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Two Separate Introductions of Asian Citrus Psyllid Populations Found in the American Continents

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ABSTRACT A phylogeographic analysis inferred from the partial mitochondrial cytochrome oxidase subunit I gene (433 bp) was performed with 22 populations of *Diaphorina citri* Kuwayama collected in the Americas and one in the Pacific. Eight populations from four countries in South America, 14 from four countries in North America, and one from Hawaii were analyzed. Twenty-three haplotypes (hp) were identified and they fell into two groups: hp1–8 were identified in South America (group 1) and hp9–23 were identified in North America and Hawaii (group 2). Hp1 and nine were present in the highest frequencies within each population and within their group, 81 and 85% for group 1 and group 2, respectively. A diagnostic nucleotide at position 48 was identified that allowed for the discrimination of the two groups; in addition, no haplotypes were shared between the two groups. An analysis of molecular variance uncovered significant genetic structure ($\Phi_{CT} = 0.733$; $P < 0.001$) between the two groups of the Americas. Two haplotype networks (ParsimonySplits and Statistical Parsimony) discriminated the two groups and both networks identified hp1 and nine as the predicted ancestral or founding haplotypes within their respective group. The data suggest that two separate introductions or founding events of *D. citri* occurred in the Americas, one in South America and one in North America. Furthermore, North America and Hawaii appear to share a similar source of invasion. These data may be important to the development of biological control programs against *D. citri* in the Americas.

KEY WORDS mitochondrial cytochrome oxidase subunit I gene, phylogeography, haplotypes, geographic structure, biological control

Diaphorina citri Kuwayama (Hemiptera: Psyllidae), of Far East origin, is a world-wide economic pest because it vectors phloem-limited bacteria belonging to the genus *Candidatus Liberibacter* that cause a deadly citrus disease known as Huanglongbing (HLB) or greening disease (Aubert 1987). Huanglongbing is considered to be one of the most serious diseases of citrus (da Graça 2008). At present, there are three known species or strains that cause HLB: *Ca.* L. asiaticus (Jagoueix et al. 1994); *Ca.* L. africanus (Garnier et al. 2000); and *Ca.* L. americanus (Coletta-Filho et al. 2005, Teixeira et al. 2005) in Asia, Africa, and America (Brazil), respectively. *Diaphorina citri* has been recorded in Brazil for the last 70 yr (Lima 1942, Catling 1970) and recently *D. citri* has expanded its range within both continents of the Americas. It has spread into states in Mexico; in the United States, including Florida, Texas, Hawaii, and California; Central American countries (e.g., Belize and Costa Rica); and South American countries (Argentina, Paraguay, Uruguay, and Venezuela) (French et al. 2001; Halbert and Manjunath 2004; Halbert and Nuñez 2004; Citrus Research Board 2008; Gomes 2008a,b,c; da Graça 2008; Hall 2008).

A phylogeographic approach was implemented by sequencing the partial mitochrondrial cytochrome oxidase subunit I gene (COI). Phylogeography is a widespread approach for delineating morphologically similar species; in addition, this approach has been used in identifying geographic origins and geographic structures of invasive species (Avise 2000; Roderick and Navajas 2003; Brown 2004; de León et al. 2004, 2006, 2008; Roderick 2004; Triapitsyn et al. 2008; de León and Sétamou 2010). Because *D. citri* has been in Brazil for the last seven decades (Lima 1942, Catling

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Table 1. List of the 22 *D. citri* populations collected in the American continent and one in the Pacific

D. citri populations	n	Haplotypes (n)	Diagnostic nt no. 48
Group 1			
South America			
1. Matõ, Sõ Paulo, Brazil	6	1(5), 2(1)[a]	G
2. Araraquara, Sõ Paulo, Brazil	6	1(6)	G
3. Fraile Pintado, Jujuy, Argentina	5	1(3), 3(1)[a], 4(1)[a]	G
4. Yuchan, Salta, Argentina	5	1(4), 5(1)[a]	G
5. Bella Vista, Corrientes, Argentina	5	1(5)	G
6. Federación, Entre Rios, Argentina	5	1(4), 6(1)[a]	G
7. Itapúa, Paraguay	8	1(6), 7(1)[a], 8(1)[a]	G
8. Itapebí, Salto, Uruguay	6	1(6)	G
Group 2			
North America			
9. Belize	6	9(6)	A
10. Costa Rica	6	9(5), 10(1)[a]	A
11. San Luis Potosí, México	5	9(5)	A
12. Akil, Yucatán, México	5	9(5)	A
13. Cd. Victoria, Tamaulipas, México	5	9(3), 11(2)	A
14. Gen. Terán, Nuevo León, México	5	9(4), 12(1)[a]	A
15. Immokalee, Florida	6	9(4), 13(2)	A
16. Weslaco, Texas	6	9(4), 14(1)[a]	A
17. Del Rio, Texas	3	9(3)	A
18. Houston, Texas	5	9(4), 15(1)[a]	A
19. Palacios, Texas	5	9(5)	A
20. San Antonio, Texas	5	9(5)	A
21. Refugio, Texas	5	9(3), 16(1)[a], 17(1)[a]	A
22. Los Angeles, California	10	9(8), 18(1)[a], 19(1)[a]	A
Pacific			
23. Hilo, Hawaii (10)	10	9(6), 20(1)[a], 21(1)[a], 22(1)[a], 23(1)[a]	A

Shown are the haplotypes identified followed in parenthesis by the number of individuals carrying that haplotype.
[a] Indicates that they are present in only single individuals and are thus considered rare haplotypes. Nucleotides (nt): G, guanine and A, adenine.

1970), we asked whether 1) *D. citri* could have invaded North America via South America, and 2) geographic structure exists (Roderick 1996). Populations of *D. citri* from four countries in South America (Brazil, Argentina, Paraguay, and Uruguay); four countries in North America (Belize, Costa Rica, Mexico, and United States); and for comparison, a population from the Pacific (Hawaii) was analyzed.

Materials and Methods

Insect Collection. Eight populations of *D. citri* (n = 46) were collected in South America from four countries (Table 1) from various citrus host plants, 14 populations (n = 75) were collected from four countries in North America, and one population (n = 10) was collected from the Pacific. Populations were collected from four provinces in Argentina, two (Jujuy and Salta) in the northern region and two (Corrientes and Entre Rios) are in the northeastern region of the country. In Brazil (São Paulo Province) and Paraguay (Itapúa Province) collections were made in the southern regions of the countries, and in Uruguay (Salto Province) collections were made in the northern region of the country. In Mexico, collections were made from four states, San Luis Potosí, located toward the center of the country, Tamaulipas and Nuevo León, in the northeastern region, and Yucatán, in the southeastern region. Six populations were collected in the state of Texas, covering the central, south central, southern, and northeastern central regions of the state. In Florida a population was collected in the southern region of the state. A population from Hilo, HI, also was included. Voucher specimens of *D. citri* used in the current study are deposited at the Entomological Laboratory of Texas A&M-Kingsville Citrus Center in Weslaco, TX.

Genomic DNA Isolation, Polymerase Chain Reaction (PCR) Amplification, and Sequencing. Total genomic DNA extraction per individual was performed as described in previous works (de León et al. 2008). Briefly, individual specimens were homogenized on ice in 1.5-ml microfuge tubes in 60 μl of lysis buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 7.5], 1% IGEPAL CA-630) with two 20-s burst with 10-min intervals on ice (Pellet Pestle Motor, Bel-Art Products, Pequannock, NJ). To avoid cross contamination between samples, a sterile plastic pestle was used per individual insect. The final DNA pellet was resuspended in 61 μl of TE [Tris-HCl (pH 7.5), 1 mM EDTA (pH 7.5)].

The general primers C1-J-1718 (forward: 5′-GGAGGATTTGGAAATTGATTAGTTCC-3′) and C1-N-2191 (reverse: 5′-CCCGGTAAAATTAAAATATAAACTTC-3′) of Simon et al. (1994) were used (Tm 58°C; 2.0 mM $MgCl_2$; 2 U *Taq*DNA Polymerase; 40 cycles) to amplify the COI partial gene from *D. citri.* Amplification products (518 bp) were subcloned with the TOPO Cloning Kit (Invitrogen Life Technologies, Carlsbad, CA), plasmid minipreps were prepared by the QIAprep Spin Miniprep Kit (Qiagen Inc, Valencia, CA), and sequencing was performed by Davis Se-

quencing (Davis, CA) as described previously (de León et al. 2008). Only a few individuals amplified with the current primers, so these few COI sequences were aligned and new 'in-house' primers were designed that included the DNA barcoding region. The new primers Dc-F/R (Dc = *Diaphorina citri; F* = forward [GGAGCTCCCGATATAGCTTTCCC] and R = reverse [GGTATAAGATTGGGTCTCCACCTC]) (Tm 60°C; 1.5 mM $MgCl_2$; 40 cycles) amplified (433 bp) well across all *D. citri* populations. The COI partial *D. citri* sequences were deposited in GenBank. The accession numbers for the 23 *D. citri* COI haplotypes are HM347565-HM347587. We acknowledged *D. citri* COI sequences deposited in GenBank that ranged in size from ≈612–812 bp. An alignment with GenBank accession number FJ190278 (812 bp) with our current 433 bp COI fragment demonstrated that there was only a 10-bp overlap at the 3′-end of the 433-bp fragment to that of the 5′-end of the 812-bp fragment (FJ190278). Therefore, a direct comparison of the COI sequences in the current study and the COI sequences of *D. citri* deposited in GenBank could not be made.

Genetic Diversity and Genetic Population Structure. Genetic tests were calculated with the program Arlequin version 3.11 (Excoffier et al. 2005). Haplotype/gene (h) diversity (Nei 1987) and nucleotide diversity (π) (Nei and Jin 1989) indices were calculated using two genetic distance methods (pairwise difference and Kimura-2P [K2P] distance model [Kimura 1980]). The data were tested for selective neutrality using Tajima's *D*-test (Tajima 1989*Fs*-test (Fu 1997) under the infinite site model with 10,000 simulations. Significantly negative values for the neutrality tests could suggest the presence of selection or the occurrence of population growth. Hierarchical genetic structure was estimated by analysis of molecular variance (AMOVA) by the method of Excoffier et al. (1992). The pairwise difference and K2P methods were used to estimate genetic distances. Levels of significance of Φ_{ST}-statistics were determined by 16,128 random permutations. Because no differences were seen between the two genetic distance methods (pairwise difference and K2P) in the current study, we only report the results with the pairwise difference method.

Two network programs were used for the intraspecific analyses of the evolutionary relationships among haplotypes. A 95% confidence ParsimonySplits network with 1,000 bootstrap replications was performed with the program SplitsTree4 (Huson 1998, Huson and Bryant 2006). Parsimony splits construct split networks based on character data (Bandelt and Dress 1994). Split networks provide an implicit picture of evolutionary relationships (Huson and Bryant 2006). A genealogical analysis that includes the incorporation of haplotype frequencies was performed with the program TCS 1.21 (Clement et al. 2000) by the method of Templeton et al. (1992). The statistical parsimony network was performed at the 95% confidence level.

Results

Genetic Diversity. A 433-bp COI fragment was generated in all individuals (n = 132) with the newly designed primers. Size differences in the COI partial gene were not observed between individuals of any population, making the alignment of the DNA sequences straightforward. The phylogeographic analysis uncovered haplotype variation in populations of *D. citri* (Table 1). In total, 23 haplotypes (hp) were identified in the populations, indicating that ≈17% of individuals carried a different haplotype and these haplotypes fell into two groups. Haplotypes 1–8 were identified in populations from South America (group 1), whereas hp9–19 were identified in populations from North America (group 2). Haplotypes 9 and 20–23 were identified in Hawaii, and this population showed great affinity to group 2. In South America, hp1 was present in the highest frequency within each population and within the group (39 out of 46 individuals [85%]); likewise, in North America, hp9 was observed with the highest frequency (64 out 76 individuals [84%]). With Hawaii included, the rate was similar at 81%. This is an important observation because it is suggested that the haplotype with the highest frequency may be the ancestral or founding haplotype (Crandall and Templeton 1993). It is also possible that some of these rare or singleton haplotypes could be artifacts of the PCR and cloning process. Haplotypes were unique to each group as sharing of haplotypes was not observed between the two *D. citri* groups, indicating strong geographic structure. Group-specific diagnostic nucleotides were identified that allowed the discrimination of the two groups. At nucleotide number 48, all individuals (n = 46) within group 1 contained a guanine (G), whereas, all individuals (n = 86) within group 2 contained an adenine (A). This change in nucleotide led to a change in a restriction enzyme site that may allow the design of diagnostic assays to discriminate the two groups. Restriction enzyme *Tso*I specifically only cuts the COI gene in individuals for group 1, whereas restriction enzyme *Hin*4I specifically only cuts in individuals from group 2. The current analysis was performed to identify diagnostic nucleotides, so all of the variable sites found throughout the COI gene that lead to the description of different haplotypes are not shown here.

Eight polymorphic sites were identified in South America and 12 were identified in North America. Equally low genetic diversity levels were identified within each group (excluding Hawaii): h = 0.28 ± -0.09 and 0.29 ± 0.07 for group 1 and group 2, respectively, and $\pi = 0.0008 \pm 0.0001$ and 0.0009 ± 0.001 for group 1 and group 2, respectively. The tests for selective neutrality were both significantly negative for each group: Tajima's D for group 1 was -2.24 ($P < 0.001$) and for group 2 was -2.35 ($P < 0.001$), and Fu's Fs for group 1 was -8.45 ($P < 0.001$) and for group 2 was -13.32 ($P < 0.001$). The values for the entire American hemisphere (including Hawaii) were -2.37 ($P < 0.001$) (Tajima's D) and -26.71 ($P < 0.001$) (Fu's

Table 2. Analysis of molecular variance (AMOVA) of *D. citri* populations from the American continent and the Pacific

Source of variation	d.f.	Sum of squares	Variance components	%variation	Fixation indices
Among groups	1	30.176	0.500	70.95	$\Phi_{CT} = 0.709^a$
Among pops. within groups	21	4.709	0.007	0.59	$\Phi_{SC} = 0.020^b$
Within pops.	109	21.850	0.200	28.46	$\Phi_{ST} = 0.715^a$
Total	131	56.735	0.704	100.00	

[a] $P < 0.001$.
[b] equals ns, non-significant.
d.f., degrees of freedom.

Fs), rejecting the null hypothesis of neutral evolution for the COI marker.

Genetic Population Structure. The significance of the observed genetic structure in *D. citri* populations was verified by analysis of molecular variance (AMOVA). The AMOVA analysis of all populations was carried out based on the identification of the two groups and the results are shown in Table 2. The highest source of variation was seen among groups ($\Phi_{CT} = 0.709$; $P < 0.001$). A similar value was obtained when the Hawaii population was excluded from the analysis ($\Phi_{CT} = 0.733$; $P < 0.001$). This fixation index value indicates that ≈71–73% of the variance is distributed among groups, whereas ≈26–28% is distributed within populations.

Analyses of evolutionary relationships among haplotypes were performed with two phylogenetic network programs. A 95% confidence ParsimonySplits network is shown in Fig. 1A. The phylogenetic network identified two *D. citri* groups, one in South America (group 1) and one in North America and Hawaii (group 2), confirming the results seen in Tables 1 and 2 . The following haplotypes were identified at the nodes of the network of each group, hp1 in group 1 and hp9 in group 2. These results implicitly suggest that each of these haplotypes is the ancestral or founding haplotype within each group. The haplotypes branching out of the nodes are considered recent evolutionary derivatives of each ancestral haplotype (Huson and Bryant 2006). The second analysis was a genealogical analysis that includes the incorporation of haplotypes frequencies. A 95% confidence statistical parsimony network is shown in Fig. 1B. The haplotypes with the greatest area were hp1 and 9, whereas most other haplotypes are considered recent evolutionary derivatives of these haplotypes. These results suggest that hp1 and nine are each the ancestral haplotypes within their respective group (Crandall and Templeton 1993). The two founding haplotypes were separated by a one mutational event. The rare haplotypes in each group differed by two steps, but the rare haplotypes between groups differed by three steps; therefore, greater differences between groups are seen than within groups, in agreement with the results on Table 2. In addition, the results of the two types of haplotype networks were in accord with each other.

Discussion

The current phylogeographic analysis inferred from COI sequence data suggests that two separate intro-

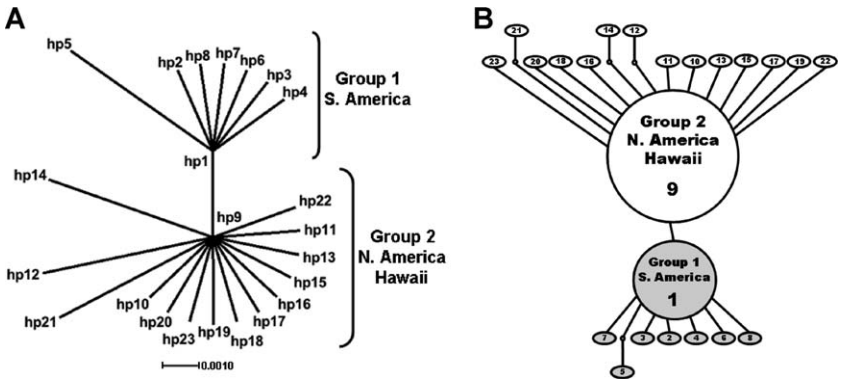

Fig. 1. Networks showing the evolutionary (A) and genealogical (B) relationships among COI haplotypes identified in *D. citri* populations from the Americas and Hawaii. A). The 95% confidence ParsimonySplits network was based on 1000 bootstrap replications. Haplotype 1 and 9 at the nodes of the network are implicitly considered ancestral, for group 1 (South America) and group 2 (North America and Hawaii), respectively. The haplotypes branching out are considered recent derivatives of the ancestral haplotypes. B). The 95% confidence statistical parsimony network includes the incorporation of haplotypes frequencies. The area of each oval is directly proportional to the frequency of each haplotype and the empty small circles are putative unsampled haplotypes. Each line represents a single mutational step. Haplotypes 1 and 9 are considered the ancestral or founding haplotypes of their respective group.

ductions occurred in the Americas. In addition, the population of *D. citri* from Hawaii was more closely related to the North American Group. Predicted ancestral haplotypes were identified by two separate phylogenetic networks with (TCS) and without (SplitsTree) the incorporation of haplotype frequencies. The analysis with the TCS program identified the ancestral haplotypes because each was present in the highest frequency within each population and within their respective group. Of great importance is the fact that haplotype sharing was not observed between the two groups. In addition, a group-specific diagnostic nucleotide at position 48 of the COI partial gene was uncovered that led to the identification of restriction enzymes that cut at group specific sites.

Significantly negative neutrality tests (Tajima's *D* and Fu's *Fs*) were obtained within each *D. citri* group, rejecting the null hypothesis of neutral evolution of the COI partial gene. The low genetic diversity (h and π) level seen here is consistent with recent introductions events and could suggest a single introduction of haplotypes per American continent. Each group of *D. citri* in the Americas appears to show ongoing radiation as evidenced by its low genetic diversity levels and expanding range, as would be expected for invading populations (recent colonization) in the process of establishment. It is interesting to speculate whether the two groups identified in the *D. citri* populations could be lineages rather than groups, however, more research is required to explore this possibility and these plans are in progress.

The results of the AMOVA analysis confirmed that the highest source of variation was identified among groups, demonstrating significant geographic structure at the COI gene. Furthermore, the data suggests highly restricted gene flow between the populations of the two groups, whereas it suggests high gene flow within the populations of each group. The two haplotype networks discriminated the *D. citri* populations into two groups. In addition, the two networks were in congruence, suggesting that both hp1 and nine were the ancestral or founding haplotypes in their respective group.

The current information is important because of the fact that *D. citri* has been in Brazil for the last 70 yr (Lima 1942, Catling 1970). The pattern in the data suggest that *D. citri* populations did not invade North America via South America, but rather, each continent may have been invaded from a different Asian source. An alternative explanation is that hp9 in North America derived from hp1 in South America. For *D. citri* from North America to have derived from South America would imply that the substitution would have had to occur immediately before transferring to North America, and that the North American transfer was most likely a point source introduction, such as a single female or a small population, because we did not find hp9 in South America. What is highly unlikely is the accumulation of 17 new substitutions in the last 20 yr since the North American invasion. This would be a mutation rate of ≈2% in 20 yr. Standard mitochondrial lore (Brower 1994) would require an estimated one million years for that many mutations.

Future plans include collecting world-wide populations of *D. citri* to determine the origin that invaded the Americas. Pinpointing the precise source of invasion is critical for collection of natural enemies in the native range of the pest. Natural enemies have usually co-evolved with the target pest and therefore, usually have highly specialized host-finding abilities that may increase the potential success of a biological control program (Narang et al. 1993, Unruh and Woolley 1999, Brown 2004, de León et al. 2004, Roderick 2004). This was successfully performed for the glassy-winged sharpshooter (*Homalodisca vitripennis*) (Hemiptera: Cicadellidae) that invaded California (de León et al. 2004). If multiple invasions indeed are confirmed, natural enemies [e.g., *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae)] of *D. citri* may need to be collected from different Asian sources for each American continent.

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