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

isofemale line belongs to clade 2. Analysis of the isofemale lines with the second diagnostic assay (ISSR-PCR) confirmed the above findings (Figure 2). The current results confirm the utility of our developed molecular diagnostic markers.

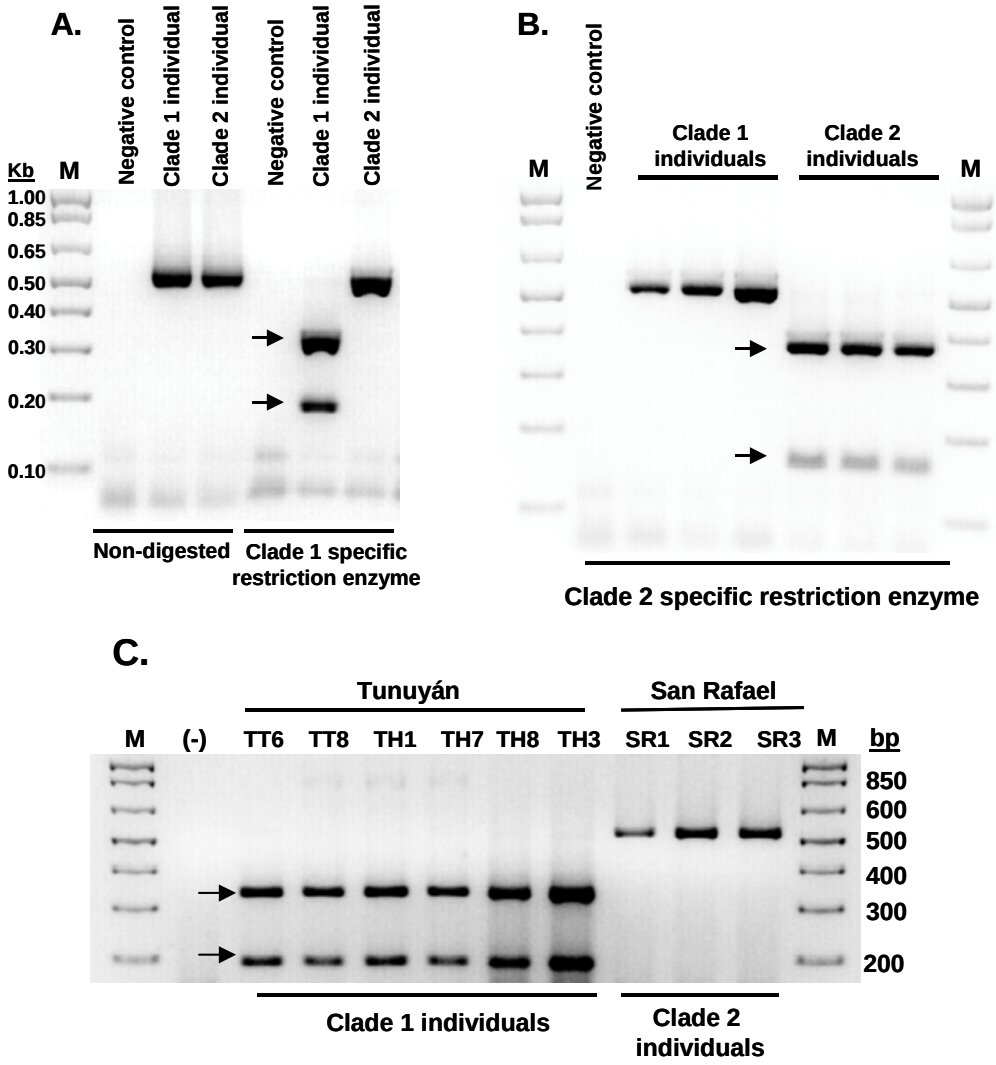

Figure 1. Representative example of the developed polymerase chain reaction-restriction fragment length polymporphism (PCR-RFLP) diagnostic assay. A. Digestion with the clade 1-specific restriction enzyme produces two bands: 193- and 325-bp, **B.** Digestion with the clade 2-specific restriction enzyme also generates two bands with the following sizes: 157- and 361-bp., and **C.** PCR-RFLP diagnostic assays of **'isofemale lines'** using the clade 1- specific restriction enzyme. TT, individuals from Tunuyán emerging from *T. rubromarginata* (Proconiini tribe); TH, individuals from Tunuyán emerging from *Hortensia similis* (Cicadellini tribe); and SR, individuals from San Rafael emerging from *T. rubommarginata*. **M**, 1.0 Kb Plus DNA Ladder.

Preliminary Reproductive compatibility studies

Field host range tests indicated that *G. tuberculifemur* has a limited development on some Cicadellini hosts showing a broader host range in the field than in the laboratory (where *G. tuberculifemur* only developed on Proconiini sharpshooters). The conflicting host ranged results could be due to the existence of sympatric cryptic species of *G. tuberculifemur* in the test area. Genotyped isofemale lines belonging to clade 1 (Tunuyán) and clade 2 (San Rafael), both of Mendoza Province, were carried out to evaluate mating compatibility. Direct and reciprocal crosses were performed: ♀San Rafael x ♂Tunuyán and ♀Tunuyán x ♂San Rafael and their controls (at least 3 replicates each and between 13-20 eggs per cross). Results are shown as mean percentage of individuals emerging from eggs parasitized by *G. tuberculifemur* females (Figure 3). Interspecific crosses produced only male offspring, whereas the intraspecific control crosses produced both males and females or fertile offspring, indicating that the populations from San Rafael and Tunuyán, which are about 100 km apart from each other, were reproductively incompatible and therefore reproductively isolated. More work is needed to complete these studies, which are in progress. Together, both our molecular data (de León et al. 2006) and the preliminary crossing studies strongly suggest that *G. tuberculifemur* either exists as a cryptic species complex or a new species has been identified. In addition, preliminary morphological work of individuals from the two clades suggests some slight differences (unpublished data, S.

Triapitsyn). Genotyping of *G. tuberculifemur* colonies reared and maintained at both Riverside, CA (UC-Riverside) and Edinburg, TX (USDA, APHIS) confirmed that both colonies or isofemale lines belong to clade 1.

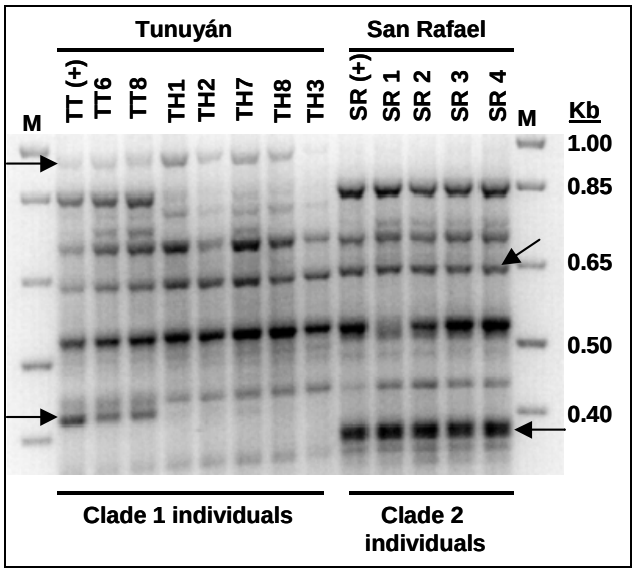

Figure 2. ISSR-PCR DNA fingerprinting using a 5'-anchored ISSR primer (Zietkiewicz et al. 1994) of 'isofemale lines' created after molecular characterization of *G. tuberculifemur* was complete. TT, individuals from Tunuyán emerging from *T. rubromarginata* (Proconiini tribe); TH, individuals from Tunuyán emerging from *Hortensia similis* (Cicadellini tribe); and SR, individuals from San Rafael emerging from *T. rubommarginata*. **M**, 1.0 Kb Plus DNA Ladder. Arrows point to banding pattern differences.

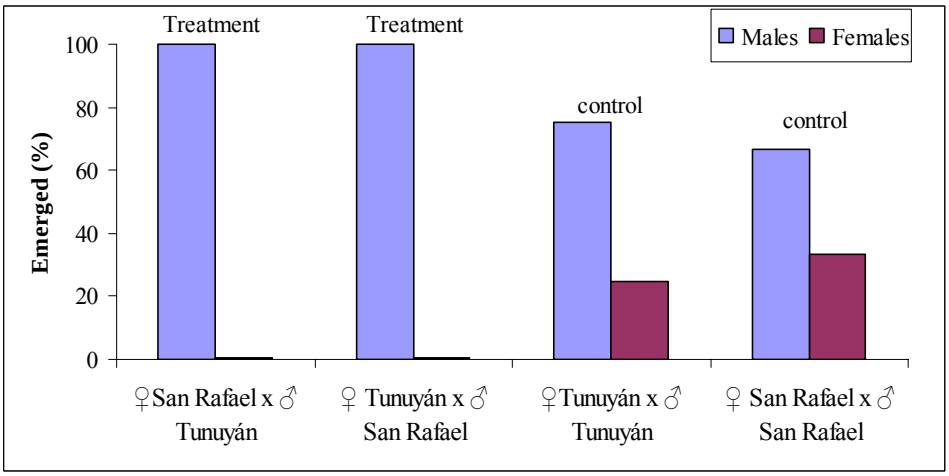

Figure 3. Preliminary hybridization studies (above). Mean percentage of female and male offspring emerging from eggs parasitized by G. tuberculifemur females obtained from different crosses with isofemale lines created from two populations from South America, Tunuyán and San Rafeal, belonging to two well-supported distinct clades (de León et al. 2006).

REFERENCES

CDFA. 2005. GWSS Biological Control, Spring Report. California Department of Food and Agriculture.

de León, J. H., G. A. Logarzo, and S. V. Triapitsyn. 2006. 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).

Jones, W. A. 2001. Classical biological control of the glassy-winged sharpshooter, pp. 50-51 In Tariq, M.A., Oswalt, S, Esser, T. [eds.], Proceedings of the Pierce's Disease Research Symposium, 5-7 December 2001, Coronado Island Mariott Resort, San Diego, CA. Organized by the California Department of Food and Agriculture. Copeland Printing, Sacramento, CA.

Jones, W. A., G. A. Logarzo, E. G. Virla, and E. Luft. 2005. Environmental risk assessment of egg parasitoids from South America: nontarget field and laboratory host range in Argentina and the U. S., pp. 343-344 In Tariq, M.A., Blincoe, P., Mochel, M., Oswalt, S., Esser, T. [eds.], Proceedings of the Pierce's Disease Research Symposium, 5-7 December 2005, San Diego Mariott Hotel & Marina, San Diego, CA. Organized by the California Department of Food and Agriculture. Copeland Printing, Sacramento, CA.

Logarzo, G. A., E. G. Virla, S. V. Triapitsyn, and W. A. Jones. 2004. Biology of *Zagella delicata* (Hymenoptera: Trichogrammatidae), an egg parasitoid of the sharpshooter *Tapajosa rubromarginata* (Hemiptera: Clypeorrhyncha: Cicadellidae) in Argenatina. Florida Entomol. 87: 511-516.

Logarzo, G., S. V. Triapitsyn, and W. A. Jones. 2003. New host records for two species of *Gonatocerus* (Hymenoptera: Mymaridae), egg parsitoids of proconiine sharpshooters (Hemiptera: Clypeorrhyncha: Cicadellidae), in Peru. Florida Entomol. 86: 486-487.

Logarzo, G. A., E. G. Virla, and W. A. Jones. 2005. Egg parasitoids from Argentina, potential candidates for the biological control of glassy-winged sharpshooter *Homalodisca coagulata* (Cicadellidae) in the United States, pp. 115-116 In Hoddle, M.S. [ed.], Second International Symposium on Biological Control of Arthropods volume III. USDA Forest Service Publication FHTET-2005-08.

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.

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.

Virla, E. G., G. A. Logarzo, W. A. Jones, and S. Triapitsyn. 2005. Biology of *Gonatocerus tuberculifemur* (Hymenoptera: Mymaridae), an egg parasitoid of the sharpshooter, *Tapajosa rubromarginata* (Hemiptera: Cicadellidae). Florida Entomol. 88: 67-71.

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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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.