IS THE GLASSY-WINGED SHARPSHOOTER PARASITOID *GONATOCERUS MORRILLI* ONE SPECIES OR A COMPLEX OF CLOSELY RELATED SIBLING SPECIES?

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ABSTRACT
The aim of the present study was to determine whether the egg parasitoid *Gonatocerus morrilli* Howard is one species or a complex of closely related sibling species. To unravel their identity we sampled specimens from Texas (TX), California (CA), Veracruz in Mexico (MX), and Tucuman in Argentina (AG) and compared them using three approaches: 1) morphological differences; 2) molecular differences in ribosomal regions: ITS1, ITS2 and 28SD2, and the mitochondrial cytochrome oxidase I (CO1); and 3) by performing cross mating compatibility studies between parasitoids from the four regions. According to the obtained large differences in sequences and the reproductive incompatibility between the four populations, these near *morrilli* populations are best treated as distinct species.

INTRODUCTION
The glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say) (Hemiptera: Cicadellidae), is an exotic pest in southern California (CA) and is the main vector strains of the bacterium *Xylella fastidiosa* Wells *et al.*, that causes the Pierce's disease in grapevines (*Vitis vinifera*) (Freitag *et al.,* 1952; Davis *et al.,* 1978; Blua *et al.*, 1999). In CA, GWSS were first observed in Orange and Ventura Counties during 1990. GWSS occurs naturally from Florida to Texas and northeastern Mexico (Young, 1958; Triapitsyn & Phillips, 2000). It was probably introduced from the southeastern United States as eggs on imported plants (Sorensen and Gill, 1996). A major Pierce's disease epidemic was first noticed in CA in 1997 (Blua *et al.,* 1999) and during a survey in 2000 up to 87% of grapevines in Temecula were infected (Perring *et al.,* 2001). Since then, GWSS has been observed as far north as Sacramento County (California Department of Food and Agriculture, 2003) suggesting that this pest is continuing to spread in CA. To perform a successful classical biological control program, species of GWSS egg parasitoids are currently being prospected for in the native range of GWSS and promising species are being released in CA (Triapitsyn *et al*., 1998; Triapitsyn & Hoddle, 2001). One of the promising GWSS egg parasitoids is the small (1.5-2mm) egg parasitoid *Gonatocerus morrilli* Howard (Hymenoptera: Mymaridae). This parasitoid occurs in the native range of GWSS in the southeastern United States (Huber, 1998). The outcome of classical biological control programs often depends on correct identification of both the pest insect and its parasite. Misidentification has negatively affected several incipient biological control programs (Messing & Aliniazee, 1988; Gordh & Beardsley, 1999). Hence, to avoid misidentification of cryptic species it is important to design molecular markers to correctly identify species (Stouthamer *et al.*, 2000; de Leon *et al.*, 2005) when consistent distinguishing morphological characters are difficult or expensive to ascertain. *Gonatocerus morrilli* has been imported from Texas (TX) and has been released in CA since 2001 with the assumption that this is one species and not an aggregation of morphologically very similar sibling species. However, light microscopy suggests that differences between the different *G. morrilli* populations may exist, which may indicate the existence of a species complex. Indeed, a closely related species has recently been found in CA (de Leon *et al.*, 2005). Here, we sort to determine whether *G. morrilli* is one species or that it is in fact a group of closely related similar looking species. Specimens from the southeastern U.S., California, Mexico, and Argentina were received from collaborators; if possible, colonies were established. We tried to determine their relationship using three approaches: (1) comparing molecular features by extracting DNA and sequencing of four different gene regions of the mitochondrial and ribosomal DNA, (2) comparing morphological characters, and (3) by investigating whether the different populations are reproductively compatible by conducting mating experiments between the geographic populations (Vickerman *et al.*, 2004). The outcome of these three approaches was evaluated to determine whether *G. morrilli* is a valid species as such or if it is better treated as a complex of closely related species.

OBJECTIVES
To determine the species status of geographically different *Gonatocerus morrilli* populations by 1) morphology, 2) sequencing of two Internally Transcribed Spacer regions (ITS1 and ITS2), the mitochondrial gene cytochrome oxidase I (CO1) and the ribosomal D2 gene, and 3) by crossing compatibility studies with four different geographic populations of which we maintain colonies.

RESULTS AND CONCLUSIONS

Comparisons in morphology

The MX near *morrilli* female differs from all other groups by showing a distinctive fifth funicle segment of the female antenna. This segment is partially brown (basally) and white (apically). *Gonatocerus morrilli* and the CA near *morrilli* have a completely white fifth funicle segment. *Gonatocerus* sp. 6 from Argentina differs from all groups in having the entire funicle of the female antenna dark brown. *Gonatocerus morrilli* and the CA near *morrilli* show more consistent differences when compared: the submedial carinae on the propodeum are close to each other in *G. morrilli* but conspicuously more apart in the CA near *morrili.*

Comparisons in sequences

The levels of genetic divergence between *G. morrilli* (from Texas, TX), the two near *morrilli* populations we studied (from Riverside County, California, CA, and from Veracruz Mexico, MX) and *Gonatocerus* sp. 6 from Argentina are summarized in Table 1.

The differences in the two spacer-regions between the group *G. morrilli*-MX and CA-ARG were very large which made it impossible to align them properly. However, the two spacer-regions within these two groups could be aligned. The CO1 and D2 genes could be aligned for all groups since these are conservative genes. For the ITS1, ITS2, CO1 and D2, the MX type differed from *G. morrilli* with resp. 29%, 29%, 5% and 3%. The CA type differed from the Arg type 6%, 8%, 2% and 1%, respectively. Intragroup variation was <1.5% for both the ITS1 and ITS2 regions, <0.9% for the CO1 gene and no variation was found within the groups using the D2 gene. For each region at least 8 individuals were sequenced.

As shown in Table 1, divergences between the different near *morrili* populations and *G. morrilli* are high. Intragroup variation is minimal, despite the fact the we sequenced *G. morrilli* from very different areas of their distribution. The obtained sequences from *G. morrilli* originating from Florida (Fl), northeastern MX and from our TX colony differ <1.5% at most. These large differences and the homogenous near *morrilli* groups might indicate that *G. morrilli* is not a monotypic species, but indeed, that it is better treated as a complex of closely related species.

Table 1. The percentage difference between *G. morrilli* from TX and the near-*morrilli* from MX, Arg. and CA as measured for the ITS1 and ITS2 region, the CO1 gene and the D2 gene.

	ITS1	**ITS2**	**CO1**	**D2**
	G. morrilli	*G. morrilli*	*G. morrilli*	*G. morrilli*
near morrilli MX	29%	29%	5%	3%
Gonatocerus sp. 6 from Argentina	x	x	6%	8%
near morrilli CA	x	x	6%	8%

Comparisons in reproductive compatibility

To test whether the four types were reproductively compatible, we performed mating experiments as described in Vickerman *et al.* (2004). We did the following crosses: ♀ *G. morrilli* (Gm) x ♂ MX, ♀ Gm x ♂ CA, ♀ Gm x ♂ Arg and all the reciprocal crosses to test for unidirectional incompatibility. At least 13 replicates were used per crossing. In addition, we performed control crosses for each group: ♀ Gm x ♂ Gm, ♀ MX x ♂ MX, ♀ Arg. x ♂ Arg and ♀ CA x ♂ CA (at least 10 replicates each) and virgin females (10 replicates each) were set up to determine whether the females used in the crossings were virgins and whether a species could be infected with an endosymbiont like *Wolbachia* (Stouthamer *et al.* 1999). All virgin females produced only sons, proving that they were unmated and that they reproduce arrhenotokously. All interspecific crosses produced only sons while the intraspecific control crosses produced both males and females as shown in Table 2.

Table 2. The sex ratios of the produced offspring per crossing measured as the proportion females. At least 10 replicates per crossing were performed. Differences between interspecific crosses were highly significant (Kruskall-Wallis, H=102.75, df=11, P=<0.0001, followed by individual Mann-Whitney U-tests, $P \leq 0.0001$)

	Gm	MX	Arg	Ca
Gm	0.86			
MX	0.03	0.77		
Arg	0	0	0.81	
CA	0	0	0	0.86

Since all interspecific crosses produced only sons, the different geographic populations are mutually incompatible with each other. The observed highly significant differences in reproductive compatibility between the control crossings and the interspecific crossings confirm our findings after analysis of the molecular data, namely that these four different taxa are best treated as four different species instead of local forms of the species *G. morrilli.*

Our findings can be of importance considering the introduction of *G. morrilli* in CA as a natural enemy of the glassy-winged sharpshooter. A major reason for introduction of *G. morrilli* was to enrich the local *G. morrilli* populations by alleged increasing genetic variability (Pilkington *et al., in press*). However, since the different species, which we assume them to be, do not successfully interbreed, the genetic variability of the CA species will not increase and indeed, as far as we are aware of, nothing is known of interspecific competition in the field between the different *G. morrilli* types. Since the different species, which we assume them to be, do not successfully interbreed, the genetic variability of the CA species will not increase.

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