DISCOVERY OF A CRYPTIC SPECIES COMPLEX IN *GONATOCERUS MORRILLI*, A PRIMARY EGG PARASITOID OF THE GLASSY-WINGED SHARPSHOOTER

Project Leaders:
Jesse H. de León, Walker A. Jones, and Mamoudou Sétamou
USDA, ARS
Beneficial Insects Research Unit
Weslaco, TX 78596

David J.W. Morgan
CDFA, PDCP
Mount Rubidoux Field Station
Riverside, CA 92501

Reporting Period: The results reported here are from work conducted fiscal year 2004 to fiscal year 2005.

ABSTRACT
We investigated the differentiation and reproductive isolation among different geographic populations of *Gonatocerus morrilli*, an egg parasitoid of the glassy-winged sharpshooter (GWSS) (*Homalodisca coagulata* Say) (Homoptera: Cicadellidae), to confirm previous observations that there may exist a cryptic species complex. Two mitochondrial genes [cytochrome oxidase subunits I (COI) and II (COII)] and the internal transcribed spacer region 2 (ITS2) of several individuals per population were sequenced. *Gonatocerus morrilli* populations from Texas (TX), Florida (FL), California (CA), and an outgroup (*G. ashmeadi*) were analyzed. For comparison, a population from Argentina identified as near *G. morrilli* (=*G. annulicornis*) was also included. For all three sequence fragments, percentage sequence divergence (%D) demonstrated that both the TX and FL populations (TX/FL) were closely related and therefore, determined to be the same species; in constrast, the %D between TX/FL and CA fell within the range of the outgroup, making the CA population a novel species (nov. sp. *G. morrilli*). Neighbor-joining distance trees clustered the TX/FL and CA populations or species into two well supported distinctive clades. The *G. morrilli* (nov. sp.) was more closely related to *G. annulicornis* than to the TX/FL species. Mating studies demonstrated that the populations or species from CA and TX were reproductively incompatible, producing no female offspring in both direct and reciprocal crosses; whereas, the heterogamic crosses between TX and FL produced fertile offspring and relative compatibility indices similar to the homogamic crosses. These results are important to the PD/GWSS biological control program in California.

INTRODUCTION
Accurate identification of natural enemies is critical to the success of classical biological control programs, as it is essential for 1) selecting the most suitable natural enemy, 2) evaluating establishment, dispersal, and efficacy of natural enemies, and 3) improving mass production. Lack of proper identification procedures has affected several projects (Rosen 1977, Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993, Miller and Rossman 1995, Schauff and LaSalle 1998, Gordh and Beardsley 1999, Unruh and Woolley 1999). Phylogenetics has become a widespread approach for delineating and identifying morphologically similar or cryptic species. Correct identification of the pest is also extremely important in biological control. Geographic populations of the same species may differ in relevant biological characteristics of importance to biological control. In addition, pin-pointing the native origin of an exotic pest is crucial for collection of natural enemies in the native range of the pest (Rosen 1977, Narang et al. 1993, Unruh and Woolley 1999, Brown 2004, Roderick 2004). We demonstrated that the GWSS that invaded CA is of TX origin, but more than one founding event occurred (de León et al. 2004a). Our data also showed that GWSS populations in the U. S. were genetically distinct, clustering into two main groups or clades, a 'southeastern' and a 'southwestern and western' clade. Similarly, molecular studies of *Gonatocerus morrilli* Howard (Hymenoptera: Mymaridae) populations from CA and TX using inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting and amplification of the internal transcribed spacer 2 region (ITS2) showed that these populations were highly differentiated (G_{ST} = 0.92) with restricted gene flow (de León et al. 2004b, de León et al. 2005). These results strongly suggested that *G. morrilli* exists in nature as a cryptic species complex. Furthermore, the different sizes of the ITS2 amplification fragments between the geographic populations raised concerns over the reproductive compatibility of these populations and its implications in a biological control program.

OBJECTIVES
The objective of the present study was to confirm whether *G. morrilli* exists in nature as a cryptic species complex. We extended our previous observations (de Leon et al. 2004b) by implementing a phylogenetic approach by sequencing two mitochondrial genes [cytochrome oxidase subunit I and II genes (COI) and (COII)] and one ribosomal DNA spacer region fragment (ITS2). Reproductive compatibility studies were performed with populations of *G. morrilli* from three origins: California, Florida, and Texas.

RESULTS AND CONCLUSIONS

ISSR-PCR DNA fingerprinting

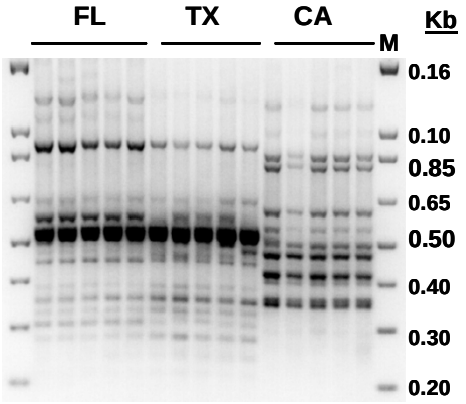

Figure 1. ISSR-PCR

Previously, we (de León et al. 2004b) determined that *G. morrilli* populations from TX and CA had different ISSR-PCR banding patterns, suggesting that these populations were reproductively isolated. In the present study a population from FL was included and we asked whether ISSR-PCR was a suitable method to predict the species status of this *G. morrilli* population. Figure 1 shows the results of this analysis where five randomly chosen field collected individuals per population from TX, CA, and FL were analyzed. The results showed that the population of *G. morrilli* from FL had the same ISSR-PCR banding pattern as the population from TX, whereas the CA population had a banding pattern that differed from TX and FL. Recently (de León et al. 2004, de León and Jones 2005) and in the current report, we observed a powerful correlation in DNA banding patterns and distinct species with the ISSR-PCR DNA fingerprinting method with Mymaridae egg parasitoids. The method has been used to distinguish about ten *Gonatocerus* egg parasitoid species (unpublished data). In the present study, based on ISSR-PCR banding patterns, we were able to predict the species status of the *G. morrilli* population from FL. The results demonstrated populations from FL and TX as distinct from the CA populations. Even though ISSR-PCR markers are scored as dominant, the ISSR-PCR technique using 5'-anchored or compound ISSR primers is still a very sensitive and useful technique because it targets random SSR or microsatellites (Zietkiewicz et al. 1994, de León and Jones 2004). An additional advantage is that the same ISSR primer can be rapidly applied across several different orders (e.g., insects, plants, fungi, bacteria) without prior knowledge of DNA sequences (de León, unpublished data), a capability not found with microsatellites. Banding patterns are consistent because the anchors serve to fix the annealing of the primer to a single position of the target site, thus resulting in a low level of slippage during amplification (Zietkiewicz et al. 1994, reviewed in Karp and Edwards 1997).

Sequence divergence in gene fragments (COI, COII, and ITS2) in *G. morrilli* geographic populations

Levels of genetic divergence in the gene fragments among populations were determined by calculating the pairwise estimates for genetic distance. Sequences were aligned with the program ClustalX and the neighbor-joining trees were reconstructed with the phylogenetic program PAUP 4.0. Dendrograms for the gene fragments are shown in Figures. 2 (COI), 3 (COI), and 4 (ITS2). Trees display branch lengths (below branches, underlined) and bootstrap values (above branches) as a percentage of 1000 replications. For all three gene sequence fragments, percentage sequence divergence (%D) demonstrated that both the TX and FL populations (TX/FL) were closely related and therefore, determined to be the same species; in constrast, the %D between TX/FL and CA fell within the range of the outgroup, making the CA population a novel species (nov. sp. *G. morrilli*) (data not shown). Neighbor-joining distance trees clustered the TX/FL and CA populations or species into two well supported distinctive clades. The *G. morrilli* (nov. sp.) was more closely related to *G. annulicornis* than to the TX/FL species. Sequence data from the mitochondrial COI and COII partial genes and the ITS2 rDNA fragment indicate that the studied populations of *G. morrilli* contain two distinct evolutionary groups. Populations from TX and FL formed one well-supported clade, while populations from CA formed another well-supported clade. Variation between the two clades with all three genes was greater between clades than within them.

Reproductive compatibility studies

Mated *G. morrilli* females from the various crosses successfully parasitized eggs of *H. coagulata*, but the percentages varied significantly with treatment ($F = 12.54$, $df = 5, 82$, $P < 0.0001$). Nearly all *H. coagulata* eggs exposed were successfully parasitized in all the direct and reciprocal crosses, except for the ♀TX × ♂CA treatment for which only 65% of eggs were successfully parasitized (Figure 5a). The crosses ♀CA × ♂CA and ♀CA × ♂TX yielded the longest immature developmental period for males; the lowest periods were obtained for immatures from ♀FL × ♂TX, ♀TX × ♂FL and ♀TX × ♂TX. Percentage of females produced varied significantly with treatment ($F = 115.05$, $df = 5, 82$, $P < 0.0001$). The sex ratios of *G. morrilli* progeny produced from the homogamic (♀CA × ♂CA and ♀TX × ♂TX) and the heterogamic (♀TX × ♂FL and ♀FL × ♂TX) crosses were female-biased and similar with > 70% of female offsprings (Fig 5b). In contrast, the heretogamic cross ♀CA × ♂TX and its reciprocal cross ♀TX × ♂CA did not produce any female progeny. Relative to their ♀TX × ♂TX homogamic cross, the relative compatibility indices (ratio between the proportion of females in heterogamic and homogamic cross) of ♀TX × ♂CA and ♀TX × ♂FL were 0 and 0.95, respectively. Similarly, the relative compatibility index of the ♀CA × ♂TX was 0. The immature developmental time of *G. morrilli* within eggs of *H. coagulata* significantly varied with treatment ($F = 212.04$, $df = 5, 1018$, $P < 0.0001$) but not with sex ($F = 0.08$, $df = 1,1018$, $P = 0.78$). For females, the longest immature developmental time was recorded for ♀CA × ♂CA, whereas no significant differences were recorded for the three crosses ♀FL × ♂TX, ♀TX × ♂FL and ♀TX × ♂TX (Fig. 5c).

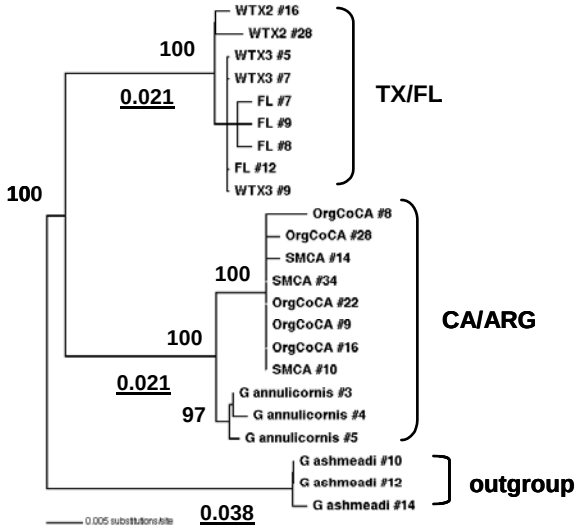

Figure 2. COI

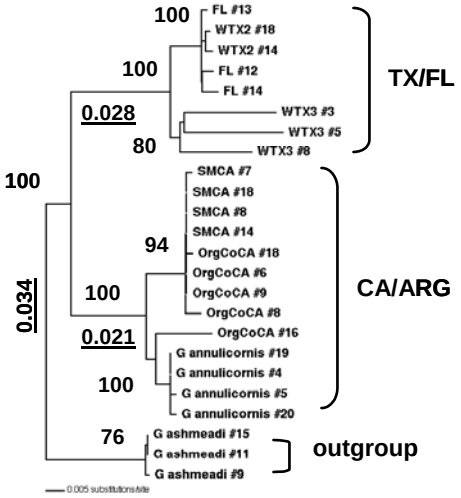

Figure 3. COII

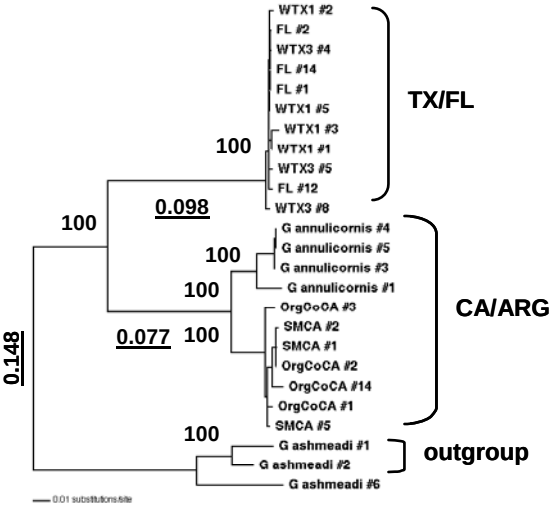

Figure 4. ITS2

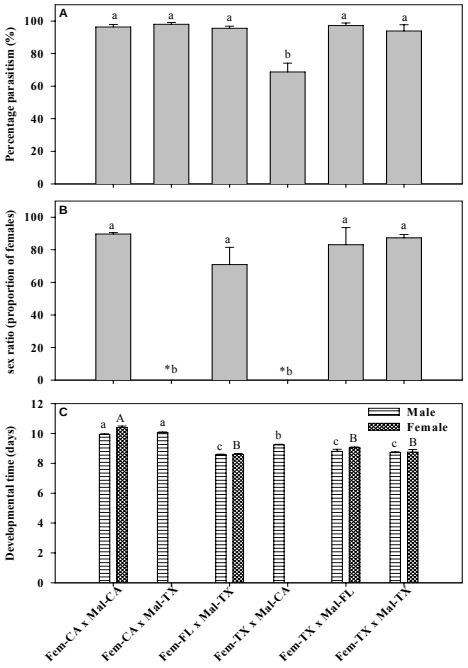

Figure 5. Crossing studies

REFERENCES

Brown, J. K. 2004. Tracing the origin of cryptic insect pests and vectors, and their natural enemies. In: *Genetics, Evolution, and Biological Control* (eds Ehler LE, Sforza R, Mateille T), pp. 113-132. CABA International, Wallingford, UK.

de León, J. H, and W. A. Jones. 2004. Detection of DNA polymorphisms in *Homalodisca coagulata* (Homoptera: Cicadellidae) by Polymerase Chain Reaction-based DNA fingerprinting methods. Ann. Entomol. Soc. Am. 97: 574-585.

de León, J. H., W. A. Jones, and D. J. W. Morgan. 2004a**.** 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., W. A. Jones, and D. J. W. Morgan. 2004b**.** 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., and W. A. Jones. 2005. Genetic differentiation among geographic populations of *Gonatocerus ashmeadi* (Hymenoptera: Mymaridae), the predominant egg parasitoid of *Homalodisca coagulata* (Homoptera: Cicadellidae). 9pp. J. Insect Sci. 5:2, Available online: insectscience.org/5.2.

de León, J. H., W. A. Jones, Mamoudou Sétamou, and D. J. W. Morgan. 2005. *Gonatocerus morrilli* (Hymenoptera: Mymaridae), a primary egg parasitoid of the glassy-winged sharpshooter (*Homalodisca coagulata*) exists in nature as a cryptic species complex. (Submitted).

Gordh, G., and J. W. Beardsley. 1999. Taxonony and biological control. In: *Handbook of Biological Control* (eds Bellows TS, Jr., Fisher TW), pp. 45-56. Academic Press, San Diego.

Karp, A., and J. Edwards. 1997. DNA markers: a global overview. In: *DNA markers- protocols, applications, and overviews* (eds Caetano-Anolles G, P. M. Gresshoff PM), (eds.), pp. 1-13. Wiley-Liss, Inc., New York, NY.

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.

Miller, D. R., and A. Y. Rossman. 1995. Systematics, biodiversity, and agriculture. BioScience 45: 680-686.

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.

Roderick, G. K. 2004. Tracing the origins of pests and natural enemies: Genetic and statistical approaches. In: *Genetics, Evolution, and Biological Control* (eds Ehler LE, Sforza R, Mateille T), pp. 97-112. CABA International, Wallingford, UK.

Rosen, D. 1977. The importance of cryptic species and specific identifications as related to biological control. In: Biosystemtics is agriculture. *Beltsville Symposium of Agricultural Research* (ed Romberger JA), pp. 23-35. Allanheld, Osmun, Monteclair, NJ.

Schauff, M. E., and J. LaSalle. 1998. The relevance of systematics to biological control: protecting the investment in research. In: *Pest Management- Future Challenges*, vol. 1., pp. 425-436. Proceedings of the 6th Australian Applied Entomological Conference, 29 September-2 October, Brisbane, Australia.

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, Lisa A. Ledezma, and Rosie Ruiz for their excellent technical assistance.