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Genetic differentiation among geographic populations of *Gonatocerus ashmeadi*, the predominant egg parasitoid of the glassy-winged sharpshooter, *Homalodisca coagulata*

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Abstract

The aim of genetically comparing different populations of the same species of natural enemies is to identify the strain that is most adapted to the environment where it will be released. In the present study, Inter-Simple Sequence Repeat-Polymerase Chain Reaction (ISSR–PCR) was utilized to estimate the population genetic structure of *Gonatocerus ashmeadi* (Girault) (Hymenoptera: Mymaridae), the predominant egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera:Cicadellidae), the glassy-winged sharpshooter. Six populations from throughout the U.S. and a population from Argentina identified as near *G. ashmeadi* were analyzed. Four populations (California; San Antonio, Texas; Weslaco, Texas [WTX-2]; and Florida) were field collected and two (Louisiana and Weslaco, Texas [WTX-1]) were reared. Three ISSR–PCR reactions were pooled to generate 41 polymorphic markers among the six U.S. populations. Nei's expected heterozygosity values (*h*), including the reared population from Louisiana, were high (9.01–14.3%) for all populations, except for a reared population from WTX-1 (2.9%). The total genetic diversity value (Ht) for the field populations was high (23%). Interestingly, the Florida population that was collected from one egg mass (siblings) generated the greatest number of polymorphic markers (20) and was observed with the highest gene diversity value (14.3%). All populations, except WTX-2 generated population-specific markers. Comparison of genetic differentiation estimates, which evaluate the degree of genetic subdivision, demonstrated good agreement between G_{ST} and θ values, 0.38 and 0.50, respectively for field populations, and 0.44 and 0.50, respectively for all populations. Genetic divergence (D) indicated that the WTX-1 population was the most differentiated. Average D results from the Argentina population support the taxonomic data that it is a different species. The present results estimate the population genetic structure of *G. ashmeadi*, demonstrating genetic divergence and restricted gene flow (Nm = 0.83) among populations. These results are of interest to the Pierce's disease/glassy-winged sharpshooter biological control program because the key to successful biological control may not be in another species, but instead in different geographic races or biotypes.

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

Abbreviations:

ISSR–PCR Inter-Simple Sequence Repeat-Polymerase Chain Reaction

Introduction

Gonatocerus ashmeadi (Girault) (Hymenoptera: Mymaridae) is a primary egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the glassy-winged sharpshooter (Huber 1998). A survey conducted by Triapitsyn et al. (1998) in California, Florida, and Louisiana concluded that this mymarid wasp was the predominant or the most common natural enemy of *H. coagulata*. A biological control program is currently in progress in California against *H. coagulata* because this xylem feeding leafhopper is a serious economic pest that vectors a strain of *Xylella fastidiosa*, a bacterium that causes Pierce's disease in grapevines (*Vitis vinifera* and *V. labrusca*) (Hopkins and Mollenbauer 1973). *H. 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, *H. coagulata* have established in southern California where they pose a serious threat to the wine and table grape industry (Sorensen and Gill 1996).

Studies of allele or marker frequencies in naturally occurring parasitoid populations are important, not only for identifying genetic variation of potential benefit in the selection and screening of biological control organisms, but also for the detection of genetic markers indicative of specific biological traits or geographic origins. In addition, the recognition of intraspecific variation can be as crucial for the success of biological control programs as is sound species determination. Populations of parasitoids from distinct geographical regions may differ in relevant

biological characteristics of importance to biological control (Powell and Walton 1989; Narang et al. 1993b; Unruh and Woolley 1999). An aim of genetically comparing different populations of the same species of natural enemies is to identify the strain that is most adapted to the environment where it will be released (Messenger and van den Bosch 1971). In other words, the key to successful biological control may not be in another species, but instead in different geographic races or biotypes of one species (Diehl and Bush 1984). Laboratory populations of parasitoids are maintained for three main purposes: to provide material for research into their basic biology and behavior, to meet quarantine requirements, and to mass-rear insects for release in biological control programs (Unruh et al. 1983; Powell and Walton 1989; Hopper et al. 1993). Reliable methods are needed for distinguishing various exotic strains of these biological control agents from those indigenous to the U.S., including parasitoids from different states within the U.S. Release of unidentified and uncharacterized strains can make it difficult to document their establishment and dispersal. Therefore, genetic typing of strains prior to their release in the field is highly desirable (Narang et al. 1993b). Molecular methods are also needed to monitor the quality of laboratory cultures, to detect development of major deviations in genetic composition from that existing in field populations, and for the detection of cross-contamination between cultures of sibling species (Powell and Walton 1989; Menken and Ulenberg 1987; Narang et al. 1993b; Hopper et al. 1993; Unruh and Woolley 1999).

A sensitive approach for obtaining polymorphic DNA markers is based on the use of simple sequence repeats (SSR) or microsatellites. Microsatellites are ubiquitous in eukaryotic genomes and can be found in both protein-coding and noncoding regions (Tóth et al. 2000). Microsatellites are widely used as genetic markers because they are co-dominant, multiallelic, easily scored, and highly polymorphic, although drawbacks for their use are the time and cost required to characterize them (Karp and Edwards 1997). However, a DNA fingerprinting procedure, Inter-Simple Sequence Repeat-Polymerase Chain Reaction (ISSR–PCR) (Zietkiewicz et al. 1994), including adding a primer pair to the reaction (pp-ISSR–PCR) (Prevost and Wilkinson 1999; Cekic et al. 2001), permits detection of DNA variation in microsatellites without the need to isolate and sequence specific DNA fragments. The approach for this technique involves amplification with oligonucleotide primers corresponding directly to random SSR sites. This involves the use of 5'-anchored or compound ISSR primers where the anchor serves 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. Many classes of microsatellite repeat motifs have been identified, though the class most abundant in eukaryotic genomes is the CA-repeat. The presence of these repeat motifs in high copy number and their dispersion throughout the genome of all eukaryotes tested has been demonstrated by earlier studies (Hamada et al. 1982; Tautz and Renz 1984; Tóth et al. 2000). Therefore, because of their high density, a few oligonucleotides complementary to these CA-repeat motifs can be used to target a significant portion of the genome and reveal highly polymorphic banding patterns (Zietkiewicz et al. 1994).

To gain an understanding of the population genetics of *G. ashmeadi*, molecular genetic markers were developed by the ISSR–PCR DNA fingerprinting procedure. Recently, we developed DNA markers for *H. coagulata* for the purpose of estimating genetic variation in natural populations (de León and Jones 2004). We screened and compared four DNA fingerprinting procedures and determined that procedures incorporating SSR or microsatellites were the most sensitive with *H. coagulata* template. To this end, the specific objectives of the present study were to 1) estimate genetic variation or gene diversity within and among populations, 2) estimate the population genetic structure, 3) determine whether ISSR–PCR was sensitive enough to identify diagnostic markers in geographic populations, and 4) confirm the species identification of a population of egg parasitoids from Argentina identified as near *G. ashmeadi*. We demonstrated the ability of the ISSR–PCR procedure with compound and 5'-anchored ISSR primers, including combining them, to generate polymorphic markers and estimate geographic variation in *G. ashmeadi* populations.

Materials and Methods

Insect collection

G. ashmeadi were collected from *H. coagulata* egg masses from various regions of the United States by the individuals listed on Table 1. A population of wasps from Argentina classified as near *G. ashmeadi* (M02012; 30 males) was also included. S. Triapitsyn from the University of California Riverside identified this egg parasitoid. This egg parasitoid was imported to the USDA, APHIS, Arthropod Quaratine Facility, Edinburg, Texas. M02012 was the unique code associated with this imported colony. Specimens were then shipped to Weslaco in 95% non-denatured ethanol. *G. ashmeadi* from California (CA; ~10 egg masses; 17 females and 13 males), Weslaco, Texas (WTX-2; ~4 egg masses; 22 females and 9 males), San Antonio, Texas (SATX; ~10 egg masses; 23 females and 7 males), and Quincy, Florida (QFL; 1 egg mass; 10 females and 3 males) were field collected. Populations from Weslaco, Texas (WTX-1; F1/F2 generation; 23 females and 7 males) and Louisiana (LA; F5 generation; 11 females and 19 males) were reared. The Weslaco populations (WTX-1 and -2) were collected a year apart, with WTX-1 collected in 2002 and WTX-2 collected in 2003.

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) with two 20 s bursts with 10 min intervals on ice (Pellet Pestle Motor, Bel-Art Products, www.bel-art.com). To avoid cross contamination between samples, a sterile plastic pestle was used per 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 the presence of genomic DNA, amplification reactions were performed with 1 µl of stock DNA and 28S primers at a Tm of 65 °C (forward CCCTGTTGAGCTTGACTCTAGTCTGGC and reverse AAGAGCCGACATCGAAGGATC) with 1.5 mM $MgCl_2$ with the assay conditions described below.

ISSR–PCR DNA fingerprinting

ISSR–PCR amplification reactions and modification of ISSR primers are described in de León and Jones (2004). Three

Table 1. Collection of *G. ashmeadi*. The population from Argentina is classified as near *G. ashmeadi* (M02012).

Location	County	Collector(s)/Affiliation	Reared/Affiliation
California (CA)	Orange	David J. W. Morgan/CDFA	
Weslaco, TX-1 (WTX-1)	Hidalgo	William Warfield/USDA, ARS	William Warfield/USDA, ARS
San Antonio, TX (SATX)	Bexar	Walker A. Jones/USDA, ARS	
Louisiana (LA)		David J. W. Morgan/CDFA	Isabelle Lauziere/USDA, APHIS
Weslaco, TX-2 (WTX-2)	Hidalgo	William Warfield/USDA, ARS	
Quincy, FL (QFL)	Gadsden	Russell F. Mizell III	
		University of Florida	
Argentina (ARG)		Walker A. Jones/USDA, ARS	Isabelle Lauziere/USDA, APHIS
		Eduardo Virla/CONICET-PROMIMI	
		San Miguel de Tucumán, Argentina	
		Guillermo Logarzo/USDA, ARS, OIRP	
		South American Biological Control	
		Laboratory, Buenos Aires, Argentina	

reactions for this study included: (a) ISSR–PCR p-6, (b) pp-ISSR–PCR #4 and (c) #9 (Table 1). The reactions were performed with 5'-anchored or compound ISSR primers that included either a single primer or a primer pair: (a) $HVH(TG)_7T$, (b) $KKVRVRV(TG)_6$ and $CCAG(GT)_7$, and (c) $CCAG(GT)_7$ and $T(GT)7(AT)_2$. The use of these primers in their original forms are reported in several sources (Wu et al. 1994; Zietkiewicz et al. 1994; Fisher et al. 1996; Witsenboer et al. 1997). Notations: K = G/T, V = G/C/A, R = G/A, 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], 2.0 mM $MgCl_2$, and 0.01% gelatin), 0.25 mM deoxynucleotide triphosphates, 0.25 µM ISSR primer(s), 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, Tm (Table 1) 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, www.bio-rad.com).

Data analysis

Data were analyzed with the pooled products of the three amplification reactions. Bands observed in each lane were compared with all the other lanes of the same gel and only reproducible bands were scored as present (1) or absent (0). Each ISSR–PCR reaction generated unique banding patterns. Only bands unique to each reaction were scored and in cases where a similar sized band was generated by more than one reaction, a band from only one reaction was chosen. 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, www.invitrogen.com) according to the algorithm provided in the Quantity One software. Genetic variation was analyzed with the programs POPGENE 3.2 (Yen et al. 1996) and Tools for Population Genetic Analyses (TFPGA, ver. 1.3, Miller 1997) as previously performed (de León et al. 2004). Marker frequencies were estimated based on Lynch and Milligan's (1994) Taylor expansion estimate. 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 applied and compared.

Results and Discussion

ISSR–PCR marker heterozygosity

A total of 41 polymorphic markers were generated in the six populations of *G. ashmeadi* (163 individuals) from the U.S. with the ISSR–PCR reactions (Table 2), 34 or 83% of these markers tested neutral based on the Ewens-Watterson analysis (Manly 1985). Reactions a, b, and c generated several polymorphic markers each. Results of G^2-contingency tests indicated significant heterogeneity of marker frequency across all U.S. populations for 31 of 41 markers and for 25 of 34 markers for the field populations. Also shown on Table 2 are the number of population-specific or rare markers identified in each geographic population, the ISSR–PCR reaction that generated the markers, the estimated size of the markers (bp), and the estimated frequency of each marker. All populations, except WTX-2, were associated with population-specific markers. Reared populations, WTX-1 (F1/F2) and LA (F5) produced two and five population-specific markers, respectively. Rare markers can be an indication of population subdivision. It was surprising that WTX-2 was not found associated with any population-specific markers since it was field collected from about ten different egg masses. Absence of rare or population-specific markers can indicate a genetic bottleneck in recent history of a population. This occurs when a

Table 2. Summary of ISSR–PCR markers generated in six populations of *G. ashmeadi* from the U.S. For the genetic analyses the products of all three reactions were pooled. Heterozygous frequencies (Heter. freq.) were determined by G^2-continency tests for all populations. Population-specific or private markers and their estimated frequencies (Est. freq.) are shown for each individual population. The population from Argentina identified as near *G. ashmeandi* (M02012) is included separately.

Reaction (Tm°C)	No. markers generated			Pop.	Heter. freq.
a) ISSR-PCR p-6 (50)	19			fc	73.50%
b) pp-ISSR-PCR #4 (51)	15			All	75.60%
c) pp-ISSR-PCR #9 (51)	12				
Total	41				

Pop.	Reaction	Marker No.	Est. Size (Kb)	No. Ind. Present	Est. freq.
CA	b	10	0.794	13	0.2472
	a	14	0.499	4	0.0691
WTX-1	b	25	0.642	27	0.6838
	c	27	0.996	1	0.0168
SATX	c	35	0.82	2	0.0339
LA	a	32	0.936	8	0.1437
	a	33	0.762	2	0.0339
	c	36[a]	0.915	11	0.2042
	c	37[a]	0.883	19	0.3945
	c	48[a]	0.903	8	0.1437
QFL	a	17	0.405	1	0.0392
	a	24	0.429	1	0.0392
	a	38	0.603	1	0.0392
	a	39	0.547	1	0.0392
	b	40	0.249	1	0.0392
ARG	a	41	0.538	30	1.0000
	b	42	0.677	30	1.0000
	b	43	0.284	30	1.0000
	b	44	0.263	30	1.0000
	b	45	0.177	30	1.0000
	b	46	0.191	14	0.2572
	c	47	0.53	30	1.0000

[a]Set of three closely spaced markers that together could be considered diagnostic.

population has undergone a drastic contraction, and its numbers rebuilt from a small number of individuals, a common occurrence in insect colonies (Munstermann 1994); however none of the populations were colonized in our study. Only seven (17%) of the 41 markers were shared among all *G. ashmeadi* populations. None of the same fixed markers were identified across all *G. ashmeadi* populations, though each population was associated with fixed or monomorphic markers. The following markers were present at high frequency: #10 and 25, 36, and 37 for CA, WTX-1, and LA, respectively. Together, markers # 36, 37, and 48 can be considered diagnostic for the LA population. Seven fixed markers (#41-47) were identified in ARG that can be considered diagnostic.

Genetic diversity

Within populations, gene diversity values (*h*) were observed ranging from 2.9 to 14.3% with WTX-1 (F1/F2) having the lowest and QFL having the highest value (Table 3). In general, the two Weslaco populations (WTX-1 and -2) were found to have the lowest *h* values. No significant differences in *h* were seen between the two Weslaco populations ($t = 1.49$, df = 58, $P > 0.05$), but significant differences ($P < 0.05$) were observed between WTX-1 and the rest of the U.S. populations. Interestingly, no significant differences in *h* were observed between the reared LA (F5) and the rest of the field populations. The fact that QFL was associated with an *h* value of 14.3% was surprising since this population was from a single egg mass. Overall, the field populations and all the U.S. *G. ashmeadi* populations together had an *h* value of 23 and 20.8%, respectively. The number of polymorphic markers ranged from 12 to 20 with WTX-1 and -2 having the lowest and QFL the highest. Percentage of polymorphic markers (%P) ranged from 29.3 to 58.8%, but overall, 100% of the ISSR–PCR markers were polymorphic, including the field populations analyzed separately. The two Weslaco

Table 3. Single-populations descriptive statistics for *G. ashmeadi* from the U.S. and genetic variation statistics for all loci. No. M, number of monomorphic markes; No. P., number of polymorphic markers; % P, percentage polymorphic loci; Polym. Ratio, number of polymorphic markers per number of insects; *h* (SD), gene diversity (standard deviation) (Nei 1973, 1977, 1978). One-tail unpaired *t* test performed for *h* values.

Pop.	No. Insects	No. M	No. P	Total # markers	% P	Polym. ratio	h (SD)
CA	30	5	16	21	39.2	0.53	0.1329 (0.182)[a]
WTX-1	30	7	12	19	29.3	0.40	0.0290 (0.158)
WTX-2	30	6	13	19	31.7	0.43	0.0901 (0.160)
SATX	30	5	16	21	39.0	0.53	0.1123 (0.170)[a]
LA	30	5	17	22	41.5	0.57	0.1252 (0.182)[a]
QFL	13	1	20	21	58.8	1.54	0.1431 (0.199)[a]
fc	103	0	34	34	100	0.33	0.2300 (0.184)
All	163	0	41	41	100	0.25	0.2082 (0.187)
ARG	30	11	8	19	16.7	0.27	0.0434 (0.127)

[a] Significantly different from WTX-1, $P < 0.05$; df = 58.

populations were associated with the lowest %P and QFL with the highest. It is interesting to note that even though both LA and WTX-1 were reared, WTX-1 is presented with a significantly ($P < 0.05$) lower *h* value. On the other hand, the Weslaco populations contain the same number of monomorphic markers (7 vs. 6), polymorphic markers (12 vs. 13), total number of markers (19 each), and %P (29.3 vs. 31.7%), but a three-fold difference in *h* (2.9 vs. 9.0%) (non-significant) is seen. If a decrease in total number of markers occurs, a genuine loss of genetic material is indicated (Munstermann 1994), but this situation is not seen between the two Weslaco populations. These results may indicate a real genetic difference between the two populations, including the possibility of sympatric strains. The highest polymorphic ratio (1.54) was seen with QFL, this value was about three- and four-fold higher than CA, SATX, LA and WTX-1 and -2, respectively. This ratio also demonstrates the high diversity in QFL; this is an interesting result since all individuals are assumed to be siblings (from the same egg mass).

ISSR–PCR differentiation among U.S. G. ashmeadi populations

Tables 4 and 5 present the results from the different approaches used to apportion variation into within- and among-populations levels. Simultaneous exact tests for population differentiation (Table 4) indicate that highly significant differences in marker frequencies exist among the six populations; significant differences are also seen among the field populations analyzed separately. In addition, pairwise exact tests showed highly significant differences in marker frequencies between populations, including between WTX-1 and -2. These statistically significant tests suggest that discrete subpopulations exist. Total genetic diversity (Ht) values, 23% for field and 20.9% for all populations (Table 5) are in agreement with the *h* values on Table 3. The average genetic diversity within populations (Hs) value for the field populations is 14.4%. Table 5 also shows a comparison of other genetic differentiation estimates, G_{ST} and θ, which evaluate the degree of genetic subdivision among populations. Good agreement was seen between G_{ST} and θ values, respectively for field and for all populations. The G_{ST} values, for field and all populations indicate that about 38 and 44% of the variance is distributed among populations, and 62 and 56% is distributed within populations, respectively. The θ values show that about 50% of the variance is seen among populations in both field and all populations. The indirect estimate of gene flow, Nm base on G_{ST}, demonstrated low values for both field and all U.S. populations.

Table 4. Exact tests (X^2) for population differentiation. Pairwise and simultaneous (simul) analyses (Raymond and Roussett 1995) of all populations of *G. ashmeadi* from the U. S., analysis of field collected (fc) populations is included separately. df, degrees of freedom.

Pop.	X^2
df = 82	
CA vs. WTX-1	320.5***
CA vs. WTX-2	300.4***
CA vs. SATX	287.3***
CA vs. LA	323.0***
CA vs QFL	202.8***
WTX-1 vs. WTX-2	277.1***
WTX-1 vs. SATX	285.2***
WTX-1 vs. LA	277.5***
WTX-1 vs. QFL	273.6***
WTX-2 vs. SATX	211.9***
WTX-2 vs. LA	264.6***
WTX-2 vs. QFL	156.9***
SATX vs. LA	272.9***
SATX vs. QFL	161.4***
LA vs. QFL	188.5***
Simul.	676.2***
fc (df = 68)	485.2***

*** $P = 0.000$

Table 5. Nei's analysis of gene diversity in populations of *G. ashmeadi* from the U. S. Ht, total genetic diversity (SD); Hs, average genetic diversity within populations (SD); fc, field collected; G_{ST} (mean), coefficient of gene differentiation (Nei 1987); θ (mean), theta (SD) is analogous to F_{ST} (Weir and Cockerham 1984; Weir 1990, 1996) and Nm, gene flow (Slatkin and Barton 1989).

Ht	Hs	G_{ST}	θ	Nm
fc				
0.2312	0.1442	0.3761	0.4957	0.8295
(0.032)	(0.016)		(0.077)	
All				
0.2087	0.1161	0.4438	0.4927	0.6267
(0.034)	(0.013)		(0.057)	

These values indicate restricted gene flow among the populations. Overall, genetic differentiation measurements (G^2 tests, exact tests, G_{ST}, θ, and Nm) indicate genetic divergence among *G. ashmeadi* populations from the U.S.

Genetic relatedness among G. ashmeadi *populations from the U.S.*

Since the levels of genetic divergence among populations can also be summarized by calculating pairwise estimates for genetic distance, we used the procedures of Nei (1978) and Reynolds et al. (1983) (Table 6). Average genetic divergence (D) among both field [Nei = 0.1702 (0.1021–0.2230); Reynolds = 0.6208 (0.4069–0.8138)] and all populations [Nei = 0.1304 (0.0715–0.2024); Reynolds = 0.6512 (0.3705–0.8890)] was high. We compared the level of genetic divergence between the field populations and the WTX-1 and LA reared populations and found mean D values of 0.1806 (Nei) and 0.8589 (Reynolds) and 0.1065 (Nei) and 0.5371 (Reynolds), respectively. These results indicate that WTX-1 is more diverged than LA. A comparison of Nei's genetic distance within the Texas populations, WTX-2 vs. WTX-1 (0.1391) and WTX-2 vs. SATX (0.1286), showed that divergence is slightly higher between the Weslaco populations. Sympatric species tend to have higher levels of genetic differentiation; more work is needed to confirm this possibility. The divergence between ARG (near *G. ashmeadi*) and all U.S. *G. ashmeadi* populations was very high, 0.3633 (Nei) and 1.6093 (Reynolds), respectively. These results support the taxonomic data that ARG (near *G. ashmeadi*) is another species. Dendrograms based on Nei's genetic distance are shown on Fig. 1 with all populations including ARG (Fig. 1A) and the field populations analyzed separately (Fig. 1B). At least two main clusters are identified on the dendrogram with ARG clustered as an outlier (Fig. 1A). Within a second cluster or all *G. ashmeadi* from the U.S., WTX-1 appears to be the most differentiated (Fig. 1A). The CA population appears to form a second subcluster and the two southeastern populations, LA and QFL form a single cluster showing their genetic similarity. The WTX-1 and -2 populations are distributed in different clusters. Also within Texas (Fig. 1B), WTX-2 and SATX show divergence as they appear on a separate cluster. It is interesting to note that this same pattern of differentiation is seen with *H. coagulata* within Texas (de León et al. 2004).

In general, genetic variation of Hymenoptera as measured by isozyme electrophoresis is approximately one-third of that found in diploid insects (Berkelhamer 1983; Graur 1985; Unruh et al. 1986; Powell and Walton 1989; Owen 1993; Unruh and Woolley 1999), though there have been limited reports of high genetic diversity (Sheppard and Heydon 1986; Boato and Battisti 1996). Berkelhamer (1983) demonstrated that Hymenoptera were associated with low gene diversity values 0.037 (3.7%) vs. 51 diploid insects 0.135 (13.5%). On the other hand, Sheppard and Heydon (1986) demonstrated mean expected gene diversity values of 0.121 to 0.144 (12.1 to 14.4%) in sawflies and Boato and Battisti (1996) demonstrated a mean expected gene diversity value of 0.197 (19.7%) in *Cephalcia* populations. Though a direct comparison of mean expected gene diversity values can not be made between enzyme electrophoreses studies and PCR methods incorporating SSR, in the present study with *G. ashmeadi* we observed individual mean expected gene diversity values ranging from 2.9 to 14.3%, but a total genetic diversity value (Ht) of 23.1% for the field populations. This Ht value falls within the range described by Boato and Battisti (1996) for *Cephalcia* populations. PCR-based techniques, such as,

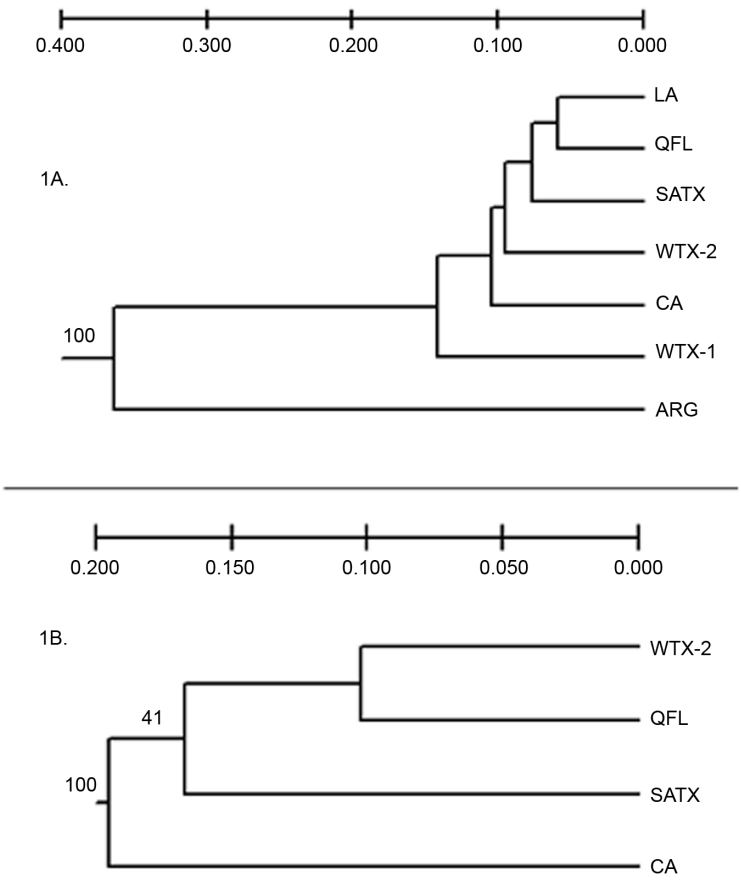

Figure 1. Dendrograms based on Nei's genetic distance (1987) by the method of UPGMA (Sneath and Sokal 1973). Relationships (A) showing the six U.S. geographic populations of *Gonatocerus ashmeadi* and a population classified as near *G. ashmeadi* (M2012) from Argentina performed by ISSR–PCR DNA fingerprinting. Field collected populations were also analyzed separately (B). Genetic distances are indicated above the dendrograms and bootstrap support values are indicated at the nodes.

Table 6. Nei's unbiased (1987) genetic distance (below diagonal) and Reynold *et al*. (1983) genetic distance (above diagonal). Six populations of *G. ashmeadi* from the U.S. Field populations were also analyzed separately.

Pop.	CA	WTX-1	WTX-2	SATX	LA	QFL
CA	*****	0.8682	0.6818	0.6441	0.6275	0.4227
WTX-1	0.2024	*****	0.8080	0.8703	0.6871	0.8890
WTX-2	0.1341	0.1391	*****	0.7213	0.6663	0.5322
SATX	0.1384	0.1789	0.1286	*****	0.4842	0.4956
LA	0.1422	0.1335	0.1233	0.0890	*****	0.3705
QFL	0.0896	0.2020	0.0890	0.0951	0.0715	*****

Pop.	CA	WTX-2	SATX	QFL
CA	*****	0.8138	0.8075	0.4559
WTX-2	0.2215	*****	0.7741	0.4069
SATX	0.2330	0.2015	*****	0.4666
QFL	0.1308	0.1021	0.1328	*****

RAPDs have been described to have generated many polymorphisms in four parasitic hymenoptera (Narang et al. 1993a) and in 40 *Trichogramma* isofemale lines that revealed several RAPD polymporphic markers (Vanlerberghe-Masutti 1994); but these studies did not report expected mean gene diversity values and therefore, direct comparisons are difficult to make. In a social wasp, *Polistes annularis*, the SSR technique identified polymorphic microsatellite loci that produced a mean observed heterozygous value of 0.62 (Hughes and Queller 1993); the SSR and ISSR–PCR methods are different techniques though are similar in that they both target SSR.

The ISSR primers that were utilized in this study contained CA-repeat motifs in their sequence (see Materials and Methods). Recently, in a genetic study of *H. coagulata*, we demonstrated high numbers of polymorphic markers with various ISSR primers containing CA-repeat motifs in their sequences (de León and Jones 2004). Based on a survey of SSR or microsatellites in different genomes by Tóth et al. (2000) and earlier studies (Hamada et al. 1982; Tautz and Renz 1984), the most frequent repeat motif in Arthropoda (mainly *Drosophila*) in all regions of the genome, which include intergenic regions, introns, and exons is the dinucleotide repeat AC. This dinucleotide repeat motif is found in greater frequency in intergenic regions and introns than in exons. The number of polymorphic markers produced in the present study therefore confirmed the utility of using ISSR primers containing these repeat motifs. The ISSR–PCR method has been utilized successfully 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). pp-ISSR–PCR has been used successfully to fingerprint potato cultivars (Prevost and Wilkinson 1999) and for genetic linkage analysis in the seasonal flowering locus in *Fragaria* (Cekic et al. 2001).

To our knowledge, the present report represents the first study of the population genetics of the Hymenopteran *G. ashmeadi* performed with the ISSR–PCR DNA fingerprinting method, including adding a second ISSR primer to the reaction (pp-ISSR–PCR). The ISSR–PCR procedure is a sensitive method and requires no prior knowledge of DNA sequence information (Zietkiewicz et al. 1994; reviewed in Karp and Edwards 1997). Amplification of *G. ashmeadi* template with the ISSR–PCR procedure (Table 2) produced a high quantity of polymorphic markers. Within the six U.S. populations of *G. ashmeadi*, 41 polymorphic markers were generated with the three pooled reactions. The high number of polymorphic markers produced is a good indication that a significant portion of the genome was targeted.

In summary, the major observations of this study were that 1) among *G. ashmeadi* populations, based on genetic differentiation measurements (exact test, G_{ST}, θ), pronounced genetic structure was identified, 2) the mean expected gene diversity value for LA (F5) did not differ from field populations, whereas WTX-1 was observed with a significantly lower mean expected gene diversity value as compared to field populations (except WTX-2), and 3) QFL generated the most polymorphic markers (20) with only 13 individuals, even though they were all siblings or from one egg mass. This is an interesting result, since it may be assumed that siblings are not associated with high variability or have isofemale line characteristics. These results indicate that *G. ashmeadi* parasitoid siblings somehow manage to maintain their genetic diversity. Further studies are required to confirm this observation in this species and other *Gonatocerus* species. Variation within 10 male individuals (*Anaphes* sp. nov.) was demonstrated with RAPD markers by Landry et al. (1993), but they were not from the same egg mass; 4) based on genetic distance or average divergence, WTX-1 appeared to be the most differentiated population. Within Texas, field populations WTX-2 and SATX appeared on separate clusters, indicating that these populations are differentiated even though they are within the same state; and 5) The ARG population (near *G. ashmeadi*, M02012) is confirmed to be a different species. More research is required to confirm these results, sequencing of standard genes (e. g., mitochrondrial cytochrome oxidase [COI and II]) and ITS fragments are in progress.

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