Research Article

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Global genetic variation in the Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Liviidae) and the endosymbiont *Wolbachia*: links between Iran and the USA detected

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Abstract

BACKGROUND: The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is one of the most serious pests of citrus in the world, because it transmits the pathogen that causes citrus greening disease. To determine genetic variation among geographic populations of *D. citri*, microsatellite markers, mitochondrial gene cytochrome oxidase I (mtCOI) and the *Wolbachia*–*Diaphorina*, *wDi*, gene *wsp* sequence data were used to characterize Iranian and Pakistani populations. Also, a Bayesian phylogenetic technique was utilized to elucidate the relationships among the sequences data in this study and all mtCOI and *wsp* sequence data available in GenBank and the *Wolbachia* database.

RESULTS: Microsatellite markers revealed significant genetic differentiation among Iranian populations, as well as between Iranian and Pakistani populations ($F_{ST} = 0.0428$, $p < 0.01$). Within Iran, the Sistan–Baluchestan population is significantly different from the Hormozgan (Fareghan) and Fars populations. By contrast, mtCOI data revealed two polymorphic sites separating the sequences from Iran and Pakistan. Global phylogenetic analyses showed that *D. citri* populations in Iran, India, Saudi Arabia, Brazil, Mexico, Florida and Texas (USA) are similar. *Wolbachia*, *wDi*, *wsp* sequences were similar among Iranian populations, but different between Iranian and Pakistani populations.

CONCLUSION: The South West Asia (SWA) group is the most likely source of the introduced Iranian populations of *D. citri*. This assertion is also supported by the sequence similarity of the *Wolbachia*, *wDi*, strains from the Florida, USA and Iranian *D. citri*. These results should be considered when looking for biological controls in either country.

Keywords: microsatellite markers; mtCOI; *wsp*; citrus greening disease; Huanglongbing; biosecurity

1 INTRODUCTION

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is the vector of *Candidatus* Liberibacter spp., the bacterium which causes Huanglongbing (HLB), throughout Asia and America (North, Central and South America).[1–5] Huanglongbing or citrus greening is the most devastating of all citrus diseases worldwide.[2–5] The Asian citrus psyllid has invaded many countries in the Arabian Peninsula, Indian Subcontinent, South East and South West Asia, the USA, Central and South America and the Indian Ocean islands of Mauritius and Réunion.[3–5] The first report of *D. citri* in Iran was in December 1997 in Sistan–Baluchestan province.[6] By 2005, large populations of *D. citri* were found in the major lime [*Citrus aurantifolia* (Christm.) Swing] growing region, Hormozgan province, followed by citrus greening disease pathogen which was collected from Sistan–Baluchistan and Hormozgan provinces.[1,7] Moreover, this pest has been established in Kerman and Fars provinces.[8]

Invasive pest species have rapid evolutionary events,[9] and introduced species that rapidly colonize broad areas lead to a higher occurrence of short-term stochastic or deterministic evolutionary changes, possibly resulting in speciation.[10,11] Information on the genetic variation can be used to identify the geographic origin of the invaders and this is valuable when looking for effective biological control agents, and understanding basic biology and ecology.[12]

Genetic markers such as microsatellites and mitochondrial DNA sequences can be used to estimate many parameters of

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interest to ecologists, such as migration rates, population size, bottlenecks and kinship.[13] In recent years, microsatellites have been successfully used in the genetic differentiation and gene flow of different insects.[14–16] The relationship among collections of *D. citri* from 15 countries worldwide has been investigated using mitochondrial cytochrome oxidase I (mtCOI) by Boykin *et al*.[12] They suggested the existence of eight haplotypes (*Dcit-1* to *Dcit-8*) of *D. citri* globally.[12] Moreover, they revealed two major haplotype groups, South West Asia (SWA) and South East Asia (SEA).[12] The recent invasion into the New World (USA and Mexico) originated from the SWA group only, while in Brazil (in separate locations) both the SEA and SWA groups were found.[12] Saha *et al*[17] reported that the *Wolbachia–Diaphorina citri*, *wDi*, was most closely related to the SWA group also. However, there has been no analysis from Iran. Boykin *et al*[12] suggested a need to utilize other genetic markers such as microsatellites for a better survey of *D. citri* diversity. In another study, 12 polymorphic microsatellite markers were developed for *D. citri*.[18]

Many insect species depend on obligate bacterial mutualism for their survival, viability and reproduction.[19] Among the best studied is *Wolbachia*, an obligate intracellular parasite that can be transmitted maternally from infected females to their progeny causing various reproductive abnormities in its host.[20] The *wsp* gene, which is associated with coding surface proteins of *Wolbachia*, evolves at a much faster rate than the genes *16S* or *ftsZ*, and has been used to characterize *Wolbachia* strains.[21] Because *Wolbachia* endosymbionts and their hosts have a co-evolutionary relationship, genetic differences among symbionts from different host populations are valuable in assessing host–population genetics.[22]

Despite the economic importance of *D. citri*, the genetic variation in this species has not been studied in different geographic areas in Iran. Moreover, little is known about the source of introduced populations, which are important for establishing a management strategy for this pest. The objective of this study was to determine the genetic variation among Asian citrus psyllid populations from different geographic locations in Iran and Pakistan, using simple sequence repeat (SSR) markers, mtCOI and *wsp* sequence analyses. In addition, it was to determine the probable source of the Iranian populations of *D. citri* based on a global phylogeny analysis of mtCOI and *wsp* sequences obtained in the current study with those available in GenBank (http://www.ncbi.nlm.nih.gov/).

2 MATERIALS AND METHODS

2.1 Psyllid samples

During 2011, six geographical populations of *D. citri* were collected on lime, *Citrus aurantifolia* (Christm.) Swingle, from orchards in major citrus-growing areas in Iran and Pakistan (Fig. 1 and Table 1). Moreover, *D. citri* mtCOI and *wsp* sequences deposited in GenBank were included in our global analyses (Table 2).

2.2 DNA extraction

Adults were killed and stored in absolute ethanol. For each population, DNA was extracted from single specimens using the NucleoSpin® Tissue XS kit Düren, (Germany).

2.3 Amplification and sequencing

2.3.1 Amplification of microsatellite loci and genotyping

Initially, six SSR primer pairs including *Dci01*, *Dci02*, *Dci03*, *Dci04*, *Dci06* and *Dci08* were tested,[4] of which the latter three were

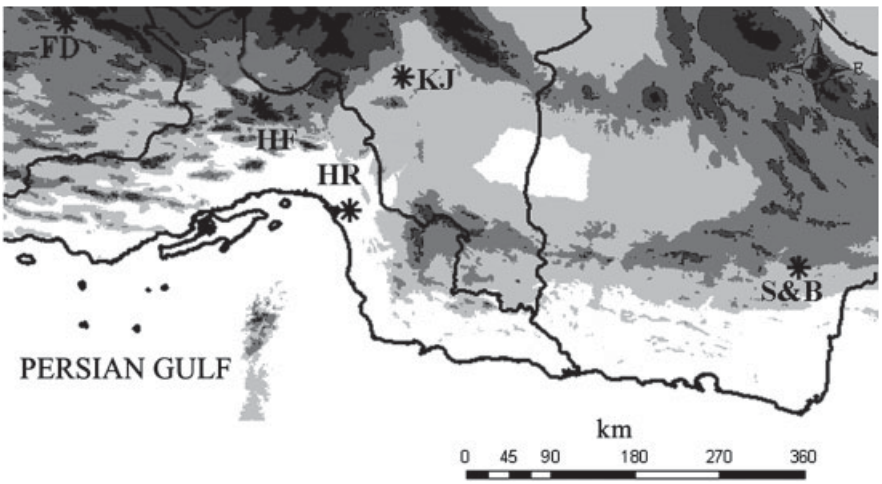

Figure 1. Distribution of sample locations in the south of Iran. See Table 1 for abbreviations.

Table 1. Collection site, code and geographic coordination of sampled *D. citri* populations in this study.

Province	Region	Code	Latitude	Longitude	Altitude (m)
Kerman	Jiroft	KJ	28°16′0.8″N	57°36′7.26″E	508
Hormozgan	Rudan	HR	27°10′3.6″N	57°9′40.70″E	370.5
Hormozgan	Fareghan	HF	28°02′1.5″N	56°14′32.2″E	1100
Fars	Darab	FD	28°46′52.9″N	54°22′13.6″E	1094
Sistan & Baluchestan	Sarbaz	S&B	26°3′26.72″N	61°24′25.4″E	300
Pakistan	Punjab	P	–	–	50–380

Table 2. Accession numbers for mtCOI and *wsp* gene sequences obtained from GenBank and the *Wolbachia* database.

Gene	Locality	Accession No.	Reference
mtCOI	Florida, St. Lucie, Co.	FJ190167	Boykin *et al.*[12]
	Florida, Palm Beach, Co.	FJ190172	
	Florida, Brevard, Co.	FJ190211	
	Florida, Miami-Dade, Co.	FJ190256	
	Florida, Broward, Co.	FJ190310	
	Florida, Indian River, Co.	FJ190187	
	Florida, Okeechobee, Co.	FJ190207	
	Florida, Martin, Co.	FJ190221	
	Florida, Collier, Co.	FJ190372	
	Texas Hidalgo, Co.	FJ190177	
	Brazil Sao Paulo	FJ190228	
	Brazil Sao Paulo	FJ190321	
	Puerto Rico,Univ. of PR	FJ190260	
	Indonesia, Java	FJ190263	
	Indonesia, Bali	FJ190266	
	Vietnam, Hanoi	FJ190272	
	Taiwan, Taipei	FJ190283	
	Pakistan, Punjab	FJ190288	
	Thailand, Hat Yai	FJ190293	
	China, Fuzhou	FJ190357	
	China, Gangzhou	FJ190366	
	Mexico, Akil Yucatan	FJ190300	
	Mexico, Nuevo Leon	FJ190306	
	Mauritius	FJ190312	
	Réunion	FJ190317	
	Saudi Arabia	FJ190337	
	India	FJ190342	
	Guadeloupe	FJ190346	
wsp	USA, Florida	AF217721	Jeyaprakash and Hoy[20] Meyer and Hoy[39]
	China, Beihai	GQ385974.1	Wang[40]
	China, FuZhou	GU480071.1	
	China, Shenzhen	GU480072.1	
	Brazil	584[a]	Guidolin and Consoli[41]
	Brazil	531[a]	
	Brazil	584[a]	
	Brazil	585[a]	
	Brazil	588[a]	
	Uruguay	583[a]	

[a] Code numbers in the *Wolbachia* database.

selected for subsequent experiments (the others were difficult to amplify). For the selected primers, a fluorescent-labelled primer (FAM; Sigma-Aldrich Co., Gillingham, UK) was incorporated for genotyping reactions according to the method used by Boykin *et al*.[18] A 20-μL polymerase chain reaction (PCR) was carried out in an Applied Biosystems 2720 thermal cycler using 2 μL reaction buffer (10×; Invitrogen, CA, USA), 2 μL (0.2 mM) of dNTPs, 0.5 μL (0.25 μM) primers, 1 μL (5 ng/μL) genomic DNA and 0.28 μL (5 U/μL) of *taq* DNA polymerase (5PRIME®, Gaithersburg, USA). PCR conditions were as follows: initial denaturation at 94 °C (2 min), followed by 35 cycles of denaturation at 94 °C (20 s), annealing at 47, 45 and 55 °C (10 s) for *Dci04*, *Dci06* and *Dci08*, respectively, extension at 65 °C (50 s), and 5 min of final extension at 65 °C .The PCR products were visualized on agarose gels (2%) stained with SYBR® Safe DNA gel stain (Invitrogen). All amplifications were diluted 1 : 150 with distilled water, and then the ratio of 1 : 10 was obtained. The lengths of the PCR products were estimated on a 3130xl Genetic Analyzer. Allele size was given by GENEMAPPER® software v. 3.0 (Applied Biosystems, CA, USA).

2.3.2 Mitochondrial COI and wsp Wolbachia DNA amplification and sequencing

PCR was performed with the primer pair *wsp*–81 F (forward: 5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and *wsp*–691R (reverse: 5′-AAAAATTAAACGCTACTCCA-3′),[23] to amplify the *wsp* gene of the *Wolbachia* DNA. In order to amplify part of cytochrome oxidase subunit I (COI), the forward primers DCITRI COI-L (5′-AGGAGGTGGAGACCCAATCT-3′) and DCITRI COI-R (5′-TCAATTGGG GGAGAGTTTTG-3′) were used.[12] Amplification of COI and *wsp* fragments was performed in a MASTERCYCLER®, Eppendorf Thermal Cycler. The PCR temperature profile for amplification of *wsp* was as follows: 35 cycles of 94 °C denaturation (30 s), 55 °C annealing (1 min), 72 °C extension (5 min) with an initial denaturation for 5 min and a final extension for 10 min. PCR conditions for the amplification of COI were initial denaturation at 94 °C (2 min) followed by 35 cycles of 94 °C denaturation (30 s), 53 °C annealing (30 s), 72 °C extension (1 min) and a final extension at 72 °C (10 min). Amplified fragments were electrophoresed on 1.2% agarose gels at 80 V for 1 h in 1% TAE buffer (pH 8.0), stained with ethidium bromide and visualized under UV light. The PCR products were purified and sequenced by Macrogen Inc. (Seoul, Korea). Sequences of COI and *wsp* have been deposited in the GenBank database under accession numbers KC509561–KC509572 and KC539837–KC539848, respectively.

2.4 Data analysis

2.4.1 Data analysis of SSR markers

The program GDA was used to estimate descriptive statistics,[24] including the mean sample size (n) over all loci, the proportion of polymorphic loci (P), the mean number of alleles per locus (A), the number of alleles per polymorphic locus (A_p), the expected heterozygosity (H_E), observed heterozygosity (H_O) and inbreeding coefficient (f). Linkage disequilibrium was calculated using Fisher's exact test implemented in GENEPOP v. 1.2.[25] Moreover, deviations from Hardy–Weinberg equilibrium (HWE) were examined in GENEPOP v. 1.2 using a Markov chain method.[26] Micro-Checker v. 2.2.3 was used to evaluate the presence of null alleles.[27] Analysis of molecular variance (AMOVA) was performed in ARLEQUIN v. 3.1,[28] to compute F_{ST} and pairwise Slatkin's linearized F_{ST} values.[29] The population structure was analysed by Bayesian cluster techniques using STRUCTURE software v. 2.3.3.[30] The STRUCTURE analysis was done using both the admixture model and the correlated allele frequencies with a burn-in period of 50 000 iterations and 500 000 Markov chain Monte Carlo (MCMC) repetitions. For the whole data set, 10 runs were carried out for each value of K from 1 to 10 and used the DK method of Evanno *et al*[31] to choose the most likely K value. STRUCTURE HARVESTER, a web-based program was used for collating results generated by the program STRUCTURE.[32] To test the hypothesis of isolation by distance, the software NTSYS was used to compute the Mantel correlation coefficient among populations.[33] The program BOTTLENECK[34] was used to test for recent bottlenecks. Geographic distances were obtained with a field GPS unit, and pairwise geographic distances were calculated using geographic information systems (ARC-INFO software).

2.4.2 Data analysis of COI and wsp sequences

Raw sequences of COI and *wsp* were edited manually to remove primers, and then aligned using ClustalX in MEGA v. 5.[35] The obtained sequences were also subjected to a BLAST search on the National Center for Biotechnology Information (NCBI) database to compare them with published sequences. Genetic diversity was initially examined in terms of the number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (Pi) and the average number of pairwise nucleotide differences (κ) between collecting locations, which calculated using DnaSP v. 5.0.[36]

In order to identify the probable source of the Iranian populations of *D. citri*, the COI sequences obtained in this study and those deposited in the GenBank by Boykin *et al*[12] (Table 2) were included in a global phylogenetic analysis. The mtCOI sequence of the Hackberry petiole gall psyllid, *Pachypsylla venusta* (Osten-Sacken) (accession number NC-006157) was chosen as the outgroup for the analysis.[12] A Bayesian analysis was conducted using MrBayes v. 3.2,[29] using parallel implementation on the BeSTGRID computer cluster.[37] Analyses were run for 10 million generations with sampling every 1000 generations. Each analysis consisted of two independent runs, each utilizing three cold and one hot chain. Convergence between runs was monitored by finding a plateau in the likelihood score (standard deviation of split frequencies < 0.0015) and the potential scale reduction factor (PSRF) approaching one. Convergence of other parameters within the runs was also checked using Tracer v. 1.5.4,[38] with ESS values above 200 for each run. The first 12.5% of each run was discarded as 'burn-in' for the estimation of consensus topology and the posterior probability of each node.

The sequences of the *wsp* gene obtained in this study were separately compared with sequences from Florida, China, Brazil and Uruguay strains deposited in the NCBI and *Wolbachia* database (http://pubmlst.org/wolbachia/wsp/) (Table 2).[20,39–41] This was achieved utilizing the Bayesian methods described above.

3 RESULTS

3.1 Genetic variation and population structure based on the SSR markers

A total of 180 individuals belonging to the six geographic populations was successfully genotyped. The mean number of alleles per population was 6.00, 6.00, 6.33, 6.66, 7.00 and 8.33 for FD, HF, HR, KJ, S&B and P, respectively. The mean number of alleles per locus was 7, 13 and 29 for *Dci04*, *Dci06* and *Dci08*, respectively. The average total number of alleles per locus was 16.33. Linkage disequilibrium analysis did not show a significant association between pairs of loci, so it was likely that the three loci represented independent information across the samples. Significant departures from HWE were observed at locus 3, *Dcit08* (Table 3). The results did not show a significant heterozygote excess; therefore, the heterozygote deficiency was associated with the observed departures from HWE, as indicated by the positive inbreeding coefficient (Table 3). The Micro-Checker analysis showed that null alleles might be present at this locus ($p < 0.01$), but further analyses are needed to verify the actual presence.

AMOVA among geographic populations revealed that 4.28, 30.03 and 65.69% of the total variance could be explained as among population ($p < 0.01$), within population and within individual variations, respectively (Table 4).

Pairwise F_{ST} analysis was calculated for each pair of populations over three loci (Table 5). There was a significant difference between Pakistani populations and all Iranian populations. Interestingly, within the Iranian populations, Sistan–Baluchestan was significantly different from the Fareghan (in Hormozgan province) and Darab (in Fars province) populations. Data obtained by STRUCTURE analysis, showed that the highest L (K) averaged over replicates running for each value of K was observed for K = 2 (−759.260000), which confirmed two main clusters, Pakistani and Iranian populations. The matrix of M values (migration rate) based on Slatkin,[42] showed a high level of migration between populations with an average of 2.063. The maximum and minimum levels of migration occurred between HR and KJ, and P with HF and FD populations, respectively (Table 5). The Mantel test did not show any significant correlation between geographic and genetic distances ($p = 0.061$). The Mantel test gave a matrix correlation coefficient (r) of 0.66, suggesting poor correlation between the two matrices. The bottleneck tests did not detect any recent bottlenecks in the populations. In the BOTTLENECK program, the SIGN TEST Probability (under the TPM) was 0.21286, 0.67022, 0.37371, 0.64897, 0.40064 and 0.06820 for populations P, S&B, KJ, HR, HF and FD, respectively.

Table 3. Descriptive statistics for *D. citri* populations, using three microsatellite loci.

Locus	Descriptive statistic	Population P	S&B	KJ	HR	HF	FD
	n	10	10	10	10	10	10
	A_p	7	4	4	5	2	3
Dci04	H_O	0.50	0.50	0.40	0.40	0.70	0.30
	H_E	0.84	0.50	0.60	0.66	0.47	0.42
	f	0.42	0	0.35	0.41	−0.50	0.30
	n	10	10	10	10	10	10
	A_p	7	7	8	6	7	7
Dci06	H_O	0.80	0.80	0.50	0.70	0.70	0.50
	H_E	0.82	0.87	0.84	0.82	0.80	0.81
	f	0.02	0.09	0.41	0.16	0.13	0.39
	n	10	10	10	10	10	10
	A_p	12	10	8	8	9	8
Dci08	H_O	0.50	0.60	0.40	0.30	0.70	0.30
	H_E	0.94	0.91	0.85	0.87	0.84	0.81
	f	**0.48**	**0.35**	**0.54**	**0.66**	**0.18**	**0.64**

n, mean sample size over all loci; A_p, number of alleles per locus; H_E, expected heterozygosity; H_O, observed heterozygosity; f, inbreeding coefficient. f-values in bold represent significant deviations from HWE.

Table 4. Analysis of molecular variance to compare the genetic variation in microsatellite data.

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation	p
Among populations	5	12.875	0.052	4.28	< 0.01
Within populations	54	82.700	0.366	30.03	> 0.05
Within individuals	60	48.000	0.800	65.69	< 0.01
Total	119	143.575	1.28		

3.2 Population variation based on COI and *wsp* sequences

Aligned mtCOI sequences were 796 bp long. Two polymorphic sites and distinct haplotypes of *D. citri* were identified among

Table 5. Matrix of Slatkin linearized F_{ST} as $t/M = F_{ST}/(1 - F_{ST})$.[29] Pairwise population differentiation estimates (F_{ST}) are averaged over three loci between geographic populations of *Diaphorina citri* (above the diagonal); the matrix of M values between *D. citri* populations are given below the diagonal.

Population	P	S&B	KJ	HR	HF	FD
P		0.080*	0.082**	0.099**	0.174**	0.170**
S&B	6.22		0.017	0.024	0.059*	0.054*
KJ	6.081	28.623		0.0002	0.054	0.014
HR	5.027	20.847	Infinite		0.041	0.009
HF	2.868	8.393	9.294	12.162		0.051
FD	2.945	9.322	34.706	56.309	9.900	

*p-values significant at the 0.05 level. **p-values significant at the 0.01 level.

all COI sequences: haplotype A including all Iranian populations and haplotype B including the Pakistani population. The values of h, Hd, Pi and κ were estimated to be 2, 0.33, 0.0008 and 0.66, respectively.

Global relationships based on the COI dataset indicated that all Iranian *D. citri* sequences were clustered distinctly from the Pakistani sequences (Fig. 2). Iranian *D. citri* populations were similar to populations from India, Saudi Arabia, Brazil, Mexico and the USA (Florida and Texas) (Fig. 2), and are characterized as haplotype 1 in Boykin *et al*,[12] whereas the Pakistani populations mentioned are haplotype 6.

The aligned *wsp* sequence was a 450-bp fragment in all populations, of which 445 bp remained constant across all the fragments sequenced. Analysis of *wsp* sequences revealed that two different strains of *Wolbachia* infected Pakistani and Iranian populations (Fig. 3). The difference between these two strains was 5 bp, which resulted in four codon substitutions and subsequent alteration in the quantity of amino acids, including aspartic acid, glutamic acid, lysine, leucine and methionine in the two populations.

Global Bayesian phylogenetic analysis of the *Wolbachia* strains indicated the presence of a similar strain infecting *D. citri* populations in Iran and Florida (Fig. 3), but as mentioned above, the Pakistani population has been infected with a separate strain. Moreover, different *Wolbachia* strains associate with the populations from China, Brazil and Uruguay (Fig. 3), compared with the Iran/Florida and Pakistan strains. Interestingly, similar phylogenetic relationships were recovered in the analysis of mtCOI (Fig. 2).

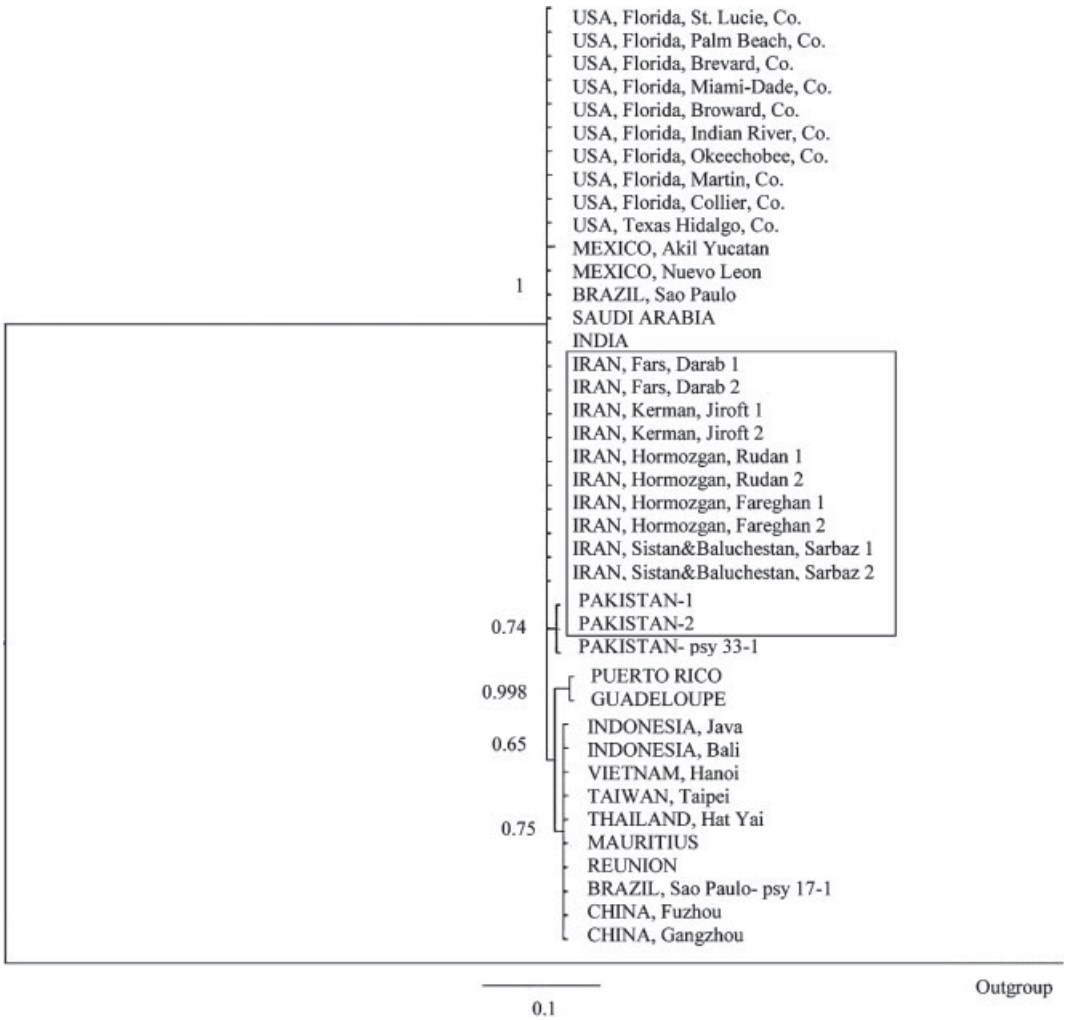

Figure 2. A Bayesian analysis of *D. citri* COI sequences obtained from the present study and Boykin *et al*.[12] using MrBayes v. 3.2. The boxes show COI genes sequenced in this study. See Table 2 for other sequences.

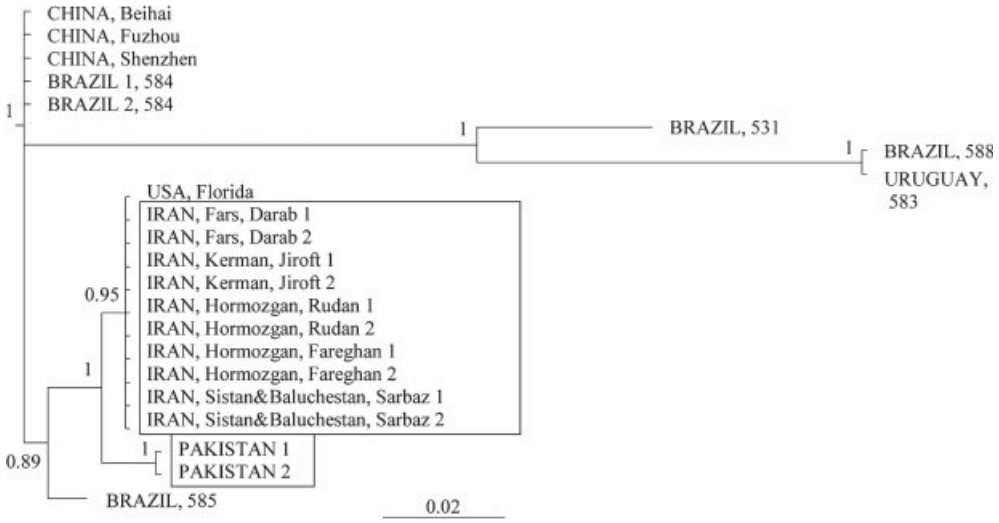

Figure 3. A Bayesian analysis of *Wolbachia* associated with *D. citri* in Iran compared with data available in GenBank and the *Wolbachia* database (See Table 2 for more information). The boxes show *wsp* genes sequenced in this study.

4 DISCUSSION

Based on the results presented here, there is a strong association between the *Wolbachia* strains and mtCOI haplotypes of *D. citri*. Boykin *et al*[12] showed that South West Asia and South East Asia haplotypes did not co-occur in any of the collection sites (except Réunion) and concluded the possible existence of some factors that limited the capacity of these two groups to co-occur. Similar findings were reported using *Wolbachia* analyses from *Diaphorina* collections in Saha *et al*.[17] The current study provides further evidence, i.e. haplotype 1 (Iran) and haplotype 6 (Pakistan) did not co-occur in any of the collection sites. Differences between the Iranian and Pakistani haplotypes suggest that there may be some degrees of reproductive isolation between these haplotypes and that might have been the case for a considerable time. The main explanation for this phenomenon in this study may be due to the endosymbiotic bacterium *Wolbachia*–*Diaphorina*, *wDi*.

Wolbachia is associated with reproductive alteration in its hosts, including cytoplasmic incompatibility (CI).[19,43] CI has two forms, unidirectional and bidirectional. Typically, bidirectional incompatibility occurs when a male and a female harbor different strains of *Wolbachia* that are mutually incompatible.[43] The infection of different populations with different *Wolbachia* strains leads to reproductive isolation between populations.[15] Our results based on the different *wsp* sequences of Iranian and Pakistani populations suggest that such a phenomenon may occur in these haplotypes. It is possible that the Iranian haplotype is infected with one strain of *Wolbachia*, *wDi-SWA*, while a different strain has been spread to the Pakistan haplotype, *wDi-SEA*. Hence, any immigrant into a population with a different mtCOI haplotype would be limited. The data must be interpreted with caution because we only sequenced the *wsp* in two individual insects from each population, and therefore, more evidence is needed to confirm this hypothesis.

These findings are based on global samples of *Wolbachia* (Fig. 3) that support two hypotheses: (1) there is a strong association between the mtCOI gene and *Wolbachia* strains. This is highlighted in Figs 2 and 3 in which Iranian *D. citri* were found to be most closely related to those from Florida (Fig. 2), and similarly, samples from Iran and Florida were found to be infected with the same *Wolbachia* strain, *wDi-SWA* (Fig. 3); (2) all *D. citri* populations of mtCOI haplotype 1 appear to be infected with the similar *Wolbachia* strain. [12] Boykin *et al*[12] showed that only Réunion Island had haplotypes from both the SEA and SWA groups. It is possible that all psyllids tested from Réunion Island populations in Boykin's study were doubly infected with two strains of *Wolbachia* compatible with SWA and SEA *D. citri* haplotypes. It has been documented that a host species can be infected with multiple strains of *Wolbachia*.[44] Keller *et al*[44] showed that CI varied among populations of *Chelymorpha alternans* Boh. They showed that doubly infected males from a western Panama population, Remedios, caused very little CI when crossed with singly infected females from Gamboa and Remedios populations. We suggest testing these hypotheses in future studies.

4.1 Differences between the populations from Iran and Pakistan

The observed departures from HWE were due to heterozygote deficiency. A heterozygote deficit (also known as homozygote excess) occurs when there are more homozygotes than expected under HWE. Heterozygote deficiency, the more common direction of HWE deviation, can be due to inbreeding or selection for or against a certain allele and the Wahlund effect.[13] Both of these causes of the heterozygote deficit should affect all loci.[13] The presence of a null allele is another common cause of a heterozygote deficit in most microsatellite studies.[13,14] In this study, the heterozygote deficit was not observed across all loci, and was indicative of the probable presence of null alleles at some loci. This is not surprising, because microsatellite null alleles have been found in a wide range of taxa.[14]

Microsatellite markers showed that all Iranian psyllid populations were genetically different from the Pakistani population. The highest L (K) in STRUCTURE was for K = 2, which was also supported by COI and *wsp* sequence data. Determination of the M-value showed that gene flow between the Pakistani and Iranian populations was much lower than that within Iranian populations. The obtained sequences from the Pakistani population in this study were identical to the haplotype Dcit-6 assigned by Boykin *et al*.[12] Such a result was observed between the Iranian populations and populations from India, Saudi Arabia, Mexico, Brazil, Florida and Texas (haplotype Dcit-1).[12] The mitochondrial haplotype network

constructed by Boykin *et al*[12] for *D. citri* suggested a basal and thus ancestral position for haplotype Dcit-1. Moreover, some studies suggested a southwestern Asia, i.e. India (haplotype Dcit-1), origin for *D. citri*.[3,5,45] Knowledge from this research may facilitate the identification of effective biological control agents for growers in Florida and Iran alike. It would be advantageous for growers in Iran and the USA to compare control strategies because it appears that the *D. citri* in these two countries are similar (Figs 2 and 3). Several studies have shown that *Tamarixia radiata* varies significantly in effectiveness depending on geographical location, for instance, it has been successfully introduced into Réunion Island, Taiwan, Mauritius, Guadeloupe, Florida, the Philippines and Indonesia (East Java), but generally has provided little biological control in Florida, whereas it significantly reduced populations of *D. citri* in Réunion Island.[3,5] In Florida, *T. radiata* populations from Taiwan and Vietnam were originally released, but other haplotypes of *T. radiata* might be more effective.[5] However, our study has identified that Iranian *D. citri* populations are genetically similar to the *D. citri* haplotype 1 mtCOI. In theory, biological control agents from these regions, i.e. countries containing *D. citri* haplotype 1 in SWA group, should be better adapted to Iranian *D. citri* populations than agents from other regions (e.g., SEA group), and future efforts to identify effective biological control should focus on these areas. The authors also suggest that more sampling from these countries and utilizing other genetic markers such as microsatellites needs to be conducted to better survey diversity.

The fact remains that HLB has not been adequately controlled in any part of the world.[2] Controlling psyllid vectors to reduce transmission has been recommended as a major method for controlling this disease.[2] As a novel method, controlling disease-vectoring insects by manipulation of *Wolbachia* strains have been done for some insects.[46–50] For instance, the bacterium *Wolbachia* has been manipulated as a potential vehicle for introducing disease-resistance genes into mosquitoes to make them resistant to the human pathogens they transmit, also it is demonstrated that *Wolbachia* induces the expression of antimicrobial genes in the *Drosophila*.[46,50] In *D. citri* it is shown that the *Ca*. Liberibacter titer within the insect have a strong positive relationship with *Wolbachia*.[51] Therefore, it is suggest that *Wolbachia*/pathogen/vector interactions be examined for further understanding.

4.2 Differences among Iranian populations

Surprisingly, microsatellite data revealed significant differentiation between the Iranian populations. However, the majority of genetic variation was among individuals within populations and overall, there was a significant difference ($p < 0.05$) between the S&B) population and either the HF or FD population. Although, the geographic distance from the latter two populations to the S&B population was greater than that for other populations, there was no significant correlation between the genetic and geographic distance matrices among populations (Mantel test, $r = 0.665$). Furthermore, a similar *wsp* sequence in these populations might rule out the effect of reproductive isolation caused by *Wolbachia*, suggesting that it might be due to the effect of isolation induced by other microorganisms.[52–55] Moreover, it has been demonstrated that the lost alleles correspond to those that occurred at the lowest frequencies in the populations with the rarest allels (indigenous populations), as would be expected following a population bottleneck.[15] Although the tests did not detect any recent bottlenecks in the studied populations, a reduction in the number of alleles at locus 1 (*Dci04*) in the HF and FD populations has clearly occurred (Table 3). The program BOTTLENECK is not considered to be an inerrable method, particularly when dealing with a small number of loci.[15] Therefore, regarding the reduction in the number of alleles, it is probable that a bottleneck is in process and with further loci, might be detected. Anthropogenic influences on the orchard agroecosystem, such as using unsuitable doses of insecticides, as outlined in Dorn and Gu,[56] and other pest management techniques can affect the structure of *D. citri* populations. Dorn and Gu,[56] showed that low dosages of azinphosmethyl significantly increased the locomotor activity of *Cydia pomonella* L. in the laboratory.

The existence of genetically distinct populations of *D. citri* might have important consequences for the management of this pest. For instance, it is possible that populations of *D. citri* in different regions vary in terms of their ability to transmit '*Ca. Liberibacter asiaticus*'. More studies are needed to effectively design pest management programmes for this highly invasive pests. Understanding the underlying genetic variation in the pest is the best first step as this information can greatly reduce the time and effort that goes into looking for biological controls for a given region.

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