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


**Project Leaders:**

Jesse H. de Leon
USDA, ARS
Beneficial Insects Res. Unit
Weslaco, TX 78596

Guillermo A. Logarzo
USDA, ARS, SABCL
Buenos Aires, Argentina

Serguei V. Triapitsyn
Deparment of Entomology
University of California
Riverside, CA 92521

**Cooperator:**

David J.W. Morgan
Calif. Dept. of Food & Agric.
Mt. Rubidoux Field Station
Riverside, CA 92501



**Reporting period:** The results reported here are from work conducted fiscal year 2005 to fiscal year 2006.



## 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

this analysis are shown on Figure 1. Several banding patterns were identified among the populations with some band sharing and in certain populations (Tartagal and Santa Clara) there was extensive variation. These ISSR-PCR results are clearly the first indication that genetic differences exist among the *G. metanotalis* populations from Argentina.

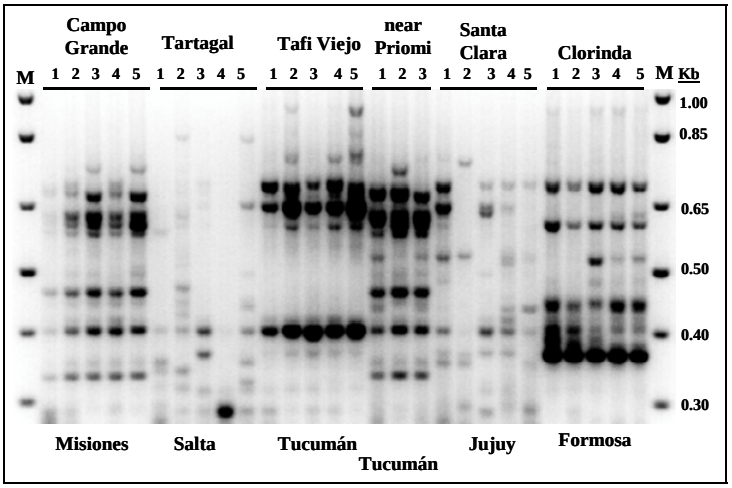


**Figure 1. ISSR-PCR DNA fingerprinting of *G. metanotalis* populations from Argentina.** Reactions were performed with genomic DNA from 3-5 separate individuals and a 5'-anchored ISSR primer (Zietkiewicz et al. 1994, de León et al. 2004). **M:** 1.0 Kb Plus DNA Ladder.

**Phylogeographic analysis of populations of *G. metanotalis***

Levels of divergence in the COI partial gene among populations from Argentina were determined by calculating the pairwise estimates for genetic distance (Table 1). Individuals clustered into three main distinct clades or groups (see Figure 2 for assignments) and individuals from each clade were pooled to calculate the pairwise estimates. The intra-clade divergence (%D) ranged from 0.0-1.0, whereas the inter-clade %D ranged from 2.4-4.6. The present results clearly indicate a deep divergence among the three main groups.

**Table 1. Pairwise sequence distances (range) of the mitochondrial COI partial gene from geographic populations of *G. metanotalis* from Argentina showing percentage divergence (%D).** The alignment program ClutstalW from DNAStar was utilized for this analysis. To account for intra- and inter-populational variation, several individuals (3-4) were included. Populations from Argentina included: Campo Grande (Misiones Province); Tartagal (Salta); Tafi Viejo (Tucumán); near PROIMI (Tucumán); Santa Clara (Jujuy); and Clorinda (Formosa). Individuals clustered into three clades, see Figure 2 for assignments. *Gontacerus ashmeadi* (G. ash) (CA) were utilized as an outgroup.

| Species/clades | Clade 1 | Clade 2 | Clade 3 | *G. ash* |
|---|---|---|---|---|
| Clade 1 | 0.0-1.0 | | | |
| Clade 2 | 2.4-3.6 | 0.0-0.8 | | |
| Clade 3 | 3.6-4.6 | 3.8-4.6 | 0.0-0.6 | |
| *G. ash* | 9.0-9.9 | 8.6-9.2 | 8.2-8.6 | 0.0-0.2 |

A neighbor-joining distance tree showed that individuals clustered into three well-supported distinct clades with very strong bootstrap values (100%) (Figure 2), uncovering haplotype or phylogeographic structure. With the exception of one population (Campo Grande), all of the individuals clustered into one of the three clades. These results suggest that three sympatric strains of *G. metanotalis* may be present in Campo Grande, although collection of more specimens are needed to confirm this observation. It is interesting to note that within clade 1, there appears to be a further subdivision with the appearance of three subclades. This observation would agree with the results of the ISSR-PCR experiment (Figure 1) that shows about five total banding patterns within the *G. metanotalis* populations.

**Phylogenetic relationships among *Gonatocerus* Nees 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, Unruh and Woolley 1999); therefore, a molecular systematic approach was undertaken with various named and two unnamed *Gonatocerus* species, along with the six *G. metanotalis* populations from Argentina

(Figure 3). These species were also included to test the support for the species groups considered. The topology of the neighbor-joining distance tree was supported by very strong bootstrap values, 100% support was seen for all species or taxonomic units, confirming species boundaries (Triapitsyn 2006, Triapitsyn et al. 2006). The phylogenetic analysis again clustered *G. metanotalis* individuals from Argentina into three main distinct clades as seen in Figure 2. Because each *Gonatocerus* species formed its own unique clade or taxonomic unit, the possibility exists that each *G. metanotalis* clade represents a different strain. This observation is also supported by the results of the ISSR-PCR experiment (Figure 1). In addition, because of the deep divergence among *G. metanotalis* clades, the possibility that a cryptic species complex was identified within *G. metanotalis* in Argentina is high. Another possibility is that we may be uncovering different species**.** Phylogenetic analyses of *Gonatocerus* species inferred by COI sequence data have been reported elsewhere (PD reports 2006 and de León et al. 2006b), but are expanded here.

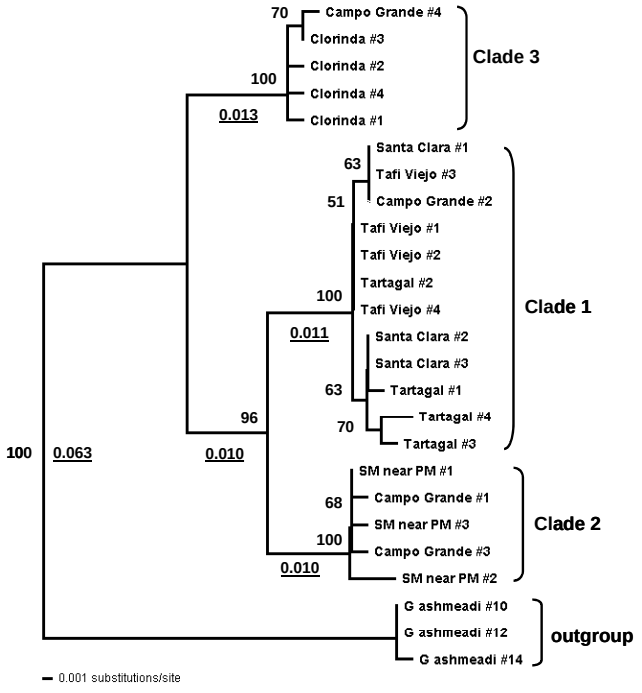


**Figure 2.**

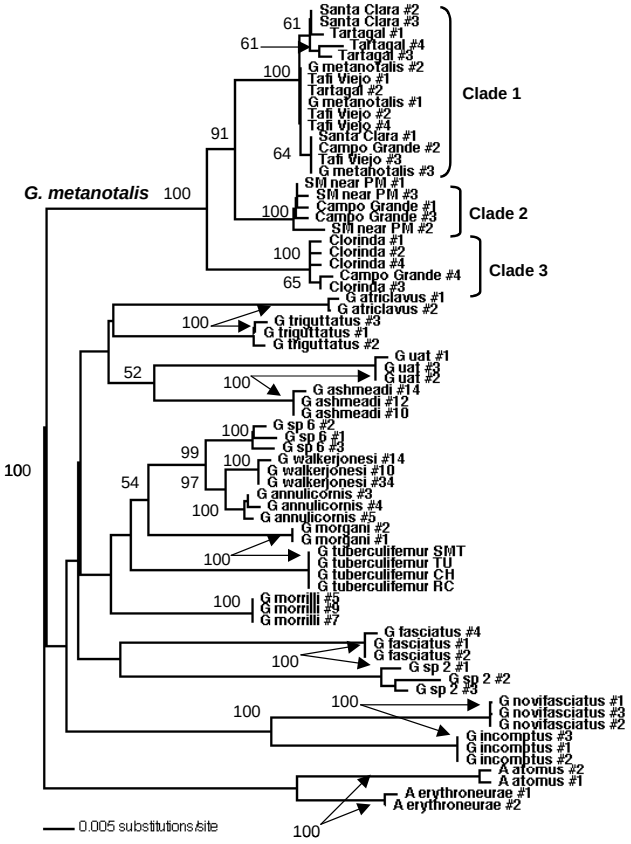


**Figure 3.**

**Figure 2.** Phylogram inferred by the COI partial gene from geographic populations of *G. metanotalis* from Argentina. Analysis was performed with the alignment program ClustalX and the neighbor-joining distance tree utilizing the uncorrected 'p' genetic distance was reconstructed with the phylogenetic program PAUP 4.0b10. To account for intra- and inter-populational variation, several individuals (3-4) were included. *G. ashmeadi* (CA) were utilized as an outgroup. 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. metanotalis* populations from Argentina. Neighbor-joining distance phylogram inferred by the COI partial gene. Anaylsis was performed as described on Figure 2. Two *Anagrus* species (mymarid genus) are included as outgroups.

**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. Molecular distinction between populations of *Gonatocerus morrilli*, egg parasitoids of the glassy-winged sharpshooter *Homalodisca coagulata*, from Texas and California: Do cryptic species exist? 7pp. J. Insect Sci. 4:39, Available online: insectscience.org/4.39.

de León, J. H., W. A. Jones, M. Sétamou, and D. J. W. Morgan. 2006a. Genetic and hybridization evidence confirms that a geographic population of *Gonatocerus morrilli* (Hymenoptera: Mymaridae) from California is a new species: Egg

parasitoids of the glassy-winged sharpshooter *Homalodisca coagulata* (Homoptera: Cicadellidae). Biol. Control 38: 282-293.
de León, J. H., G. A. Logarzo, and S. V. Triapitsyn. 2006b. 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 M. S. Hoddle [ed], California Conference on Biological Control. July 11-12, 2000. Riverside, CA.
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, 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. 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.
Morgan, D. J. W., S. V. Tripitsyn, R. A. Redak, L. G. Bezark, and M. S. Hoddle. 2000. Biological control of the glassy-winged sharpshooter: current status and future potential, pp. 167-171 In M. S. Hoddle [ed], California Conference on Biological Control July 11-12, 2000. Riverside, CA.
Narang, S. K., W. J. Tabachnick, and R. M. Faust. 1993. Complexities of population genetic structure and implications for biological control programs. In: Applications of Genetics to Arthropods of Biological Control Significance (eds Narang SK, Barlett AC, Faust RM), pp. 19-52. CRC Press Inc, Boca Raton, Florida.
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., D. B. Vickerman, J. M. Heraty, and G. A. Logarzo. 2006. A new species of *Gonatocerus* (Hymenoptera: Mymaridae) parasitic on proconiine sharpshooters (Hemiptera: Cicadellidae) in the New World. Zootaxa 1158: 55-67.
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.
Unruh, T. R., and J. B. Woolley. 1999. Molecular Methods in Classical Biological Control. In: Biological Control (eds Van Driesche RG, Bellows TS, Jr.), pp. 57-85. Chapman and Hall, NY.
Zietkiewicz, E., A. Rafalski, and D. Labuda. 1994. Genomic fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20: 176-183.

**FUNDING AGENCIES**
Funding for this project was provided by the USDA Agricultural Research Service.

**Additional note:** We acknowledge Marissa González and Lisa A. Ledezma (current address: USDA, APHIS, PPQ-Edinburg, TX) and Laura Varone and Vanina Varni (USDA, SABCL-Argentina) for their excellent technical assistance.

# 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


**Project Leaders:**

Jesse H. de Leon
USDA, ARS
Beneficial Insects Res. Unit
Weslaco, TX 78596

Guillermo A. Logarzo
USDA, ARS, SABCL
Buenos Aires, Argentina

Serguei V. Triapitsyn
Deparment of Entomology
University of California
Riverside, CA 92521

**Cooperator:**

David J.W. Morgan
Calif. Dept. of Food & Agric.
Mt. Rubidoux Field Station
Riverside, CA 92501



**Reporting Period:** The results reported here are from work conducted fiscal year 2005 to fiscal year 2006.



## 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 erythroneurae*) (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 beloning 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 Aliniazee 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 erythroneurae* Trjapitzin & 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.