DEVELOPMENT OF MOLECULAR DIAGNOSTIC MARKERS FOR GLASSY-WINGED AND SMOKETREE SHARPSHOOTERS FOR USE IN PREDATOR GUT CONTENT EXAMINATIONS

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ABSTRACT

To aid in identifying key predators of Proconiini sharpshooter species present in California, we developed and tested molecular diagnostic markers for the glassy-winged sharpshooter *Homalodisca coagulata* (Say) and smoke-tree sharpshooter *Homalodisca liturata* (Ball) (Homoptera: Cicadellidae: Proconiini). Two different types of markers were compared, those targeting single-copy sequence characterized amplified regions (SCAR) and mitochondrial markers targeting the multi-copy cytochrome oxidase subunit genes I (COI) and II (COII). A total of six markers were developed, two SCAR and four mitochondrial COI or COII markers. Specificity assays demonstrated that SCAR marker HcF5/HcR7 was *H. coagulata*-specific and HcF6/HcR9 was *H. coagulata*/*H. liturata*-specific. COI (HcCOI-F/R) and COII (HcCOII-F4/R4) markers were *H. coagulata*-specific, COII (G/S-COII-F/R) marker was *H. coagulata*/*H. liturata*-specific, and lastly, COII marker (Hl-COII-F/R) was *H. liturata*-specific. Sensitivity assays using genomic DNA showed the COI marker to be the most sensitive marker with a detection limit of 6 pg of DNA. This marker was 66-fold more sensitive than marker Hl-COII-F/R that showed a detection limit of 400 pg of DNA. In addition, the COI marker was 4.2-fold more sensitive than the COII marker. In predator gut assays, the COI and COII markers demonstrated significantly higher detection efficiency than the SCAR markers. Furthermore, the COI marker demonstrated slightly higher detection efficiency over the COII marker. Lastly, we describe the inclusion of an internal control (28S amplification) for predation studies performing predator gut analyses utilizing PCR. This control was critical in order to monitor reactions for PCR failures, PCR inhibitors, and for the presence of DNA.

INTRODUCTION

Effective control of *H. coagulata* requires an area-wide, multi-tactic pest management program. A major component of such an approach is the exploitation of the pest's natural enemies, which, when utilized to their greatest potential, can increase the effectiveness of other control tactics. A classical biological control program is currently in progress in California against *H. coagulata*, utilizing parasitoid species that attack *H. coagulata* egg masses (CDFA 2003, Triapitsyn et al. 1998). However, little is known about the predaceous enemies that feed on eggs, nymphs, or adult *H. coagulata* (Triapitsyn et al. 1998). Direct visual field observations of predation are difficult to obtain and the field study of insect predation has often relied on indirect techniques for measurement and analysis. A sensitive approach to detect prey in predator gut contents is the use of monoclonal antibodies (MAb) in enzyme-linked immunoassays (ELISA) (Hagler et al. 1991, 1993, Hagler and Naranjo 1994). More recently, PCR-based methods have been developed that allow for rapid detection of prey in predator gut contents (reviewed in Symondson 2002, Harper et al. 2005). These methods include, 1) sequence characterized amplified region-polymerase chain reaction assays (SCAR-PCR), where RAPD-PCR species-specific bands are excised from gels and sequenced, and primers are designed toward those DNA fragments (Agustí et al. 1999, de León et al. 2005 submitted), 2) targeting genes that are present in the cell in high copy number, such as, mitochondrial genes (COI and COII) (Agustí et al. 2003, Chen et al. 2000, de León et al. 2005 submitted) and internal transcribed spacer regions (ITS1), and 3) a sensitive and efficient multiplex PCR procedure incorporating fluorescent markers (Harper et al. 2005).

OBJECTIVES

The aim of this work was to develop species-specific molecular diagnostic markers that were specific toward the invasive *H. coagulata* and the closely related *H. liturata*. Ultimately, the markers developed here will be used to detect *H. coagulata* and/or *H. liturata* remains in the guts of field-collected predators (Fournier et al., unplubl. data). Identifying the key predators of these sharpshooters will help towards establishing a conservation or augmentation biological control program, and will be useful in identifying the impact of natural enemies in field studies. In addition, these markers will be useful in identifying any life stage of *H. coagulata* and/or *H. liturata*, even before they emerge from egg masses, thus saving time and money required to rear these insects to the adult stage for morphological identification.

RESULTS AND CONCLUSIONS

Homalodisca coagulata, H. liturata, and *H. coagulata/H. liturata* molecular diagnostic markers

RAPD-PCR DNA fingerprinting was performed with various sharpshooters and an *H. coagulata*-specific band (674-bp) was excised, sequenced, and SCAR markers were designed toward it (data not shown). Both *H. coagulata*/*H. liturata*-(HcF6/HcR9) and *H. coagulata*-specific (HcF5/HcR7) primer sets were designed from this sequence and they produced amplification products of 166- and 302-bp sizes, respectively. Table 1 shows the optimized amplification reaction conditions for each diagnostic primer set and the name, the expected amplification product size, the $MgCl_2$ concentration, the annealing

temperature (Tm), and the number of cycles. The amplification reaction conditions are highly specific to each primer set in order to prevent cross-reactivity with any of the non-target species. If the specific reaction conditions are modified, those new conditions must be tested with all species of interest to test for cross-reactivity. An *H. coagulata*-specific primer set was developed toward the COI sequence, whereas, two sets of primers were developed toward the COII sequence, an *H. coagulata*/*H. liturata*- and an *H. coagulata*-specific set. Lastly, a COII-specific primer set was developed toward *H. liturata*.

Species specificity of the molecular diagnostic markers

To test the specificity of the diagnostic markers, amplification assays were performed with stock genomic DNA from various sharpshooter species, several of them present in California, along with lacewing, earwig, and ground beetle predators. The results of the specificity assays for all six diagnostic markers that were designed toward the RAPD-PCR fragment and the COI and COII partial sequences are given in Figure 1. For the size of the expected amplification products and the specific reaction conditions of each marker refer to Table 1. As seen each diagnostic marker was highly specific toward its target(s) (Figure 1). All diagnostic markers amplified DNA fragments of the correct size and none crossed-reacted with other sharpshooter species or the predators of interest. The internal control, 28S amplification is seen across all samples indicating that PCR inhibitors or failures did not play a role in the reactions (Figure 1G).

Detection of *H. coagulata* DNA in predator guts

The results of the amplification assays of predators from the laboratory feeding trails showed that all diagnostic markers, *H. coagulata*/*H. liturata*-, *H. coagulata*-, and *H. liturata*-specific detected prey in predator gut contents (Figure 2, A-E). As demonstrated, amplification was not seen in predators not fed on *H. coagulata*, whereas the 28S amplifications (Figure 2G) were positive. The marker set targeting the COI gene, HcCOI-F/R exhibited the highest sensitivity (6.0 pg of DNA); whereas marker Hl-COII-F/R was the least sensitive (400 pg of DNA), a 66-fold difference. The difference in sensitivity between *H. coagulata* COI (HcCOI-F/R) and COII (HcCOII-F4/R4) was about 4.2-fold. The sensitivity limits for each marker is shown on Table 2 in brackets.

Efficiency of molecular diagnostic markers at detecting *H. coagulata* remains in the guts of predators

Between the two SCAR markers, marker HcF6/HcR9 was slightly more efficient than marker HcF5/HcR7, a significant difference was seen with lacewing at the 0 h time point (Table 2). Marker HcF6/HcR9 produced an amplification product size of 166-bp, whereas, marker HcF5/HcR7 produced one of 302-bp; a difference of 136-bp. In lacewings at the 0 h retention interval the percentage detection was 8.3 and 58.0% for marker HcF5/HcR7 and HcF6/HcR9, respectively. Since the detection efficiency was low for SCAR markers we did not further test the rest of the time intervals. Detection of *H. coagulata* in earwig gut contents was equally low with both SCAR markers. *Homalodisca coagulata* could not be detected in ground beetles whether it fed on one or two *H. coagulata* adults using SCAR marker HcF5/HcR7; whereas, SCAR marker HcF6/HcR9 was more efficient. The detection efficiency was slightly higher for ground beetles that fed on two *H. coagulata* adults (25.0%) than on one (9.10%) at 0 h digestion (Table 2).

The detection efficiency of the markers targeting the multi-copy mitochondrial genes (COI, II) was significantly higher than the single-copy SCAR markers (Table 2). This was observed even though the number of amplification cycles was higher with the SCAR markers. In general, the detection efficiency of COI (197-bp) was better than COII (295-bp) using markers HcCOI-F/R and HcCOII-F4/R4, respectively. At the 0 and 8 h time point of lacewings that fed on *H. coagulata* eggs, the COI marker detection efficiency was 91.7 and 86.4% efficient as compared to 83.3 and 47.6% for COII, respectively. Only the 8 h time point was highly significant. The detection efficiency was the same for both the COI and COII markers at the 4, 16, and 24 h retention intervals. The detection efficiency of earwigs that fed on *H. coagulata* eggs at 0 h was significantly higher for the COI marker (87.5%) and than the COII marker (25.0%). A similar, but non-significant pattern was also observed in ground beetles that fed on adult *H. coagulata*. The detection efficiency was slightly higher with the COI marker in ground beetles that fed on one adult *H. coagulata*, 33.3% for COII as compared to 54.5% for COI. The detection efficiency reached 100.0% for the COI marker with ground beetles that fed on two *H. coagulata* adults. In both types of markers (SCAR and mitochondrial), a direct correlation between detection efficiency and amplification product size was observed.

For lacewings that fed on *H. liturata* eggs the detection efficiency was between 80-90% at 0 h with both *H. liturata*- and *H. coagulata*/*H. liturata*-specific markers. The *H. coagulata*/*H. liturata*-specific COII marker (G/S-COII-F/R) that produced an amplification product size of 178-bp was slightly more efficient than the COII marker (Hl-COII-F/R) that produced a size of 295-bp; 90 and 80%, respectively.

Table 1. Summary and optimized conditions of diagnostic primer sets showing primer name, sequence, DNA fragment size, $MgCl_2$ concentration, annealing temperature (Tm), cycle number, and species specificity. F, forward; R, reverse; COII, mitochondrial cytochrome oxidase subunit gene II; COI, mitochondrial cytochrome oxidase subunit gene I; Hc, *H. coagulata*; Hl, *H. liturata*; G/S, *H. coagulata/H. liturata*.

Primer name	Frag. size	$MgCl_2$ (mM)	Tm (°C)	Cycle number	Designed toward
SCAR					
HcF5/ HcR7	302-bp	2.0	65	45	Hc
HcF6/ HcR9	166-bp	2.0	59	45	Hc/Hl
Mitochondrial					
HcCOII-F4/R4	295-bp	1.6	55	35	Hc
G/S-COII-F/R	178-bp	1.5	56	30	Hc/Hl
HcCOI-F/R	197-bp	1.4	60	31	Hc
Hl-COII-F/R	295-bp	1.6	56	33	Hl

Table 2. Detection efficiency of molecular diagnostic markers in predators; small scale analysis. The specificity of the marker and the expected size of the DNA fragment are included below the marker name. Lacewings and earwigs fed on *H. coagulata* eggs and ground beetles fed on *H. coagulata* adults. Individual lacewings for retention the time experiment fed on 2 to 3 eggs, as did lacewing feeding on *H. liturata* eggs. Individual earwigs fed on 5 to 20 eggs. np, not performed; n/a, not applicable. Numbers in parenthesis are number of individuals tested. Shown in brackets are the sensitivity limits of the diagnostic markers measure as pg of DNA. Statistics were performed with Fisher's Exact Test using two-sided *p*-values. Hc, *H. coagulata*; Hl, *H. liturata*

	HcF5/R7[c] Hc 302-bp	HcF6/R9[c] Hc/Hl 166-bp	HcCOII-F4/R4[d] Hc 295-bp	G/S-COII-F/R[d] Hc/Hl 178-bp	HcCOI-F/R Hc 197-bp	Hl-COII-F/R Hl 295-bp
Hc	[100]	[50]	[25]	[50]	[6]	[400]
Lacewing[a]						
0 h	8.3% (12)	58.0% (12)	83.3% (12)[h]	83.3 % (12)	91.7% (12)[e, h]	n/a
4 h	np	np	27.3% (11)	18.2% (11)	27.3% (11)	n/a
8 h	np	np	47.6% (21)	86.4% (22)	86.4% (22)	n/a
16 h	np	np	37.5% (8)	50.0% (8)	37.5% (8)	n/a
24 h	np	np	9.10% (11)	9.10% (11)	9.10% (11)	n/a
Lacewing[b]	25.0% (4)	50.0% (4)	50.0% (4)	50.0% (4)	50.0% (4)	n/a
Earwig[b]	12.5% (8)	12.5% (8)	25.0% (8)	25% (8)	87.5% (8)[g, j]	n/a
Beetle[b]:						
1 adult GWSS	0.0% (11)	9.10% (11)	33.3% (12)	16.7% (12)	54.5% (11)[e, j]	n/a
2 adult GWSS	0.0% (8)	25.0% (8)	87.5% (8)	100.0% (8)	100.0% (8)[e, h]	n/a
Hl						
Lacewing[a] n/a	80.0% (10)	n/a	90.0% (10)	n/a	80.0% (10)	

[a]Crude DNA extract procedure; [b]Qiagen preparation of DNA of insects at 0 h; [c]Primers designed toward same SCAR sequence; [d]Primers designed toward same COII (*H. coagulata*) sequence; [e]Not significantly different from HcCOII-F4/R4 (COII), $P = 1.0000$; [f]Very significantly different from COII, $P < 0.001$; [g]Significantly different from COII, $P < 0.05$; [h]Extremely significantly different from HcF5/R7, $P < 0.001$; [i]Very significantly different from HcF5/R7, $P < 0.005$; [j]Significantly different from HcF5/R7, $P < 0.05$

Figure 1

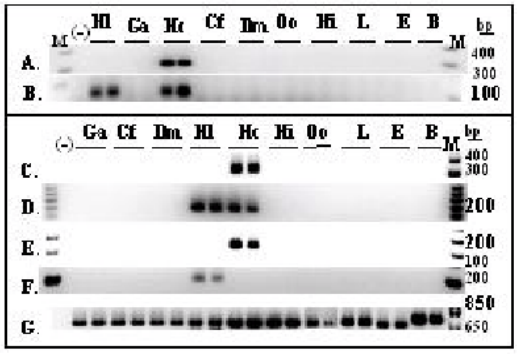

Figure 2

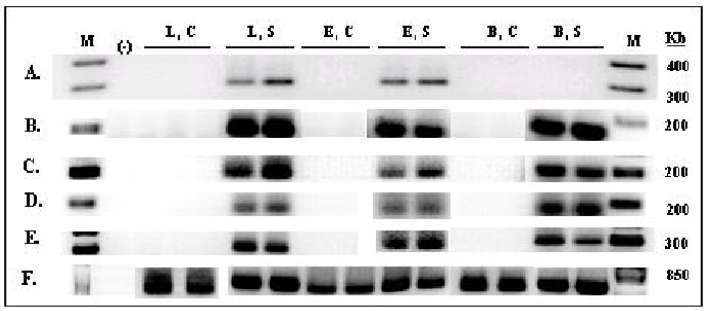

Figure 1. Specificity of molecular diagnostic markers. **A and B).** RAPD-PCR DNA fingerprinting was performed with the following sharpshooters: *Homalodisca liturata* (**Hl**); *Graphocephala atropunctata* (**Ga**); *H. coagulata* (**Hc**); *Carneocephala fulgida* (**Cf**); *Draeculacephala minerva* (**Dm**); *Oncometopia orbona* (**Oo**); and *H. insolita* (**Hi**). The optimal amplification conditions for all reactions are listed on Table 1. **A).** *H. coagulata*-specific marker, HcF5/HcR7 (302-bp) and **B).** *H. coagulata*/*H. liturata*-specific marker, HcF6/HcR9 (166-bp). Predators included in the analysis were: *Chrysoperla carnea* [green lacewing larvae (L); *Forficula auricularia* [earwig (E)]; and *Calosoma sp*. [ground beetle (B)]. **C).** *H. coagulata*-COII-specific primers, HcCOII-F4/R4 (295-bp). **D).** *H. coagulata*/*H. liturata*-COII-specific primers, G/S-COII-F/R (178-bp). **E).** *H. coagulata*-COI-specific primers, HcCOI-F/R (197-bp). **F).** *H. liturata*-COII-specific primers, Hl-COII-F/R (295-bp); **G)** 28S internal control. **M**: 1.0 Kb Plus DNA Ladder.

Figure 2. Detection of *H. coagulata* eggs or adults in predator gut contents by diagnostic amplification assays. (-), negative control (no DNA template); C, control (not fed on *H. coagulata*); S, sample (fed on *H. coagulata*). Lacewings and earwigs fed on *H. coagulata* eggs and ground beetles fed on *H. coagulata* adult(s). **A)** HcF5/HcR7 (*H. coagulata*-specific; 302-bp); **B)** HcF6/HcR9 (*H. coagulata* /*H. liturata*-specific; 166-bp); **C)** HcCOII-F4/R4 (*H. coagulata*-COII-specific; 295-bp); **D)** G/S-COII-F/R (*H. coagulata* /*H. liturata*-specific; 178-bp); **E)** HcCOI-F/R (*H. coagulata*-COI-specific; 197-bp); **F)** 28S internal control.

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