DEVELOPMENT OF MOLECULAR MARKERS TOWARD GLASSY-WINGED SHARPSHOOTER (GWSS) EGG PARASITOIDS TO AID IN BOTH IDENTIFYING KEY PREDATORS OF THE GWSS EGG STAGE AND IN TAXONOMIC IDENTIFICATIONS

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ABSTRACT

To aid in identifying key predators of the glassy-winged sharpshooter (GWSS) egg stage, we developed 'one-step' molecular diagnostic markers specific toward the internal transcribed spacer 2 region (ITS2) of five primary GWSS egg parasitoid species belonging to the genus *Gonatocerus* Nees. In addition, these markers will also serve to aid taxonomic identifications, especially before the egg parasitoids emerge from GWSS egg masses. The molecular markers were designed toward the following species: *G. triguttatus* (Gtri-F/R); *G. walkerjonesi* (wjca-F2/R); *G. morrilli* (gmtx-F/R); *G. ashmeadi* (Gash-F/R); and *G. morgani* (Gmorg-F/R). Cross-reactivity assays with 21 *Gonatocerus* species, each with specific assay conditions, demonstrated that the developed markers were highly specific toward the species that they were designed for, as cross-reactivity was not observed with non-target species. One of the markers (Gtri-F/R) was tested in predator gut content assays. Positive reactions were observed in gut assays in which lacewings were allowed to feed on parasitized (*G. triguttatus*) GWSS eggs. In addition, positive banding was also seen with GWSS parasitized eggs alone, that is before the egg parasitoids emerged from the GWSS egg masses. The current results confirm the specificity and utility of the various one-step molecular diagnostic markers. In addition to the predation studies, these markers should be useful to the biological control program.

INTRODUCTION

The glassy-winged sharpshooter, GWSS, *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)] (Hemiptera: Cicadellidae) was reported in California (CA) USA in 1989 (Sorenson and Gill 1996) and since then it has spread throughout CA (Blua et al. 2001), posing a serious threat to the wine and table grape industry. The GWSS that invaded CA have their origins in Texas USA (de León et al. 2004), an observation that was later confirmed by Smith (2005). A biological control program has been put into place to try to control this devastating pest (CDFA 2003). To effectively control GWSS, an area-wide pest management approach is required. However, limited knowledge is available pertaining to the GWSS generalist predator complex. At present, the two most common methods used to study predation by gut content analysis are immunoassasys (ELISAs) (Hagler 1998; Fournier et al. 2006, 2007) that detect prey-specific proteins and polymerase chain reaction (PCR)-based markers targeting prey DNA (Agustí et al. 2003; Greenstone and Shufran 2003; de León et al. 2006; Fournier et al. 2006, 2007). Furthermore, some prey specific ELISAs are also life-stage specific, for example, toward the egg stage of GWSS (Hagler et al. 1991, 1993; Fournier et al. 2006). Combing this type of ELISA with a PCR-based analysis could provide a more accurate definition of a predation event (Fournier et al. 2007).

OBJECTIVES

Our research is in the initial stages. We plan to identify key predators of the GWSS egg stage [e.g., targeted (effective) biological control] and GWSS parasitoid complex [e.g., non-targeted (interference) biological control]. We will also identify and quantify egg parasitism for several of the key native and exotic GWSS parasitoid species. Another objective of our research is to aid in taxonomic identifications, a request from the CDFA (D. Morgan). Our first immediate goal is to develop 'one-step' molecular diagnostic markers toward the internal transcribed spacer region 2 (ITS2) of several Gonatocerus species (5): *G. triguttatus* (Girault), *G. walkerjonesi* S. Triapitsyn; *G. morrilli* (Howard); *G. ashmeadi* (Girault); and *G. morgani* S. Triapitsyn (Hymenoptera: Mymaridae). Our second immediate goal is to begin to test the utility of the developed markers by examining the gut contents of predators (lacewings) feeding on parasitized (*G. triguttatus*) GWSS egg masses. Along with these DNA markers, future studies will involve using an established GWSS-egg specific ELISA (Hagler et al. 2003; Fournier et al. 2006).

RESULTS AND CONCLUSIONS

Development and specificity of the 'one-step' species-specific molecular markers directed toward the ITS2 rDNA fragments

ITS2 rDNA sequences from several *Gonatocerus* Nees species (21) from both North and South America were aligned with the program DNAStar (DNAStar, Inc; Madison, WI) using the ClustalW algorithm (Higgins et al. 1994). Five primer sets were designed that discriminated the species of interest from each other and from the rest of the *Gonatocerus* species,

including the most important species present in California. To determine the specificity of the five molecular diagnostic markers, we tested specific amplification assay conditions and screened a total of 21 *Gonatocerus* GWSS egg parasitoid species for cross-reactivity. Figure 1: **A**, Gtri-F/R (*G. triguttatus*); **B**, wjca-F2/R (*G. walkerjonesi*); **C**, gmtx-F/R (*G. morrilli*); **D**, Gash-F/R (*G. ashmeadi*); and **E**, Gmorg-F/R (*G. morgani*) shows that all of the developed markers amplified DNA fragments of the correct size, and all were highly specific as cross-reactivity with the specific amplification assay conditions was not seen with any of the *Gonatocerus* species tested, including all species belonging to the *morrilli* subgroup of the *ater* species group of *Gonatocerus* (Triapitsyn 2006). Markers for *G. morrilli* and *G. walkerjonesi* have recently been reported (de León and Morgan 2007), but the current study expands on those results.

Fig. 1.

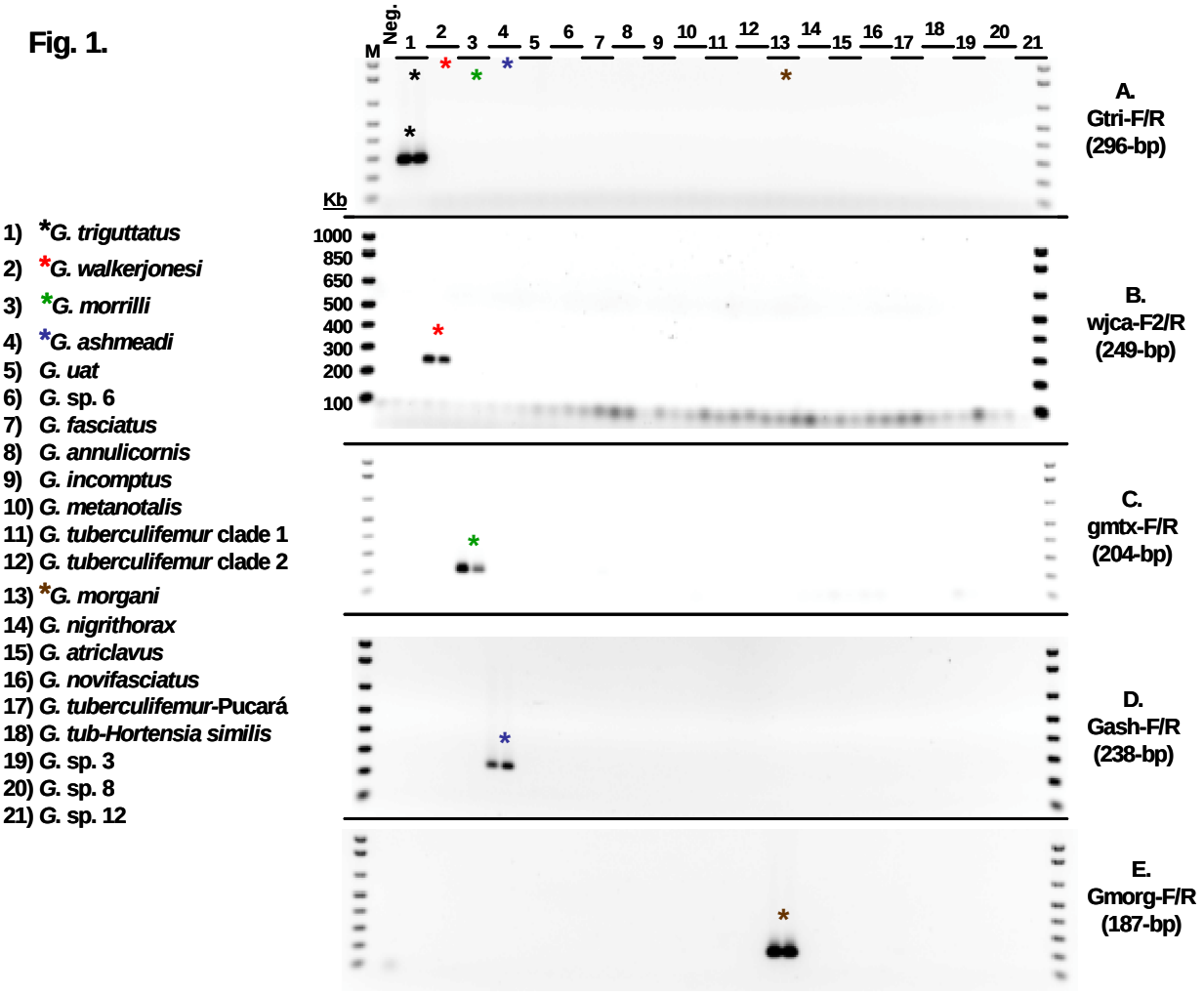

Figure 1. Specificity assays using the developed 'one-step' species-specific ITS2 diagnostic markers. A) Gri-F/R (*G. triguttatus*) (Expected size: 296-bp); *B) wjca-F2/R (*G. walkerjonesi*) (249-bp); *C) gmtx-F/R (*G. morrilli*) (204-bp); D) Gash-F/R (*G. ashmeadi*) (238-bp); and E) Gmorg-F/R (*G. morgani*) (187-bp). Total genomic DNA per *Gonatocerus* species (21) was used in amplification assays to test for cross-reactivity of the markers, each with specific assay conditions. Neg., negative control (no template DNA). M, 1.0-Kb Plus DNA Ladder. *Previously reported with 16 *Gonatocerus* species (de León and Morgan 2007).

Detection of parasitized GWSS eggs in predator gut contents

For this particular experiment, lacewings were allowed to feed (4 h) on GWSS egg masses parasitized by *G. triguttatus*, a primary egg parasitoid of GWSS native to the southeastern U.S. (Texas) and northeastern Mexico and imported into CA as a biological control agent of GWSS (Triapitsyn 2006). Figure 2A demonstrates that the Gtri-F/R markers were able to produce a positive band using parasitized (*G. triguttatus*) GWSS egg masses alone, that is, before the egg parasitoids emerged from the GWSS egg masses. Furthermore, a positive reaction was generated in the gut contents of lacewings that were allowed to feed on parasitized (*G. triguttatus*) GWSS egg masses. These reactions were highly specific, as the controls produced negative banding with this specific marker. The controls included: 1) GWSS egg alone, 2) starved lacewings alone, and 3) lacewings fed on non-parasitized GWSS eggs.

These same samples were also assayed with the GWSS-specific COI marker (HcCOI-F/R) [life-stage independent (de León et al. 2006)] (Figure 2B). As expected, positive reactions were generated with the lacewings that fed on non-parasitized GWSS eggs. Positive reactions were also produced with the parasitized (*G. triguttatus*) GWSS eggs alone. These results confirm that both the GWSS egg DNA and the egg parasitoid DNA can be detected using our specific molecular markers. The detection of the GWSS egg DNA probably depends on the age of the parasitized egg, in other words, as the egg parasitoid embryo is developing with time, the less likely it will be to detect GWSS remains or DNA. Slight detection of GWSS DNA was detected in lacewings allowed to feed on parasitized GWSS eggs. As previously shown, the GWSS-specific marker (HcCOI-F/R) is highly specific (de León et al. 2006).

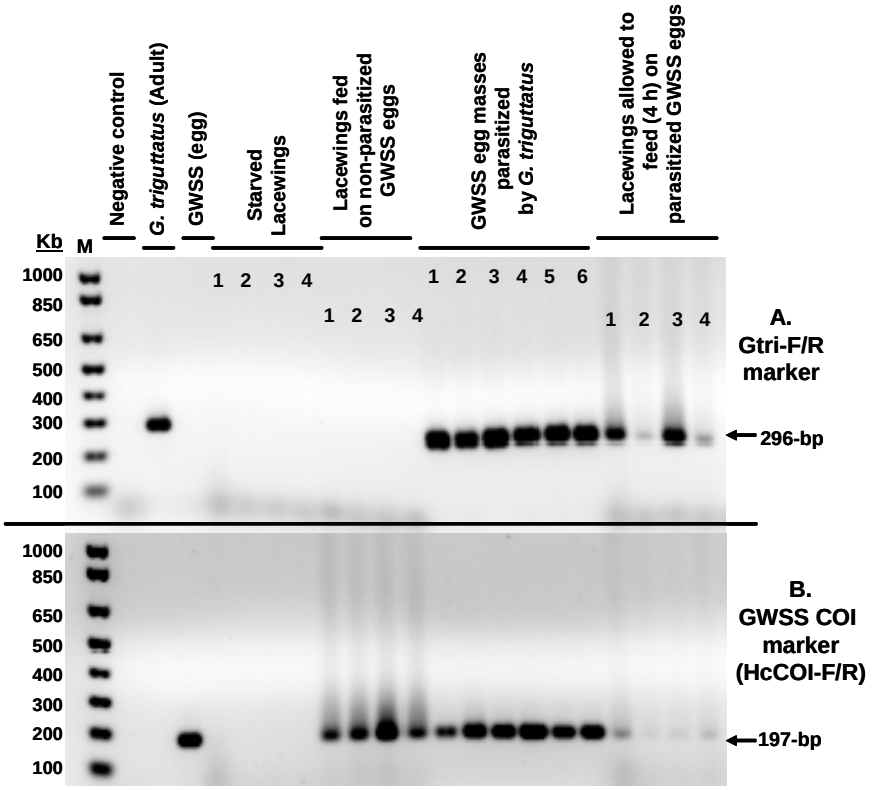

Figure 2. Detection of parasitized (*G. triguttatus*) GWSS eggs in predator (lacewings) gut contents. Diagnostic amplification assays were performed with A) *G. triguttatus* markers (Gtri-F/R) (296-bp) and B) GWSS mitochondrial cytochrome oxidase subunit I gene (COI) markers (HcCOI-F/R) (197-bp); this marker was developed by de León et al. (2006). The test predators included were third-instar green lacewings, *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae). Experimental conditions on shown above the gel. M, 1.0-Kb Plus DNA Ladder.

Our proposed research addresses every 'high priority' research recommendation for biological control research set forth by the National Academies, National Research Council review of the GWSS/Pierce's disease research effort. The committee strongly recommended that biological control efforts (predators and parasitoids) focus on the establishment of protocols for the effective selection of natural enemies, the development of strategies that will increase the success of biological control agents, and the rigorous evaluation of the effectiveness of natural enemies.

The National Research Council has identified the lack of information on GWSS natural enemies as a critical weakness with a strong recommendation to develop more information on this topic (NRC 2004). The experiments in this study are designed to advance our ability to precisely identify the key GWSS parasitoids and predators and identify the non-target impact of the GWSS generalist predator complex.

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