# EVALUATION OF MOLECULAR MARKERS FOR DISCRIMINATING *GONATOCERUS MORRILLI*: A BIOLOGICAL CONTROL AGENT IMPORTED FROM THE ORIGIN OF THE GLASSY WINGED SHARPSHOOTER

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## ABSTRACT

We examined the utility of molecular markers for discriminating between two very closely related species, *Gonatocerus morrilli* (*G. morrilli*) (Howard), imported from Texas and *Gonatocerus walkerjonesi* S. Triapitsyn (*G. walkerjonesi*), native to California, to determine whether post-release *G. morrilli* specimens could be detected and discriminated in the field. We started by analyzing post-release specimens collected in 2002 and 2003. Amplification size of the internal transcribed spacer region (ITS2) demonstrated that all of the specimens were of the *G. walkerjonesi* ITS2 genotype. ISSR-PCR DNA fingerprinting experiments of specimens from the original *G. morrilli* 'release' colony showed that the DNA banding patterns were superimposable to that of the native *G. walkerjonesi*, confirming a colony contamination. A new *G. morrilli* colony was initiated in the spring of 2005, and we continued to survey random post-release specimens from the 2004-2006 collections. As expected, from 2004 and most of 2005, only the *G. walkerjonesi* ITS2 genotype was detected. In the fall of 2005 and in the spring and fall of 2006, we detected the *G. morrilli* ITS2 genotype at sites where the new colony was previously released. Analyses with two newly developed 'one-step' species-specific ITS2 diagnostic markers were in agreement with the results of the markers described above, demonstrating their usefulness in aiding the biological control program. *G. morrilli* is now one of the most recovered imported natural enemies in certain regions of California.

## INTRODUCTION

In the last 20 years, the glassy-winged sharpshooter (GWSS) has established and spread in southern California, where it poses a serious threat to the wine and table grape industry. Recently, we demonstrated that the GWSS that invaded California is of Texas USA origin (de León et al. 2004a), an observation that was later confirmed by Smith (2005). Pinpointing the origin of a pest and examining the role that natural enemies play in that region can aid in designing biological control strategies (Scheffer and Grissell 2003, Roderick 2004). Natural enemies have usually co-evolved with the target pest in the area of origin and therefore have highly specialized host-finding abilities that may increase the potential success of a biological control program (Scheffer and Grissell 2003, Roderick 2004, Brown 2004, Narang et al. 1993).

Initially, populations of *G. morrilli* from Texas and California were identified as a single species (Phillips et al. 2001). The California population of *G. morrilli* was identified as such because it was similar in several morphological features, including the antennae, coloration, and the wings, and also because it keyed to *G. morrilli* in Huber's key (Huber 1988, Triapitsyn 2006). Populations of *G. morrilli* imported from Texas had supposedly been released in California since 2001. It had therefore been difficult to distinguish between the native and imported natural enemies to determine their establishment. Molecular studies by de León et al. (2004a,b; 2006) strongly suggested that geographic populations of *G. morrilli* could be cryptic species. Molecular diagnostic markers were developed that distinguished the native and imported natural enemies from California and Texas. The two types of molecular markers were: 1) size differences in the internal transcribed spacer region (ITS2) fragment and 2) different inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting banding patterns (de León et al. 2004a). These studies prompted a closer morphological analysis of the populations of *G. morrilli* (Triapitsyn 2006). Based on these observations combined with molecular and hybridization studies (de León et al. 2004a, 2006), the population of *G. morrilli* from California was determined to be a new species, *G. walkerjonesi* S. Triapitsyn (Triapitsyn 2006). *G. walkerjonesi* and *G. morrilli* belong to the *morrilli* subgroup of the *ater* species group of *Gonatocerus*. The development and use of molecular diagnostic markers is very important for reasons discussed extensively in the literature (Powell and Walton 1989, Narang et al. 1993, Karp and Edwards 1997, Unruh and Woolley 1999, MacDonald and Loxdale 2004).

## OBJECTIVE

The objective of the current study was to evaluate the utility of various types of molecular markers in detecting and discriminating *G. morrilli* populations released in California against GWSS. Two previously developed molecular markers were tested: ISSR-PCR DNA fingerprinting and amplification size of the ITS2 (de León et al. 2004a, 2006). In addition, two newly developed 'one-step' species-specific primer sets targeting the ITS2 fragment of each parasitoid species were also evaluated (de León and Morgan 2007). Randomly chosen post-release specimens from collections made by the California Department of Food and Agriculture (CDFA) from 2002-2006 from several counties in southern California were analyzed.

**RESULTS AND CONCLUSIONS**

***The utility of the ITS2 size fragment in discriminating* G. morrilli *and evaluating post-release specimens***

Releases and post-release collections were made by the CDFA from several locations in southern California, a total of 329 specimens were analyzed in 19 total locations (data not shown) (see Table in current poster presentation). The expected ITS2 fragment sizes were: *G. morrilli* (TX) = 1063-1067 bp and *G. walkerjonesi* (CA) = 851-853 bp (Figure 1). Based on the size (851-853 bp) of the ITS2 fragments, all the individuals from the representative San Juan Capistrano, CA location corresponded to the *G. walkerjonesi* ITS2 genotype. Analyses of the remaining specimens (total of 280) from the various locations showed the same trend, that is, 100% of the individuals were of the *G. walkerjonesi* ITS2 genotype and none were of the *G. morrilli* ITS2 genotype.

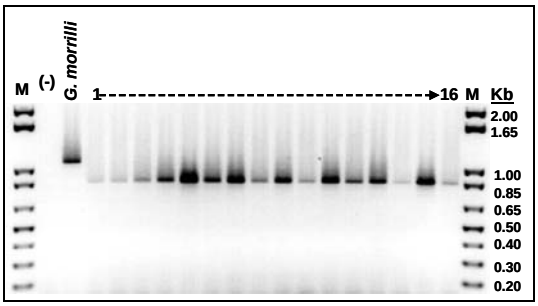


**Figure 1.** Representative example of the utility of the size of the ITS2 rDNA fragment. The ITS2 size was used as a molecular tool for discriminating and evaluating post-release specimens of *G. morrilli.* The ITS2 rDNA fragment was amplified with standard primers from 16 individuals from randomly chosen post-release specimens collected in San Juan Capistrano, CA. The size of the expected ITS2 amplification products are as follows: *G. morrilli* = 1063-1067 bp and *G. walkerjonesi* = 851-853 bp. The *G. morrilli* (TX) is included as a control. (-), negative control (no template DNA). **M**: 1.0 Kb Plus DNA Ladder.

***Molecular analysis of the original* G. morrilli *'release' colony by ISSR-PCR DNA fingerprinting***

Since egg parasitoids recovered from the 2002-2003 collections were only of the *G. walkerjonesi* ITS2 genotype, a possibility existed that the released *G. morrilli* did not establish or the released colony was contaminated. ISSR-PCR DNA fingerprinting of several individuals per colony were performed to answer this question. As controls, *G. morrilli* and *G. walkerjonesi* were included. The ISSR-PCR DNA banding pattern of the original *G. morrilli* 'release' colony was superimposable to that of the native California *G. walkerjonesi* species, demonstrating that the individuals from the 'release' colony were not *G. morrilli* (Figure 2). These results were in accordance with those seen with the post-release specimens from the 2002 and 2003 collections based on ITS2 fragment size (Figure 1).

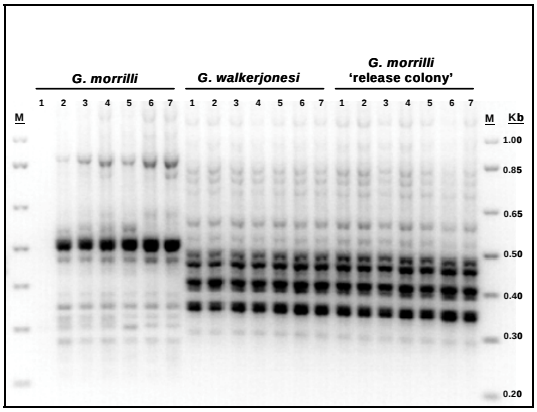


**Figure 2.** Evaluation of the original *G. morrilli* colony that was used for releases in California. ISSR-PCR DNA fingerprinting was utilized as a molecular tool to evaluate the *G. morrilli* 'release' colony. *G. morrilli* and *G. walkerjonesi* were included as controls. **M:** 1.0 Kb Plus DNA Ladder.

***Evaluation of post-release specimens from the 2004-2006 collections by ITS2 size fragment***
After realizing that the original *G. morrilli* 'release' colony used for previous releases were *G. walkerjonesi*, J. de León (ARS-Weslaco) sent the CDFA *G. morrilli* from Texas, the origin of GWSS. The CDFA began releases with the new *G. morrilli* colony in the summer of 2005. We continued surveying *G. morrilli* specimens from post-release collections from 2004 through 2006 by amplification of the ITS2 fragment. For the whole year of 2004 and most of 2005, we only detected the *G. walkerjonesi* ITS2 genotype in most locations. However, in three locations where CDFA made releases from the new *G. morrilli* release colony, this species was detected in the fall of 2005 and in the spring and fall of 2006 (data not shown) (see Table on current poster).

***Development, specificity, and utility of the 'one-step' species-specific ITS2 markers for* G. morrilli *and* G. walkerjonesi, *respectively***
To determine the specificity of the two molecular diagnostic markers, we tested specific amplification assay conditions and screened a total of 16 *Gonatocerus* Nees GWSS egg parasitoid species for cross-reactivity. Figures 3A (gmtx; *G. morrilli*) and 3B (wjca; *G. walkerjonesi*) show that the markers were specific. 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).

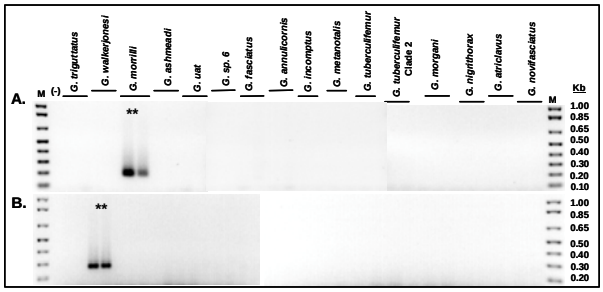


**Figure 3.** Specificity assays using the 'one-step' species-specific ITS2 diagnostic markers: A) gmtx (*G. morrilli*) and B) wjca (*G. walkerjonesi*). Genomic DNA from two individuals per *Gonatocerus* species (16) was used in amplification reactions to test for cross-reactivity of the two diagnostic markers with specific assays conditions. The species are listed on the figure. Expected fragment sizes: gmtx = 204 bp and wjca = 249 bp. **(-)**, negative control (no template DNA). **M**, 1.0 Kb Plus DNA Ladder.

***Molecular analysis of the original* G. morrilli *'release' colony used before the summer of 2005 with the ITS2 species-specific markers***
To test the utility of the newly developed 'one-step' diagnostic markers, we analyzed the same individuals from the previous *G. morrilli* release colony (those shown in Figure 2). Amplification with the 'gmtx' marker showed positive banding in only the control *G. morrilli* (TX) species (Figure 4A) but not in the control *G. walkerjonesi* species. Amplification with this marker of the *G. morrilli* 'release' colony also produced negative banding, confirming that the original release colony was not *G. morrilli*. Analysis of the same colony with the 'wjca' marker showed negative banding in the control *G. morrilli* species and positive banding in both the control *G. walkerjonesi* species and in the original 'release' colony (Figure 4B), confirming the results of ISSR-PCR DNA fingerprinting experiment and unambiguously confirming that the original 'release' colony was contaminated with the native species (*G. walkerjonesi*) from California.

***The utility of the 'one-step' ITS2 species-specific markers in discriminating and evaluating post-release* G. morrilli *specimens in California***
To confirm the usefulness of these diagnostic markers, we randomly screened post-release specimens of *G. morrilli*, including the specimens that tested positive by utilizing the amplification size of the ITS2 fragment. Amplification with the 'gmtx' marker produced positive banding in the three locations (Figure 5A, Lanes e, g, and h) that tested previously positive, whereas amplification with the 'wjca' marker tested positive only with the *G. walkerjonesi* specimens (Figure 5B, Lanes a-d and f), confirming the results inferred by amplification of the ITS2 region. In early 2007, the new *G. morrilli* release colony was tested with the 'gmtx" marker and the results showed that the colony was indeed *G. morrilli*, confirming the purity of the colony (results not shown).

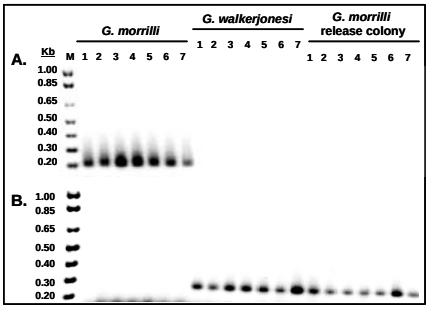


**Figure 4.** Evaluation of the original *G. morrilli* 'release' colony by the 'one-step' ITS2 markers: A) gmtx and B) wjca. *G. morrilli* and *G. walkerjonesi* were included as controls. Seven individuals per colony were included. **M**, 1.0 Kb Plus DNA Ladder.

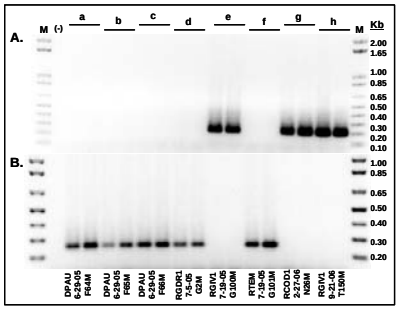


**Figure 5.** Representative example of the utility of the 'one-step' species-specific markers: A) gmtx and B) wjca were used for detecting and discriminating post-release random specimens in California. Two individuals per collection site were included. **M**, 1.0 Kb Plus DNA Ladder.

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