PROGRESS ON RESOLVING THE *GONATOCERUS TUBERCULIFEMUR* COMPLEX: NEITHER COI NOR ITS2 SEQUENCE DATA ALONE CAN DISCRIMINATE ALL THE SPECIES WITHIN THE COMPLEX, WHEREAS, ISSR-PCR DNA FINGERPRINTING CAN

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ABSTRACT

We utilized two molecular methods to aid in resolving the *Gonatocerus tuberculifemur* (*G. tuberculifemur*) complex, potential glassy-winged sharpshooter (GWSS) biological control candidate agents from South America. The two methods used were DNA sequencing of both the mitochondrial cytochrome oxidase subunit I gene (COI) and the ribosomal internal transcribed spacer region 2 (ITS2) and inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting. COI sequence variation, as shown previously, was able to discriminate *G. tuberculifemur* individuals belonging to clades 1 and 2, but *G. tuberculifemur* individuals from clade 2 were not discriminated from the *G. tuberculifemur* specimens collected from the type locality (Pucará) or *G.* sp 3. *G. tuberculifemur* individuals emerging from *Hortensia similis* (*H. similis*) (Cicadellini leafhopper) formed a new clade (Y). On the other hand, ITS2 rDNA sequence data could not discriminate *G. tuberculifemur* individuals belonging to clades 1 and 2. However, ITS2 was able to discriminate *G.* sp 3 and *G. tuberculifemur* specimens from the type locality (Pucará), forming two new clades, Z and X, respectively. Specimens emerging from *H. similis* were also discriminated by ITS2 analysis. Interestingly, the separation of all of the species or strains within the *G. tuberculifemur* complex was accomplished by ISSR-PCR DNA fingerprinting. No single gene (COI and ITS2) sequenced was able to discriminate all of the species within the *G. tuberculifemur* complex. Based on the current data, it appears that there could be four species within the *G. tuberculifemur complex*. 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

The GWSS, *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae) that invaded California (CA) USA have their origins in Texas USA (de León et al. 2004), an observation that was later confirmed by Smith (2005). GWSS is a serious economic pest that poses a serious threat to the wine and table industry, therefore, a biological control program is in place to try to control this devastating pest (CDFA 2003). Currently, our research is focused on developing a neoclassical biological control program against GWSS in CA (de León et al. 2006a,b,c,d, 2007, Triapitsyn et al. 2007a,b). Egg parasitoids of closely related hosts belonging to the sharpshooter tribe Proconiini [*Tapajosa rubromarginata* (Signoret)] were sought from regions in South America where climate types and habitats were similar to CA (Jones 2001, Logarzo et al. 2003, 2004, 2005). Prospective egg parasitoid candidate agents were identified by S. Triapitsyn (UC-Riverside) among several *Gonatocerus* Nees species (Hymenoptera: Mymaridae) reared from *T. rubomarginata* (Logarzo et al. 2005, Virla et al. 2005), including specimens emerging from a different host tribe (Cicadellini). Among the species identified was *Gonatocerus tuberculifemur* (Ogloblin), one of the most promising biological control agents from South America because of its unique climate match to CA and not to the southeastern U.S., as it would not be predicted to migrate to the southeastern U.S. and attack non-target native leafhoppers. This is very important because it can reduce the risk factors of releasing this egg parasitoid in CA. However, it appears that *G. tuberculifemur* is part of a species complex involving several species [e. g., *G. tuberculifemur* clades 1 and 2 (de León et al. 2006a,d, 2007, Triapitsyn et al. 2007b), *G. tuberculifemur* collected from the type locality (Pucará, Neuquen Province, Argentina), *G. tuberculifemur*-like individuals emerging from a different host tribe (e. g., *Hortensia similis*), and *G.* sp 3 (de León et al. 2006b)].

OBJECTIVES

The aim of the present study was to continue resolving the *G. tuberculifemur* complex. Two molecular methods, ISSR-PCR DNA fingerprinting and DNA sequencing of both the mitochondrial cytochrome oxidase subunit I gene (COI) and the ribosomal internal transcribed spacer region 2 (ITS2) were utilized and compared. Included in the analyses were *G. tuberculifemur* individuals belonging to both 'clade 1' and 'clade 2' (de León et al. 2006a,d, 2007), *G.* sp 3 (de León et al. 2006b), *G. tuberculifemur*-like individuals emerging from *H. similis* (Cicadellini leafhopper); and *G. tuberculifemur* individuals collected from the type locality (Pucará, Neuquén Province, Argentina). Included in the analyses to provide phylogenetic support were several species belonging to the *morrilli* subgroup of the *ater* species group of *Gonatocerus* (de León et al. 2007, Triapitsyn 2006).

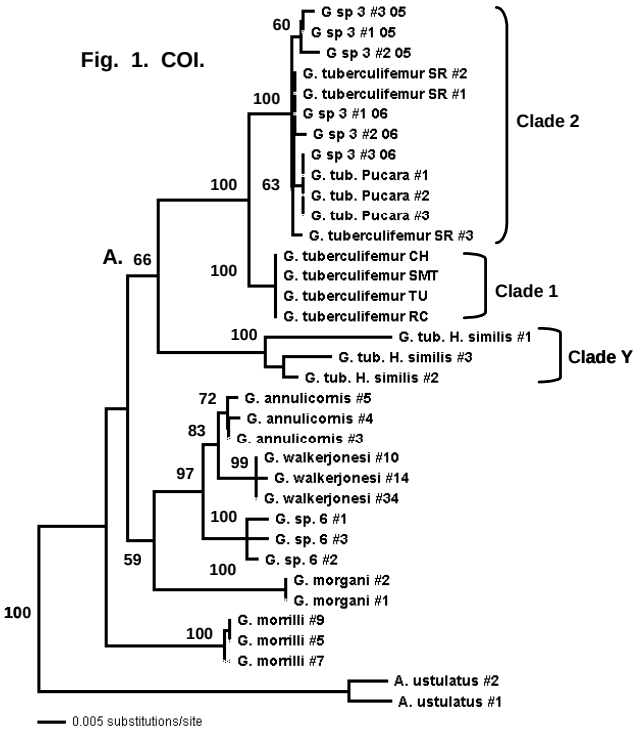
RESULTS AND CONCLUSIONS

Phylogenetic analysis of individuals belonging to the* G. tuberculifemur *complex inferred from COI sequence data

Ten *Gonatocerus* species (or strains) were included in this study. A total of 33 ingroup specimens were analyzed and two specimens from *Anagrus ustulatus* (Haliday) (also a mymarid species) were included as an outgroup. Many species formed their own taxonomic unit or distinct clade, confirming the species boundaries shown recently, including the two clades (1 and 2) of *G. tuberculifemur* (de León et al. 2006c,d, 2007, Triapitsyn 2006) (Figure 1). In addition, a new clade (Y) was observed in *G. tuberculifemur* individuals emerging from the host *H. similis*, suggesting that clade Y individuals are a valid species. However, COI sequence variation was unable to discriminate *G. tuberculifemur* clade 2 individuals (San Rafael) from those of *G*. sp 3, as shown previously (de León et al. 2006b), and *G. tuberculifemur* (G. tub Pucará) individuals collected from the type locality (Pucará). The current data also suggests that individuals from the *G. tuberculifemur* complex (main clade 'A') are related or could belong to the *morrilli* subgroup of the *ater* species group of *Gonatocerus*. Very strong bootstrap support (100%) was seen within the clades (1, 2, and Y) of the *G. tuberculifemur* complex.

Phylogenetic analysis of individuals belonging to the* G. tuberculifemur *complex inferred from ITS2 sequence data

The results of this analysis are shown on Figure 2. Again, the species boundaries of many of these species utilizing the ITS2 rDNA fragment were confirmed (de León et al. 2006c), with very strong support (96-100%). Clade Y (G. tub *H. similis*) was again observed based on ITS2 phylogenetic analysis, confirming the results of the COI analysis. ITS2 sequence variation was able to discriminate individuals from both *G*. sp 3 (clade Z) and G. tub Pucará (clade X). However, ITS2 was unable to discriminate individuals of *G. tuberculifemur* belonging to clades 1 and 2. This is an interesting observation because COI sequence data and ISSR-PCR DNA fingerprinting were both able to discriminate these two clades (de León et al. 2006d, 2007); in addition, preliminary cross-mating data suggest that individuals from the two clades of *G. tuberculifemur* are reproductively incompatible (de León et al. 2006a). Furthermore, morphological analyses uncovered some differences between the two clades [Triapitysn et al. 2007 (submitted)]. Taken together, all of these data highly suggest that the individuals of *G. tuberculifemur* belonging to the two clades are valid species.



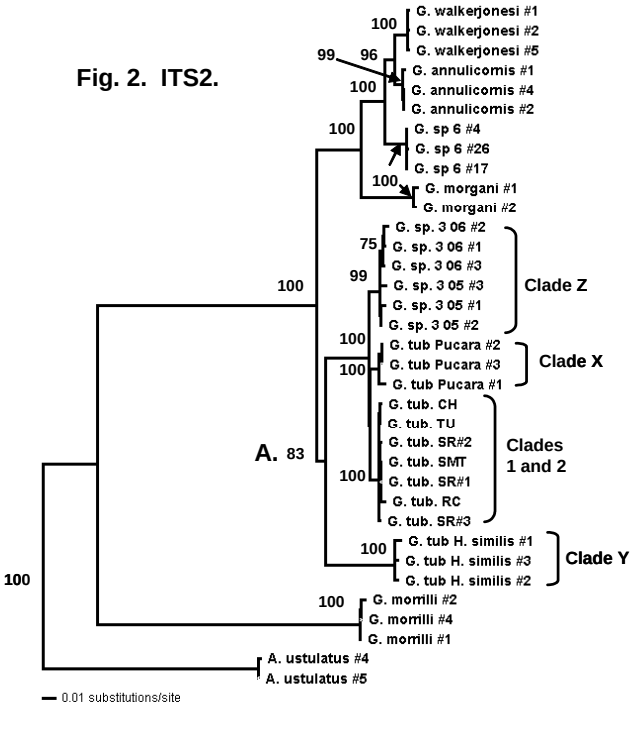


Figure 1 (COI) and **Figure 2** (ITS2) show phylograms of egg parasitoid species belonging to the *G. tuberculifemur* complex inferred from COI and ITS2 sequence data, respectively. Analyses were performed with the alignment program ClustalX and the neighbor-joining distance trees utilizing the uncorrected 'p' genetic distance were reconstructed with the phylogenetic program PAUP 4.0b10 for Macintosh (PPC). The trees display bootstrap values, as percentage of 1000 replications. Collections of *G. tuberculifemur* (G. tub) were from: San Rafael (SR) (Mendoza Province), these individuals belong to 'clade 2' (de León et al. 2006d, 2007). RC, Rio Colorado (Rio Negro); SMT, San Miguel de Tucumán (Tucumán); TU, Tunuyán (Mendoza); and CH, Chile; these individuals belong to 'clade 1' (de León et al. 2006d, 2007). *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)] (de León et al. 2006b). *G. tuberculifemur*-like individuals emerged from *H. similis* (Cicadellini leafhopper) (G. tub *H. similis*); the rest emerged from *T. rubromarginata*. *G. tuberculifemur* individuals from Pucará (G. tub Pucará) are from the type locality. Main clade 'A' are individuals from the *G. tuberculifemur* complex.

ISSR-PCR DNA fingerprinting of individuals belonging to the G. tuberculifemur complex

The results of this experiment are shown on Figure 3. ISSR-PCR uncovered fixed banding pattern differences in all of the species or strains belonging to the *G. tuberculifemur* complex: G. tub Pucará (clade X); G. tub *H. similis* (clade Y); results for *G*. sp 3 (clade Z) are shown elsewhere (de León et al. 2006b); and *G. tuberculifemur* clades 1 and 2, shown previously (de León et al. 2006d, 2007). Not sharing bands among species is usually an indication of reproductive isolation. Cross-mating studies and morphological analyses are in progress to confirm the current molecular results.

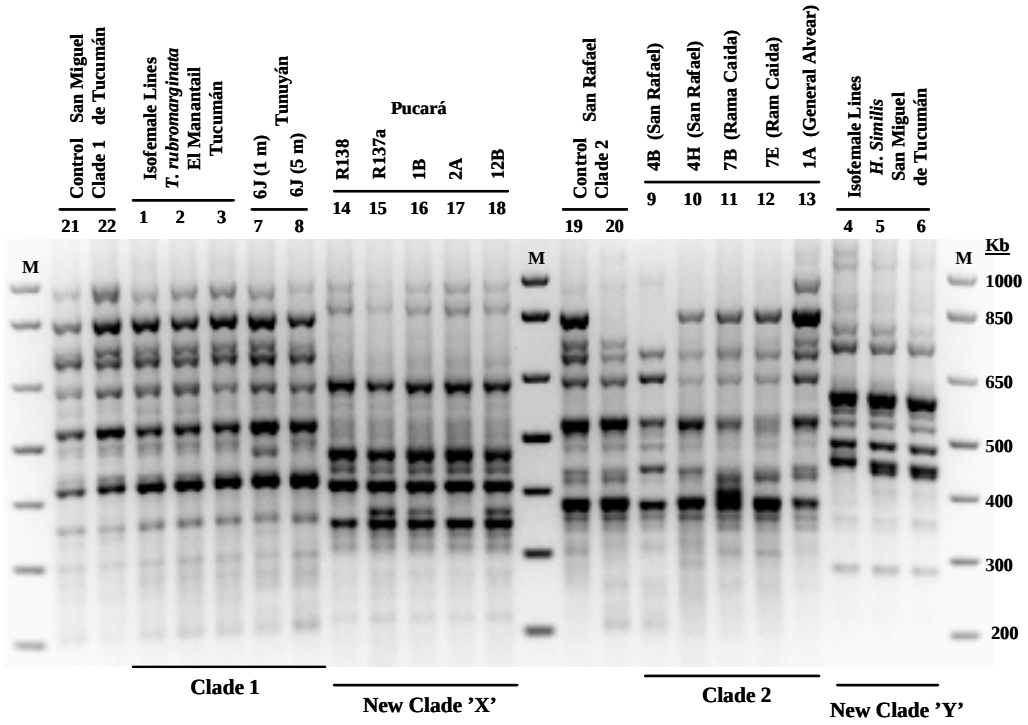


Figure 3. ISSR-PCR DNA fingerprinting of various species within the *G. tuberculifemur* complex. Reactions were performed with total genomic DNA from 2-5 separate individuals and a 5'-anchored ISSR primer (de León et al. 2004, 2007). Specimens from San Miguel de Tucumán and El Manantial are from Tucumán Province; Pucará individuals are from Neuquén Province (type locality); and San Rafael, Rama Caida, and General Alvear individuals are from Mendoza Province. *G*. sp. 3 specimens are not included [see de León et al. 2006b]. *G. tuberculifemur*-like individuals emerged from *H. similis*, a different host tribe (Cicadellini); the rest emerged from *T. rubromarginata* (Proconiini host tribe). **M**, 1.0-Kb Plus DNA Ladder.

REFERENCES

CDFA. 2003. Pierce's Disease Program Report to the Legislature. California Department of Food and Agriculture.

de León, J. H., W. A. Jones, and D. J. W. Morgan. 2004. Population genetic structure of *Homalodisca coagulata* (Homoptera: Cicadellidae), the vector of the bacterium *Xylella fastidiosa* causing Pierce's disease in grapevines. Ann. Entomol. Soc. Am. 97: 809-818.

de León, J. H., G. A. Logarzo, and S. V. Triapitsyn. 2007. Molecular characterization of *Gonatocerus tuberculifemur* (Ogloblin) (Hymenoptera: Mymaridae), a prospective *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae) biological control candidate agent from South America: Divergent clades. Bull. Entomol. Res. (in press).

de León, J. H., G. A. Logarzo, and S. V. Triapitsyn. 2006a. 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, pp. 44-47. *In* Proceeding, Pierce's Disease Research Symposium, 27-29 November 2006, San Diego, CA. Complied by Tom Esser, M. Athar Tariq, Raygina Medeiros, Melinda Mochel, and Sean Veling, Sacramento, CA.

de León, J. H., G. A. Logarzo, and S. V. Triapitsyn. 2006b. 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, pp. 48-51. *In* Proceeding, Pierce's Disease Research Symposium, 27-29 November 2006, San Diego, CA. Complied by Tom Esser, M. Athar Tariq, Raygina Medeiros, Melinda Mochel, and Sean Veling, Sacramento, CA.

de León, J. H., G. A. Logarzo, and S. V. Triapitsyn. 2006c. 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 fragment sizes, pp. 56-59. *In* Proceeding, Pierce's Disease Research Symposium, 27-29 November 2006, San Diego, CA. Complied by Tom Esser, M. Athar Tariq, Raygina Medeiros, Melinda Mochel, and Sean Veling, Sacramento, CA.

de León, J. H., G. A. Logarzo, and S. V. Triapitsyn. 2006d. Genetic characterization of *Gonatocerus tuberculifemur* from South America uncovers divergent clades: Prospective egg parasitoid candidate agent for the glassy-winged sharpshooter in California, pp. 40-43. *In* Proceeding, Pierce's Disease Research Symposium, 27-29 November 2006, San Diego, CA. Complied by Tom Esser, M. Athar Tariq, Raygina Medeiros, Melinda Mochel, and Sean Veling, Sacramento, CA.

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 Marriott Resort, 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 Argentina. 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 parasitoids 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.

Smith, P. T. 2005. Mitochondrial DNA variation among populations of the glassy-winged sharpshooter, *Homalodisca coagulata*. 8pp. J. Insect Sci. 5:41, available online: insectscience.org/5.41.

Triapitsyn, S.V. 2006. A key to the Mymaridae (Hymenoptera) egg parasitoids of proconiine sharpshooters (Hemiptera: Cicadellidae) in the Nearctic region, with description of two new species of *Gonatocerus*. Zootaxa 1203: 1-38.

Triapitsyn, S. V., G. A. Logarzo, J. H. de León, and E. G. Virla. 2007a. A new species of *Gonatocerus* (Hymenoptera: Mymaridae) from Argentina, with taxonomic and molecular data on *G. tuberculifemur* and the related forms. Submitted to Zootaxa (in review).

Triapitsyn, S. V., G. A. Logarzo, E. G. Virla, and J. H. de León. 2007b. A new species of *Gonatocerus* (Hymenoptera: Mymaridae) from Argentina, an egg parasitoid of *Tapajosa rubromarginata* (Hemiptera: Cicadellidae). Zootaxa (in press).

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.

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