GENETIC CHARACTERIZATION OF *GONATOCERUS TUBERCULIFEMUR* FROM SOUTH AMERICA UNCOVERS DIVERGENT CLADES: PROSPECTIVE EGG PARASITOID CANDIDATE AGENT FOR THE GLASSY-WINGED SHARPSHOOTER IN CALIFORNIA

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ABSTRACT

In present study we genetically characterized the prospective South American egg parasitoid candidate, *Gonatocerus tuberculifemur* (Ogloblin) of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)] for a neoclassical biological control program in California. Two molecular methods, inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting and a phylogeographic approach inferred by the mitochondrial cytochrome oxidase subunit I gene (COI). Five geographic populations from South America were analyzed; in addition, a phylogenetic analysis was performed with several named and two unnamed *Gonatocerus* Nees species. DNA fingerprinting uncovered a fixed geographic banding pattern difference in the population from San Rafael, Mendoza Province, Argentina. The COI analysis uncovered haplotype or geographic structure in *G. tuberculifemur*. A neighbor-joining distance tree clustered the populations into two well-supported distinct clades with very strong bootstrap values (96-100%) with the population from San Rafael clustering into a separate clade than the rest of the South American populations. No haplotype sharing was observed between individuals from the two clades. A phylogenetic analysis performed by the neighbor-joining method of 15 *Gonatocerus* Nees species confirmed species boundaries and again uncovered two distinct clades in *G. tuberculifemur* with very strong bootstrap support (96-100%). The two molecular methods were in accord and the evidence is suggestive of a species level divergence. Because *G. tuberculifemur* is under consideration as a potential biological control agent for the invasive GWSS in California, understanding possible cryptic variation of this species is critical.

INTRODUCTION

Uncertainty exists as to whether egg parasitoids native to California will be as effective against the glassy-winged sharpshooter (GWSS) *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)]as they are in their co-evolved native range (Jones 2001, Logarzo et al. 2003, 2004, Virla et al. 2005). As a consequence, 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 Argentina and Chile during 2000 through 2005, two prospective egg parasitoid candidate agents were identified among several *Gonatocerus* Nees species reared from *T. rubomarginata* (Jones et al. 2005, Logarzo et al. 2005, Virla et al. 2005). The egg parasitoid candidates from South America were identified by S. V. Triapitsyn (UC-Riverside) as *Gonatocerus tuberculifemur* and *G. metanotalis* (Ogloblin) (Hymenotpera: Mymaridae). *Gonatocerus tuberculifemur* is now being permitted for release in California (CDFA 2005). Mymarid wasps are the best-known egg parasitoids for controlling populations of leafhoppers (Huber 1986, Döbel and Denno 1993). 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. Lack of proper identification procedures has affected several projects (Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993).

OBJECTIVE

The aim of the present study was to survey molecular methods useful in egg parasitoid identification and discrimination and investigate the possibility that *G. tuberculifemur* (Ogloblin) could exist as a cryptic species complex. In addition, perform a phylogenetic analysis with several 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 and Chile with 5-9 separate individuals from pooled egg masses per location. Locations included were: Argentina: Rio Colorado (RC) (Rio Negro Province), San Rafael (SR) (Mendoza), San Miguel de Tucumán (SMT) (Tucumán), and Chile: Jalsuri (CH). Previously, we have demonstrated a positive correlation between ISSR-PCR banding patterns and species distinction (de León and Jones 2004, de León et al. 2004a,b, 2006). In addition, we have utilized the method to distinguish about 8 *Gonatocerus* species. As a first approach, we asked whether the ISSR-PCR method was suitable to distinguish geographic populations of *G. tuberculifemur* from Argentina and Chile. The results of this analysis are shown on Figure 1. Three geographic- or population-specific bands were identified within the San Rafael population, as indicated by the arrows. Slight variation was seen within the rest of the populations, but in general, similar banding patterns were observed within these

populations, demonstrating their genetic similarity. The Tunuyán (TU) (Mendoza Province) population was not available at the time of this experiment.

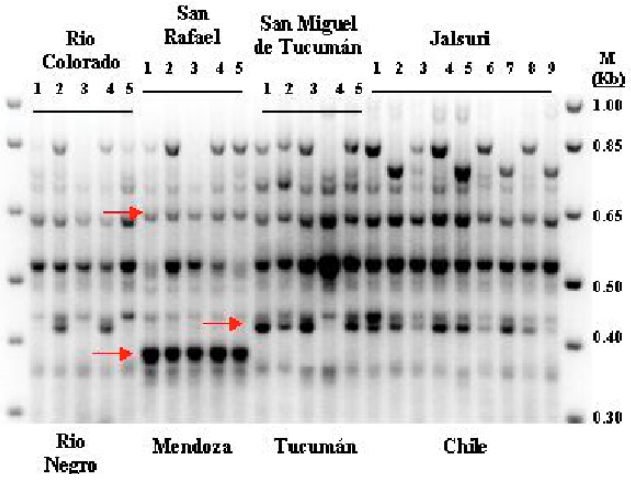

Figure.1. ISSR-PCR DNA fingerprinting of *G. tuberculifemur* populations from Argentina and Chile. Reactions were performed with genomic DNA from 5-9 separate individuals and a 5'-anchored ISSR primer (Zietkiewicz et al. 1994, de León et al. 2004b). Arrows point out the banding patterns differences. M: 1.0 Kb Plus DNA Ladder.

Phylogeographic analysis of populations of *G. tuberculifemur*

Levels of genetic divergence (%D) in the COI partial gene among populations were determined by calculating the pairwise estimates for genetic distance (Table 1). Individuals from each clade were pooled to calculate the pairwise estimates. The intra-populational and -specific variation (0.0-0.6%) was small within each clade and species. The %D within each *G. tuberculifemur* clade was 0.0-0.6, whereas between them was 1.4-2.2. A neighbor-joining distance tree showed that individuals clustered into two well-supported distinct clades with very strong bootstrap values of 96-100%, with all of the San Rafael individuals forming a distinct clade (Figure 2). Though the %D was moderate between the two *G. tuberculifemur* clades, it corroborates with the results seen in Figures 1 and 2 and Table 1, showing a very clear genetic distinction between individuals from the two clades. In addition, haplotype or phylogeographic structure was uncovered in these populations.

Phylogenetic analysis of several named and two unnamed* Gonatocerus *species

Resolution of relationships requires information about variability not only at the level of populations within a species but also between species (Narang et al. 1993); therefore, a molecular systematic approach inferred by the COI gene was undertaken with various named *Gonatocerus* species, along with *G. tuberculifemur* populations from South America. The named *Gonatocerus* species were also included to test the support for the species groups considered. A total of 48 ingroup specimens were analyzed and four specimens from two *Anagrus* Haliday species (also a mymarid genus) were included as outgroups. Each named *Gonatocerus* species formed its own distinct clade or taxonomic unit (Figure 3), confirming the species boundaries of Triapitsyn (2006). For each taxonomic unit, the neighbor-joining distance tree was supported by very strong bootstrap values (96-100%). The specimens of *G. tuberculifemur* again formed two distinct clades among the named *Gonatocerus* species. All specimens from San Rafael clustered into clade 2, whereas the rest of the populations from South America all clustered into clade 1, suggesting that *G. tuberculifemur* contains two distinct lineages. Each of the two unnamed *Gonatocerus* species (*G*. sp. 2 and *G*. sp. 6) from South America also clustered into distinct clades, suggesting that indeed they are separate species.

Two molecular methods were employed to genetically characterize the candidate GWSS egg parasitoid species, *G. tuberculifemur* from South America. ISSR-PCR DNA fingerprinting identified fixed geographic-specific variation in the population from San Rafael (Mendoza). 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, 2006). Similarly, the phylogeographic approach inferred by the COI partial gene, detected two well-supported clades in South America. The two molecular methods were in accord and the results are suggestive of a species level divergence. More work is needed to determine whether these two genetically distinct *G. tuberculifemur* clades are actually cryptic or different species. Hybridization and morphological studies are in progress.

Table 1. Pairwise sequence distances (range) of mitochondrial COI partial gene fragments from geographic populations of *G. tuberculifemur* showing percentage divergence. The alignment program ClustalW from DNAStar was utilized for this analysis. To account for intra- and inter-populational variation, several individuals (3-6) were included (15 total). Argentina: Rio Colorado (Rio Negro Province); San Rafael (Mendoza); Tunuyán (Mendoza); San Miguel de Tucumán (Tucumán); and Chile: Jalsuri. Refer to Figure 2 for assignments. *G. annulicornis* (G. ann) (Argentina, South America) and *G. morrilli* (G. mor) (Texas USA, North America) were included as outgroups.

Species/clades	Clade 1	Clade 2	*G. ann*	*G. mor*
Clade 1	0.0-0.6			
Clade 2	1.4-2.2	0.0-0.6		
G. ann	5.0-5.6	5.2-5.8	0.2-0.4	
G. mor	5.6-6.0	6.2-6.7	4.6-4.8	0.0-0.0

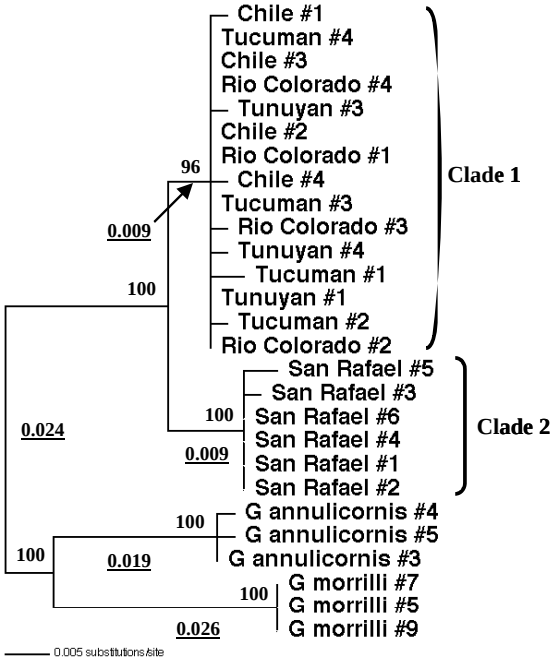

Figure 2.

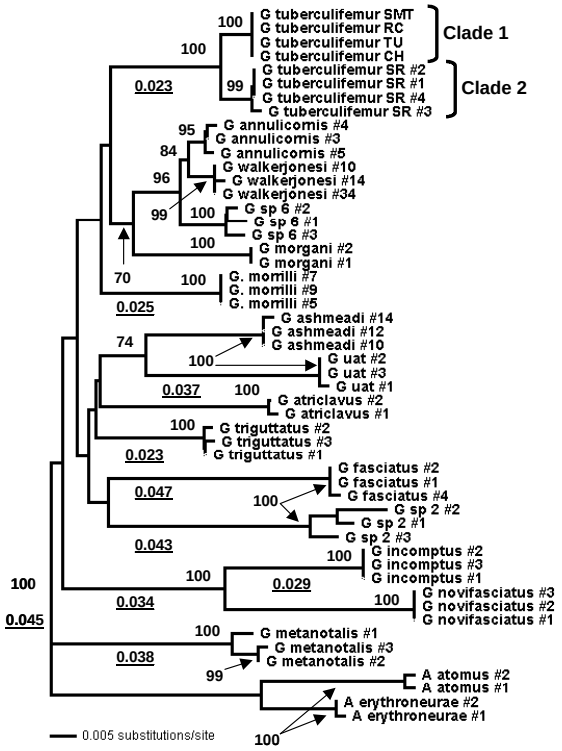

Figure 3.

Figure 2. Phylogram of the COI partial gene from geographic populations of *G. tuberculifemur* from Argentina and Chile. Analysis was performed with the alignment program ClustalX and the neighbor-joining distrance tree utilizing the uncorrected 'p' genetic distance 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.

Figure 3. Phylogenetic relationships of several named and two unnamed *Gonatocerus* Nees species along with *G. tuberculifemur* geographic populations from South America. Neighbor-joining distance phylogram inferred by the COI partial gene. Anaylsis was performed as described on Figure 2. Two *Anagrus* species (mymarids) were included as outgroups.

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PRELIMINARY EVIDENCE FROM REPRODUCTIVE COMPATIBILITY STUDIES SUGGESTS THAT *GONATOCERUS TUBERCULIFEMUR* EXISTS AS A CRYPTIC SPECIES COMPLEX, OR A NEW SPECIES IS IDENTIFIED: DEVELOPMENT AND UTILITY OF MOLECULAR DIAGNOSTIC MARKERS

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ABSTRACT

Recent work uncovered divergent clades or distinct lineages in populations of *Gonatocerus tuberculifemur* from South America. *G. tuberculifemur* 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)]. In the present study, we developed molecular diagnostic markers by two approaches to distinguish field-collected populations of *G. tuberculifemur* for reproductive compatibility studies. The two diagnostic assays were: polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the mitochrondrial cytochrome oxidase subunit I gene (COI) and inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting. Clade-specific restriction enzymes generated bands of the correct size with high specificity. Analysis of two isofemales lines created from freshly field-collected populations belonging to clade 1 (Tunuyán) and clade 2 (San Rafael) showed that both of our developed molecular diagnostic markers correctly genotyped these isofemale lines, confirming the utility of our diagnostic markers. Based on our molecular work, we predicted that *G. tuberculifemur* individuals belonging to the two distinct clades would not hybridize. Preliminary mating compatibility studies between these two isofemale lines demonstrated that our prediction was indeed correct. Interspecific crosses produced only male offspring, whereas, the intraspecific control crosses produced both males and females or fertile offspring. Taken together, both our molecular work and the preliminary reproductive compatibility studies strongly suggest that *G. tuberulifemur* either exists as a cryptic species complex or a new species is identified. Since *G. tuberculifemur* is under consideration as a biological control agent against the invasive GWSS in California, understanding cryptic variation of this species is critical.

INTRODUCTION

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 South America 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). One candidate was identified by S. Triapitsyn (UC-Riverside) as *Gonatocerus tuberculifemur* (Ogloblin) (Hymenotpera: Mymaridae) and is now being permitted for release in California (CDFA 2005). Identifying the correct natural enemy is critical to the success of classical biological control programs. Lack of proper identification procedures has affected several projects (Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993).

OBJECTIVES

1. Develop molecular diagnostic markers by two methods, ISSR-PCR DNA fingerprinting and PCR-RFLP of the COI gene to distinguish *G. tuberculifemur* isofemale lines belonging to the two distinct well-supported clades [accompanying report and de Leon et al. (2006)]
2. After freshly collecting and creating isofemale lines, genotype them to determine which clade they belong to, and initiate reproductive compatibility studies with individuals from the distinct two clades.

RESULTS AND CONCLUSIONS

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism diagnostic assays

Restriction enzyme maps of the partial COI gene were generated from individuals belonging to the two clades of *G. tuberculifumer* [accompanying report and de León et al. (2006)] and clade-specific restriction enzymes were identified that distinguished individuals from the two clades. The results of this experiment are shown on Figure 1. Digestion with the clade 1- and clade 2-specific restriction enzymes generated bands of the expected sizes (Figures 1A and 1B) with high specificity. After the completion of all molecular work, including the development of the diagnostic markers, the next step was to determine the utility of the markers. Based on our molecular work, we predicted that *G. tuberculifemur* individuals from clade 1 and clade 2 were not reproductively compatible. To initiate these studies, fresh field collections of *G. tuberculifemur* were made in both Tunuyán and San Rafael**,** populations belonging to clade 1 and clade 2, respectively and isofemales lines were created. PCR-RFLP diagnostic assays confirmed that the isofemale lines carried the correct genotypes (Figure 1C), that is, as predicted from our molecular work the Tunuyán isofemale line belongs to clade 1 and the San Rafael