Genetic differentiation between eastern populations and recent introductions of potato psyllid (*Bactericera cockerelli*) into western North America

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Abstract

Although tomato psyllid, *Bactericera cockerelli* (Sulc) (Homoptera, Psyllidae), annually causes significant losses in potato and tomato crops in eastern Mexico and the central United States, infestations in western North America have been historically rare. However, substantial populations appeared in 2001 in western North America and caused losses in tomato production exceeding 80%; losses in 2004 reached 50%. To determine if these new outbreaks were the result of a simple range expansion or the evolution of a new *B. cockerelli* biotype, inter simple sequence repeat (ISSR) markers, as well as mitochondrial gene cytochrome oxidase I (COI), internal transcribed spacer 2 (ITS2), and *wsp* sequence data were used to characterize populations of the psyllid. Western populations from Baja, Mexico, Orange County, and Ventura County were compared with populations from central USA (Colorado and Nebraska) and eastern Mexico (Coahuila). Based on ISSR markers, the psyllid populations clustered into two groups, with one group including populations from western North America and the other group including populations from central USA and eastern Mexico. For COI comparisons, there was one base-pair difference found in the 544 bp-long COI fragments, but the populations again segregated along the same geographic lines. Two strains of *Wolbachia* were identified, the maximal differences between *wsp* clones from all populations was 5 bp for strain Bac1 and 23 bp for strain Bac2 out of a 555-bp fragment. The ISSR data, therefore, were consistent in indicating the development of a new psyllid biotype that has adapted to western North America rather than a simple range expansion, but the other genetic data sets were less conclusive.

Introduction

In the past few years, major outbreaks of tomato psyllid [*Bactericera cockerelli* (Sulc) (Homoptera, Psyllidae)] have occurred in Ontario, Canada (Ferguson et al., 2002), in Washington State (McGuire, 2002), in the central United States (Zink, 1998; Al-Jabar, 1999), and in California and Baja (Mexico) (Liu & Trumble, 2004, 2005). However, infestations in western North America have been historically rare. Although early reports stated that this pest was only found in Utah, Colorado, and parts of Wyoming (Richards, 1928), sporadic populations were reported in California in the 1930s and 1940s (Pletsch, 1947).

Relatively little information is available on the movement patterns of this pest within North America. Romney (1939) indicated that the tomato psyllid developed large spring migratory populations in the misnamed 'overwintering breeding sites' in southern Arizona from January through May on wild *Lycium* spp. Typically, psyllids then migrated to Colorado, Nebraska, and other northern states. Pletsch (1947) later pointed out that the annual originating populations occurred much further south, and migration from southern Texas (near the Rio Grande River) and even further south in the east coast area of Mexico provided the individuals that inoculated the 'overwintering breeding sites' each year in Arizona and New Mexico. Thus, given the initial outbreaks in 2001 in southern California, populations along the west coast of North America could have originated in Baja (Mexico) (possibly from movements of transplants from the Mexican mainland) or from much further east near southern Texas. The location of the originating populations could be important. If movement

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occurs from transplants transported from the western Mexican mainland to Baja (Mexico), cultural and chemical strategies could be used to break the transport cycle. If both the California and Baja (Mexico) populations simply migrate from breeding sites in Arizona and New Mexico, interfering with the migration pattern will not be possible.

The now standard taxonomic tools of DNA analyses have been successfully used to genetically document population biotypes (Frohlich et al., 1999; Shufran et al., 2000, and references therein). Numerous studies have used the mitochondrial gene cytochrome oxidase I (COI) and the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) to identify genetic relationships between species. Studies at within-species level using COI and ITS regions are more limited: Genetic variation in COI and ITS regions have been used to simply characterize intraspecific variation (Palenko et al., 2004), explain the population changes due to geographic isolation (Leebens-Mack & Pellmyr, 2004), document hydrographic separation of subterranean and surface habitats (Verovnik et al., 2004), and classify individuals into strains (Gomez & Gonzalez, 2004), subspecific clades (Shufran, 2003), or haplotypes (Tuda et al., 2004).

Inter simple sequence repeat (ISSR) markers make use of anchored primers to amplify regions between microsatellite sites and can generate larger numbers of polymorphisms per primer than the randomly amplified polymorphic DNA (RAPD) technique (Zietkiewicz et al., 1994). The ISSRs are dominant markers interpreted in a diallelic fashion, either 'band present' or 'band absent' (Wolfe et al., 1998). The ISSR markers are used extensively in plant studies for assessing hybridization (Wolfe et al., 1998), for fingerprinting species, subspecies, or cultivars (Provan et al., 1996; Wolff & Morgan-Richards, 1998; McGregor et al., 2000), and for characterizing plant populations (Parsons et al., 1997). However, published characterizations of animal populations using the ISSR are rare.

Insects of the suborder Sternorrhyncha (Homoptera), including psyllids, aphids, mealy bugs, and whiteflies, feed primarily or exclusively on plant phloem sap, which is deficient in essential amino acids (Sandstrom & Moran, 1999). Thus, endosymbionts associated with these insects often have functions related to host nutrition. A recent study on psyllids showed that the phylogenetic tree derived from the 16S-23S ribosomal DNA of endosymbionts agreed with the phylogram derived from the host nuclear gene *wingless*, a result consistent with a single infection of a psyllid ancestor and subsequent cospeciation of endosymbionts and hosts (Thao et al., 2001). *Wolbachia*, a common, maternally inherited endosymbiont, has evolved a number of reproductive manipulations of its hosts, including cytoplasmic incompatibility, parthenogenesis induction, feminization, and male killing, thereby influencing the evolution of host-sex determination, eusociality, and speciation (Stouthamer et al., 1999). A *wsp* gene coding for a surface protein of *Wolbachia* evolves at a much faster rate than other genes such as 16S or *ftsZ*, and has been useful in characterizing *Wolbachia* strains (Van Meer et al., 1999). This coevolutionary relationship between the *Wolbachia* endosymbionts and their hosts makes the genetic differences between symbionts from different host populations valuable in assessing host-population genetics.

The primary goal of this study was to test the hypothesis that the tomato psyllids found in the west coast of North America were from a different population than occurs in central/eastern North America. By identifying potential genetic differences among psyllid populations from different geographic locations using ISSR markers as well as COI, ITS2, and *wsp* sequence data, we hoped to determine whether the psyllid outbreak patterns on the west coast of North America were the result of simple migration or represented a potential range expansion associated with a new biotype.

Materials and methods

Sampling

Psyllids were collected from each of six sites, including western populations from San Quintin (Baja, Mexico, at 29°48′N in August 2003), southern California (Santa Ana, Orange County, at 33°45′N in August 2003), and central California (Camarillo, Ventura County, at 34°12′N in September 2004), and populations from the central USA (Fort Collins of Colorado at 40°45′N in October 2004; and Columbus of Nebraska, at 41°28′N in August 2003) and eastern Mexico (Arteaga, Coahuila, Mexico, at 18°50′N in November 2004). The geographic distances between populations ranged from 250 km to over 1200 km.

DNA extraction and PCR amplification

DNA was extracted from 95% ethanol-preserved adults using the modified cetyltrimethylammonium bromide method according to Navajas et al., 1998). Fifty extractions (50 individuals) of each population were used for the ISSR polymerase chain reactions (PCR). Several individuals of each population were used to amplify ITS2 and COI fragments; other individuals were used for *wsp* fragment amplification. ITS2, COI, and *wsp* were subsequently sequenced. ITS2 (AY971897 to AY971914), COI (AY971885 to AY971890), and *wsp* sequences (AY971915 to AY971950) were deposited in GenBank. Internal transcribed spacer 2 and COI analyses were conducted to substantiate that populations were all from the same species: large differences would indicate multiple species.

The *wsp* region of the *Wolbachia* DNA was amplified with the primers *wsp*-81F (forward) and *wsp*-691R (reverse) (Braig et al., 1998). The COI region was amplified using the

forward primer (5′-CAACACCTATTCTGATTTTTTGG) modified from the primer Jerry (mtD-08) according to Simon et al. (1994), and the reverse primer UEA9 (equivalent of C1-N-2753) (5′-TGTTGAGGAAAAAATGTTAG-GTTTAC) (Lunt et al., 1996). The forward primer (5′-GATCGATGAAGACCGCAGC), modified according to known 5.8S sequences and White et al. (1990), and the reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC) (White et al., 1990) were used to amplify the ITS2 region of rDNA.

Initially, 100 ISSR primers purchased from the Biotechnology Laboratory, University of British Colombia (UBC set no. 9), were screened, and three of the primers produced clear and reproducible bands, viz., 818 (5′-CACACACA-CACACACAG), 829 (5′-TGTGTGTGTGTG TGTGC), and 847 (5′-CACACACACACA CACARC). These primers were selected for subsequent experiments.

Polymerase chain reaction was performed in a volume of 25 μl containing 2.5 μl buffer (Promega 10 ×), 0.2 mM of dNTPs, 0.2 μM primers, 2 μl genomic DNA, and 0.5 unit of Taq DNA polymerase (Promega, Madison, WI, USA). A control PCR tube containing all components, but no genomic DNA, was run with each primer to check for contamination. DNA amplification was performed in a Programmable Thermal Cycler (Mastercycler®, Eppendorf, New York, NY, USA). Inter simple sequence repeat reactions were run as follows: 94 °C (2 min) for initial strand separation, then 37 cycles of 94 °C (0.5 min), 56–61 °C (primer 818 at 61, 829 at 56, and 847 at 59.5 °C) (45 s), 72 °C (1.5 min), and 72 °C (20 min) for the final extension. Internal transcribed spacer 2 reactions were conducted under the following conditions: 95 °C (4 min), then 36 cycles of 95 °C (45 s), 53 °C (1 min), 72 °C (1.5 min), and followed by one cycle of 72 °C (10 min). For *wsp*, reactions were run as follows: 94 °C (5 min), then 35 cycles of 94 °C (30 s), 55 °C (1 min), 72 °C (1min), and 72 °C (5 min) for the final extension. For COI, reactions were run as follows: 95 °C (5 min), then 36 cycles of 95 °C (30 s), 46.4 °C (1 min), 72 °C (1 min), and followed by one cycle of 72 °C (10 min).

Electrophoresis, detection, and processing of PCR products

The amplified products for ISSR were electrophoresed on 1% agarose gels at 84 V for 1 h 15 min with Tris-acetate-ethylenediaminetetraacetic acid buffer (pH 8.0). After staining with ethidium bromide, the banding patterns were visualized under UV light (254 nm). In preliminary studies, the repeatability of ISSR was examined by both repeating the PCRs of 20 samples and running the same PCR product twice in separate lanes; results indicated that the patterns of ISSR products were highly reproducible. The three primers produced 161 bands across the 300 individuals examined from six populations. The size of the fragments ranged from 0.35 to 2.2 Kb.

The amplified fragments for COI were purified, and then sent to an automated sequence facility (Core Instrumentation Facility, University of California, Riverside, CA, USA). The ITS2 and *wsp* PCR products were purified, ligated into a T vector and cloned in *Escherichia coli* cells. *Escherichia coli* colonies containing a plasmid with an insert of the correct size were checked by PCR using the primers mentioned previously. The PCRs of the successfully transformed colonies were then purified and sent to an automated sequence facility (Core Instrumentation Facility). Sequencing was conducted both with the forward and reverse vector primers to check for accuracy of the sequencer. Three clones of ITS2 for each population were sequenced.

Initially, 10 *wsp* clones each for populations from Baja (Mexico), Ventura County, and Orange County were sequenced. There potentially could be many *Wolbachia (*maybe of different strains) in each individual psyllid, so there could be many *wsp* fragments in each DNA extraction. A '*wsp*' clone therefore represents a single *wsp* fragment from a DNA extraction, which is cloned to produce numerous copies. Two strains of *Wolbachia* clones (named *wsp* Bac1 and *wsp* Bac2 hereafter) were identified with approximately 110 bp differences between strains and with negligible within-strain differences. Thus, only two clones of different strains needed to be sequenced for the remaining three psyllid populations for comparison, and 36 *wsp* sequences were therefore reported in the GenBank. The restriction enzyme *Spe*I (New England Biolabs, Boston, MA, USA) was found to recognize the site 5′ . . . A^{∇} CTAGT . . . 3′, and cut only the strain *wsp* Bac1 into two fragments. Thus, the digestion of clones with *Spe*I produced two bands for the strain *wsp* Bac1, and one for *wsp* Bac2. By digesting clones using *Spe*I and subsequent sequencing, the same two strains of *Wolbachia* were identified in populations from Nebraska, Colorado, and Coahuila (Mexico). Twenty *wsp* clones each from all populations were used to find the proportion of the two *Wolbachia* strains within an individual.

Data analysis

The ISSR band profiles were image analyzed using Kodak Digital Science 1D Image Analysis Software (Eastman Kodak, New York, NY, USA), and scored for presence (= 1) or absence (= 0) for each DNA sample, excluding smeared and weak bands. The binary data matrix was analyzed using POPGENE (Yeh et al., 1997), assuming a Hardy–Weinberg equilibrium. The percentage of polymorphic loci (P), the mean expected heterozygosity (H) (Nei, 1973), and Shannon's information index of diversity (I) were used to quantify genetic diversity of each population. Nei's gene diversity statistic was used to assess genetic differentiation among populations (Nei, 1973). F_{ST}, an inbreeding coefficient, is

Table 1 Gene diversity and gene flow estimates of six tomato psyllid, *Bactericera cockerelli*, populations[a]

Population	Gene diversity (H)	Shannon's information index (I)	Percentage of polymorphic loci (P)	Coefficient of gene differentiation (G_{st})	Gene flow (Nm)
Ventura County	0.0550	0.0979	80.75		
Orange County	0.0588	0.1027	83.23		
Baja (Mexico)	0.0498	0.0925	75.16		
Nebraska	0.0523	0.0949	60.25		
Coahuila (Mexico)	0.0551	0.0976	71.43		
Colorado	0.0573	0.0998	78.88		
Group 1	0.0542	0.1190	98.14	0.0082	60.3
Group 2	0.0456	0.1027	93.17	0.0142	34.6

[a]Group 1 = populations from Ventura County, Orange County, and Baja (Mexico). Group 2 = populations from Coahuila (Mexico), Colorado, and Nebraska (USA).

a statistic that compares the level of genetic variation within two or more subpopulations relative to all subpopulations combined. G_{ST} is Nei's coefficient of gene variation. The amount of gene flow among populations was calculated using $Nm = (1/G_{ST} - 1)/4$ after Slatkin & Barton (1989). Analysis of molecular variance (AMOVA) was conducted to calculate variance components among and within populations using ARLEQUIN (Schneider et al., 2000).

An agglomerative technique, the unweighted pair group method with arithmetic averages (UPGMA) in PHYLIP 3.5 (Felsenstein, 1993), was used to construct dendrograms based on pairwise genetic distances (Nei, 1972). Because the clustering pattern showed two groups of populations, the six sample populations were divided into two groups, with group 1 including populations from Orange County, Ventura County, and Baja (Mexico), and group 2 including the populations from the central USA and eastern Mexico.

Results

Population variation based on the ISSR markers

Gene diversity and gene flow estimates are shown in Table 1. The populations from Orange County had slightly higher gene diversity as shown by higher values of H, I, and P (H = 0.06, I = 0.1, P = 83). The lowest gene diversity was found in the Nebraska and Baja populations (H = 0.05, I = 0.09, and P<75). Higher gene differentiation was found in populations from group 2 as indicated by higher G_{ST} value of 0.0142, compared to 0.0082 in group 1. A higher level of gene flow occurred in populations of group 1 with an Nm of 60.3, whereas the value was only 34.6 in group 2.

Analysis of molecular variance (Table 2) using the ISSR data set indicated that 97.7% of the total variance could be explained as within-population differences, 1.2% as among-population differences within groups, and only 1.1% variance was accounted for by differentiation between groups (P<0.001). Pairwise comparisons of genetic distance (F_{ST}) showed no significant genetic differences between the Ventura population and the Baja population, or between the Orange County and Baja populations, but significant genetic differences (P<0.001) were found between all the other populations.

Comparison of datasets

The ITS2 was an 807-bp-long fragment in all populations, 786 of which remained constant across all the clones sequenced (Table 3). The amplified COI fragment was 544 bp long, 543 bp of which were all the same for all the populations; the single base pair difference did cause an amino acid change. Thus, because there was little or no

Table 2 Summary of molecular variance (AMOVA) based on *Bactericera cockerelli* ISSR markers

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation (%)	P[a]
Among groups	1	24.75	0.08607	1.14	<0.001
Among populations within groups	4	47.36	0.08908	1.18	<0.001
Within populations	294	2171.42	7.38578	97.68	<0.001
Total	299	2243.53	7.56093		

[a]Levels of significance based on 1000 iteration steps.

Table 3 Comparison of datasets in the molecular characterization of *Bactericera cockerelli* populations

	No. of characters	No. of constant[a]	% variation	Maximal difference between variants (bp)
ITS2 sequences	807	786	2.9	17
COI sequences	544	543	0.2	1
wsp Bac1	552	546	1.1	5
wsp Bac2	555	521	6.1	23
ISSR	161	9	94	–

[a]Similar number of constant for the ISSR markers means the bands were evenly distributed among populations.

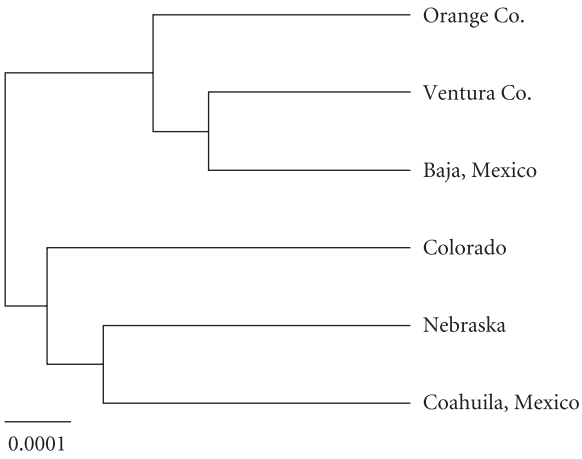

Figure 1 UPGMA dendogram of *Bactericera cockerelli* populations based on the ISSR markers.

difference identified by ITS2 and COI, all populations regardless of geographic location can be considered to belong to the same species.

From all the *wsp* sequences, two strains of *Wolbachia* were identified and the difference between them was about 110 bp. All individual psyllids for which *wsp* DNA was sequenced were doubly infected. For the *wsp* strain Bac1, a 552 bp fragment was amplified for each of the populations, 546 bp of which remained constant. However, the Strain Bac2 was either 552 or 555 bp long, 521 bp of which were identical across all the clones sequenced. In all psyllid populations, the *Wolbachia* strain *wsp* Bac2 dominated over *wsp* Bac1, with *wsp* Bac1 accounting for 10% of the clones in Nebraska, 20% in Colorado, 40% in Orange County, and 30% in Coahuila (Mexico), Ventura County, and Baja (Mexico).

Table 3 further shows that the greatest variation between populations (94%) was found using the ISSR method, the least (0.2%) using COI sequences, with *wsp* and ITS2 sequences intermediate. Similar amounts of variation were identified using *wsp* sequences (1.1% for Bac1 or 6.1% for Bac2) and ITS2 (2.9%). The maximal difference between *wsp* clones from all populations was 23 bp for strain Bac2 and 5 bp for strain Bac1, compared to 17 bp between ITS2 variants.

Cluster analysis

The UPGMA dendrograms based on pairwise genetic distances are shown in Figure 1. The dendrogram based on the genetic distance calculated from the ISSR markers indicated that all the populations clustered into the same two geographic groups identified previously. In group 1, the population from Baja (Mexico) first clustered together with that of Ventura County, then with that of Orange County. As to group 2, the population of Coahuila (Mexico) was more closely related to that of Nebraska than with that of Colorado.

Discussion

Population variation

The psyllid populations from Coahuila (Mexico), Colorado, and Nebraska were clustered genetically (Figure 1). This result was not surprising because there is considerable evidence that psyllids migrate northward in spring every year from the southern Texas border near the Gulf of Mexico to Nebraska and other northern states on the warm monsoon winds from the Gulf of Mexico (Pletsch, 1947). Wallis (1946) and Al-Jabar (1999) stated that the evidence for this annual migration was (1) airplane trapping of psyllids at up to 1524 m a.s.l.; (2) coincidental capture with sugar beet leafhoppers, *Circulifer tenellus* (Baker), a species with previously documented migration from southern areas; (3) a heavier pattern of infestations in areas closer to breeding sites near the border between Texas and Coahuila (Mexico); (4) intolerance of high temperature and disappearance from breeding sites during the summer; and (5) cage studies showing the species cannot withstand the winter temperatures that occur in north-central states such as Nebraska.

The populations of Baja, Orange County, and Ventura County were also genetically similar. This result is consistent with our hypothesis that psyllids in Orange County and Ventura County originated from Baja (Mexico). This pattern has been seen for other insects and appears to be related to a weather condition known as the Catalina Eddy, a frequently occurring low-pressure system centered near Catalina Island, CA, that creates winds that sweep north from western Mexico to southern California (Trumble & Baker, 1984; Wiesenborn et al., 1988). This conclusion is also supported by the determination that gene flow among populations in group 1 was much higher than that among

the populations in group 2. This increased gene flow could be explained by relatively short geographic distances among the populations and the likelihood that airborne transport could occur in both directions (north to south and vice versa) across the spring, summer, and fall seasons for group 1. Group 2 populations tend to migrate only northward, with the winter breeding areas becoming unsuitable for habitation in late spring through the summer, thereby creating a barrier to southward movement. In addition, the psyllids are now overwintering in Ventura County, CA (J Trumble, pers. obs.).

Our *Wolbachia* DNA assays showed that all psyllids tested were double infected with both strains of *Wolbachia* identified. Because different strains of *Wolbachia* may exist in a host species for particular climates (Keller et al., 2004), we had anticipated that these differences would be greater. However, despite the fast-evolving nature of the *wsp* gene and environmentally selective pressures imposed on psyllid populations in the two geographic groups, the two strains of *Wolbachia* showed few differences among populations, although differences were less in individuals from group 1 as compared to group 2 for both strains. The maximal difference between any two populations was only 5 bp for strain *wsp* Bac1 and 23 bp for *wsp* Bac2 based on a 555-bp fragment. Some differences were observed for proportions of *Wolbachia* strains in group 2 populations (10–30%), but the ratios of the strains were more balanced in group 1 populations (30–40%). Because we only sequenced the *wsp* in a single insect from each population, these data are much less conclusive than the other data sets, and should be interpreted with caution.

Because of the rapidly changing nature of microsatellite sites, the ISSR technique is more sensitive to genetic variation among populations (Parsons et al., 1997; Jian et al., 2004). In our study, 60–83% of the loci found were polymorphic. However, COI and ITS2 sequences showed much smaller differences among the populations, as is indicative of a single species, but did support the hypothesis that psyllid populations from group 1 locations may be diverging into separate biotypes. The observation that tomato psyllids are now overwintering in Ventura County, CA since 2002 indicates that there has been a geographic range expansion that would be consistent with the development of a new biotype adapted to coastal conditions of western North America.

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