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Source: Journal of Economic Entomology, 104(1):279-287. 2011.
Published By: Entomological Society of America
DOI: 10.1603/EC10112
URL: http://www.bioone.org/doi/full/10.1603/EC10112

Genetic Structure of *Graphocephala atropunctata* (Hemiptera: Cicadellidae) Populations Across Its Natural Range in California Reveals Isolation by Distance

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J. Econ. Entomol. 104(1): 279–287 (2011); DOI: 10.1603/EC10112

ABSTRACT The genetic structure of 23 populations of *Graphocephala atropunctata* (Signoret) (Hemiptera: Cicadellidae: Cicadellinae), a vector of the plant pathogenic bacterium *Xylella fastidiosa* Wells et al., was investigated using ribosomal 28S and mitochondrial cytochrome C oxidase I gene sequences. The 28S sequences were identical across all *G. atropunctata* specimens and populations, but 16 mitochondrial haplotypes were detected and significant interpopulation differences were found in the distribution of these haplotypes. Pairwise estimates of F_{st} correlated positively with geographical distance between populations, a phenomenon known as genetic isolation by distance. Thus, despite potential for widespread movement of *G. atropunctata* through nursery and agricultural industries, isolated populations of *G. atropunctata* have remained genetically distinct, suggesting that negligible numbers of *G. atropunctata* are actually transported or population interbreeding rarely occurs. The phylogenetic relationship between *G. atropunctata* and two additional congeners, *Graphocephala cythura* Baker and *Graphocephala flavovittata* Metcalf, which have overlapping distributions with *G. atropunctata*, also was investigated. Although 28S sequences of *G. flavovittata* were strikingly similar to those of *G. atropunctata*, mitochondrial DNA (mtDNA) suggests that both species are genetically distinct from *G. atropunctata*.

KEY WORDS *Graphocephala flavovittata*, *Graphocephala cythura*, sharpshooters, cytochrome C oxidase I, 28S

The blue-green sharpshooter, *Graphocephala atropunctata* (Signoret) (Hemiptera: Cicadellidae: Cicadellinae), is a xylem-feeding leafhopper that vectors the bacterium *Xylella fastidiosa* Wells et al., the causative agent of Pierce's disease in grape (*Vitis* spp.) vines. *G. atropunctata* is native to the western United States, with a range that extends as far south as Nicaragua in Central America (McKamey 2007). *G. atropunctata* is common throughout California, and although it is found primarily on wild grape and blackberry (*Rubus* spp.), in humid riparian areas, it also feeds on a wide variety of native and exotic plants (Severin 1949). Polyphagy, wide distribution, and transmission of *X. fastidiosa* combine to make *G. atropunctata* a perennial threat to the California grape industry, especially in the Napa Valley area of northern California, where it is the primary vector of Pierce's disease (Purcell 1976).

Much of the research conducted on *G. atropunctata* regarding life history, disease transmission, and control strategies has focused on populations from northern California (Severin 1949, Purcell 1976, Feil et al. 2000) with the assumption that results apply to all populations found throughout California. However, *G. atropunctata* occupies a north–south latitudinal range of ≈1,000 km across California and has limited natural dispersal abilities (Severin 1949). Thus, opportunities for interbreeding to occur between highly isolated populations across this vast range may be restricted, resulting in limited gene flow and the development of significant differences in reproductive behavior, biology, and ecology between isolated populations.

One readily observable difference between widely disparate populations of *G. atropunctata* is their morphology, in particular their color, which varies dramatically from northern to southern California (Fig. 1). Typically, *G. atropunctata* from northern California are dark green with faint markings on the body, whereas southern California populations are bright blue with pronounced markings on the head, scutellum, and wings (Severin 1949). Whether these differences in morphology are an expression of phenotypic plasticity or are indicative of genetic differences is currently unknown. However, this latter possibility could have important implications for the direction of current research and the tactics presently used throughout California for control of this vineyard pest.

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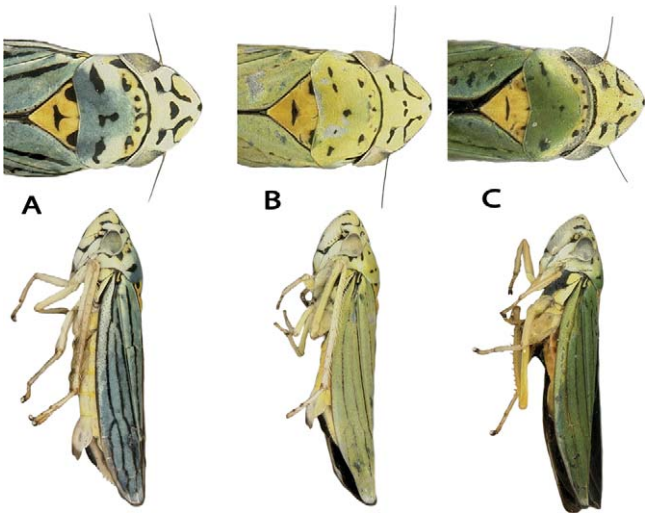

Fig. 1. Photograph of three *G. atropunctata* specimens illustrating the differences in morphology among populations in California. (A) Southern California, Laguna Beach. (B) Central California, Berkeley. (C) Northern California, Redding. (Online figure in color.)

Its large range, disjunct distribution, and occupation of isolated habitats, suggest that populations of *G. atropunctata* across California may have quantifiable population-level genetic differences. Given its status as a significant pest, such genetic differentiation may have important ramifications for the control of any given population. We examined differences among Californian populations of *G. atropunctata* along a latitudinal cline of ≈1,000 km, by using DNA sequence data from nuclear and mitochondrial genes to assess gene flow between study populations. This work had three main objectives. The first was to examine DNA sequences from different *G. atropunctata* populations for evidence of the existence of cryptic species. Second, we sought to examine molecular level differences in *G. atropunctata* populations across California to ascertain the likely level of interpopulation movement and gene flow. Third, we compared *G. atropunctata* to two other species of *Graphocephala*, *Graphocephala cythura* Baker and *Graphocephala flavovittata* Metcalf (Fig. 2), to determine molecular similarity between these three species. The results of these studies are presented here.

Materials and Methods

Specimen Collections. *G. atropunctata* were collected between July 2007 and January 2008 from locations throughout California across a latitudinal range of ≈1,000 km. Adult specimens of *G. atropunctata* were collected from 23 locations across coastal and inland southern California, the central coast, the central valley, the San Francisco Bay area, Napa and Sonoma valleys, and coastal and inland northern California (Table 1; Fig. 3). Sample locations were separated by at least 3 km. Two additional species of *Graphocephala* were collected for DNA comparison to *G. atropunctata.* Two specimens of *G. cythura* were collected in Fullerton, CA, and three specimens of *G. flavovittata* were collected in Uruapan, Michoacán, Mexico (Table 1; Fig. 2). All specimens collected for study were preserved in 95% ethanol, and kept at −20°C until used for DNA analyses. Voucher specimens of each *Graphocephala* species were deposited in the University of California–Riverside Entomology Museum (Table 1).

Fig. 2. Photographs of *G. cythura* (A) and *G. flavovittata* (B) illustrating morphology, in particular, color and body markings, for comparison with *G. atropunctata* (see Fig. 1). (Online figure in color.)

Table 1. Collection and voucher information for populations of *Graphocephala* spp. studied

Collection date (mo/d/yr)	GPS coordinates	Elevation (m)	California county[a]	Species	Map reference[b]	UCRC ENT accession no.
7/15/2007	33° 32′33″ N 117° 47′07″ W	0	Orange	*G. atropunctata*	S	223957, 223956, 223955, 015808*, 015809*
7/17/2007	33° 29′29″ N 117° 15′09″ W	306	Riverside	*G. atropunctata*	T	223954, 223953, 015819*
7/30/2007	37° 52′39″ N, 122° 14′40″ W	309	Alameda	*G. atropunctata*	J	223946, 223945, 223944, 015810*, 015811*
7/30/2007	37° 53′08″ N, 122° 15′42″	146	Alameda	*G. atropunctata*	I	223943, 223942, 223941
7/30/2007	38° 30′29″ N, 122 53′09″ W	30	Sonoma	*G. atropunctata*	H	223940, 223939, 223938, 015812*, 015813*
7/30/2007	38° 35′39N, 122° 55′0″ W	155	Sonoma	*G. atropunctata*	G	223937, 223936, 223935
7:1:2007	40° 40′60″ N, 122° 39′00″ W	767	Shasta	*G. atropunctata*	C	223930, 223929, 223928
7/31/2007	38° 53′48″ N, 123° 12′46″ W	280	Mendocino	*G. atropunctata*	F	223933
7/31/2007	39° 11′08″ N, 123° 01′35″ W	419	Lake	*G. atropunctata*	E	223952, 223951, 223950
8/1/2007	40° 42′53″ N, 122° 38′04″ W	430	Shasta	*G. atropunctata*	B	223949, 223948, 223947, 015815*
8/1/2007	40° 40′10″ N, 122° 55′11″ W	516	Trinity	*G. atropunctata*	D	223927, 223926, 223925
8/1/2007	40° 55′01″ N, 122° 23′33″ W	360	Shasta	*G. atropunctata*	A	223922, 23923, 223924, 015814*
8/11/2007	32° 52′44″ N, 116° 54′16″ W	197	San Diego	*G. atropunctata*	R	223932, 223931
8/13/2007	34° 24′52″ N, 119° 44′12″ W	42	Santa Barbara	*G. atropunctata*	O	223964, 223965, 015816*
8/15/2007	35° 12′08″ N, 120° 42′56″ W	31	San Luis Obispo	*G. atropunctata*	N	223961
8/22/2007	33° 04′54″ N, 117° 03′37″ W	123	San Diego	*G. atropunctata*	V	223934, 015818*
8/23/2007	33° 00′35″ N, 117° 14′23″ W	5	San Diego	*G. atropunctata*	W	223921, 223920, 223919
8/23/2007	33° 21′40″ N, 117° 12′17″ W	162	San Diego	*G. atropunctata*	U	223918, 223917, 015817*
8/26/2007	34° 02′15″ N, 118° 44′59″ W	40	Los Angeles	*G. atropunctata*	P	015820*
8/29/2007	33° 41′06″ N, 117° 39′41″ W	260	Orange	*G. atropunctata*	Q	223962, 223963,
9/1/2007	35° 15′10″ N, 120° 52′30″ W	54	San Luis Obispo	*G. atropunctata*	M	223958
8/31/2007	35° 28′28″ N, 120° 51′01″ W	89	San Luis Obispo	*G. atropunctata*	L	223960, 223959
10/28/2007	36° 00′36″ N 121° 31′05″ W	23	Monterey	*G. atropunctata*	K	223916, 223915, 223914
11/24/2007	33° 53′16″ N, 117° 53′03″ W	80	Orange	*G. cythura*	X	223910
1/13/2008	19° 28′18″ N 102° 26′43″ W	1,675		*G. flavovittata*		223913, 223912, 223911

An asterisk indicates that the specimen is sequenced and deposited in GenBank.
[a] *G. flavovittata* was collected in Michoacán, Mexico.
[b] See Fig. 3.

DNA Extraction. DNA was extracted from a single tibia of individual specimens using a chelex extraction method (Walsh et al. 1991). Single *G. atropunctata* were removed from alcohol and allowed briefly to air-dry. A mesothoracic tibia was dissected out, transferred to a 0.5-μl microcentrifuge tube containing 2 μl of proteinase-K, and ground up using a micropestle. Then, 100 μl of a 5% chelex-100 (Bio-Rad Laboratories, Hercules, CA) suspension (in water) was added, and the tubes were incubated at 55°C for 1 h, and then for a further 10 min at 99°C to inactivate the proteinase-K. Tubes were then spun in a microcentrifuge at 14,000 rpm for 4 min, pelleting the chelex and insect debris, and the supernatant (containing the extracted DNA) was transferred to a new 0.5-μl microcentrifuge tube and stored at −20°C.

Amplification of Extracted *G. atropunctata* DNA. Polymerase chain reaction (PCR) was used to amplify a 532-bp section of the D2 region of 28S ribosomal RNA by using the PCR primers 28sF3633 (5′-TACCGTGAGGGAAAGTTGAAA-3′) and 28sR4076 (5′-AGACTCCTTGGTCCGTGTTT-3′) (Choudhury and Werren 2006). PCR was performed in 25-μl reactions containing 2 μl of DNA template from extraction (concentration not determined), 1× Thermopol PCR buffer (New England Biolabs, Ipswich, MA), 200 μM dNTP, 0.2 μM of each primer, and 1 U of *Taq* polymerase (New England Biolabs). PCR was performed in a Mastercycler 5331 or Mastercycler ep gradient S thermocycler (Eppendorf North America Inc., New York, NY) programmed for 94°C for 2 min, followed by 38 cycles of 94°C for 30 s, 58°C for 50 s, 72°C for 90 s, and a final extension for 10 min at 72°C.

A section of the cytochrome C oxidase subunit I (COI) of mitochondrial DNA also was amplified for each specimen. Initially, the PCR primer pair LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (Folmer et al. 1994) was used. PCR was performed in 25-μl reactions containing 2–4 μl of DNA template (concentration not determined), 1× Thermopol PCR buffer, 200 μM dNTP, 0.2 μM of each primer, and 1 U of *Taq* polymerase (New England Biolabs). PCR was performed in an Eppendorf thermocycler programmed for 94°C for 2 min, three cycles of 94°C for 30 s, 45°C for 50 s, 72°C for 40 s; 35 cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 40 s; and a final extension at 72°C for 2 min.

As our study progressed, the initial COI primer set became increasingly problematic and amplifications deteriorated to the point where PCR product that once appeared as a "crisp" single band on an agarose gel instead became a general "smear" of approximately the right size. Ordering new, replacement PCR primers made no difference to the quality of our amplifications and sequencing became impossible. We believe this observed deterioration was an example of "genotyping crash," which has been reported from laboratories that amplify DNA with the same pair of primers over an extended period (e.g., Han et al. 2006). To overcome this problem, specimens that could not be successfully amplified and sequenced

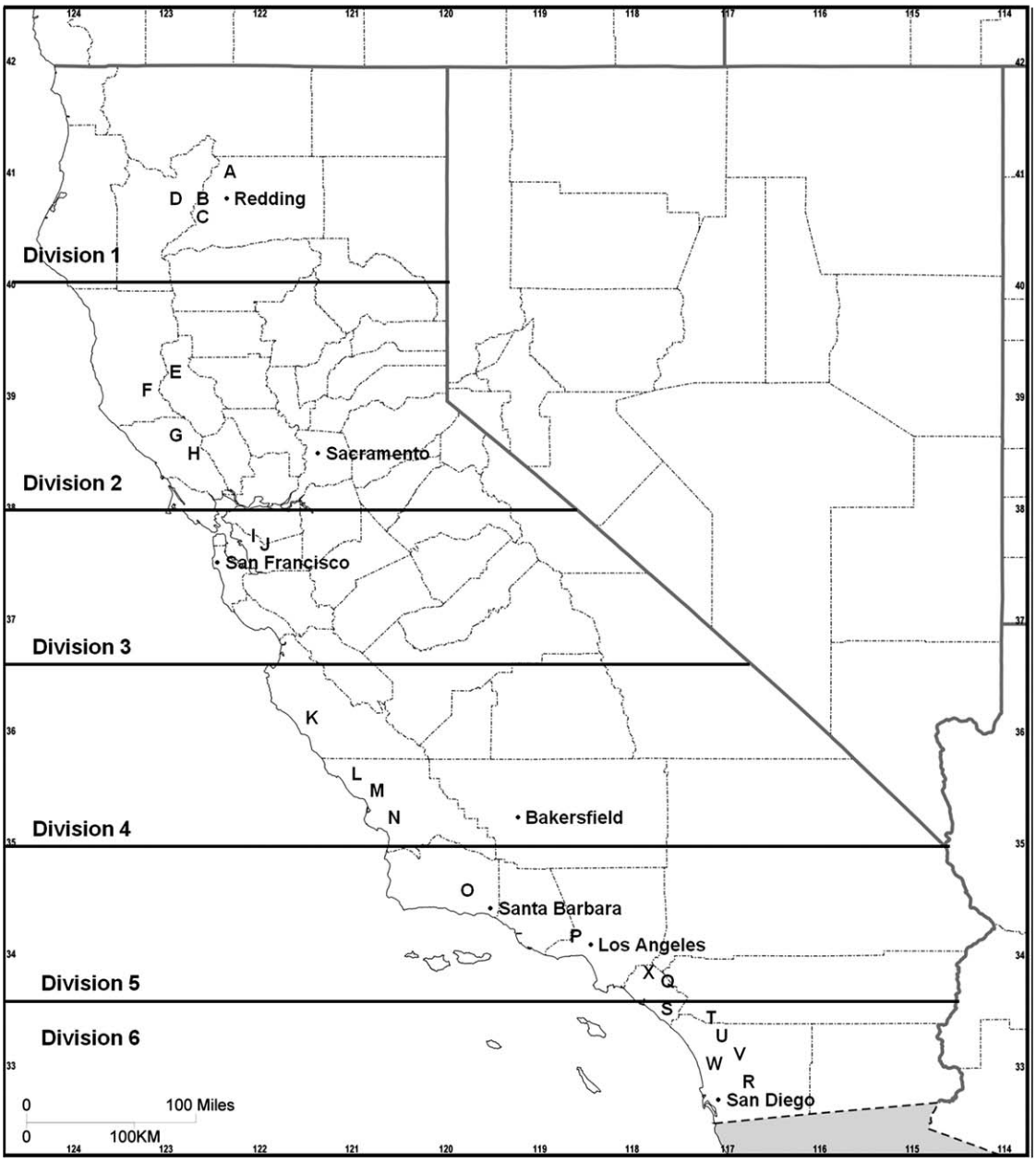

Fig. 3. Map of collection sites in California for *G. atropunctata* and *G. cythura*. See Table 1 for map legend with corresponding collection information.

with the original primers, were instead amplified using a set of internal primers *G. atropunctata* COI-F2 (5′-TCGAATTGAAYTWGCWCAGC-3′) and *G. atropunctata* COI-R2 (5′-AGCTCCTGCYAAWACWGGTA-3′), under identical PCR conditions.

Cleaning and Sequencing. PCR products were visualized after electrophoresis on 1% agarose gels stained with ethidium bromide, purified using the Wizard PCR Preps DNA purification system (Promega, Madison WI), and sequenced in both directions at the University of California–Riverside Genomics Institute Core Instrumentation Facility. Sequences were trimmed (removing the primers), aligned manually using BioEdit version 7.0.9.0 (Hall 1999), and deposited in GenBank (Benson et al. 2000).

Genetic Analyses. COI sequences were translated using the EMBOSS-Transeq website (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) to confirm the absence of nuclear pseudogenes (Song et al. 2008), and trimmed to match the length of those produced by the internal primer amplifications, resulting in a matrix of 70 sequences, each 465 bp. The number of *G. at-*

ropunctata mitochondrial haplotypes and haplotype diversity were calculated using DnaSp software, version 4.10.7 (Rozas et al. 2003), and a haplotype network was constructed using the statistical parsimony method of Templeton et al. (1992) in the software program TCS, version 1.21 (Clement 2000). COI sequences of specimens from the 23 original collection locations (Table 1) were arbitrarily grouped according to collection latitude, creating six geographically defined *G. atropunctata* population groups (Fig. 3). Genetic differences between these population groups were approximated by calculating estimates of F_{st} for each pair of groups by using the program ARLEQUIN (Schneider et al. 2000). The significance of F_{st} values was evaluated by permuting the haplotypes (1,000 permutations) between groups. Sequential Bonferroni corrections were applied. Partitioning of genetic variation was estimated using the analysis of molecular variance (AMOVA) implemented in ARLEQUIN. Genetic variation was partitioned into two levels: 1) within populations of *G. atropunctata* and 2) among populations of *G. atropunctata.* The significance of population differentiation was evaluated using the permutation method (1,000 permutations) invoked in ARLEQUIN. To test for isolation by distance, a Mantel test seeking a correlation between linearized F_{st} and the natural log of geographic distance (Rousset 1997) separating the six *G. atropunctata* population groups was implemented using the ISOLDE program in Genepop, version 4.0.10 on the web (http://genepop.curtin.edu.au/index.html; Raymond and Rousset 1995, Rousset 2008). Number of permutations was 1,000.

The relationship between *G. atropunctata* and the other two species, *G. cythura* and *G. flavovitatta*, was investigated by reconstructing a maximum-likelihood tree. 28S and COI sequences were concatenated, producing an aligned matrix of 19 haplotypes, each 997 bp. No gaps were necessary in either the 28S (532 bases) or the COI (465 bases) partition and the resulting matrix was deposited in TreeBASE, version 2 (Piel et al. 2009; http://treebase.org). Analysis of the partitioned data set was conducted using RAxML 7.0.4 (Stamatakis et al. 2005) and run on the CIPRES web portal (http://www.phylo.org/sub_sections/portal/) with the rapid bootstrap search algorithm (RBS) (Stamatakis et al. 2008), in which bootstrap analyses are conducted first with 100 repetitions, followed by fast and then slow searches on the sampled trees to find the best-known likelihood tree.

Results

Sequences of 28S rDNA were identical for all 70 *G. atropunctata* individuals sampled across the 23 California collections (GenBank accession FJ890817). Sixteen different mitochondrial haplotypes (GenBank accessions FJ890820–FJ890835) were found among the 70 specimens (Fig. 4). Thirty-three specimens shared the most common haplotype that is only present in central and northern California, and no other *G. atropunctata* haplotype differed from that by >4 bp (<1%). Northern California (37°52′ N to 40°55′ N) possessed six distinct haplotypes, whereas central and southern California (32° 52′ N to 36° 00′ N) possessed ten distinct haplotypes (Fig. 4). *G. atropunctata* specimens could be assigned to different populations by their state at specific single nucleotide polymorphisms (Table 2).

Estimates of F_{st} between our geographically defined populations of *G. atropunctata* ranged from 0.0016 to 0.5990 and were significantly different from zero for 11 of 15 pairwise population comparisons (Table 3). Those without significant differences were typically populations of *G. atropunctata* that were relatively close to each other (i.e., < 200 km). Within *G. atropunctata*, the highest pairwise F_{st} difference was found between the populations that were geographically furthest from one another (i.e., divisions 1 and 6; Fig. 3; Table 3). A similar pattern was evident in the results of the AMOVA, with the majority of molecular variation (65.03%; $df = 64, P < 0.001$) expressed within populations but a still highly significant amount due to interpopulation differences (34.97%; $df = 5$, $P < 0.001$). Finally, the Mantel test revealed a strong positive correlation between the genetic and geographic distances separating the six *G. atropunctata* population groups across California ($r = 0.782, P = 0.004$) (Fig. 5).

Comparison with the other *Graphocephala* species revealed that 28S sequences of *G. flavovittata* were very similar to those of *G. atropunctata.* Of the three *G. flavovittata* specimens collected in Mexico, one had a 28S sequence identical to that of the California *G. atropunctata*, and the other two differed by only a single nucleotide at the 252 bp (GenBank accession FJ890819). In contrast, *G. cythura* had nine nucleotide differences (≈1.7% sequence divergence) in 28S compared with *G. atropunctata* (GenBank accession FJ890818). COI sequences showed greater divergence. *G. flavovittata* possessed two COI haplotypes (GenBank accessions FJ890837–FJ890838), differing from each other by a single nucleotide, but differing from the most similar *G. atropunctata* haplotype at 41 nucleotide positions (≈9% sequence divergence). *G. cythura* was represented by a single COI haplotype that differed from the most similar *G. atropunctata* haplotype at 65 nucleotide positions (≈14% sequence divergence) (GenBank accession FJ890836). These patterns were reflected with strong branch support (>90%) in the RAxML maximum-likelihood phylogenetic reconstruction (Fig. 6).

Discussion

The blue-green sharpshooter is a major vector of Pierce's disease in grape vines and occupies a fragmented range throughout California. This study revealed the existence of 16 mitochondrial haplotypes in specimens of *G. atropunctata* across California. COI sequence data for 70 specimens seems to indicate a clear geographic signal with unique single nucleotide polymorphisms characterizing populations from south central coast, central to northern California, and southern California (Table 2).

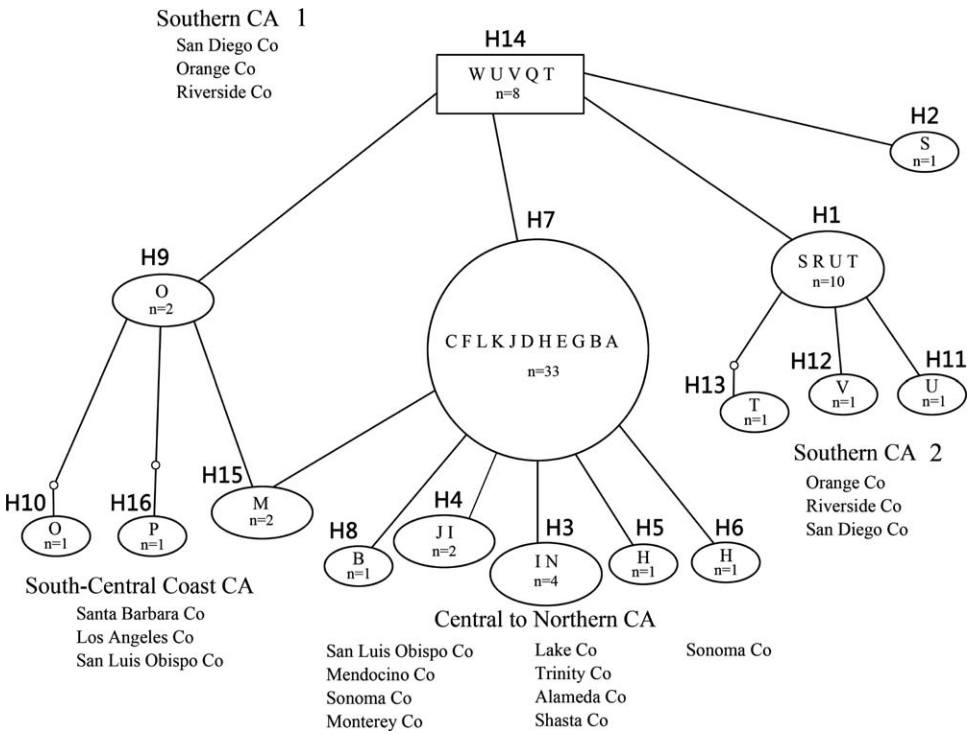

Fig. 4. Haplotype network for the COI sequences of *G. atropunctata* sampled across California. Each haplotype is represented by a rectangle or oval. The rectangular haplotype is that assigned the highest outgroup probability. Size of each haplotype is indicative of the number of specimens sharing that haplotype; also given inside each haplotype. Also included within each haplotype are the collection sites where that haplotype was detected (see Table 1 and Fig. 3 for explanation of alpha codes and geographic locations). Small circles represent hypothetical haplotypes not detected in our sample and lines between haplotypes represent a mutation in a single base pair.

Comparison of six geographically defined population groups revealed significant differences in the distribution of the 16 mitochondrial haplotypes; 11 of 15 pairwise F_{st} estimates were significantly different from zero, indicating limited gene flow between the populations (Table 3). Estimates of F_{st} also were strongly correlated with geographic distance (Fig. 5), suggesting that one major factor limiting gene flow between populations is the distance separating them. In *Macrosteles fascifrons* (Stål) (Hemiptera: Cicadellidae), a leafhopper that has a similar extended range across the west coast of the United States, Beirne (1956) noted that different populations were biologically isolated from one another as a result of their low relative dispersal rates across a large geographic range. The small size and relatively weak flying abilities of *G. atropunctata*, coupled with a lack of contiguous habitat, may promote a similar pattern of isolation by distance. In our study, the four pairwise population comparisons where significant gene flow was evident represented near neighboring population groups (separated by <200 km): divisions 1 and 2, divisions 2 and 4, divisions 3 and 4, and divisions 5 and 6 (Fig. 3). Contrary to what we might expect under isolation by distance, gene flow also seemed to be restricted between two pairs of neighboring population groups (divisions 2 and 3, divisions 4 and 5; Fig. 3), both with a minimum separation distance of only 90 km. This suggests that other barriers may play a role in limiting gene flow between these populations, such as differ-

Table 2. Specific combinations of single nucleotide polymorphisms in the COI sequence correlate with the geographic location in California where *G. atropunctata* specimens were collected

Pop[a]	Position in COI sequence		
	239	395	440
Southern California 1	A	G	C
Southern California 2	A	A	C
South central coast	A	G	T
Central to northern California	G	G	C

[a] For population designations, see Fig. 4, the haplotype diversity network.

Table 3. Pairwise estimates of F_{st} between six geographically defined *G. atropunctata* population groups (see Fig. 3)

	1	2	3	4	5
2	0.0016				
3	0.4312**	0.2510*			
4	0.2604**	0.0876	0.0313		
5	0.5731**	0.4318**	0.2131**	0.2485**	
6	0.5990**	0.4690**	0.2639**	0.2993**	0.0111

*, $P < 0.05$; **, $P < 0.001$.

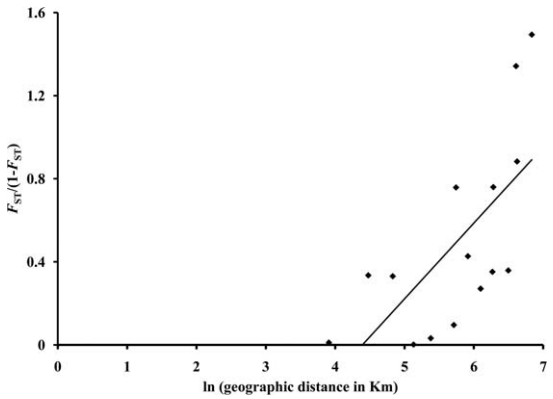

Fig. 5. Correlation between genetic differences and geographical distance between populations of *G. atropunctata* in California. There is a highly significant relationship between geographic distance between populations and genetic differences in *G. atropunctata* ($r = 0.782$, $P = 0.004$).

ences in terrain, habitat availability, mating behavior, and weather patterns (especially wind). Regardless, the distribution of COI haplotypes suggests that gene flow between geographically isolated populations of *G. atropunctata* is limited. Such a lack of gene flow may provide the conditions necessary for genetic divergence and the evolution of reproductive incompatibility between *G. atropunctata* populations. This possibility deserves investigation.

Caution should be exercised when considering the apparent genetic structuring of *G. atropunctata* as suggested by our data. One important weakness is that inferences about population structure rely on a single mtDNA marker. The genealogy of any single gene may be different from the true history of the species and this is compounded for mtDNA because, due to their uniparental mode of inheritance, mitochondria are not subject to recombination (Ballard and Whitlock 2004, Rubinoff et al. 2006). As such, the distribution of mitochondrial haplotypes may be influenced by fac-

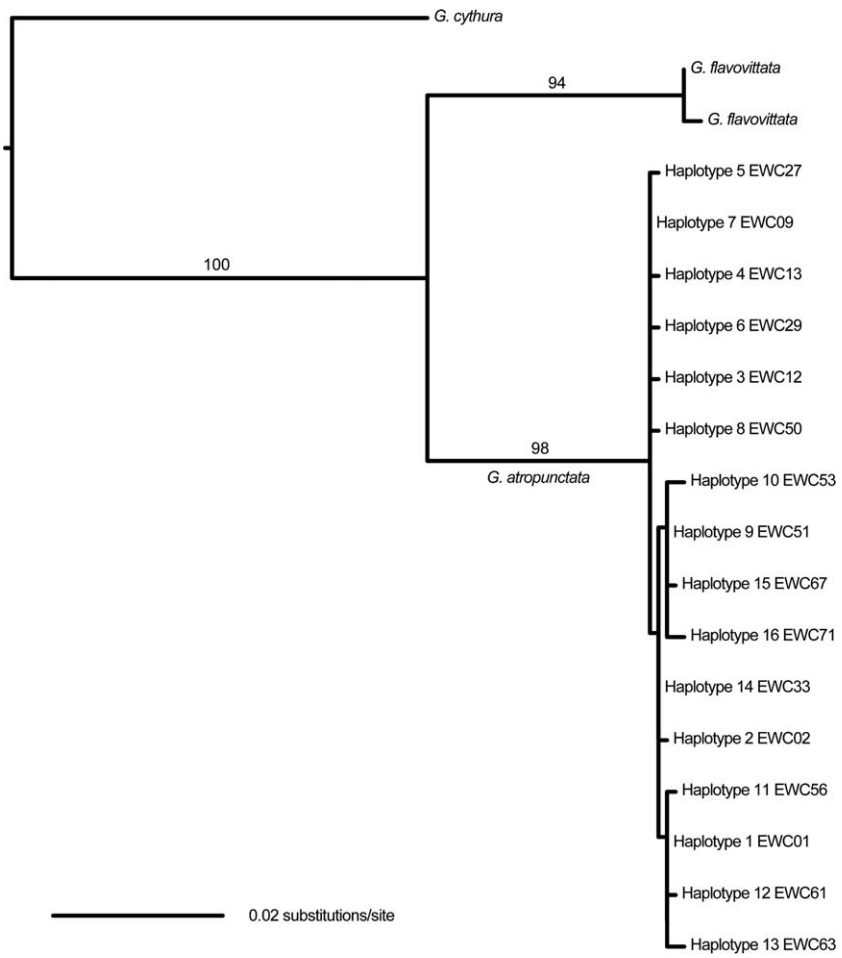

Fig. 6. Best-known likelihood tree based on a partitioned nucleotide model for concatenated 28S and COI sequences depicting the relationship between the 16 *G. atropunctata* haplotypes and two additional species, *G. flavovittata* and *G. cythura* (study accession, http://purl.org/phylo/treebase/phylows/study/TB2:S10976). Bootstrap support (expressed as a percentage) is given above the three main branches only.

tors such as differences in dispersal (or incidental movement) between the sexes. In many species, males disperse in search of mating opportunities, but females are more sedentary. If this were the case mtDNA might show evidence of strong population isolation where in fact none is present (i.e., the mixing of nuclear genes is common). However, both sexes of *G. atropunctata* are weak dispersers (Severin 1949), and it seems unlikely that males of this species are more likely to be transported than females. One solution to the problems associated with using mtDNA would be to include a variable nuclear marker. If a similar pattern of population structure was revealed, this would add strong support to our inferences from mtDNA (Rubinoff and Holland 2005, Rugman-Jones et al. 2010). We examined sequences of a section of 28S rDNA, but this proved to be conserved across all our populations. A more variable region such as the internal transcribed spacers of rDNA may have been a better choice for these analyses.

A further problem with our study arises from the small numbers (just two or three) of individuals sampled from each population. These low numbers reflected low relative abundance and subsequent difficulty of capture at collection sites. F_{st} is an estimate of the proportion of genetic diversity due to differences in the frequency of alleles (or in this case haplotypes) among populations. Therefore, as a result of sampling error, the smaller a population sample is, the less likely it is that the sample haplotype frequencies will truly reflect the underlying frequencies of haplotypes in the population. We have attempted to minimize this problem by grouping populations according to collection latitude. In the context of our study, we believe this is justified because grouping neighboring populations is more likely to obscure, rather than reveal, larger scale population structure. However, even after consolidation, the best sampled latitudinal division (Fig. 3) was still populated by sequence data from only 17 individuals. Of the 16 mtDNA haplotypes detected in our *G. atropunctata* specimens, 12 were apparently unique to a particular division. This undoubtedly contributed to some very high intraspecific pairwise estimates of F_{st} (Table 3), and these should be regarded with some caution. More comprehensive sampling within *G. atropunctata* populations across California may have shown at least some of these "unique" haplotypes to have been distributed across other divisions.

Despite clear morphological differences between *G. atropunctata* specimens from different areas in California (Fig. 1), the 28S sequences of all *G. atropunctata* were identical. It was however, surprising to find that the 28S sequences of *G. flavovittata* specimens were nearly identical to *G. atropunctata* with one of three specimens matching exactly, and the other two having only a single nucleotide substitution. The similarity of their 28S sequences may suggest that these two species have diverged relatively recently, may potentially interbreed, or, may in fact be synonymous. The two species have partial overlapping geographic ranges in Mexico, and morphologically, adult *G. flavovittata* and *G. atropunctata* are very similar (Figs. 1 and 2). The nymphs also look remarkably similar in both body type and color (Oman 1949). However, the degree of divergence detected between the COI sequences of the two species (≈9%) was much higher than that detected among the California *G. atropunctata* haplotypes (<1%) and much higher than the 3% level suggested as a species boundary by proponents of DNA barcoding (Hebert et al. 2003). Unfortunately, we were only able to obtain a very small number of *G. flavovitta* specimens, and those were collected in Mexico. The possibility remains that the observed levels of mtDNA divergence are actually intraspecific and simply an extension of isolation by distance in *G. atropunctata.* A larger sample of *G. flavovittata* specimens, and from areas where *G. atropunctata* and *G. flavovittata* occur in sympatry, coupled with reciprocal crossing experiments will be needed to confirm whether *G. atropunctata* and *G. flavovittata* are truly different species.

The existence of genetically distinct populations of *G. atropunctata* could have important management consequences for grape producing regions outside of northern California where control strategies have been developed for this pest (Severin 1949, Purcell 1976, Feil et al. 2000). For example, it is possible that regionalized populations of *G. atropunctata* may differ in their transmission efficiency of *X. fastidosa*, thus affecting their status as a grape pest in different areas. In addition, the reproductive behavior, population phenology, host plant preferences, and natural enemy complexes associated with *G. atropunctata* could vary, which also may influence pest status and affect the efficacy of control programs.

In summary, this work had three main objectives: 1) to determine whether isolated *G. atropunctata* populations in California distributed over an ≈1,000-km latitudinal cline are the same species; 2) to examine genetic variation in *G. atropunctata* populations as a measure of population movement across California; and 3) to compare *G. atropunctata* to two other species of *Graphocephala* to determine the specific status of these three species. Based on DNA sequences, it seems that across California, *G. atropunctata* is probably a single species. However, mtDNA sequences suggest that gene flow between distant populations is limited. This may indicate low levels of dispersal, mixing or interbreeding between populations which could promote speciation. In addition, based on COI sequences, *G. atropunctata* exhibited large differences from the other *Graphocephala* species. However, 28S sequences of *G. atropunctata* and *G. flavovittata* were almost identical and further research is needed to verify the possibility of a species complex involving these two species.

Acknowledgments

We thank those who assisted with field collections, including Christina Wistrom, Betsy Boyd, Justin Nay, Christina Hoddle, Aaron Ballman, and Alexander Purcell, as well as all landowners for allowing collecting on their property. Mike Lewis, Amy Truong, and Ruth Vega were invaluable with

greenhouse and laboratory assistance. In addition, we thank Chris Dietrich for sharpshooter identifications, John Heraty for help with the maximum-likelihood analysis, and Doug Yanega and the University of California–Riverside museum staff for efforts with vouchering specimens. Finally, we are indebted to two anonymous reviewers whose thorough and insightful criticism greatly improved this article. This work was funded in part by the Temecula Valley Wine Society Nancy Johnson Memorial Scholarship.

References Cited

Ballard, J.W.O., and M. C. Whitlock. 2004. The incomplete natural history of mitochondria. Mol. Ecol. 13: 729–744.

Beirne, B. P. 1956. Leafhoppers (Homoptera: Cicadellidae) of Canada and Alaska. Can. Entomol. 88: 1–180.

Benson, D., A. I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, B. A. Rapp, and D. L. Wheeler. 2000. GenBank. Nucleic Acids Res. 28: 15–18.

Choudhury, R., and J. H. Werren. 2006. Unpublished primers. (http://troi.cc.rochester.edu/~wolb/FIBR/protocols.html).

Clement, M. 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9: 1657–1659.

Feil, H., W. Feil, and A. Purcell. 2000. Effects of temperature on the flight activity of *Graphocephala atropunctata* (Hemiptera: Cicadellidae). J. Econ. Entomol. 93: 88–92.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome *C* oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3: 294–299.

Hall, T. A. 1999. BioEdit: a user friendly biological sequence alignment and analysis program from Windows 95/98/NT. Nucleic Acids Symp. 41: 95–98.

Han, D., R. Chen, E. Hill, M. Tilley, and H. Gu. 2006. Cause and solutions to the polymerase chain reaction smear problem in genotyping. Anal. Biochem. 353: 296–298.

Hebert, P.D.N., A. Cywinska, S. L. Ball, and J. R. deWard. 2003. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B 270: 313–321.

McKamey, S. 2007. Taxonomic catalogue of the leafhoppers (Membracoidea). Part 1 Cicadellinae. Mem. Am. Entomol. Inst. 78: 1–394.

Oman, P. W. 1949. The Nearctic leafhoppers: a generic classification and check list. The Entomological Society of Washington, Washington DC.

Purcell, A. H. 1976. Seasonal changes in host plant preference of the blue-green sharpshooter *Hordnia circellata.* Pan-Pac. Entomol. 52: 33–37.

Piel, W., L. Chan, M. Dominus, J. Ruan, R. Vos, and V. Tannen. 2009. TreeBASE v. 2: a database of phylogenetic knowledge. e-BioSphere 09 International Conference on Biodiversity Informatics, London, United Kingdom.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Hered. 86: 248–249.

Rousset, F. 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. Genetics 145: 1219–1228.

Rousset, F. 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Mol. Ecol. Resour. 8: 103–106.

Rozas, J. J., C. Sanchez-Del Barrio, X. Messegue, and R. Rozas. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496–2497.

Rubinoff, D., and B. S. Holland. 2005. Between two extremes: mitochondrial DNA is neither the panacea nor the nemesis of phylogenetic and taxonomic inference. Syst. Biol. 54: 952–961.

Rubinoff, D., S. Cameron, and K. Will. 2006. A genomic perspective on the shortcomings of mitochondrial DNA for "barcoding" identification. J. Hered. 97: 581–594.

Rugman-Jones, P. F., M. S. Hoddle, and R. Stouthamer. 2010. Nuclear-mitochondrial barcoding exposes the global pest western flower thrips (Thysanoptera: Thripidae) as two sympatric cryptic species in its native California. J. Econ. Entomol. 103: 877–886.

Schneider, S., D. Roessli, and L. Excoffier. 2000. Arlequin: a software for population genetics data analysis. Version 2.000. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva, Switzerland.

Severin, H. 1949. The life history of the blue-green sharpshooter, *Neokolla circellata.* Hilgardia 19: 187–189.

Song, H., J. E. Buhay, M. F. Whiting, and K. A. Crandall. 2008. Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. Proc. Natl. Acad. Sci. U.S.A. 105: 13486–13491.

Stamatakis, A., T. Ludwig, and H. Meier. 2005. Raxml-iii: A fast program for maximum likelihood-based inference of large phylogenetic trees. Bioinformatics 21: 456–463.

Stamatakis, A., P. Hoover, and J. Rougemont. 2008. A rapid bootstrap algorithm for the RAxML web servers. Syst. Biol. 57: 758–771.

Templeton, A. R., K. A. Crandall, and C. F. Sing. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data III. Cladogram estimation. Genetics 132: 619–633.

Walsh, P. S., D. A. Metzger, and R. Higuchi. 1991. Chelex® 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10: 506–513.

Received 24 March 2010; accepted 4 November 2010.