ISSR-PCR DNA FINGERPRINTING UNCOVERS DISTINCT BANDING PATTERNS IN *GONATOCERUS* SPECIES 3 (*G.* sp. 3) INDIVIDUALS EMERGING FROM DIFFERENT HOST TRIBES: A PROSPECTIVE EGG PARASITOID CANDIDATE AGENT FOR THE GLASSY-WINGED SHARPSHOOTER IN CALIFORNIA

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ABSTRACT

We started work to genetically characterize a prospective glassy-winged sharsphooter (GWSS), *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)] egg parasitoid biological control candidate agent from South America known as *Gonatocerus* species 3 (*G*. sp. 3). This species is morphologically very similar to *G. tuberculifemur*, another prospective agent from South America. We asked two questions, 1) are *G*. sp. 3 and *G. tuberculifemur* the same species and 2) are two collections of *G*. sp. 3 individuals emerging from different host tribes (Proconiini and Cicadellini) genetically distinct. Or, in both cases, are we seeing genetic variation of the same species. Two molecular methods were utilized to begin to study these species, the very sensitive inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting and mitochondrial cytochrome oxidase subunit I gene (COI) variation. ISSR-PCR analysis performed together on both *G*. sp. 3 and *G. tuberculifemur* uncovered the following: 1) as previously shown, *G. tuberculifemur* geographic populations were genetically distinct, 2) *G*. sp. 3 and *G. tuberculifemur* were very clearly distinct, and 3) banding patterns differences (about four bands) distinguished the two collections of *G*. sp. 3. A single most parsimonious tree clustered the current specimens in the following fashion: 1) as previously shown, the geographic populations of *G. tuberculifemur* clustered into two well-supported distinct clades with very strong bootstrap values (90-99%), and 2) the *G*. sp 3 collections clustered along with clade 2 (San Rafael population) of the *G. tuberculifemur* populations, though one *G*. sp. 3 collection (Jan 05; Proconiini host) forms a unique clade with moderate bootstrap support (63%). Even though, the divergence between the two *G*. sp. 3 collections was very small, the two shared no haplotypes. The current results confirm that ISSR-PCR DNA fingerprinting using a 5'-anchored ISSR primer is an excellent molecular diagnostic tool for distinguishing *G*. sp. 3 from both clades of *G. tuberculifemur*. COI sequence variation effectively distinguished *G*. sp. 3 from *G. tuberculifemur* individuals from clade 1, though it did not effectively separate *G*. sp. 3 from *G. tuberculifemur* individuals from clade 2 (San Rafael population). We conclude that based on ISSR-PCR analysis, *G*. sp. 3 and *G. tuberculifemur* and both collections of *G*. sp. 3 are clearly genetically distinct. The only way to confirm whether these specimens are actually cryptic or different species is by performing hybridization studies. These molecular results are important to the biological control program in California.

INTRODUCTION

Beginning in 2000, egg parasitoids of closely related hosts belonging to the sharsphooter tribe Proconiini [*Tapajosa rubromarginata* (Signoret)] were sought from regions in South America where climate types and habitats were similar to California for a neoclassical biological control program (Jones 2001, Logarzo et al. 2003, 2004, 2005). In surveys conducted in Argentina and Chile during 2000 through 2005, prospective egg parasitoid candidate agents were identified among several *Gonatocerus* Nees species (Hymenotpera: Mymaridae) reared from *T. rubomarginata* (Jones et al. 2005, Logarzo et al. 2005, Virla et al. 2005). Several unnamed egg parasitoid candidate agents within the genus *Gonatocerus* from South America were identified by S. Triapitsyn (UC-Riverside). Phylogenetic analysis inferred by COI sequencing on two of the unnamed species (*G*. sp. 2 and *G*. sp. 6) are reported in an accompanying report and elsewhere (de León et al. 2006b). The data suggests that these unnamed species are valid species or taxonomic units. A third unnamed species known as *G*. sp. 3 was also identified that is morphologically very similar or almost identical to another South America species, *G. tuberculifemur* (Ogloblin) (S. Triapitsyn, unpublished data). Since this and other South American species are prospective biological control agents, molecular studies are critical to help resolve the taxonomic status of this and other species. Identifying the correct natural enemy is critical to the success of classical biological control programs, since lack of proper identification procedures has affected several projects (Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993).

OBJECTIVES

The aim of the present study was to survey molecular methods to study *G*. sp. 3. Morphologically, this species is almost identical or very similar to *G. tuberculifemur* (S. Triapitsyn, unpublished data). The first objective was to begin to gain insights as to whether *G*. sp. 3 and *G. tuberculifemur* are distinct species or whether variation of the same species exists, and the second objective was to determine whether collections *G*. sp. 3 emerging from different host tribes (Proconiini and Cicadellini) are genetically distinct.

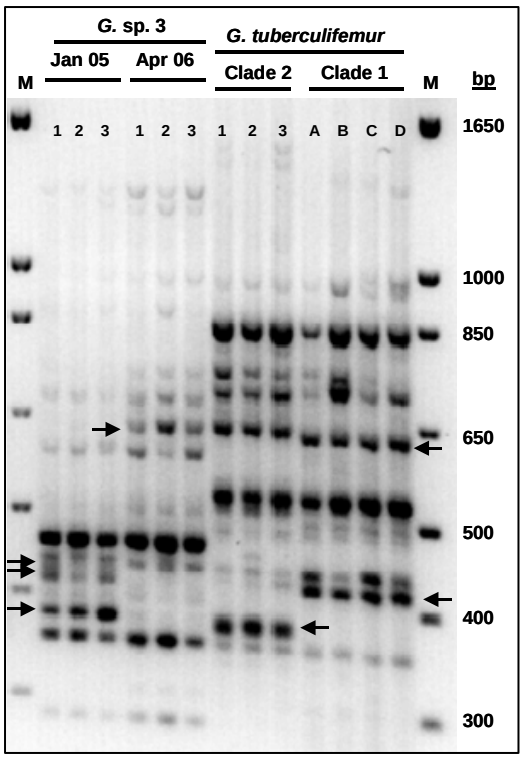

Figure 1. ISSR-PCR DNA fingerprinting of two *G*. sp. 3 populations from San Miguel de Tucumán, Tucumán Province, Argentina. Reactions were performed with genomic DNA from 3-4 separate individuals and a 5'-anchored ISSR primer (Zietkiewicz et al. 1994, de León et al. 2004b). *G*. sp. 3 are from two different collection dates, January 2005 [emerged from *T. rubromarginata* (Proconiini leafhopper) and April 2006 emerged from *Plesiommata mollicella* (Cicadellini leafhopper)]. *G. tuberculifemur* collections are as follows- Clade 2 are from San Rafael (SR) (Mendoza Province) Argentina and Clade 1 are: A, Rio Colorado (RC) (Rio Negro); B, San Miguel de Tucumán (SMT) (Tucumán); C, Chile (CH); and D, Tunuyán (TU) (Mendoza). Arrows point out the banding patterns differences. M: 1.0 Kb Plus DNA Ladder.

RESULTS AND CONCLUSIONS

ISSR-PCR DNA fingerprinting

Previously (de León et al. 2006b) and in an accompanying report, we demonstrated molecular differences in populations of *G. tuberculifemur* from South America. ISSR-PCR DNA fingerprinting uncovered fixed geographic variation and COI variation uncovered divergent clades in *G. tuberculifemur*. Since it is thought that *G*. sp. 3 could actually be *G. tuberculifemur*, we asked two questions: 1) based on ISSR-PCR, are *G*. sp. 3 and *G. tuberculifemur* genetically distinct, and 2) based on ISSR-PCR, are there distinct differences in *G*. sp. 3 individuals emerging from different hosts tribes [San Miguel de Tucumán (Tucumán)]. The results of this experiment are shown on Figure 1 and we make three observations: 1) as previously shown (de León et al. 2006b) the two clades of *G. tuberculifemur* are distinguished, see arrows that point to different bands; 2) very clear banding pattern differences between *G*. sp. 3 and *G. tuberculifemur*, and 3) banding pattern differences between the two collections of *G*. sp. 3, about four bands distinguish the two collections. These results are very reproducible.

Phylogeographic analysis between the two collections of G. sp. 3 and among geographic populations of *G. tuberculifemur* inferred by the mitochondrial COI partial gene

The single most parsimonious tree is supported by strong bootstrap values, 63-99% for the ingroups, and 100% for the outgroups (*G. annulicornis* and *G. morrilli*) (Figure 2). As shown previously (de León et al. 2006b) and in an accompanying report, populations of *G. tuberculifemur* clustered into two well-supported clades (90-99%). In general, the two collections of *G*. sp. 3 clustered with the *G. tuberculifemur* clade from San Rafael, though *G*. sp. 3 from the Jan 05 collection emerging from the Proconiini host appears to be slightly diverged as seen by a distinct clade with moderate bootstrap support (63%). It is interesting to note that even though, based on COI analysis, the divergence between the two collections of *G*. sp. 3 is very small, they did not share haplotypes.

Two molecular methods were employed to genetically study a GWSS candidate agent, G. sp. 3 from South America. ISSR-PCR DNA fingerprinting identified fixed geographic-specific variation among the G. tuberculifemur populations (de León et al. 2006b) and fixed genetic variation between the two collections of G. sp. 3 emerging from different hosts. Even though ISSR-PCR markers are scored as dominant, the method is still extremely sensitive and an excellent first approach to detect genetic differences among species, especially haplodipoid species (de León and Jones 2004, de León et al. 2004a,b, 2006a,b). A phylogenetic approach inferred by the COI partial gene, detected two well-supported clades in G. tuberculifemur (de León et al. 2006b). However, in the present situation with G. sp. 3, the COI gene was less sensitive than ISSR-PCR. A possible explanation for the COI sequence analysis being less sensitive than ISSR-PCR could be that G. sp. 3 diverged a very short time ago from G. tuberculifemur and the COI gene is not yet variant enough at this time to properly distinguish them. Time since divergence is a very significant factor as reviewed in Roderick and Navajas (2003). Another explanation is that the two collections of G. sp. 3 emerged from different hosts. More work and an increased number of specimens are needed to determine whether G. sp. 3 and G. tuberculifemur are actually different species and whether the two collections of G. sp. 3 are different strains or whether what we are seeing is just genetic variation of the same species. To resolve these issues, crossing studies should answer our questions. Hybridization and morphological studies are presently being planned. For now, we can positively state that G. sp. 3 and G. tuberculifemur and the two collections of G. sp. 3 are clearly genetically distinct by ISSR-PCR DNA fingerprinting. COI variation effectively distinguished G. sp. 3 from G. tuberculifemur individuals from clade 1, but not clade 2. So, the following questions arise from these studies: Is ISSR-PCR using a 5'-anchored ISSR primer more sensitive than DNA sequencing and can ISSR-PCR detect differences in individuals of the same species emerging from different hosts, or rather do the different hosts change their genetic structure? The ISSR-PCR technique targets random simple sequence repeats or microsatellites targeting the whole genome, thus revealing highly polymorphic banding patterns (Zietkiewicz et al. 1994). Therefore, increased power to resolve genetic relationships comes with information from many loci within the nuclear DNA (de León and Jones 2004, de León et al. 2004a,b, 2006,

Zietkiewicz et al. 1994). The results of the present study are important to the biological control program in California against the invasive GWSS.

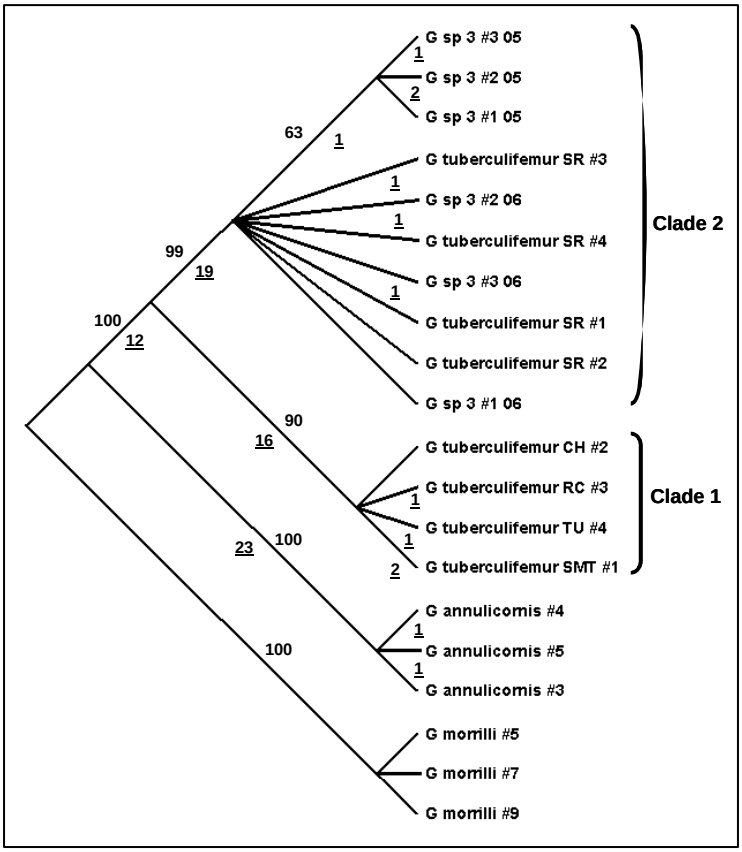

Figure 2. Slanted cladogram inferred by the COI partial gene from two collections of *G.* sp. 3 from Argentina (San Miguel de Tucumán, Tucumán Province). Analysis was performed with the alignment program ClustalX and the single most parsimonious tree was reconstructed with the phylogenetic program PAUP 4.0b10. *G. annulicornis* and *G. morrilli* were included as outgroups. The tree displays branch lengths (below branches, underlined) and bootstrap values (above branches), as percentage of 1000 replications. Tree length = 57 steps; consistency index (CI) = 0.982; and retention index (RI) = 0.994. Included for comparison are populations of *G. tuberculifemur* from South America: SR, San Rafael; CH, Chile; RC, Rio Colorado; TU, Tunuyán; and SMT, San Miguel de Tucumán.

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GENETIC STUDIES OF *GONATOCERUS METANOTALIS* POPULATIONS FROM ARGENTINA UNCOVER DIVERGENT CLADES: A PROSPECTIVE EGG PARASITOID CANDIDATE AGENT FOR THE GLASSY-WINGED SHARPSHOOTER IN CALIFORNIA

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ABSTRACT

Two molecular methods were utilized to genetically distinguish geographic populations of *Gonatocerus metanotalis* (Ogloblin) (Hymenoptera: Mymaridae) from Argentina and to begin to test the possibility that this South American species could exist as a cryptic species complex. *Gonatocerus metanotalis* is a prospective egg parasitoid candidate agent for a neoclassical biological control program in California against the invasive glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)]. Six populations were analyzed: Campo Grande (Misiones Province), Tartagal (Salta), Tafí Viejo (Tucumán), near PROIMI (Tucumán), Santa Clara (Jujuy), Clorinda (Formosa). As a first approach, inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting was performed with a 5'-anchored ISSR primer. Several distinct banding patterns were identified among the populations with some band sharing and in certain populations (Tartagal and Santa Clara) there was extensive variation. Next, a phylogeographic analysis inferred by the mitochondrial cytochrome oxidase subunit I (COI) gene was performed. A neighbor-joining distance tree clustered the *G. metanotalis* populations into three main distinct clades supported by very strong bootstrap values (100%), uncovering haplotype or phylogeographic structure. With the exception of one population (Campo Grande), all individuals from the populations fell into one of the three clades. Individuals from Campo Grande clustered into the three clades, suggesting that three sympatric strains may be present in this location. A phylogenetic analysis performed by the neighbor-joining algorithmic method along with other named and two unnamed *Gonatocerus* Nees species (15) confirmed species boundaries and again uncovered three main distinct clades in *G. metanotalis*. Very strong bootstrap support (100%) was seen for both the *G. metanotalis* clades and for all of the *Gonatocerus* species. Understanding possible cryptic variation in this prospective GWSS egg parasitoid candidate agent is critical to the biological control program in California.

INTRODUCTION

A biological control program is currently in progress in California against the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)] (Hemiptera: Cicadellidae) (CDFA 2003). Biological control is an important component of the management of the GWSS in California (Morgan et al. 2000, Jones 2001). Uncertainty exists as to whether egg parasitoids native to California will be as effective against the GWSS as they are in their co-evolved native range (Jones 2001, Logarzo et al. 2003, 2004, Virla et al. 2005). Beginning in 2000, egg parasitoids of closely related hosts belonging to the sharsphooter tribe Proconiini [*Tapajosa rubromarginata* (Signoret)] were sought from regions in South America where climate types and habitats were similar to California for a neoclassical biological control program (Jones 2001, Logarzo et al. 2005). In surveys conducted in South America during 2000 through 2005, prospective egg parasitoid candidates were identified among several *Gonatocerus* Nees species reared from *T. rubomarginata* (Logarzo et al. 2005, Virla et al. 2005). One egg parasitoid candidate was identified by S. Triapitsyn (UC-Riverside) as *G. metanotalis* (Ogloblin) (Hymenotpera: Mymaridae). Molecular studies of insects are becoming increasingly important in resolving taxonomic relationships critical to the success of biological control programs. Identifying the correct natural enemy is critical to the success of classical biological control programs (Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993, Unruh and Woolley 1999).

OBJECTIVES

The aim of the present study was to survey molecular methods useful in egg parasitoid identification and discrimination and to begin to investigate the possibility that *G. metanotalis* could exist as a cryptic species complex in South America. In addition, perform a phylogenetic analysis with several named and two unnamed species within the genus *Gonatocerus* Nees to confirm species boundaries and to test the support for the species groups considered.

RESULTS AND CONCLUSIONS

ISSR-PCR DNA fingerprinting

Amplification reactions were performed with geographic populations from Argentina with 3-5 separate individuals from pooled egg masses per location. Populations included: Campo Grande (Misiones Province); Tartagal (Salta); Tafí Viejo (Tucumán); near PROIMI (Tucumán); Santa Clara (Jujuy); and Clorinda (Formosa). Previously, we demonstrated a positive correlation between ISSR-PCR banding patterns and species distinction (de León et al. 2004, 2006a,b). In addition, we have utilized the method to distinguish about 8 *Gonatocerus* species (de León et al. 2006b). As a first approach, we asked whether the ISSR-PCR method was suitable to distinguish geographic populations of *G. metanotalis* from Argentina. The results of