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Molecular distinction between populations of *Gonatocerus morrilli*, egg parasitoids of the glassy-winged sharpshooter from Texas and California: Do cryptic species exist?

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Abstract

Two molecular methods were utilized to distinguish geographic populations of *Gonatocerus morrilli* (Howard) from Texas and California and to test the possibility that this species could exist as a species-complex. Inter-Simple Sequence Repeat–Polymerase Chain Reactions (ISSR–PCR) were performed with a 5'-anchored ISSR primer. Twenty-five markers were generated with four populations (40 individuals) of *G. morrilli*. Twenty-three were polymorphic and the percentage of polymorphic loci was 92%. Most markers could be considered diagnostic since there was no band sharing between the Texas and California populations. Such differences typically are not found unless the populations are reproductively isolated. Exact tests for population differentiation indicated significant differences in marker frequencies among the populations. Comparison of other genetic differentiation estimates, which evaluate the degree of genetic subdivision, demonstrated excellent agreement between G_{ST} and θ values, 0.92 and 0.94, respectively, indicating that about 92 to 94% of the variance was distributed among populations. The average genetic divergence (D), as measured by genetic distance, was extremely high (Nei = 0.82 and Reynolds = 2.79). A dendrogram based on Nei's genetic distance separated the Texas and California populations into two clusters, respectively. Amplification of the Internal Transcribed Spacer-1 (ITS-1) region showed no size differences, whereas the ITS-2 DNA fragment varied in size between the two geographic populations. The ITS-2 fragment sizes were about 865 and 1099 base pairs for the California and Texas populations, respectively. The present study using the two molecular methods provides novel data critical to the glassy-winged sharpshooter/Pierce's disease biological control program in California.

Keywords: differentiation, DNA fingerprinting, genetic distance, grapevines, molecular markers, natural enemies, Pierce's disease, polymorphic loci, population genetics

Abbreviations:

ISSR–PCR	Inter-Simple Sequence Repeat–Polymerase Chain Reaction
ITS	Internal Transcribed Spacer

Introduction

Gonatocerus morrilli (Howard) (Hymenoptera: Mymaridae) are egg parasitoids of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the glassy-winged sharpshooter (Turner and Pollard 1959; Triapitsyn *et al*. 1998). This primary egg parasitoid species is common in the southern United States and Mexico (Huber 1988). A biological control program is currently in progress in California against *H. coagulata*, a xylem feeding leafhopper which is a serious economic pest that transmits a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's disease in grapevines (*Vitis vinifera* and *V. labrusca*) (Hopkins and Mollenhauer 1973). *Homalodisca coagulata* are native to the southern United States, ranging from Florida to Texas and northern Mexico (Young 1958; Turner and Pollard 1959; Nielsen 1968; Brlansky *et al*. 1983). Within the last 10 years, the insect has established itself in southern California where it poses a serious threat to the wine and table grape industry (Sorensen and Gill 1996).

Accurate identification of natural enemies is critical to the success of classical biological control programs. Lack of proper identification procedures has affected the early stages of several projects (Messing and Aliniazee 1988; Löhr *et al*. 1990). There is a need for molecular markers for natural enemies to provide new characters for studies of phylogenetic relatedness, for identification

of cryptic species and biotypes, and for the assessment of heritable variation for population genetics and ecological investigations (Unruh and Woolley 1999). Studies of allele or marker frequencies in naturally occurring parasitoid populations are important, not only for identifying genetic variation of potential benefit, but also for the detection of genetic markers indicative of specific biological traits or geographic origins. Furthermore, the recognition of intraspecific variation can be as crucial for the success of biological control programs as is sound species determination (Powell and Walton 1989; Narang *et al*. 1993; Unruh and Woolley 1999).

Recently, we developed DNA markers for *H. coagulata* for the purpose of estimating genetic variation in natural populations (de León and Jones 2004). Inter-Simple Sequence Repeat–Polymerase Chain Reaction (ISSR–PCR) (Zietkiewicz *et al*. 1994) was shown to be a sensitive and efficient procedure for *H. coagulata* template. This DNA fingerprinting procedure permits detection of DNA variation in simple sequence repeats (SSR) without the need to isolate and sequence specific DNA fragments. In addition, we distinguished three *Homalodisca* sharpshooter species by this method. Hedrick and Parker (1997) have shown that microsatellites are highly useful for Hymenoptera because of the much higher level of heterozygosity evident in this type of variation.

The internal transcribed spacer regions (ITS-1 and -2) have also been used extensively in the examination of the taxonomic status of species and for diagnostic purposes (reviewed in Collins and Paskewitz 1996). Stouthamer *et al*. (1999) used ITS-2 DNA fragment sizes as taxonomic characters to develop a precise identification key for sibling species of the genus *Trichogramma*. In cases where species were observed with similar sized ITS fragments these authors suggested amplification, sequencing, and restriction digestion.

The objective of the present study was to survey molecular methods useful in egg parasitoid identification and discrimination and to investigate the possibility that *G. morrilli* could exist as a species-complex in nature. The DNA fingerprinting method, ISSR–PCR was utilized with a 5'-anchored ISSR primer. Different approaches to estimate population differentiation: exact tests (Raymond and Rousset 1995), G_{ST} (Nei 1987), θ (Weir 1990, 1996), and dendrograms based on genetic distance (Nei 1987; Reynolds *et al*. 1983) by the UPGMA method (Sneath and Sokal 1973) were than applied and compared. In addition, the ITS were amplified with standard primers to determine if DNA size fragments could be used to distinguish the geographic populations of *G. morrilli*.

Materials and Methods

Insect collection

Gonatocerus morrilli were collected from *H. coagulata* egg masses. Two populations were collected five months apart in 2003 from Orange (OrCo) and San Diego (SDCo) counties of California and two were from Hidalgo county, Texas (Weslaco; Wes-2 and Wes-3). Sharpshooter egg masses laid in citrus leaves were retrieved from the field. Five individuals from Wes-2 were an F1-generation. The leaves were washed with 5% bleach then incubated at 28 °C, 16:8 L:D scotophase on damp filter papers in Petri dishes. Wasps were collected daily and stored at −80 °C in 95% ethanol prior to analysis.

Genomic DNA isolation

Genomic DNA was extracted according to standard methods (Sambrook and Russell 2001). Individual wasps were homogenized on ice in 1.5 ml microfuge tubes in 60 µl of lysis buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8.0], 1% IGEPAL CA-630]) using two 20 second grindings with 10 min intervals on ice (Pellet Pestle Motor, Bel-Art Products, Pequannock, New Jersey). To avoid cross contamination between samples, a sterile plastic pestle was used for each insect. The final DNA pellet was resuspended in 61 µl of TE (Tris-HCl [pH 7.5], 1 mM EDTA [pH 7.5]). To confirm for the present of genomic DNA, amplification reactions were performed with 1 µl of stock DNA and 28S primers at an annealing temperature of 65 °C (forward CCCTGTTGAGCTTGACTCTAGTCTGGC and reverse AAGAGCCGACATCGAAGGATC) with 1.5 mM $MgCl_2$ and the amplification conditions described below.

ISSR–PCR DNA fingerprinting

ISSR–PCR amplification reactions are described in de León and Jones (2004). The reactions were performed with the 5'-anchored primer $HVH(TG)_7T$ (Zietkiewicz *et al*. 1994). Notations: H = A/T/C, and V = G/C/A. The reactions were performed in a final volume of 20 µl with the following components: 1X PCR buffer [50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, and 0.01% gelatin], 0.25 mM deoxynucleotide triphosphates, 0.25 mM ISSR primer, 1.0 µl of stock genomic DNA and 0.05 U/µl *Taq* DNA Polymerase (New England Biolabs, Beverly, Massachusetts). The cycling parameters were as follows: 1 cycle at 94 °C for 2 min followed by 45 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min. Reactions were optimized for amount of genomic DNA, annealing temperature, $MgCl_2$ concentration, and cycle number. Negative control reactions were performed in the absence of genomic DNA. Amplification products were loaded onto 2% agarose gels and submitted to electrophoresis in 1X TBE buffer (90 mM Tris-borate, 2 mM EDTA) in the presence of 0.2 µg/ml ethidium bromide. Gels were photographed with the Chemi Doc System and markers/bands were scored via the Quantity One Software (Bio-Rad Laboratories, Hercules, California).

Internal transcribed spacer amplification, ITS-1 and -2

For ITS-1 amplification, the following primers were used: ITS5, GGAAGTAAAAGTCGTAACAAGG and RNA2, CACGAGCCGAGTGATCCACCGCTAAGAGT (White *et al*. 1990) and for ITS-2, 5.8S-F, TGTGAACTGCAGGACACATGAAC and 28S-R, ATGCTTAAATTTAGGGGGTA (Porter and Collins 1991) were used. The amplification reactions were performed as with ISSR–PCR above. The $MgCl_2$ concentration for the ITS-1 reaction was increased to 2.0 mM. The cycling parameters were as follows: 1 cycle for 3 min at 94 °C followed by 45 cycles at 94 °C for 20 s, 55 °C (ITS-1) or 45 °C (ITS-2) for 20 s, and 72 °C for 60 s.

Data analysis

Bands observed in each lane were compared with all the other lanes of the same gel and reproducible bands scored as presence (1) or absence (0). ISSR–PCR markers are treated as dominant markers (reviewed in Wolfe and Liston 1998). Fragment sizes were estimated based on the 1.0 Kb Plus DNA Ladder (Invitrogen Life Technologies,

Carlsbad, California) according to the algorithm provided in the Quantity One software. Genetic variation was analyzed using the POPGENE 3.2 genetic software program (Yen *et al*. 1996). The program estimates polymorphic loci, percentage polymporhic loci, gene diversity (*h*), total genetic diversity (Ht), average genetic diversity within populations (Hs) (Nei 1973, 1977, 1978), coefficient of gene differentiation (G_{ST}) (Nei 1987), and gene flow (Slatkin and Barton 1989). Dendrograms based on Nei's (1987) and Reynolds *et al*. (1983) genetic distances and the unweighed pair-group method with arithmetic averages (UPGMA) of Sneath and Sokal (1973) were constructed with the program Tools for Population Genetic Analysis (TFPGA, ver. 1.3, Miller 1997). Marker frequencies were estimated based on Lynch and Milligan's (1994) Taylor expansion estimate. Bootstrapping over loci was also performed with TFPGA with 1000 permutations by the algorithm of Felsenstein (1985). Exact tests (χ^2) for population differentiation (simultaneous analyses of all populations) were calculated with the TFPGA program by the method of Raymond and Rousset (1995), where a Markov Chain Monte Carlo approach was employed that gives an excellent approximation of the exact probability of the observed differences in marker frequencies. The exact tests, which included Lynch and Milligan's (1994) Taylor correction factor, were performed with 1000 dememorization steps, 20 batches, and 2000 permutations per batch. Fisher's combined probability tests (Fisher 1954; Manly 1991; Sokal and Rohlf 1995) were employed as a global test over loci to determine the overall significance. *F*-statistics (Weir and Cockerham 1984) were calculated with TFPGA and reported using the terminology of Weir (1990, 1996) where theta (θ) corresponds to F_{ST}. For dominant markers, estimates of θ by TFPGA were constructed under the assumption of Hardy-Weinberg proportions. Jackknifing/bootstrapping over loci with 5000 replications included Lynch and Milligan's (1994) Taylor expansion estimate. The pseudorandom numbers used in bootstrapping were produced by L'ecuyer's (1988) combined MLCG algorithm.

Results and Discussion

ISSR–PCR DNA fingerprinting

Figure 1 shows an example of ISSR–PCR DNA fingerprinting demonstrating the banding pattern differences between the geographic populations of *G. morrilli* from California (OrCo) and Texas (Wes-2) performed with a 5'-anchored ISSR primer. Markers ranged in size from about 200 to 900 base pairs. Overall, a total of 25 markers were generated among all four populations with a total of 40 individuals. Twenty-three were polymorphic and the percentage of polymorphic loci was 92%. Within individual populations, no diversity was seen within the California populations and some diversity was observed in the Texas populations. For the Texas populations, Wes-2 and Wes-3, 5 polymorphic markers each were generated and 20% of the markers were polymorphic. Most markers are geographic-specific and can therefore be considered diagnostic since there was no band sharing between the Texas and California populations.

ISSR–PCR differentiation among four G. morrilli populations

Exact tests (simultaneous analysis) for population differentiation indicated that highly significant differences in marker frequencies existed among the *G. morrilli* populations (χ^2 = 400.8; df = 50; *P* = 0.0000) (Table 1). Total genetic diversity (Ht) was high (0.35 or 35.0%), whereas average genetic diversity within populations was low (0.03 or 3.0%). Table 1 also shows a comparison of other genetic differentiation estimates, G_{ST} (coefficient of gene differentiation) and θ (theta, analogous to F_{ST}), which evaluate the degree of genetic subdivision among populations. Excellent agreement was seen between G_{ST} and θ values, 0.92 and 0.94, respectively. Theses values indicate that about 92 to 94% of the variance is distributed among populations. The indirect estimate of gene flow, Nm base on G_{ST}, demonstrated a low value (0.04) among the geographic populations; this value indicates highly restrictive gene flow. Overall, genetic differentiation measurements (exact tests, G_{ST}, θ, and Nm) indicate profound genetic divergence/structuring between *G. morrilli* populations from Texas and California.

Genetic relatedness among G. morrilli populations

Levels of genetic divergence among populations were also determined by calculating pairwise estimates for genetic distance by the procedures of Nei (1978) and Reynolds *et al*. (1983) (Table 2). Average genetic divergence (D) among populations was extremely high [Nei = 0.82 (0.89–1.07) and Reynolds = 2.79 (1.4–3.4)]. A

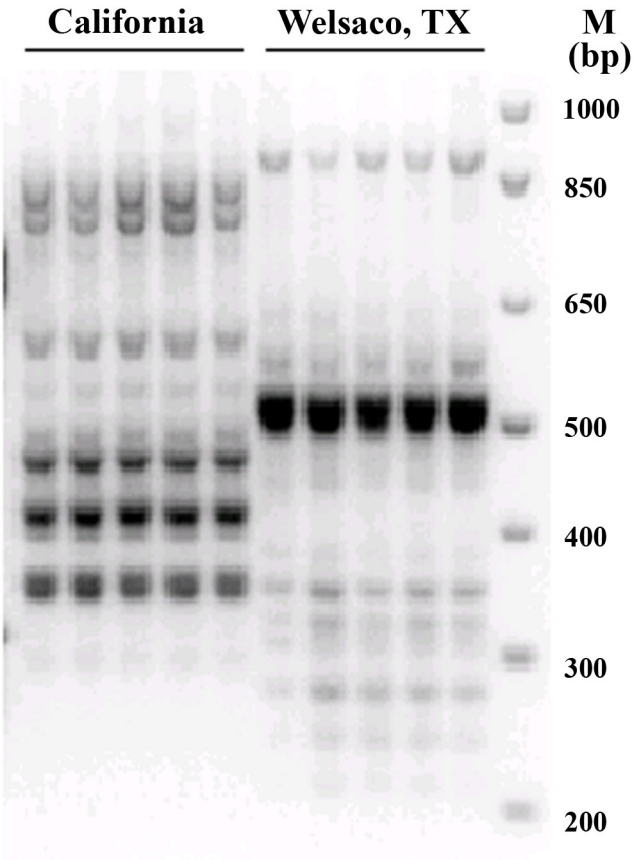

Figure 1. Representative example of ISSR–PCR DNA fingerprinting of *Gonatocerus morrilli* populations from California and Texas. Reactions were performed with genomic DNA from 5 separate individuals and the 5'-anchored ISSR primer HVH(TG)$_7$T (Zietkiewicz *et al*. 1994). M: 1.0 Kb Plus DNA Ladder.

dendrogram based on Nei's genetic distance is shown on Figure 2 with all *G. morrilli* geographic populations. Two clades are identified on the dendrogram with the California and Texas populations appearing on separate clusters. These two clusters are supported by strong bootstrap support values, 68 and 64%, respectively, for the California and Texas populations.

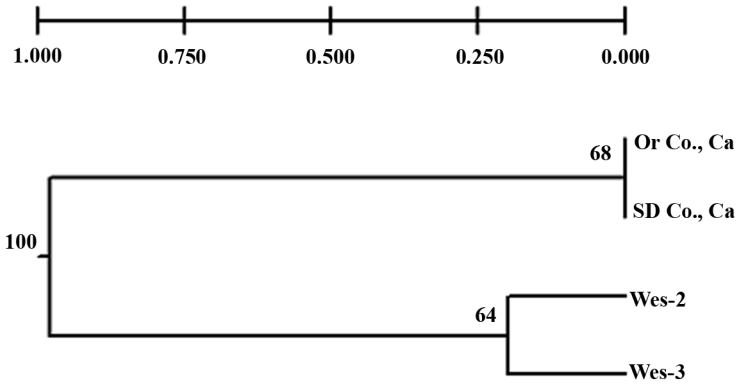

Figure 2. Dendrogram based on Nei's genetic distance (1978) by the method of UPGMA. Relationships among the four geographic populations of *G. morrilli* performed by ISSR–PCR DNA fingerprinting. Genetic distances are indicated above the dendrograms and bootstrap support values are indicated at the nodes.

Amplification of the ITS-1 and -2 regions in G. morrilli geographic populations

Monomorphic patterns were demonstrated with amplification of the ITS-1 region in all of the populations from California and Texas (~850 bp) (Figure 3); whereas, polymorphic or different DNA fragment sizes were detected within the ITS-2 region. The California populations were observed with an ITS-2 fragment size of about 865 base pairs and the Texas populations with a size of about 1099 base pairs.

Good agreement is seen between the two molecular methods and they both suggest that cryptic species may exist. The results with ISSR–PCR, demonstrating distinct banding patterns (no band sharing) between geographic populations, is not typically found unless the populations are reproductively isolated. Similar results were obtained by Hoy *et al*. (2000) with two populations of *Ageniaspis citrocola* using RAPD–PCR. The following genetic differentiation parameters, extract test, G_{ST}, θ, genetic distances, and gene flow (Nm) lend support to this observation. The extremely low value for gene flow between the populations from California and Texas lend support that these populations are isolated reproductively. Restricted gene flow usually leads to increased differentiation among populations as seen from the G_{ST} and θ values (92 to 94% of the variance is seen among populations). In addition, the divergence (D) between these populations is also high.

Methods incorporating SSR appear to be sensitive at detecting DNA polymorphisms in natural populations. Previously, we utilized ISSR–PCR to distinguish three species of *Homalodisca* sharpshooters (*H. coagulata*, *H. liturata*, and *H. insolita*) (de León and Jones 2004). Even though this method is sensitive, there are not many reports in the literature utilizing ISSR–PCR to study insect population genetics and phylogenetics. We have also had success determining the population genetic structure of *H. coagulata* representing 19 populations from through the U.S. (de León *et al*. 2004). Several investigators have used the ISSR–PCR method successfully with different organisms. For example, the method has been utilized for genetic characterization of the silkworm moth, *Bombyx mori* (Reddy *et al*. 1999), for genetic variability studies of *Biomphalaria straminea* snail complexes (Caldeira *et al*. 2001), and in many plant studies (reviewed by Wolfe and Liston 1998).

The internal transcribed spacer regions (ITS-1 and -2) have been used extensively to examine the taxonomic status of species and for diagnostic purposes, as reviewed by Collins and Paskewitz (1996). Species-diagnostic differences in sibling species of mosquitoes of the genus *Anopheles* were determined by Porter and Collins (1991). Zhu and Greenstone (1999) utilized the ITS-2 region to distinguish species and strains of the hymenopterous parasitoid

Table 1. Nei's analysis of gene diversity in populations of *Gonatocerus morrilli* from Texas and California. Ten individuals per population (40 total individuals) were subjected to ISSR–PCR DNA fingerprinting. χ^2, exact tests (simultaneous analysis) for population differentiation, df = degrees of freedom; Ht, total genetic diversity (SD); Hs, average genetic diversity within populations (SD); G_{ST} (mean), coefficient of gene differentiation; θ, theta (analogous to F_{ST}); and Nm, gene flow. $^{***}P = 0.0000$.

χ^2 (df)	Ht	Hs	G_{ST}	θ	Nm
400.8 (50)***	0.35 (0.04)	0.03 (0.00)	0.92	0.94 (0.02)	0.04

Table 2. Nei's unbiased (1978) genetic distance (below diagonal) and Reynolds *et al*. (1983) genetic distance (above diagonal). Four geographic populations of *G. morrilli*, two from Texas (Hidalgo county, Wes-2 and Wes-3) and two from California (OrCo, Orange County and SDCo, San Diego County).

Pop	OrCo	SDCo	Wes-2	Wes-3
OrCo	****	undef	3.40	2.88
SDCo	0.00	****	3.40	2.88
Wes-2	1.07	1.07	****	1.40
Wes-3	0.89	0.89	0.20	****

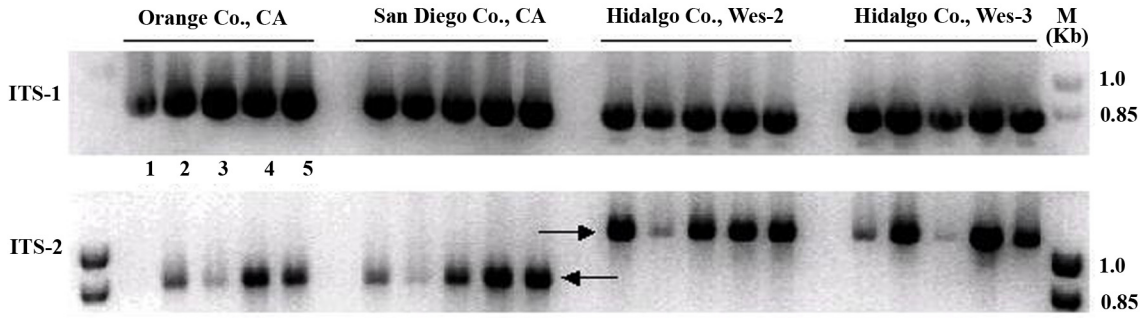

Figure 3. Amplification of the Internal Transcribed Spacer regions (ITS). The ITS-1 and -2 regions were amplified with standard ITS-specific primers (White *et al*. 1990; Porter and Collins 1991) with genomic DNA from five separate individuals from each geographic population. Arrows indicate different ITS fragment sizes. M: 1.0 Kb Plus DNA Ladder.

Aphelinus. The size of the ITS-1 fragment between the California and Texas *G. morrilli* populations did not vary, whereas the ITS-2 fragment did vary in size.

These novel observations strongly suggest that *G. morrilli* may exist in nature as a species-complex. Results from our recent study with *H. coagulata* suggest that a subset of these insects have their origin in Texas (de León *et al*. 2004). Those results together with our present results with *G. morrilli* suggest that this egg parasitoid from Texas may be a good candidate for the biological control efforts in California against *H. coagulata,* the causative agent of Pierce's disease. More research is required to confirm these results. Hybridization studies along with sequencing of standard genes [e.g., mitochrondrial cytochrome oxidases (COI and II)] and ITS fragments are in progress.

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