10 replicates and both primers failed to detect DNA from *T. citricida* (Fig. 1).

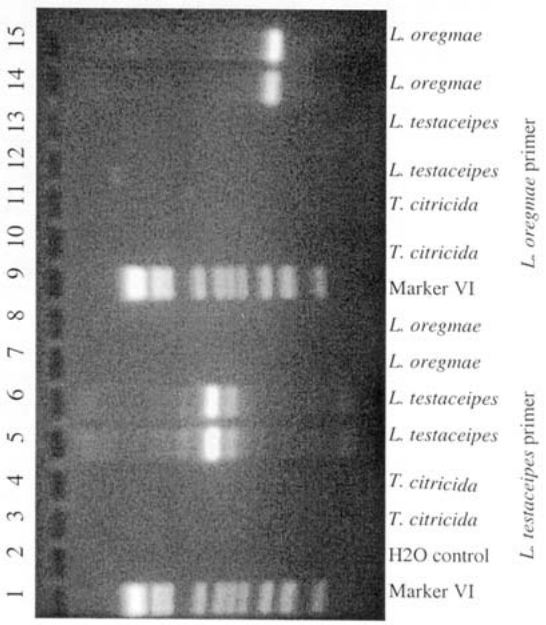

Fig. 1. High-fidelity PCR products obtained with parasitoid species-specific primers and DNA extracted from adults of *Lysiphlebus testaceipes* (520 bp), *Lipolexis oregmae*(270bp) and *Toxoptera citricida* (no bands).

Detection of Parasitoid Eggs and First-instars

As early as 6 h after exposure to *L. testaceipes* and *L. oregmae*, 34 and 46%, respectively, of the exposed third instars of brown citrus aphids produced PCR products. For both parasitoids, all aphids that contained eggs could be detected 24 h after oviposition opportunities (Fig. 2). Because parasitoid larvae contain more DNA than parasitoid eggs, all aphids containing first instars yielded PCR products with their respective primers in all trials with High-fidelity PCR (data not shown).

Other Hosts of *L. testaceipes* and *L. oregmae* and Sensitivity of the Assay

Table 2 indicates that six species of aphids commonly associated with citrus in Florida were all parasitized by *L. oregmae* when single oviposition opportunities were allowed in the laboratory. The data suggest that *L. oregmae* can parasitize and successfully complete its life cycle in all aphid hosts tested and thus may parasitize other aphid species in Florida citrus groves. This oligophagous nature of *L. oregmae* is expected (Stary & Zeleny 1983; Hoy & Nguyen 2000) and, the ability to survive on other pest aphids may be advantageous to the parasitoid. Field collections of aphids for studies on the establishment of *L. oregmae* should therefore include any aphid species encountered on plants in, and adjacent to, citrus groves, including weeds, ornamentals or vegetables.

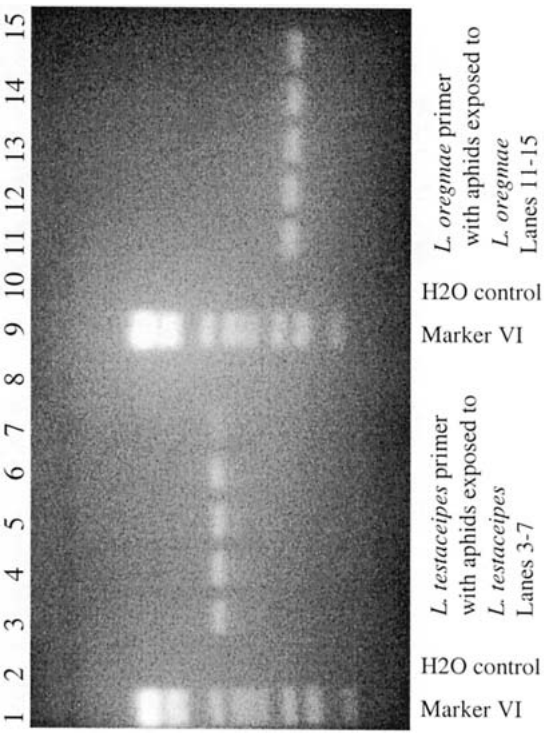

Fig. 2. Detection of eggs of *Lysiphlebus testaceipes* and *Lipolexis oregmae* within third instars of *Toxoptera citricida* with species-specific primers 24 h after exposing aphids to parasitoid females.

TABLE 2. DETECTION RATES WHEN UNPARASITIZED APHIDS WERE MIXED WITH ONE THIRD INSTAR APHID PARASITIZED[1] BY *LYSIPHLEBUS TESTACEIPES* OR *LIPOLEXIS OREGMAE*.

	No. of unparasitized aphids added to one parasitized aphid and detection rates			
Aphid species	*L. oregmae*	% Detection	*L. testaceipes*	% Detection
Aphis spiraecola (c)	45[2]	100	45	100
	54	70	54	40
	63	40	—	—
Aphis gossypii (c)	36	100	45	100
	45	40	54	40
Aphis craccivora (v)	36	100	45	100
	45	60	54	70
	54	20	63	10
Toxoptera citricida (c)	36	100	36	100
	45	40	45	50
	—	—	54	20
Toxoptera aurantii (c)	18	100	27	100
	27	70	36	40
	36	00	—	—

[1]24 h after five aphid species reared on citrus (c) or vegetables (v) were exposed to either parasitoid in the laboratory.
[2]In each trial there were 10 replicates; all trials started with 1 parasitized: 9 unparasitized and were increased serially by adding an additional 9 unparasitized aphids until detection dropped below 50%.

When a single *L. testaceipes* or *L. oregmae* egg was present in a third instar of the brown citrus aphid and 36 unparasitized third instars of the brown citrus aphid were combined, all assays were positive for their respective parasitoid (Table 2). DNA from a pooled maximum of 36 aphids thus can be used in analyses of field-collected *T. citricida* for determining presence of either parasitoid. The assay also detected eggs of both parasitoids within third instars of the other five aphid species. The maximum number of unparasitized aphid third instars that can be added to a single parasitized third instar aphid and provide 100% detection rate ranged from 18 to 45 individuals for the other five aphid species. The decline in detection was variable with aphid species; for example, *T. aurantii* detection failed when 36 unparasitized aphids were added to one parasitized aphid, while the same concentration gave 100% detection in *T. citricida* (Table 2).

Because these trials were conducted with aphids containing 24-h-old eggs, there is a high probability that eggs older than 24 h and all parasitoid larval and pupal stages will be detected because more parasitoid DNA will be present. This is substantiated by the observation that when one first instar was mixed with 500 unparasitized *T. citricida*, a PCR product was always obtained in the 10 replicates with High-fidelity PCR assay (data not shown). These data indicate the assay is suitable for qualitative analyses of large batches of field-collected aphid samples to determine whether *L. oregmae* larvae are present.

Interspecific Interactions between Parasitoids

Interspecific interactions produced single brown citrus aphids containing eggs or larvae of both *L. testaceipes* and *L. oregmae*. A PCR product specific to each parasitoid was obtained from the aphids tested (Fig. 3) and similar results were obtained in the reverse oviposition sequence (data not shown). In Figure 3, a PCR product was not obtained when the *L. testaceipes* primer was tested on aphid 6 (Fig. 3, lane 8); however, a PCR product was obtained with the *L. oregmae* primer (lane 18) suggesting that only the *L. oregmae* female oviposited and injected its DNA into aphid 6. The opposite probably happened with aphid 3, because only DNA from *L. testaceipes* was found (Fig. 3, lane 5). The experiment indicates that the assay can detect the presence of eggs of both parasitoid species when they co-occur in a single aphid. Hence, tests on field-collected material should detect the presence of either parasitoid species as early as 24 h after parasitoids oviposit.

Other Parasitoids

DNA extracted from the additional seven parasitoid species produced no PCR products with either species-specific primer. To confirm that the quality of the DNA was not an issue, we subjected the extracted DNA from each additional parasitoid species to the universal 5.8 S-F and 28 S-R primers. All PCR reactions produced bands, which confirm that the DNA was amplifiable. Absence of bands with the species-specific primers confirms the specificity of the primers for the target parasitoids.

Conclusions

This High-fidelity PCR assay is highly specific and sensitive, providing a relatively inexpensive tool for sampling large aphid populations. The standard practice of holding and monitoring field samples of foliage for up to two weeks in order to collect emerged adults is a labor-intensive and time-consuming process which can be affected by parasitoid mortality (Persad & Hoy, unpublished data). The presence of immature *L. testaceipes* and *L. oregmae* parasitoids as young as 24-h old can be determined by High-fidelity PCR and, if samples are stored for 70 h, then larger groups of up to 500 aphids can be sampled in a single assay. Currently this assay is being used to evaluate the establishment of *L. oregmae* on several aphid hosts on citrus and other plants near *L. oregmae* release sites in Florida.

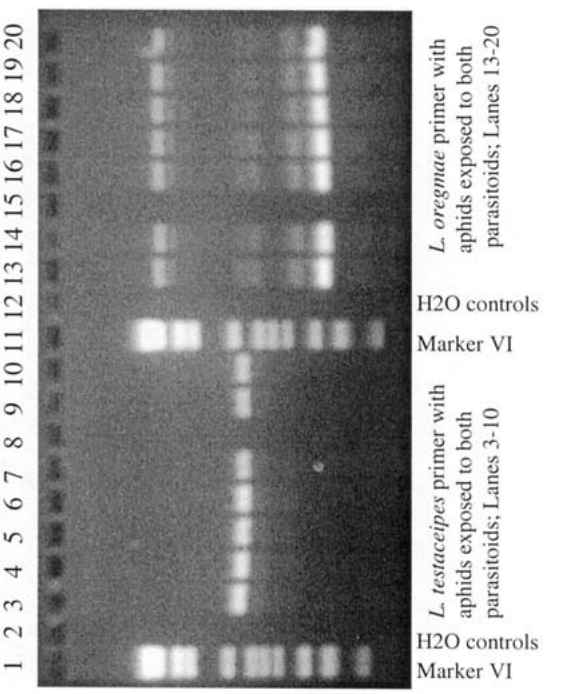

Fig. 3. The presence of both *Lysiphlebus testaceipes* and *Lipolexis oregmae* in single third instars of *Toxoptera citricida* did not affect the accuracy of the species-specific primers.

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