

**THE UTILITY OF THE INTERNAL TRANSCRIBED SPACER REGION 2 (ITS2) IN CONFIRMING SPECIES
BOUNDARIES IN THE GENUS *GONATOCERUS*: COMPARISON TO THE CYTOCHROME OXIDASE
SUBUNIT I (COI) GENE AND TAXONOMIC DATA: MOLECULAR KEY BASED ON ITS2 SIZES**

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ABSTRACT

We sequenced the nuclear ribosomal internal transcribed spacer region 2 (ITS2) from several glassy-winged sharpshooter (GWSS) [*Homalodisca vitripennis* Germar (= *H. coagulata* Say)] egg parasitoid species (Hymenoptera: Mymaridae) belonging to the genus *Gonatocerus* Nees to test the utility of this fragment to confirm species boundaries and to define phylogenetic relationships. A total of 35 specimens belonging to 10 named species, one unnamed species, and two specimens from another mymarid genus (*Anagrus erythroneuræ*) (outgroup) were analyzed. A phylogenetic tree generated using the neighbor-joining algorithmic method showed that each named *Gonatocerus* species formed its own unique taxonomic unit or clade with very strong bootstrap support (100%), confirming species boundaries. The ITS2 fragment confirmed species boundaries as well as cytochrome oxidase subunit I (COI) sequence data. Furthermore, the phylogenetic relationships among species generated by the ITS2 fragment were in excellent agreement with those delineated by taxonomic data. The current results clearly confirm the utility of the ITS2 fragment in confirming species boundaries of egg parasitoids belonging to the genus *Gonatocerus*. The results showed that the ITS2 fragment appears to be phylogenetically more informative or valuable than that inferred by COI sequence data. Since several important *Gonatocerus* species were analyzed, a molecular key based on ITS2 sizes was developed. In the event two species (e. g., *G. ashmeadi* and *G. metanotalis* and *G. walkerjonesi* and *G. annulicornis*) were found with similarly sized ITS2 fragments, inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting was performed to distinguish them. ISSR-PCR very clearly distinguished the aforementioned species, demonstrating that it is an excellent molecular diagnostic tool. The current results are important to the biological control program in California.

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 Aliniaze 1988, Löhr et al. 1990, Narang et al. 1993). Among others, DNA markers such as the nuclear ribosomal internal transcribed spacer regions (e. g., ITS2) are used to characterize parasitoid taxa because these DNA regions usually evolve relatively rapidly (Hillis and Dixon 1991, Narang et al. 1993). The ITS regions have been used extensively in the examination of the taxonomic status of species and for diagnostic purposes (Collins and Paskewitz 1996, Stouthamer et al. 1999). Many examples of phylogenetic studies inferred by the nuclear ITS regions or fragments, including different sized fragments, exists in the literature (Marinucci et al. 1999, Förster et al. 2000, Pryor and Gilbertson 2000, Alvarez and Hoy 2002, Thomson et al. 2003, de León et al. 2006a, Wagener et al. 2006), including those by Stouthamer et al. (1999).

OBJECTIVES

Sequence the nuclear ribosomal internal transcribed spacer region 2 (ITS2) from several GWSS egg parasitoid species (11) belonging to the genus *Gonatocerus* to test the utility of this rDNA fragment to: 1) confirm species boundaries and 2) define phylogenetic relationships.

RESULTS AND CONCLUSIONS

Species boundaries inferred by the ribosomal internal transcribed spacer region 2 (ITS2)

We obtained 8 of the 13 named *Gonatocerus* Nees species delineated by Triapitsyn (2006) and Triapitsyn et al. (2006) and several named and one unnamed species from South America for a total of 11 species. A total of 35 ingroup specimens were analyzed and two specimens from *Anagrus erythroneuræ* Triapitzin & Chiappini (also a mymarid species) were included as an outgroup. Each named *Gonatocerus* species formed its own taxonomic unit or distinct clade (Figure 1), corroborating the species boundaries of Triapitsyn (2006) and Triapitsyn et al. (2006). A neighbor-joining distance tree showed that each taxonomic unit was supported by very strong bootstrap values, in fact, each received 100% support. In addition, the unnamed *Gonatocerus* species (*G. sp. 6*) from Argentina also clustered into its distinct clade, suggesting that it is a separate or valid species. Analysis of several other *Gonatocerus* species inferred by the ITS2 DNA fragment are in progress to complete this project. As previously demonstrated by Vickerman et al. (2004) and Triapitsyn et al. (2006) no divergence or differences were seen in the five geographic populations [California, Texas (Weslaco and San Antonio), Florida, and Louisiana] of *G. ashmeadi*, as they all formed their unique clade.

Comparison of ITS2 DNA sequences to those of the COI partial gene sequences

To confirm the utility of using the ITS2 DNA fragment in these types of studies, we matched the mitochondrial cytochrome oxidase subunit I (COI) gene sequences to the same *Gonatocerus* species. A phylogenetic analysis of several *Gonatocerus* species inferred by the COI gene has been reported elsewhere [accompanying report and de León et al. (2006b)].

Comparison of the neighbor-joining distance tree generated by ITS2 fragments to that generated by the COI gene (Figure 2), confirms that the ITS2 fragment can be used to determine species boundaries of egg parasitoids belonging to the genus *Gonatocerus*. As with the ITS2 fragments, very strong bootstrap support (95-100%) was seen with each taxonomic unit or distinct clade generated by the COI gene (de León et al. 2006b).

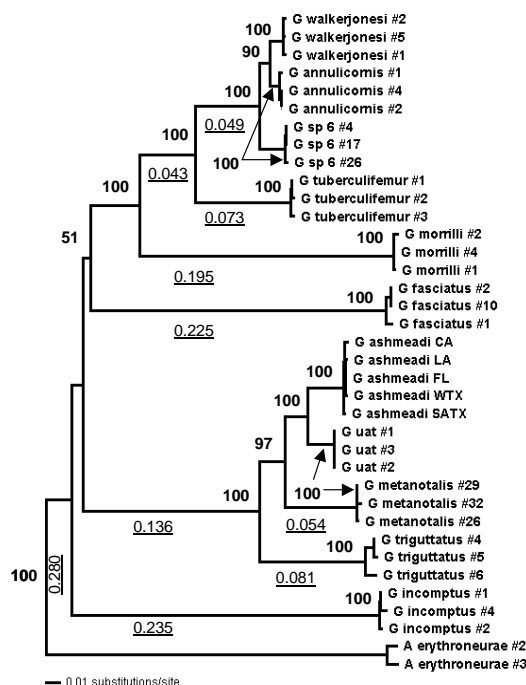


Figure 1. ITS2

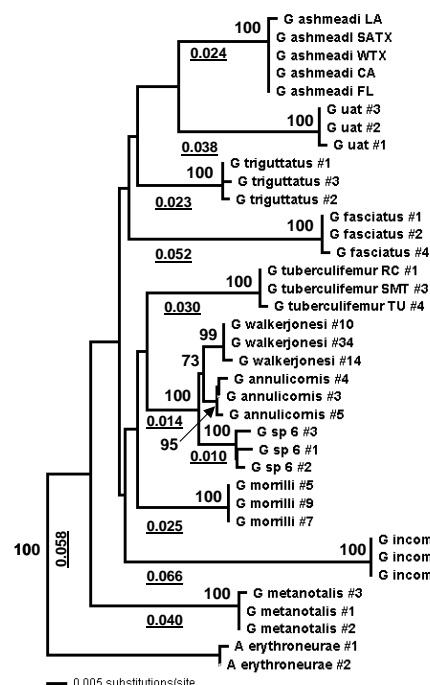


Figure 2. COI

Figure 1. (ITS2) and Figure 2. (COI) Phylograms of GWSS egg parasitoid species belonging to the genus *Gonatocerus* inferred by ITS2 and COI sequence data. Analyses were performed with the alignment program ClustalX and the neighbor-joining distance trees utilizing the uncorrected 'p' genetic distance were reconstructed with the phylogenetic program PAUP 4.0b10. *Anagrus erythroneurae* (a mymarid species) was included as an outgroup. The tree displays branch lengths (below branches, underlined) and bootstrap values (above branches), as percentage of 1000 replications.

Comparison to taxonomic data

To confirm the utility of the ITS2 fragment in defining phylogenetic relationships among egg parasitoid species within the genus *Gonatocerus*, a comparison of the topology of the phylogenetic tree was made to the morphological work delineated by Triapitsyn (2006) and Triapitsyn et al. (2006). Morphologically, almost every species in the USA has its 'look-a-like' species in the Neotropics, particularly in Argentina (S. Triapitsyn, unpublished data). The following South and North American species pair-up morphologically: respectively *G. sp. 2* and *G. fasciatus*; *G. annulicornis* and *G. walkerjonesi*; *G. metanotalis* and *G. triguttatus*; and *G. uat* and *G. ashmeadi*. *Gonatocerus morrilli* belongs to the *morrilli* subgroup of the *ater* species group of *Gonatocerus* along with the following species: *G. annulicornis*, *G. walkerjonesi*, *G. sp. 6*, and *G. morgani* (S. Triapitsyn, unpublished data and Triapitsyn 2006). In addition, *G. novifasciatus* and *G. incomptus* are also related (Triapitsyn 2006). Not all mentioned *Gonatocerus* species were analyzed at the time of this report. The phylogenetic relationships inferred by ITS2 fragment sequence data analyzed by the neighbor-joining method of the South and North American *Gonatocerus* species in the current study are all in accord with the morphological evaluations mentioned above (Triapitsyn 2006, Triapitsyn et al. 2006). Based on the phylogenetic tree, the following species paired-up in the following fashion: *G. ashmeadi* and *G. uat* (Triapitsyn et al. 2006, de León et al. 2006b); *G. walkerjonesi* and *G. annulicornis* (de León et al. 2006a); *G. morrilli* and *morrilli* group (de León et al. 2006a); *G. sp. 6* and *morrilli* group (de León et al. 2006b); and to some extent *G. metanotalis* and *G. triguttatus* (except for the color and several other differences, *G. ashmeadi*, *G. uat*, *G. triguttatus*, and *G. metanotalis* are all quite similar morphologically, and the above molecular analysis supports their close relationship. We noticed in our phylogenetic analyses inferred from both COI and ITS2 that *G. tuberculifemur* clustered with the *morrilli* group (current study and de León et al. 2006b). The topology of the phylogenetic tree generated by the COI sequence data differed slightly from that generated by the ITS2 fragment with the placement of three species: *G. fasciatus*, *G. metanotalis*, and *G. fasciatus*, thus differing from the morphological work of Triapitsyn (2006) and Triapitsyn et al. (2006).

The current results showed that the nuclear ribosomal ITS2 fragment appears to be phylogenetically more informative or valuable than that inferred by COI sequence data. A similar observation has been seen in phylogenetic studies of *Diadegma* species (Hymenoptera: Ichneumonidae) (Wagener et al. 2006).

Molecular key based on ITS2 sizes

Several of the most important egg parasitoid species were analyzed in the current study and therefore allowed for the development of a molecular key based on the specific sizes of the various ITS2 fragments. Many species have unique ITS2 sizes, but others (e. g., *G. ashmeadi* and *G. metanotalis* and *G. walkerjonesi* and *G. annulicornis*) have similar sizes (Table 1). The only concern in these cases would be if any of the South America species (*G. metanotalis* and *G. annulicornis*) were to be released in California.

ISSR-PCR DNA fingerprinting

Although there exist several approaches [e. g., PCR-RFLP (Stouthamer et al. 1999)] to distinguish the aforementioned species containing similarly sized ITS2 fragments, a sensitive diagnostic approach is by inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting using a 5'-anchored ISSR primer (Zietkiewicz et al. 1994). Therefore, we submitted the following species: *G. ashmeadi* and *G. metanotalis* and *G. walkerjonesi* and *G. annulicornis* to ISSR-PCR DNA fingerprinting and the results are shown on Figure 3. As seen from the figure, a very clear distinction is made between the species-pairs, including all four species. Unique ISSR-PCR banding patterns were obtained per species. Even though variation is seen in *G. annulicornis*, the total banding pattern is still different from the rest of the species.

The current results demonstrated that ISSR-PCR is an excellent molecular diagnostic tool with haplodiploid species, an observation seen recently with several *Gonatocerus* species (de León et al. 2005, de León et al. 2006a, b, de León and Morgan 2006).

Table 1. Molecular key based on ITS2 sizes. *Includes populations from California, Texas (Weslaco and San Antonio), Louisiana, and Florida; ^aVery close in size; ^bOverlap in size; ^cProspective GWSS egg parasitoid candidate agents from South America.

<i>Gonatocerus</i> species	<i>n</i>	ITS2 range (bp)
<i>G. ashmeadi</i>	29*	939-948 ^a
<i>G. triguttatus</i>	3	995-1006
<i>G. fasciatus</i>	3	1071-1077
<i>G. morrilli</i>	8	1063-1066
<i>G. walkerjonesi</i>	6	851-853 ^b
<i>G. tuberculifemur^c</i>	3	823-825
<i>G. metanotalis^c</i>	3	929-932 ^a
<i>G. annulicornis</i>	4	850-854 ^b
<i>G. uat</i>	4	914-915
<i>G. sp. 6^c</i>	3	867-868
<i>G. incomptus</i>	4	870-876
<i>G. novifasciatus</i>	1	762

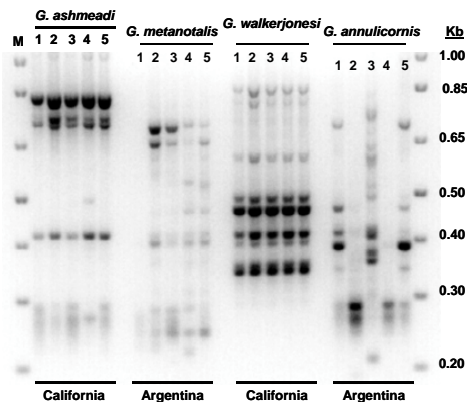


Figure 3. ISSR-PCR DNA fingerprinting of *Gonatocerus* species with similarly sized ITS2 fragments. Reactions were performed with genomic DNA from five separate individuals and a 5'-anchored ISSR primer. M, 1.0 Kb plus DNA Ladder.

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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*. Analysis 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 Aliniaze 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 haplodiploid 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.