Population Genetic Structure of *Homalodisca coagulata* (Homoptera: Cicadellidae), the Vector of the Bacterium *Xylella fastidiosa* Causing Pierce's Disease in Grapevines

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ABSTRACT In the current study, inter-simple sequence repeat (ISSR) primers (p-13 and p-15) were used to estimate the population genetic structure of the sharpshooter *Homalodisca coagulata* (Say) (Homopera: Cicadellidae). Eighteen populations from throughout the United States and a population from Tahiti, French Polynesia, were analyzed. Populations were arbitrarily assigned to three regions: southeastern, southwestern, and western. Exact tests for population differentiation indicated highly significant differences in marker frequencies among the 18 populations with both primers. Analyses of molecular variance also indicated significant geographic structuring with both primers. A dendrogram based on Reynolds coancestry distance performed with p-15 clustered the U.S. populations into two main groups. The southeastern populations were grouped into one cluster and the southwestern and western populations into a second cluster. Within the western region, dendrograms produced with p-13 and p-15 showed in both cases that two populations (Edison and Bakersfield) clustered as outliers. The average divergence (D) among all populations was 0.099. Divergence values of 0.254, 0.103, and 0.102 were observed when comparing Bakersfield and the southeastern, southwestern, and western populations, respectively. Within the western region, D values for Bakersfield were 1.8- (p-13) and 2.4-fold (p-15) higher than the D of the western populations. The present results suggest that a subset of insects in California may have their origins in the southwestern region (Texas); furthermore, these results are suggestive of more than one founding event in California and/or biotypes or geographic races.

KEY WORDS inter-simple sequence repeat-polymerase chain reaction, DNA fingerprinting, *Homalodisca coagulata*, geographic variation

Homalodisca coagulata (Say) (Homoptera: Cicadellidae) is a large xylem-feeding leafhopper that is a serious pest because it vectors a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's disease in grapevines, *Vitis vinifera* L. and *Vitis labrusca* L. (Hopkins and Mollenhauer 1973). *H. coagulata* is native to the southern United States, ranging from Florida to Texas and northern Mexico (Young 1958, Turner and Pollard 1959, Nielson 1968, Brlansky et al. 1983). However, within the last 10 yr, *H. coagulata* has established in southern California where they pose a serious threat to the wine and table grape industry (Sorensen and Gill 1996).

In classical biological control of insect pests, one approach is to import and establish natural enemies that will control pest populations (Hall and Ehler 1979, Greathead and Greathead 1992). Genetic characterization is critical to ensure that there is a single species and not a species complex involved in a biological control release program. Lack of proper identification procedures has affected the early stages of several projects (Messing and Aliniazee 1988, Löhr et al. 1990). But it is also critical that the insect pest itself be genetically characterized because populations of the same species may differ in relevant biological characteristics of importance to biological control (Narang et al. 1993). Studies of allele or marker frequencies in naturally occurring insect 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). Populations with some degree of genetic differentiation (differences in allele frequencies) may be considered as races (Narang et al. 1993).

There is a need for molecular markers for insect pests to provide new characters for studies of phylogenetic relatedness, for identification of cryptic species and biotypes and for assessment of heritable variation for population genetics and ecological in-

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Table 1. *H. coagulata* collection sites in United States

City, state	County	Location (°N, °W)	Host(s)	Collector(s)
Southeastern				
Charleston, SC (24)	Charleston	32°46′, 79°55′	Crepe myrtle, okra	Rolando Lopez
Tifton, GA (30)	Tift	31°27′, 83°30′	Pearl millet	Robert E. Lynch
Cairo, GA (30)	Grady	30°52′, 84°12′	Holly, *Ilex burfordii*	Russell F. Mizell III
Tallahassee, FL (30)	Leon	30°26′, 84°16′	Grape herbmont	Jesusa C. Lepaspi, I. Baez
Semmes, AL (25)	Mobile	30°46′, 88°15′	Hollies, *Ilex* spp.	David W. Boyd, Jr.
Onward, MS (30)	Issaquena	32°47′, 91°09′	Cottonwood trees	Nathan Schiff
Southwestern				
Temple, TX (30)	Bell	31°05′, 97°20′	*Populus fremontii, Lagerstroemia* sp.	Jack DeLoach, James L. Tracy, T. O. Robbins
Monte Alto, TX (30)	Hidalgo	26°22′, 97°58′	Grape vines	William Warfield, Jesse H. de León
Weslaco, TX (30)	Hidalgo	26°09′, 97°59′	Crepe myrtle	William Warfield, Jesse H. de León, Marissa González
San Antonio, TX (30)	Bexar	29°25′, 98°29′	Crepe myrtle	Walker A. Jones
Western				
Pauma, CA (30)	San Diego	33°19′, 116°57′	Citrus	David J. W. Morgan, Lila Higgins
San Marcos, CA (30)	San Diego	33°08′, 117°09′	Citrus, acacia, brachychitin	David J. W. Morgan, Lila Higgins
Redlands, CA (30)	San Bernardino	34°03′, 117°10′	Citrus	David J. W. Morgan, Lila Higgins
Riverside, CA (30)	Riverside	33°57′, 117°23′	Mixed citrus	David J. W. Morgan
Pomona, CA (30)	Los Angeles	34°05′, 117°44′	Citrus	David J. W. Morgan, Lila Higgins
Edison, CA (30)	Kern	35°20′, 118°52′	Orange	Gregory S. Simmons, S. Rill, A. Long
Fillmore, CA (30)	Ventura	34°23′, 118°55′	Citrus	Jason Welch, Jaime Cuevas, C. Colazzo
Bakersfield, CA (30)	Kern	35°22′, 119°01′	Citrus	Gregory S. Simmons, Isabelle Lauziere

The number of individuals per population is in parentheses.

vestigations (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). We screened and compared four DNA fingerprinting procedures and determined that procedures incorporating simple sequence repeats (SSR) or microsatellites were the most sensitive. Specifically, inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) (Zietkiewicz et al. 1994) was shown to be the most sensitive and efficient procedure with *H. coagulata* template. This DNA fingerprinting procedure permits detection of DNA variation in SSR without the need to isolate and sequence specific DNA fragments. Many classes of microsatellite repeat motifs have been identified, although 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 previous studies (Hamada et al. 1982, Tautz and Renz 1984, Tóth et al. 2000). Therefore, because of their high density, oligonucleotides complementary to these CA-repeat motifs can be used as single primers to target a significant portion of the genome and reveal highly polymorphic banding patterns (Zietkiewicz et al. 1994).

For understanding the biology of invading insect species and designing control strategies, a genetic approach to geographic or population structure is necessary (Carey 1991, reviewed in Roderick 1996 and Unruh and Woolley 1999). To this end, the objectives of the current study with *H. coagulata* were to 1) estimate genetic diversity, 2) estimate the population genetic structure, 3) ascertain whether our method was sensitive enough to determine the origin of *H. coagulata* present in California, and 4) investigate the possibility that *H. coagulata* is a species complex or a geographic race. The PCR-based DNA fingerprinting method ISSR-PCR was used with two separate compound ISSR primers that were previously shown to generate highly polymorphic markers in *H. coagulata* (de León and Jones 2004). We demonstrated and compared the ability of these two compound ISSR primers to generate neutral polymorphic markers and to estimate geographic variation in 18 natural populations of *H. coagulata* from the United States and a population from Tahiti, French Polynesia. We arbitrarily assigned the 18 populations of *H. coagulata* into three regions: southeastern, southwestern (Texas), and western (California). Different approaches to estimate population differentiation or genetic structure were applied and compared: exact tests (Raymond and Rousset 1995), Φ_{ST} (Excoffier at al. 1992), G_{ST} (Nei 1987), θ (Weir 1990, 1996), and dendrograms based on genetic distance (unweighted pair-group method with arithmetic average method) (Sneath and Sokal 1973). The presence of geographic races or biotypes within geographic populations in California may indicate that different biological control approaches may be required for *H. coagulata.*

Materials and Methods

Insect Collection. Adult *H. coagulata* were collected from various regions of the United States by the individuals listed on Table 1. Live insects were frozen overnight and then transferred to 95% nondenatured ethanol. Specimens were then shipped to Weslaco in ethanol. Specimens (15) from Tahiti, French Polynesia, were collected by Rudolph Putoa from the Min-

istry of Agriculture and Livestock. Adult *Oncometopia nigricans* (Walker) (23 individuals) (outgroup population) were collected in Raleigh, NC, by John Meyer from North Carolina State University.

Genomic DNA Isolation. High molecular weight genomic DNA was extracted according to standard methods (Sambrook and Russell 2001). Individual whole sharpshooters were homogenized on ice in 5 ml polystyrene round-bottom tubes in 2 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.1% SDS) with one 20-s burst with a tissue homogenizer (Tissue Tearor, Biospec Products, Inc., Bartleville, OK). The final DNA pellet was resuspended in 100 μl of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 7.5). The integrity of the DNA was determined by gel electrophoresis in 1% agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA) in the presence of 0.2 μg/ml ethidium bromide. The concentration of the DNA was estimated with the DNA Mass Ladder (Invitrogen, Carlsbad, CA).

ISSR-PCR. ISSR-PCR reactions were performed as described previously (de León and Jones 2004) in a final volume of 20 μl with the following components: 1× PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 0.01% gelatin), 0.25 mM deoxynucleotide triphosphates, 0.25 μM ISSR primer [p-13, $A(CA)_7(TA)_2T$ and p-15, $T(GT)_7(AT)_2$ reported in Witsenboer et al. (1997) and de León and Jones (2004)], 0.5–1.0 ng of high-molecular-weight genomic DNA, and 0.05 U/μl *Taq*DNA polymerase (New England Biolabs, Beverly, MA). The cycling parameters were as follows: one cycle at 94°C for 2 min followed by 45 cycles at 94°C for 1 min, 54°C (p-15) or 52°C (p-13) for 1 min, and 72°C for 2 min. Reactions were optimized for amount of genomic DNA, $MgCl_2$ 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 1× TBE buffer (90 mM Tris-borate, 2 mM EDTA) in the presence of ethidium bromide. Photographs of the gels were taken with the Chemi Doc System, and markers/bands were scored with the Quantity One Software (Bio-Rad, Hercules, CA).

Data Analysis. Bands observed in each lane were compared with all the other lanes of the same gel and only the most reproducible bands were scored as presence (1) or absence (0). ISSR-PCR markers are treated as dominant markers (reviewed in Wolfe and Liston 1998). Data were analyzed independently for each ISSR primer. Fragment sizes were estimated based on the 1.0-kb Plus DNA Ladder (Invitrogen) according to the algorithm provided in the Quantity One software. Genetic variation was analyzed with 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), gene flow (Slatkin and Barton 1989), the Ewens–Watterson test for neutrality (1000 simulations) (Manly 1985) described by Ewens (1972) and Watterson (1978), and the χ^2 contingency test. Dendrograms based on Reynolds et al. (1983) coancestry distance and the unweighed pair-group method with arithmetic averages (UPGMA) of Sneath and Sokal (1973) were constructed with the program Tools for Population Genetic Analyses (TPFGA, version 1.3; Miller 1997). Marker frequencies were estimated based on the Taylor expansion estimate of Lynch and Milligan (1994). Bootstrapping over loci also was performed with TPFGA with 1000 permutations by the algorithm of Felsenstein (1985). Exact tests (χ^2) for population differentiation (simultaneous analysis of all populations) were calculated with the TPFGA program by the method of Raymond and Rousset (1995), where a Markov Chain Monte Carlo approach was used that gives an excellent approximation of the exact probability of the observed differences in marker frequencies. The exact tests, which included Taylor correction factor (Lynch and Milligan 1994) were performed with 1000 dememorization steps, 20 batches, and 2,000 permutations per batch. Fisher combined probability tests (Fisher 1954, Manly 1991, Sokal and Rohlf 1995) were used as a global test over loci to determine the overall significance. *F*-statistics (Weir and Cockerham 1984) were calculated with TPFGA 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 5,000 replications included Taylor's expansion estimate (Lynch and Milligan (1994). The pseudorandom numbers used in bootstrapping were produced by the combined MLCG algorithm (L'ecuyer 1988). Analysis of molecular variance (AMOVA) was performed with 999 permutations (pairwise Φ_{ST} comparisons) with the program Genetic Analysis in Excel (GenAlEx V5) (Peakall and Smouse 2001) following the procedures of Excoffier et al. (1992), Huff et al. (1993), Peakall et al. (1995), and Michalakis and Excoffier (1996). Φ_{ST} (Phi) is the analog of the conventional F_{ST}.

Results

ISSR-PCR Marker Heterozygosity. A total of 69 and 101 polymorphic markers were generated in the 19 populations of *H. coagulata* (544 individuals) with ISSR compound primers p-15 and p-13, respectively. Seven and 10 or 10% of the markers identified were not neutral, leaving 62 and 91 neutral polymorphic markers with p-15 and p-13, respectively (Table 2). Results of chi-square (χ^2) contingency tests indicated significant heterogeneity of marker frequency across populations for 14 of 62 markers (22.6%) with p-15 and 36 of 91 markers (39.6%) with p-13. Three, 12, and eight region-specific markers were identified in the southeastern, southwestern, and western populations, respectively, with p-15, whereas, four region-specific markers were identified per region with p-13.

Genetic Diversity. Within populations of *H. coagulata,* relatively low values of gene diversity (h) were

Table 2. Nei's analyses of gene diversity in subdivided populations of *H. coagulata* from the United States analyzed by region

Region	No. insects	No. P	Polym. ratio	%P	h (SD)	Ht (SD)	Hs (SD)	χ^2 (df)
p-15								
Southeast	169	34	0.20	100	0.0562 (0.095)	0.0560 (0.009)	0.0536 (0.008)	201.90*** (72)
Southwest	120	54	0.45	100	0.0432 (0.047)	0.0432 (0.002)	0.0414 (0.002)	178.83*** (112)
West	240	46	0.19	100	0.0464 (0.049)	0.0464 (0.002)	0.0441 (0.002)	311.76*** (102)
All	529	62	0.12	100	0.0369 (0.048)	0.0369 (0.002)	0.0328 (0.001)	664.39*** (138)
p-13								
Southeast	169	67	0.40	100	0.0728 (0.092)	0.0724 (0.009)	0.0679 (0.007)	415.92*** (126)
Southwest	120	84	0.70	100	0.0722 (0.058)	0.0722 (0.003)	0.0688 (0.003)	403.77*** (178)
West	240	83	0.35	100	0.0615 (0.065)	0.0606 (0.004)	0.0555 (0.003)	640.83*** (174)
All	529	91	0.17	100	0.0583 (0.064)	0.0581 (0.004)	0.0535 (0.003)	1,279.0*** (202)

No. P, number of polymorphic loci; Polym. ratio, polymorphic ratio (number of polymorphic loci per number of insects); %P, percentage of polymorphic loci; h, average gene diversity; Ht, total genetic diversity; Hs, average genetic diversity within populations; and Extract tests (χ^2) (results over loci) (TPFGA) for population differentiation (Raymond and Rousset 1995).
*** $P = 0.0000$ (overall P value).

observed ranging from 0.0202 to 0.0669 with p-15 and 0.0258–0.0725 with p-13 (Table 3). Weslaco had the highest value (6.69%) with p-15 and Monte Alto the highest (7.25%) with p-13. The greatest number of neutral polymorphic markers was seen in individual southwestern populations, with Weslaco presenting the highest number (48) with p-15 and Monte Alto the highest (69) with p-13. Percentage neutral polymorphic markers ranged from 16.1 to 77.4% with p-15, with Weslaco containing the highest value and Semmes the lowest. The percentage of polymorphic markers with p-13 ranged from 11.0 (Tahiti) to 75.8% (Monte Alto). Individually, Monte Alto and Weslaco were the populations with the greatest genetic diversity. In contrast to the gene diversity values seen in single populations, when genetic diversity (h) was analyzed by region, the southeastern region contained the highest values (5.62%, p-15 and 7.28%, p-13), even though in general they were relatively low (Table 3). Higher values for total genetic diversity (Ht) also were seen in the southeastern region. The highest polymorphic ratios (number of polymorphic markers per number of insects) (0.47, p-15 and 0.70, p-13) were seen in the southwestern region with both primers and the lowest (0.19, p-15 and 0.35, p-13) were seen in the western region.

ISSR-PCR Differentiation among Populations. Exact tests for population differentiation (Table 2) indicated that highly significant differences in marker frequencies existed among the 18 populations with both primers, p-15 and p-13. In addition, exact tests showed significant marker frequency differences within regions, with the western region showing the highest values with both primers. Tables 4 and 5 present the results from the different approaches used to apportion variation into within- and among-population levels. AMOVA analyses (Table 4) showed a statistically significant partitioning of gene diversity

Table 3. Single-populations descriptive statistics

Population	No. P	%P	h (SD)	Population	No. P	%P	h (SD)
p-15				p-13			
Southeast				Southeast			
Charleston, SC	14	22.6	0.0304 (0.081)	Charleston, SC	18	19.8	0.0379 (0.105)
Tifton, GA	12	19.4	0.0361 (0.100)	Tifton, GA	33	36.3	0.0447 (0.091)
Cairo, GA	19	30.7	0.0387 (0.081)	Cairo, GA	39	42.7	0.0570 (0.099)
Tallahassee, FL	10	16.1	0.0215 (0.072)	Tallahassee, FL	32	35.2	0.0474 (0.097)
Semmes, AL	10	16.1	0.0242 (0.081)	Semmes, AL	37	40.7	0.0519 (0.090)
Onward, MS	18	29.0	0.0253 (0.070)	Onward, MS	46	50.6	0.0612 (0.098)
Southwest				Southwest			
Temple, TX	18	29.0	0.0317 (0.071)	Temple, TX	51	56.0	0.0699 (0.095)
San Antonio, TX	17	27.4	0.0202 (0.046)	San Antonio, TX	49	53.9	0.0540 (0.088)
Monte Alto, TX	29	46.8	0.0253 (0.034)	Monte Alto, TX	69	75.8	0.0725 (0.071)
Weslaco, TX	48	77.4	0.0669 (0.081)	Weslaco, TX	53	58.2	0.0597 (0.071)
West				West			
San Marcos, CA	23	37.1	0.0307 (0.061)	San Marcos, CA	50	54.4	0.0495 (0.077)
Pauma, CA	21	33.9	0.0345 (0.060)	Pauma, CA	43	47.3	0.0519 (0.091)
Redlands, CA	19	30.7	0.0249 (0.048)	Redlands, CA	51	56.0	0.0513 (0.076)
Pomona, CA	21	33.9	0.0318 (0.061)	Pomona, CA	50	55.0	0.0578 (0.087)
Riverside, CA	30	48.4	0.0446 (0.069)	Riverside, CA	34	37.4	0.0389 (0.076)
Fillmore, CA	30	48.4	0.0395 (0.056)	Fillmore, CA	45	49.5	0.0460 (0.077)
Edison, CA	26	41.9	0.0367 (0.066)	Edison, CA	41	45.1	0.0534 (0.091)
Bakersfield, CA	14	22.6	0.0272 (0.083)	Bakersfield, CA	38	41.8	0.0572 (0.096)
Tahiti, FP	11	17.7	0.0206 (0.051)	Tahiti, FP	10	11.0	0.0258 (0.088)

Summary of genetic variation statistics for all loci. ISSR primers used are p-15 and p-13. No. P, no. of polymorphic loci; %P, percentage of polymorphic loci; and h (SD), gene diversity (standard deviation).

Table 4. AMOVA for *H. coagulata* populations from the United States

Source	df	SS	MS	Est. var.	%D
p-15					
Among regions	2	93.43	46.71	0.244	11
Among populations/regions	15	80.24	5.35	0.122	6
Within populations	511	904.0	1.77	1.769	83
p-13					
Among regions	2	75.28	37.64	0.128	3
Among populations/regions	15	239.7	15.98	0.403	9
Within populations	511	2124.3	4.16	4.157	88

	F-statistics		
	F_{CT}	F_{SC}	F_{ST}
p-13	0.027**	0.088**	0.113**
p-15	0.114**	0.064**	0.171**

Statistics include df, degrees of freedom; SS, sum of squares; MS, mean squares; Est. var., estimated variance; and %D, distribution of total variance.

** $P = 0.001$.

among regions, 11% with p-15 and a lower value of 3% with p-13. Significant differentiation also was distributed among populations within regions, 6% with p-15 and 9% with p-13. The majority of the variance, however, was distributed within populations, 83% with p-15 and 88% with p-13. Significant population structure or differentiation was demonstrated by both primers, p-13 and p-15.

Table 5 shows a comparison of other genetic differentiation estimates, G_{ST} and θ. Excellent agreement was seen between G_{ST} and θ values for the within-population variances, 89.0 and 90.1% and 92.0 and 93.3% with p-15 and p-13, respectively. Within each region, little-to-moderate genetic differentiation was seen with G_{ST} and θ values with either primer, although the western region populations demonstrated slightly higher G_{ST} and θ values. Furthermore, the indirect estimate of gene flow, Nm based on G_{ST}, demonstrated slightly lower values with both primers. Overall, gene flow was greater among populations within regions than among regions. Together, though, these overall results indicate moderate genetic differentiation of *H. coagulata* populations, but the fact that most of the genetic variation is distributed within populations may be an indication of strong microgeographical differentiation.

Relationships among *H. coagulata* Populations. A dendrogram based on Reynolds et al. (1983) coancestry distance performed with p-15 is shown on Fig. 1A. Two main clusters were formed with the southeastern region populations (cluster B) separated from the southwestern and western region populations (cluster A). Within cluster A, clusters or subgroups c and d are formed with the Edison and Bakersfield populations forming the second separate cluster (d). Two more clusters are seen within cluster c. Western or California populations are distributed within three separate clusters within the main cluster A. Southeastern populations formed two clusters within cluster B, with Tifton and Cairo, GA, residing in one cluster. Results performed with p-13 showed a similar pattern of clustering but with some variation, in that case Weslaco and Monte Alto, TX populations were clustered within the southeastern populations, although the western and southeastern region populations were still clustered separately (data not shown). To see a clearer picture of the western region populations, analyses were performed separately from the rest of the U.S. populations with p-15 and p-13, and the results are demonstrated on Fig. 1B and C, respectively. With both primers, two main clusters (A and B) were formed. Variation in clustering of the populations is seen within cluster A between the two primers; however, results show that in both cases, the Edison and Bakersfield populations were clustered (B) as outliers. In the western region, Edison and Bakersfield are geographically isolated from the rest of the western populations and seem to be more differentiated.

Because the levels of genetic divergence among populations also can be summarized by calculating pairwise estimates for genetic distance, we used the procedure of Reynolds et al. (1983). The pairwise genetic distance values performed with p-15 are shown on Table 6 for all populations. The average divergence (D) among all *H. coagulata* populations (19) was moderate, D = 0.099. This value stayed the same when excluding the population from Tahiti, French Polynesia. Overall, the Bakersfield population was the most diverged. A comparison of Bakersfield and the average divergence of the southeastern populations was high, D = 0.254. This value was 2.6-fold higher than the overall D value of all populations. Divergence values of D = 0.103 and 0.102 were observed when comparing Bakersfield and the D of the southwestern and western populations, respectively; demonstrating the closer genetic relationship between these two regions. These results are in excellent agreement with those seen with the dendrogram on Fig. 1A. An individual comparison between Cairo and Bakersfield resulted in a high D value of 0.300. Average

Table 5. Estimates and comparison of G_{ST} (POPGENE) and θ (TFPGA) values from *H. coagulata* populations from three regions of the United States

Region	G_{ST}	θ (SD)	Nm	Region	G_{ST}	θ (SD)	Nm
p-15				p-13			
Southeast	0.0426	0.0321 (0.008)	11.24	Southeast	0.0615	0.0551 (0.008)	7.63
Southwest	0.0426	0.0376 (0.014)	11.22	Southwest	0.0465	0.0449 (0.010)	10.25
West	0.0492	0.0403 (0.016)	9.66	West	0.0843	0.0631 (0.026)	5.43
All	0.1101	0.0989 (0.048)	4.04	All	0.0799	0.0668 (0.012)	5.75

G_{ST} (mean), coefficient of gene differentiation; θ (mean), theta is analogous to F_{ST}; and Nm, gene flow.

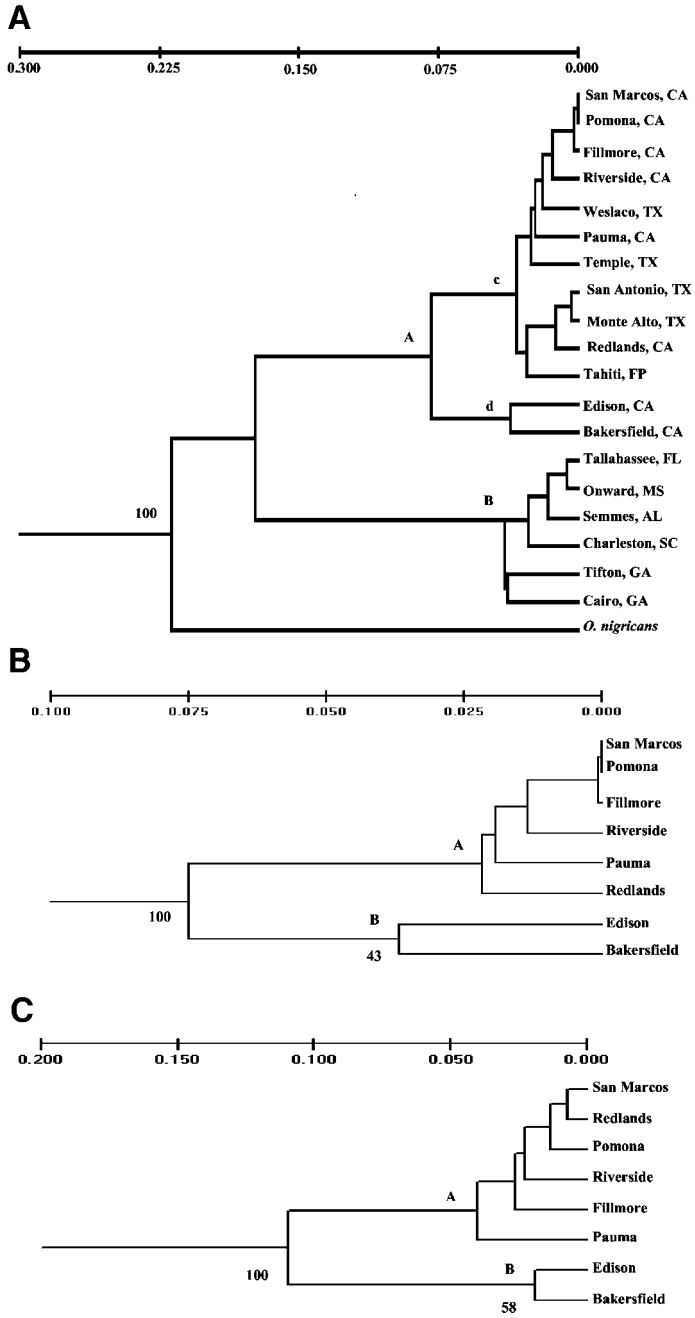

Fig. 1. Dendrograms based on Reynolds et al. (1983) coancestry distance by the method of unweighted pair-group method with arithmetic average. Marker frequencies were estimated based on Taylor expansion estimate (Lynch and Milligan (1994). Relationships (A) showing the 19 geographic populations of *H. coagulata* performed with p-15. *O. nigricans* were included as an outgroup. Western region populations were analyzed separately from the rest of the U.S. populations with p-15 (B) and p-13 (C). Genetic distances are indicated above the dendrograms and bootstrap support values (>40%) are indicated at the nodes.

divergence also was analyzed independently per region with p-13 and p-15. Results of D for within regions with p-13 were southeastern, 0.032; southwestern, 0.034; and western, 0.042; and for p-15 were southeastern, 0.059; southwestern, 0.045; and western, 0.062. The western region populations were observed with slightly higher D values with both ISSR primers. The D values, 0.027 and 0.015, in the western region without Edison and Bakersfield were lowered by 2.27- and 2.79-fold for p-13 and p-15, respectively. Within the

Table 6. Pairwise genetic distance matrix

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	******																			
2	0.0277	******																		
3	0.0269	0.0366	******																	
4	0.0175	0.0345	0.0407	******																
5	0.0354	0.0402	0.0418	0.0105	******															
6	0.0260	0.0582	0.0384	0.0058	0.0207	******														
7	0.1853	0.1762	0.2387	0.1521	0.1529	0.1664	******													
8	0.1959	0.1860	0.2539	0.1572	0.1679	0.1685	0.0333	******												
9	0.1658	0.1678	0.2258	0.1306	0.1440	0.1375	0.0354	0.0037	******											
10	0.1251	0.1184	0.1654	0.1205	0.1213	0.1241	0.0342	0.0510	0.0480	******										
11	0.1744	0.1556	0.2263	0.1457	0.1493	0.1595	0.0166	0.0149	0.0297	0.0157	******									
12	0.1567	0.1478	0.2018	0.1329	0.1126	0.1422	0.0302	0.0265	0.0272	0.0362	0.0178	******								
13	0.1852	0.1799	0.2420	0.1469	0.1589	0.1573	0.0360	0.0085	0.0153	0.0506	0.0232	0.0293	******							
14	0.1723	0.1618	0.2233	0.1431	0.1492	0.1566	0.0163	0.0169	0.0265	0.0224	−0.0039	0.0176	0.0169	******						
15	0.1440	0.1196	0.1823	0.1208	0.1228	0.1283	0.0303	0.0290	0.0296	0.0150	0.0133	0.0265	0.0291	0.0178	******					
16	0.1493	0.1424	0.1973	0.1220	0.1226	0.1324	0.0210	0.0113	0.0182	0.0231	0.0024	0.0162	0.0124	0.0012	0.0098	******				
17	0.1777	0.1850	0.2233	0.1468	0.1553	0.1562	0.0707	0.0570	0.0367	0.0565	0.0567	0.0582	0.0476	0.0459	0.0418	0.0305	******			
18	0.2582	0.2517	0.3007	0.2226	0.2477	0.2432	0.1059	0.1146	0.0967	0.0935	0.1145	0.1211	0.1008	0.0950	0.0945	0.0846	0.0353	******		
19	0.1851	0.1750	0.2208	0.1580	0.1682	0.1642	0.0723	0.0389	0.0170	0.0564	0.0501	0.0309	0.0237	0.0434	0.0450	0.0271	0.0524	0.1439	******	
20	0.2967	0.2851	0.3279	0.2885	0.2910	0.2816	0.1949	0.2008	0.1678	0.1412	0.1827	0.1655	0.1812	0.1749	0.1546	0.1491	0.1550	0.2359	0.1839	******

The procedure by Reynolds et al. (1983) was used to construct the pairwise genetic matrix, and marker frequencies were estimated based on Taylor expansion estimate (Lynch and Milligan 1994). ISSR-PCR DNA fingerprinting was performed with primer p-15 with 18 populations of *H. coagulata* from the United States and one from Tahiti, French Polynesia. 1, Charleston, SC; 2, Tifton, GA; 3, Cairo, GA; 4, Tallahassee, FL; 5, Semmes, AL; 6, Onward, MS; 7, Temple, TX; 8, San Antonio, TX; 9, Monte Alto, TX; 10, Weslaco, TX; 11, San Marcos, CA; 12, Pauma, CA; 13, Redlands, CA; 14, Pomona, CA; 15, Riverside, CA; 16, Fillmore, CA; 17, Edison, CA; 18, Bakersfield, CA; 19, Tahiti, French Polynesia; and 20, *O. nigricans*.

western region (performed with p-13), a comparison of Edison and the D of the rest of the California populations (excluding Bakersfield) gave a D value of 0.110; likewise, the same comparison with Bakersfield (excluding Edison) resulted in a D value of 0.109. Both of these values are higher (1.8-fold) than the overall D value (0.062) for the region; indicating that both Edison and Bakersfield are the most differentiated of the western populations. A similar pattern was observed when the western region populations were analyzed separately with p-15. A comparison of Bakersfield and the rest of the California populations (excluding Edison) resulted in a D value of 0.098; this is a 2.4-fold difference in divergence. The same comparison for Edison (excluding Bakersfield) resulted with a D value that was 1.26-fold higher than the D value of the western region.

Discussion

To our knowledge, the present report represents the first study of the population genetic structure of *H. coagulata* performed with the ISSR-PCR DNA fingerprinting method. 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 *H. coagulata* template with compound ISSR primers (p-13 and p-15) produced a high quantity of neutral polymorphic markers and therefore reduced the need to use multiple primers. Within the 19 populations of *H. coagulata*, 62 and 91 neutral polymorphic markers were generated by p-15 and p-13, respectively. The high number of polymorphic markers produced is a good indication that a significant portion of the genome was targeted, allowing us to analyze and compare each compound ISSR primer separately.

In general, good agreement was obtained with both ISSR primers as far as their abilities to produce polymorphic markers and estimate a genetic structure for *H. coagulata.* Low values of genetic diversity (h) in single populations were seen regardless of primer used (Table 2), so the genetic differences seen among populations were due, in part, to marker frequency differences. Support for this is seen with the various genetic analyses that were performed. Exact tests, which estimate whether significant differences in marker/allele frequencies exist among populations (Raymond and Rousset 1995), showed significant values with both primers (Table 3). These tests also demonstrated significant population differentiation within regions, with the western region populations containing the highest χ^2 values. These higher values are probably due to the fact that both Bakersfield and Edison demonstrate higher average divergence than the rest of the western regions populations. Results obtained with AMOVA analyses also demonstrated significant population differentiation at the three hierarchical levels (Table 4). Three to 11% of the total variation was attributable to differentiation among sampled regions and values of G_{ST} (8–11%) and θ (7–10%) were of comparable magnitudes (Table 5). The majority of the variance, though, was seen within populations. As expected for outcrossing species, high within-population variation estimated with fixation indices G_{ST}, and θ may be an indication of relatively restricted population differentiation. In addition, if multiple populations are founded independently, they should display more interpopulation variation in gene frequencies than the source population (Unruh and Woolley 1999). This is also an indication that these populations experienced gene flow in recent history or diverged a relatively short time ago.

The dendrogram based on Reynolds et al. (1983) coancestry distance was able to geographically distinguish the southeastern region populations from the rest of U.S. populations (Fig. 1A); the southwestern and western region populations, however, formed one cluster (A). The lack of total separation of these three regions in the dendrogram reflects the moderate (but significant) genetic differentiation among populations, corroborating the results obtained from AMOVA, G_{ST}, and θ. The inability of the dendrograms (p-15 and p-13; not shown) to separate the southwestern and western region populations (at least with our procedure), would indicate that *H. coagulata* populations from these regions are more similar to each other than to the populations from the southeastern region; but this also may be an indication that the origin of a subset of *H. coagulata* in California are from Texas or the southwestern region and not from the southeastern region (e.g., Florida). Support for this possibility also is demonstrated by the average divergence (D) of the populations. Comparison of Bakersfield and the southeastern populations produced a high D value of 0.254, whereas comparison of Bakersfield and the southwestern and western populations generated D values of 0.103 and 0.102, respectively, in agreement with results from the topology of the dendrogram (Fig. 1) showing a closer genetic relationship between these two regions. Within the western region, Edison and Bakersfield formed a cluster separate from the rest of the populations in California. This was demonstrated with the two different ISSR primers (Fig. 1B and C). Based on these dendrograms, the Edison and Bakersfield populations seemed to be the most differentiated. Average divergence comparisons also demonstrated similar patterns. Comparison of the D values (performed with p-13) for Bakersfield and Edison and the rest of the western region populations were both ≈1.8-fold higher than the D value of the region. Similarly, a divergence comparison performed with p-15 demonstrated a D difference of 2.4-fold between Bakersfield and the western region populations. The D results also were in excellent agreement with those obtained with the topology of the dendrograms (Fig. 1) and therefore confirm the utility of using dendrograms for these types of population genetic studies. It is possible that the genetic differentiation detected here may have come from two populations introduced at different times or rather, that more than one founding event is recognized. Another explanation for these observations is that *H. coagulata* is a species complex. The data also suggest that geographic races exist

within *H. coagulata.* Populations with some degree of genetic differentiation (differences in allele/marker frequencies) may be considered as races (Narang et al. 1993). In addition, the data suggest the presence of biotypes. In a laboratory colony, *H. coagulata* from Bakersfield showed a longer reproductive diapause period (6–7 wk) than *H. coagulata* from Riverside reared under the same conditions. The reproductive diapause of the Riverside colony was almost nonexistent. In addition, host oviposition preferences were also observed between these two reared colonies (R. A. Leopold, personal communication). Support for both of these observations are seen genetically in the dendrogram (Fig. 1A) that clusters the southeastern populations separately from the rest of the *H. coagulata* populations in the United States and in the western region dendrogram (Fig. 1B) separating the Bakersfield and Edison populations from the rest of the California populations. Examples of such events with other insects have been demonstrated by Alosi-Carter et al. (1996) with the bark beetle *Tomicus piniperda* (L) and Fong et al. (1995) with California grape phylloxera, *Daktulosphaira vitifoliae* (Fitch). Because we do not have *H. coagulata* from northern Mexico, we cannot rule out the possibility that *H. coagulata* from that region have not made their way into California. So, to confirm the origin of all *H. coagulata* that invaded California, specimens would need to be collected from different parts of northern Mexico.

In conclusion, we demonstrated the utility of the ISSR-PCR procedure by using compound ISSR primers containing CA-repeat motifs in estimating the population genetic structure of *H. coagulata.* This DNA fingerprinting procedure also has been used successfully in many other genetic studies, such as for genetic characterization of the silkworm, *Bombyx mori* (L.) (Reddy et al. 1999); for genetic variability studies of *Biomphalaria straminea* snail complexes (Caldeira et al. 2001); and in a host of plant studies (reviewed in Wolfe and Liston 1998). These novel data are important to the *H. coagulata*/Pierce's biological control program. Work is in progress incorporating other molecular methods to confirm if *H coagulata* is a cryptic species.

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