DEVELOPMENT AND UTILITY OF A 'ONE-STEP' SPECIES-SPECIFIC MOLECULAR DIAGNOSTIC MARKER FOR *GONATOCERUS MORRILLI* DESIGNED TOWARD THE INTERNAL TRANSCRIBED SPACER REGION 2 (ITS2) TO MONITOR ESTABLISHMENT IN CALIFORNIA

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ABSTRACT

In addition to the 'one-step' species-specific molecular diagnostic ISSR-PCR DNA fingerprinting method, we developed an additional 'one-step' molecular diagnostic marker 'gmtx' toward *Gonatocerus morrilli* (Howard) designed toward the ribosomal internal transcribed spacer region 2 (ITS2) to aid in monitoring establishment in California. *Gonatocerus morrilli*, the imported natural enemy from Texas, is very closely related to *G. walkerjonesi*, the native California species. Specificity assays with this newly developed marker and a total of 16 *Gonatocerus* Nees species demonstrated that it was highly specific toward the species that it was designed for (*G. morrilli*), as cross-reactivity was not seen with any of the tested species, including all species belonging to the *morrilli* subgroup of the *ater* species group of *Gonatocerus*. Anaysis of the 'release' 'TX/MX' colony used before the summer of 2005 with this species-specific diagnostic marker confirmed previous results that the 'release' 'TX/MX' colony was not the imported *G. morrilli*, but the native species *G. walkerjonesi*, confirming a colony contamination. Analysis of post-released *G. morrilli* collections with this diagnostic marker detected *G. morrilli* in a site where it was previously released, in accordance with our recent finding using two other diagnostic markers used in combination, ITS2 fragment size and ISSR-PCR DNA fingerprinting. The current results confirm the utility of the newly developed species-specific ITS2 molecular diagnostic marker as an excellent tool to aid in monitoring the establishment of the imported natural enemy of the glassy-winged sharpshooter, *G. morrilli*. These results and molecular tools are critical to the biological control program in California. We now have in our hands the molecular technology to evaluate the *G. morrilli* biological control program in California from start to finish, that is, monitor establishment, dispersal, and efficacy of natural enemies and improve mass rearing.

INTRODUCTION

Accurately identifying natural enemies is critical to the success of classical biological control programs and lack of proper identification procedures has affected several projects (Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993). In addition, reliable methods or molecular markers are needed for distinguishing various exotic strains of these biological control agents from those indigenous to the U. S., including parasitoids from different states within the U. S. [e. g., *G. morrilli* (Howard) and *G. walkerjonesi* (Triapitsyn) (Hymenoptera: Mymaridae)]. Molecular markers are also needed to monitor the establishment of released populations and for the detection of cross-contamination between cultures of cryptic or closely related species (Powell and Walton 1989, Menken and Ulenberg 1987, Narang *et al*. 1993, Hopper *et al*. 1993, Unruh and Woolley 1999). The ribosomal internal transcribed spacer (ITS) regions have been used extensively in the examination of the taxonomic status of species and for diagnostic purposes (reviewed in Collins and Paskewitz 1996). Stouthamer *et al*. (1999) have used ITS2 rDNA fragment sizes as taxonomic characters to develop a precise identification key for sibling species of the genus *Trichogramma*. In cases where species were observed with similar sized ITS fragments the authors suggested a two-step procedure, amplification followed by restriction digestion called polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). If considering this procedure, having several species with similarly or overlapping ITS2 fragment sizes (accompanying PD report) would require development of several species-specific 'two-step' PCR-RFLP assays. The amount of steps can increase further if more than one restriction enzyme is used. Several *Gonatocerus* Nees species have similarly or overlapping ITS2 fragments (accompanying PD report). Having a couple of 'one-step' procedures, along with a rapid crude DNA extraction method, would, of course, be desirable. In addition, confirming results with more than one molecular diagnostic marker adds confidence and accuracy to the results. Recently, we demonstrated that inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting using a 5'-anchored ISSR primer is an excellent 'one-step' molecular diagnostic tool with haplodipoid glassy-winged sharpshooter (GWSS) [*Homalodisca vitripennis* (Germar)] egg parasitoids, such as, *G. morrilli*, *G. tuberculifemur* (Ogloblin), *G. metanolatis* (Ogloblin), and *G*. sp. 3 (accompanying PD reports and de León et al. 2004a, 2006a,b). We have been using a combination of two methods to aid in detecting, distinguishing, and monitoring released populations of *G. morrilli* imported from Texas, specifically, ITS2 fragment size and ISSR-PCR DNA fingerprinting with excellent success (de León and Morgan 2005, 2006). Recent molecular genetic studies by de León et al. (2004a) and Smith (2005) demonstrated that the origin of the GWSS that invaded California was Texas and that GWSS geographic populations clustered into two distinct clades. Furthermore, scientific evidence from both of these studies also demonstrated that the GWSS that recently invaded the Pacific Island of French Polynesia (Secretariat of the Pacific Community, 2002) clustered along with the Texas and California or the 'western and southwestern' clade, strongly suggesting that GWSS invaded French Polynesia via California.

OBJECTIVE

In addition to ISSR-PCR DNA fingerprinting, develop an additional 'one-step' molecular diagnostic marker toward *Gonatocerus morrilli* based on ITS2 species-specific primers. Confirming results with two 'one-step' molecular diagnostic markers adds confidence and accuracy to the results.

RESULTS AND CONCLUSIONS

Development and specificity of the species-specific ITS2 diagnostic marker 'gmtx' for *Gonatocerus morrilli*

A species-specific marker 'gmtx' (gm = *G. morrilli* and tx = Texas) was designed toward the ITS2 rDNA fragment of *G. morrilli*, the imported egg parasitoid species from Texas. *G. morrilli* is very closely related to the native species from California, *G. walkerjonesi* (León et al. 2004a, 2006a, Triapitsyn 2006). For several years it was difficult to distinguish these two species (S. V. Triapitsyn, personal communication), therefore, making it impossible to monitor the *G. morrilli* biological control program in California. To determine the specificity of the 'gmtx' diagnostic marker (expected size = 204-bp), we tested specific amplification assay conditions, a rapid crude DNA extraction procedure, and screened a total of 16 *Gonatocerus* Nees GWSS egg parasitoid species for cross-reactivity. Figure 1, which shows 11 *Gonatocerus* species, shows that the diagnostic marker 'gmtx' was highly specific toward the species (*G. morrilli*) that it was designed for. We also screened the following species: *G. tuberculifemur* (clade 2), *G. morgani*, *G.* sp. 2, *G. atriclavus*, and *G. novifasciatus* (data not shown). Cross-reactivity with our 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*. The results demonstrated that the 'gmtx' molecular diagnostic marker was highly specific toward *G. morrilli*, making it highly suitable to use as a monitoring tool for *G. morrilli* post-released populations in California.

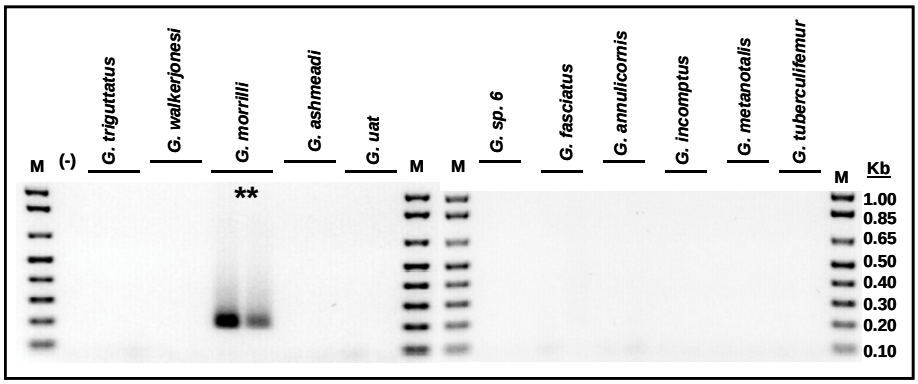

Figure 1. Specificity assays using the species-specific ITS2 molecular diagnostic marker 'gmtx'. Genomic DNA from two individuals per species was used in amplification assays to test for cross-reactivity of the 'gmtx' marker (204-bp) with specific assay conditions. The *Gonatocerus* species are listed on the figure. The following species were also tested, but not shown here: *G. tuberculifemur* (clade 2), *G. morgani*, *G.* sp. 2, *G. atriclavus*, and *G. novifasciatus*. (-), negative control, no template DNA. **M**, 1.0 Kb Plus DNA Ladder.

Molecular diagnostic analysis of the 'release' 'TX/MX' colony used before the summer of 2005 by the ITS2 rDNA species-specific marker, 'gmtx'

Previously, we demonstrated the utility of using two diagnostic marker sets in combination to genotype the 'release' 'TX/MX' colony and to detect post-released *G. morrilli* populations in California (de León and Morgan 2005, 2006). Those results demonstrated that the 'release' 'TX/MX' colony was not the imported species *G. morrilli*, but the native California species, *G. walkerjonesi*; indicating a contamination of the colony. In the current study, we again tested the same 'release' 'TX/MX' colony, along with *G. morrilli* (TX) and *G. walkerjonesi* (CA) as control species, with our newly developed 'gmtx' species-specific molecular diagnostic marker. Amplification with the 'gmtx' marker showed positive banding in only in the control *G. morrilli* (TX) (Figure 2), but not in the other control *G. walkerjonesi*. Amplification with this marker of the 'release' 'TX/MX' colony also produced negative banding. The current results with our diagnostic species-specific ITS2 marker are in accordance with our previous findings (de León and Morgan 2005, 2006), that is, the 'release' 'TX/MX' colony was not *G. morrilli*, but the native *G. walkerjonesi*, again confirming a colony contamination.

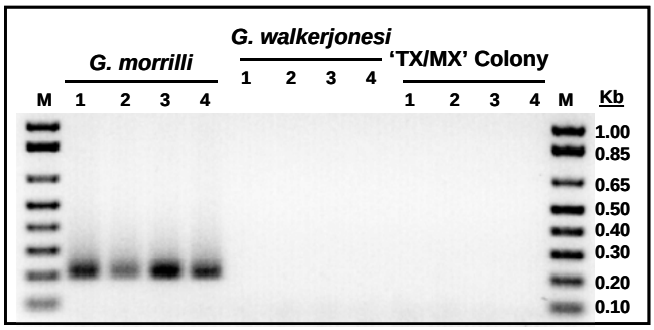

Figure 2. Evaluation of the 'release' 'TX/MX' colony that was used before the summer of 2005. *G. morrilli* (TX) and *G. walkerjonesi* (CA) are included as control species. The ITS2 species-specific diagnostic marker 'gmtx' was used in diagnostic amplification assays. Four individuals per species were included. **M**, 1.0 Kb Plus DNA Ladder.

The utility of the ITS2 species-specific molecular diagnostic marker as a tool to evaluate post-released *G. morrilli* populations in California

In the summer of 2005, after the confirmation of the colony contamination, *G. morrilli* from Texas was imported to California to restart the biological control program and shortly thereafter releases were started in California. We screened a few post-released populations from San Diego and Riverside Counties with our newly developed diagnostic marker, 'gmtx'. Amplification with 'gmtx' produced positive banding in one location (Figure 3, lane E) where *G. morrilli* (TX) was previously released, whereas, amplification was not seen in the rest of the post-released collection sites (Figure 3, lanes A, B, C, D, and F); indicating that *G. morrilli* was detected. The current results are in accordance with our previous results using other diagnostic markers (de León and Morgan 2005, 2006). The current results confirm the utility of our newly developed ITS2 species-specific molecular diagnostic marker as an excellent tool to aid in monitoring the establishment of *G. morrilli* in California. These results and molecular tools are critical to the biological control program in California. We now have the molecular technology to evaluate the *G. morrilli* biological control program in California from start to finish, that is, monitor establishment, dispersal, and efficacy of natural enemies and improve mass rearing.

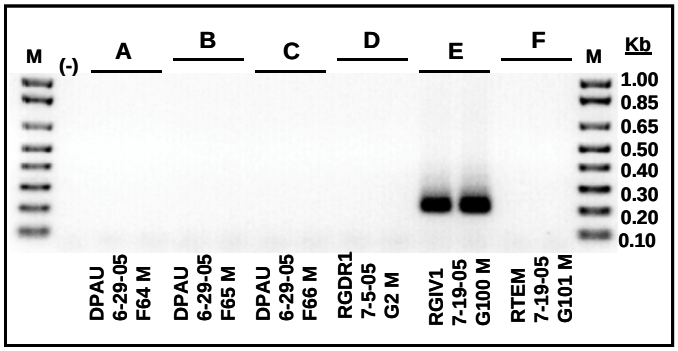

Figure 3. An example of the utility of the species-specific ITS2 diagnostic marker 'gmtx' in evaluating post-released *G. morrilli* collections. Two individuals per collection site were tested with the diagnostic marker. Collection sites: DPAU1, Pauma, San Diego County; RGDR1, Meyers St, Riverside County; RGlV1, Glen Ivy, Riverside County; RTEM2, Temecula, Riverside County. **M**, 1.0 Kb Plus DNA Ladder.

REFERENCES

Collins, F. H., and S. M. Paskewitz 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anophels* species. Insect Mol. Biol. 5: 1-9.

de León, J. H., W. A. Jones, and D. J. W. Morgan. 2004a. Molecular distinction between populations of *Gonatocerus morrilli*, egg parasitoids of the glassy-winged sharpshooter *Homalodisca coagulata*, from Texas and California: Do cryptic species exist? 7pp. J. Insect Sci. 4:39, Available online: insectscience.org/4.39.

de León, J. H., W. A. Jones, and D. J. W. Morgan. 2004b. Population genetic structure of *Homalodisca coagulata* (Homoptera: Cicadellidae), the vector of the bacterium *Xylella fastidiosa* causing Pierce's disease in grapevines. Ann. Entomol. Soc. Am. 97: 809-818.

de León, J. H., W. A. Jones, M. Sétamou, and D. J. W. Morgan. 2006a. Genetic and hybridization evidence confirms that a geographic population of *Gonatocerus morrilli* (Hymenoptera: Mymaridae) from California is a new species: Egg

parasitoids of the glassy-winged sharpshooter *Homalodisca coagulata* (Homoptera: Cicadellidae). Biol. Control 38: 282-293.
de León, J. H., G. A. Logarzo, and S. V. Triapitsyn. 2006b. Molecular characterization of *Gonatocerus tuberculifemur* (Ogloblin) (Hymenoptera: Mymaridae), a prospective *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae) biological control candidate agent from South America: Divergent clades. (submitted)
de León, J. H., and D. J. W. Morgan. 2005. Small scale post-release evaluation of a *Gonatocerus morrilli* program in California against the glassy-winged sharpshooter: Utility of developed molecular diagnostic tools, pp. 306-309. In Proceeding, Pierce's Disease Research Symposium, 5-7 December 2005, San Diego, CA. Complied by M. Athar Tariq, Stacie Oswalt, Peggy Blincoe, Amadou Ba, Terrance Lorick, and Tom Esser, Sacramento, CA.
de León, J. H., and D. J. W. Morgan. 2006. The utility of developed molecular diagnostic tools to monitor the establishment of *Gonatocerus morrilli* (Hymenoptera: Mymaridae) in the biological control program against the glassy-winged sharpshooter (*Homalodisca coagulata*) in California. (submitted)
Hopper, K. R., R. T. Roush, W. Powell. 1993. Management of genetics of biological control introductions. Ann. Rev. Entomol. 38: 27-51.
Löhr, B. A., M. Varela, and B. Santos. 1990. Exploration for natural enemies of the cassava mealybug, *Phenococcus manihoti* (Homoptera: Pseudococcidae), in South America for the biological control of this introduced pest in Africa. Bull. Entomol. Res. 80: 417-425.
Menken, S. B. J., and S. A. Ulenberg. 1987. Biochemical characters in agricultural entomology. Agr. Zool. Rev. 2: 305-353.
Messing, R. H., and M. T. Aliniazee. 1988. Hybridization and host suitability of two biotypes of *Trioxys pallidus* (Hymenoptera: Aphidiidae). Ann. Entomol. Soc. Am. 81: 6-9.
Narang, S. K., W. J. Tabachnick, and R. M. Faust. 1993. Complexities of population genetic structure and implications for biological control programs, pp. 19-52 In Narang SK, Barlett AC, Faust RM. [eds.], Applications of Genetics to Arthropods of Biological Control Significance. CRC Press Inc, Boca Raton, Florida.
Powell, W., and M. P. Walton. 1989. The use of electrophoresis in the study of hymenopteran parasitoids of agricultural pest. In: Loxdale HD, den Hollander J, editors. Electrophoretic Studies on Agricultural Pests, 443-65. Oxford, Clarendon.
Secretariat of the Pacific Community. 2002. Incursion of glassy-winged sharpshooter *Homalodisca coagulata* in French Polynesia. Pest Alert, Plant Protection Service. http://www.spc.org.nc/pps/PestAlerts/PestAlertNo24.pdf (accessed June 2005).
Smith, P. T. 2005. Mitochondrial DNA variation among populations of the glassy-winged sharpshooter, *Homalodisca coagulata*. J. Insect Sci. 5:41, available online: insectscience.org/5.41
Stouthamer, R., J. Hu, F. J. P. M. Van Kan, G. R. Platner, J. D. Pinto. 1999. The utility in internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of *Trichogramma*. BioControl 43: 421-440.
Triapitsyn, S.V. 2006. A key to the Mymaridae (Hymenoptera) egg parasitoids of proconiine sharpshooters (Hemiptera: Cicadellidae) in the Nearctic region, with description of two new species of *Gonatocerus*. Zootaxa 1203: 1-38.
Unruh, T. R., and J. B. Woolley. 1999. Molecular Methods in Classical Biological Control. In: Van Driesche RG, Bellows TS, Jr., editors. Biological Control, 57-85. Chapman and Hall, NY.
Zietkiewicz, E., A. Rafalski, and D. Labuda. 1994. Genomic fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20: 176-183.

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IDENTIFYING KEY PREDATORS OF THE GLASSY-WINGED SHARPSHOOTER IN A CITRUS ORCHARD

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ABSTRACT

Over 1,500 predators were screened for glassy-winged sharpshooter (GWSS) remains using a GWSS egg-specific monoclonal antibody (MAb) and several GWSS-specific genetic markers. Specimens were collected in 2002 and 2003 from a citrus orchard (Riverside, CA) harboring high densities of GWSS. We found that 6.2% of all specimens examined tested positive for GWSS remains. The most frequent predators to test positive included the assassin bug, *Zelus renardii* (Kolenati) (Hemiptera: Reduviidae) and the spiders *Trachelas pacificus* Chamberlin and Ivie (Araneae: Corinnidae) and *Olios* sp. (Araneae: Sparassidae) with 41, 22, and 19% of the specimens testing positive with either ELISA and/or PCR, respectively.

INTRODUCTION

Effective control of GWSS will require an areawide integrated pest management approach (AW-IPM). A major component of AW-IPM is the exploitation of the pest's natural enemies, which, when utilized to their greatest potential, can increase the effectiveness of other control tactics. Very little information exists on GWSS's predaceous natural enemies. Identifying the impact of predators can be challenging as they are usually small, elusive, nocturnal or cryptic. Direct visual field observations of predation are rare and often difficult to obtain. While predation studies using enclosures can provide some indication of predator impact, it fails to recreate natural conditions and can result in an overestimation of predation. A more valid method to qualitatively identify predators of key pests in nature is by the molecular analysis of predator gut contents for pest remains (reviewed in Sheppard and Harwood 2005). The state-of-the-art predator stomach content analyses include both MAb-based enzyme-linked immunosorbant assays (ELISA), which detect prey-specific proteins (Hagler et al. 1994ab, Schenk and Bacher 2004), and polymerase chain reaction (PCR)-based assays, which detect prey-specific DNA (Zaidi et al. 1999, Agustí et al. 2003). While DNA-based approaches reveal the prey identity at the species-level, they are unable to indicate which prey life stage is consumed. In contrast, pest-specific and life stage-specific MAbs can target a particular life stage of a given species, providing a higher level of precision to document predation (Hagler and Naranjo 1996). Combining both assays can provide a powerful tool to study predation on the GWSS.

To this end, genetic markers were designed using the cytochrome oxidase gene subunit I (COI) to detect and amplify a GWSS-specific fragment (de León et al. 2006), and a GWSS-egg specific MAb was developed to detect GWSS egg-specific protein (Hagler et al. 2002, Fournier et al. 2006).

OBJECTIVE

The main objective of this research is to identify the key predators of the different life stages of GWSS. More specifically, our aim is to determine the proportion of predators feeding on the various GWSS life stages in a citrus orchard. Using GWSS-specific ELISA and PCR assays, we examined the guts of 1,507 field-collected generalist predators. Results obtained from this research will aid in evaluating the efficacy of generalist predators for conservation biological control program.

RESULTS

Generalist arthropod predators were collected during 2002 and 2003 from a citrus orchard located at the Agricultural Operations Farm at the University of California, Riverside, CA. Collections were performed by beating the foliage or fogging the citrus trees with pyrethrum insecticide. Densities of GWSS were recorded as well (Blua and Akey, unpublished data). For each group of predators, we conducted lab trials to generate negative controls (i.e. individuals with no GWSS remains in their guts) and positive controls (i.e. individuals fed GWSS). Predators were frozen, sorted and then screened for GWSS remains with GWSS egg-specific sandwich ELISA and GWSS-specific PCR assays. Materials and methods employed were similar to the ones described in Fournier et al. (2006) and de León et al. (2006). Predators were scored positive for prey remains if the 197-bp specific GWSS DNA fragment was successfully amplified. With ELISA, specimens