Morphometric comparisons of *Diaphorina citri* (Hemiptera: Liviidae) populations from Iran, USA and Pakistan

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ABSTRACT

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), vector of citrus greening disease pathogen, Huanglongbing (HLB), is considered the most serious pest of citrus in the world. Prior molecular based studies have hypothesized a link between the *D. citri* in Iran and the USA (Florida). The purpose of this study was to collect morphometric data from *D. citri* populations from Iran (mtCOI haplotype-1), Florida (mtCOI haplotype-1), and Pakistan (mtCOI haplotype-6), to determine whether different mtCOI haplotypes have a relationship to a specific morphometric variation. 240 samples from 6 ACP populations (Iran - Jiroft, Chabahar; Florida - Ft. Pierce, Palm Beach Gardens, Port St. Lucie; and Pakistan - Punjab) were collected for comparison. Measurements of 20 morphological characters were selected, measured and analysed using ANOVA and MANOVA. The results indicate differences among the 6 ACP populations (Wilks' lambda= 0.0376, F= 7.29, P <0.0001). The body length (BL), circumanal ring length (CL), antenna length (AL), forewing length (WL) and Rs vein length of forewing (RL) were the most important characters separating the populations. The cluster analysis showed that the Iran and Florida populations are distinct from each other but separate from the Pakistan population. Thus, three subgroups can be morphologically discriminated within *D. citri* species in this study, 1) Iran, 2) USA (Florida) and 3) Pakistan population. Morphometric comparisons provided further resolution to the mtCOI haplotypes and distinguished the Florida and Iranian populations.

Keywords: Asian citrus psyllid, ACP, Citrus, Huanglongbing, HLB

INTRODUCTION

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Burckhardt & Ouvrard, 2012), is the vector of the bacteria '*Candidatus* Liberibacter spp.', the causal agent associated with Huanglongbing (HLB) or citrus greening disease (Bové, 2006; Grafton-Cardwell et al., 2013; Hall et al., 2013). Huanglongbing is considered the world's most important disease of citrus (Gottwald, 2010; Grafton-Cardwell et al., 2013, Hall et al., 2013). In southern Iran, the ACP was discovered in 1997 followed by the HLB disease in 2006 (Bové et al., 2000; Faghihi et al., 2009) and now it has established in the citrus growing regions of Hormozgan, Sistan–Baluchistan, Kerman and Fars Provinces. In the USA, it was first reported from Florida in 1998 (Bové, 2006) and now occurs from Florida to California (Boykin et al., 2012). Also it was reported from Pakistan in 1927 (Husain & Nath, 1927), and has become a serious pest in all citrus growing areas of Pakistan (Riaz et al., 2014).

Kommentar [DB1]: Why do you list these countries and not others. You should give an general overview of the known distribution.

Worldwide genetic diversity of *D. citri*, based on mitochondrial cytochrome oxidase I (mtCOI) DNA sequences, suggests the existence of eight haplotypes (Dcit-1 to Dcit-8) (Boykin et al., 2012). Haplotype-1 consists of the following countries: United States of America (USA: Florida and Texas), India, Saudi Arabia, Brazil and Mexico (Boykin et al., 2012). An additional study revealed that *D. citri* populations from Iran are genetically similar to the mtCOI Haplotype-1 group, while the Pakistan population has been designated as mtCOI haplotype-6 (Lashkari et al., 2014). Further evidence supporting the haplotype grouping comes from *Wolbachia* wsp sequences (Lashkari et al., 2014). Analysis of the *Wolbachia*, *wDi*, wsp gene sequences indicated that the Iran population was similar to the Florida population, but was different from the Pakistan population (Lashkari et al., 2014).

Kommentar [DB2]: And what about haplotypes 2-5?

Morphologically, psyllid species can be differentiated by the shape of the genal cones, the shape and coloration of the forewings, the arrangement of spinules on the forewing membrane, the shape of the female terminalia and antenna length (Hollis, 1987; Hodkinson, 2009). Six morphological measurements, including body length, wing length and width, genal process length and width, and antenna length have been used to study the morphometry of ACP populations on six Rutaceae from Mexico (García-Pérez et al., 2013). Additionally, Vargas-Madríz et al. (2013) used 4 morphological indices including body length, body width, wing length, and wing width to describe the morphometry of another psyllid species, *Bactericera cockerelli* (Hemiptera:Triozidae), on two varieties of host plant.

The purpose of this study was to morphometric comparisons of *D. citri* populations from Iran, Florida and Pakistan, using the traditional morphometric approaches, to explore whether the different mtCOI haplotypes of ACP populations correlate with specific morphometric variation.

MATERIAL AND METHODS

Psyllid samples

D. citri adults were collected from April 2013 to April 2014 in the major citrus-growing areas of Iran (Jiroft) [*Citrus sinensis* (L.) Osbeck], Iran (Chabahar) [*Citrus sinensis* (L.) Osbeck], Florida (USDA ARS colony, Ft. Pierce, St. Lucie County) [*Citrus macrophylla* Wester], Florida (Palm Beach Gardens, Palm Beach County) [*Murraya paniculata* (L.) Jack.], Florida (Port St. Lucie, St. Lucie County) [*Murraya paniculata* (L.) Jack.] and Pakistan (Punjab) [*Citrus sinensis* (L.) Osbeck]. The collected specimens were preserved in 96% ethanol.

Morphometric analysis

Kommentar [DB3]: processes. Inconsistent with table 1.

Kommentar [DB4]: Is this about psyllids in general or Diaphorina? Quite a fe species do not have genal processes, or a pattern on the forewing or lack surface spinules.

Kommentar [DB5]: wrong reference

Kommentar [DB6]: Something is missing here.

A total of 240 female adults (40 adults from each population) were randomly selected. In order to calculate the morphometric information of the specimens, each insect was dissected to separate the different structures. The selected specimens were placed individually in 1.5 ml tubes containing 96% ethanol. Twenty standard morphological characters (Table 1) were selected to survey the morphometric variation among the 4 populations (Hollis, 1987; Ossiannilsson, 1992; Mifsud & Burckhardt, 2002; Hodkinson, 2009; García-Pérez et al., 2013). Descriptions of the characters are given in Table 1 and Fig.1. The body structures (except wings) were mounted on slides with glycerin and photographs were taken of each structure/specimen using a digital camera coupled with a stereomicroscope with 40X magnification. The right forewing of each specimen was slide-mounted using Euparal as the mounting medium. All measurements (mm) were performed with National Instruments Vision Assistant Software, version 2012 (National Instruments Corporation 2012).

Kommentar [DB7]: wrong reference

Kommentar [DB8]: relevant publications are also:
- Burckhardt D. 1986. The mediterranean species of Diaphorina Löw (Homoptera, Psylloidea). Phytophaga, Palermo 2 (1984): 1-29.
- Olivares, T. & Burckhardt, D. 1997. Jumping plant-lice of the New World genus Calinda (Hemiptera: Psyllodea: Triozidae). Revue suisse de Zoologie 104(2): 231-344.
- Burckhardt D. & Basset Y. 2000. The jumping plant-lice (Hemiptera, Psylloidea) associated with Schinus (Anacardiaceae): systematics, biogeography and host plant relationships. Journal of Natural History 34: 57-155.

Kommentar [DB9]: Why didn't you include the male terminalia?

Statistical Methods

Data were analyzed using analysis of variance (ANOVA) to compare different populations for each character, and pairwise comparisons based on Tukey's HSD (Honest Significant Difference) test were calculated only after a significant ANOVA was found. A multivariate analysis of variance (MANOVA) was done for the comparison of the group means of all variables. The Wilks' lambda test was applied as the statistical significance of the MANOVA. Moreover, the Canonical Variate Analysis (CVA) was used to determine the relative importance of characteristics as discriminators between groups. Mahalanobis distances (D2) were calculated between all populations' centroids using a pooled variance covariance matrix. All statistical analyses were conducted using the SAS statistical program (SAS Institute, 2003). The UPGMA

Kommentar [DB10]: Did you perform these analyses only for females? Why?

(Unweighted Pair Group Method with Arithmetic Mean) hierarchical cluster analysis (Sneath & Sokal, 1973) based on squared Euclidean distances and the mantel tests were performed with NTSYS-pc program (Rohlf, 1993). Geographic distances among locations were measured using the Google Earth.

RESULTS

According to univariate analysis, 15 morphological characters were found to be significantly different among the 4 ACP populations (α= 0.01). These included body length (BL) (F = 78.07, df = 5, P < 0.0001), Vertex width (VW) (F = 4, df = 5, P = 0.0019), antenna length (AL) (F = 11.63, df = 5, P < 0.0001), forewing length (WL) (F = 28.50, df = 5, P < 0.0001) and width (WW) (F = 11.19, df = 5, P < 0.0001), Rs vein length of forewing (RL) (F = 26.92, df = 5, P < 0.0001), length of the line connecting apices of vein Rs and Cu1a of forewing (RC) (F = 7.27, df = 5, P < 0.0001), length of the line connecting the base and apex of vein M3+4 of forewing (b) (F = 6.38, df = 5, P < 0.0001), length of the line connecting apices of veins M1+2 and M3+4of forewing (c) (F = 9.69, df = 5, P < 0.0001), length of the line connecting apices of vein Cu1a and Cu1b of forewing (d) (F = 15.58, df = 5, P < 0.0001), length of widest perpendicular distance to d in cell cu1 (e) (F = 3.85, df = 5, P = 0.0025), metatibial length (ML) (F = 5.19, df = 5, P = 0.0002), female proctiger length (FP) (F = 14.69, df = 5, P < 0.0001), circumanal ring length (CL) (F = 15.74, df = 5, P < 0.0001) and female subgenital plate length (SL) (F = 8.94, df = 5, P < 0.0001) (Table 2).

The results from the HSD post-hoc test revealed that in most variables, the ACP populations from Iran and Florida were clearly similar and differentiated from the Pakistan population (Table 2). The smallest significant value of the following characters were found in the

Pakistan population ($P \leq 0.01$): body length (BL), antenna length (AL), metatibial length (ML) , female proctiger length (FP), circumanal ring length (CL) and female subgenital plate length (SL). There was no statistical difference between populations with regards to the following characters: head width (HW), vertex length (VL), genal process length (GL), genal process width (GW) and length of the line connecting the base and apex of vein M1+2 of forewing (a).

The MANOVAs of the ACP populations revealed a significant difference among the size variables of the populations (Wilks' lambda= 0.0376, F= 7.29, $P < 0.0001$). The shortest Mahalanobis distance (D2 = 0.720) was between the two populations from Florida (Palm Beach Gardens and Port St. Lucie), whereas the longest distance was between the populations from Pakistan and Florida (Palm Beach Gardens) (D2 = 36.756) (Table 3). The cluster analysis revealed three major clusters. The first contained samples from Iran (Jiroft, Chabahar), the second contained those from Florida (Ft. Pierce, Port St. Lucie, Palm Beach Gardens) and the third one contained the Pakistan population.

The canonical discriminant analysis indicated that the first two canonical variables (CVA1 and CV2) described 65.15 % and 24.85 % of the total variance, respectively. The first and second together (CVA1+CVA2) equaled 90 % (Table 4). The body length (BL), circumanal ring length (CL), antenna length (AL), forewing length (WL) and Rs vein length of forewing (RL) contributed most to this variation based on the first canonical axes (CVA1). Other characteristics also contributed, but to a lesser extent (Table 4).

The Mantel test showed that there was not significant correlation between geographic and morphological distances ($r = 0.535$, $p = 0.999$). Therefore, geographical distances did not impact the morphological differentiation found between the populations.

DISCUSSION

The morphometric analyses of the ACP populations from Iran, USA (Florida) and Pakistan indicated the existence of three groups within the populations analyzed. The first group included populations from Iran (Jiroft and Chabahar), the second from USA (Florida), and the third was represented by a population from Pakistan. These results support similar findings from wing structures of ACP from Iran and Pakistan (Lashkari et al., 2013). The results presented here also support previous findings indicating that *D. citri* populations in Iran and Florida are separated from Pakistan populations based on a global phylogenetic ~~analyses~~ analysis of mtCOI, and *Wolbachia* wsp sequences Lashkari et al. (2014). Prior molecular based studies showed that all Iranian populations of ACP are genetically similar to the Florida populations indicating a link between the ACP in Iran and the USA (Florida) (Lashkari et al., 2014). In contrast, the morphometric data provides further resolution to the previous molecular research, which indicated that the Iran and Florida populations were indistinguishable using mtCOI. It is not uncommon for molecular and morphologically derived phylogenies to differ. It has been shown that the morphological and molecular changes may be independent; responding to different evolutionary pressures (Hillis & Moritz, 1990). For example, Bomfim et al. (2011) showed a size similarity between populations of *Anastrepha pickeli* Lima (Diptera: Tephritidae) from Brazil, Bolivia and Paraguay using the traditional morphometry, but molecular phylogenetic analysis based on sequencing of a fragment of 1,050 bp within the mitochondrial COI gene indicated that the populations clustered into three clades. Also, Gómez-Palacio et al. (2012) showed intralineage variability related to the genetic and morphological differences between *Rhodnius pallescens* (Hemiptera: Reduviidae) populations. By analyzing the mitochondrial cyt *b* gene, they discovered two evolutionary lineages, the Colombian (Colombia-North and South) and the

Central American (Colombia-West and Panama-West). A morphometric analysis, on the other hand, found a significant size differences between the Colombia-North and Colombia-South populations, while no differences were found between the populations from Panama-West and Colombia-South. Understanding the link between morphological and molecular characters is of vital importance for designing diagnostic tests for highly invasive species to aid global biosecurity (Boykin et al., 2012).

The Mantel test results showed that the separation of the Iran and Florida populations in this study was not due to the geographic distance. So, the differences may be attributed to variation in both environmental and genetic factors. García-Pérez et al. (2013) have ~~showed~~ shown the separation of host-associated populations of ACP. They showed that the host ~~plant~~ species or variety can influence morphometric traits of different host associated populations of ACP (García-Pérez et al. 2013). They indicated that the largest ACP populations were associated with *C. sinensis* (L.) Osbeck cv. 'Marrs', *C. sinensis* (L.) cv. 'Valencia' and *Murraya paniculata* (L.) Jack, while~~,~~ the smallest sizes were found in males collected from *Citrus limetta* Risso, *C. sinensis* (L.) 'Selection 8' and *C. paradisi* Macfad. In this study the populations from Iran that were collected from the same host ~~plant~~ species (*C. sinensis*) were clustered together and the Florida populations that were collected from *M. paniculata* were clustered together, while the Florida population collected from *C. macrophylla* was separate; but Pakistan population, that has a same host ~~plant~~ species with Iran populations, is clustered distinctly. It may be state other effective variables on the detected variation.

In the current study, the body length (BL), circumanal ring length (CL), antenna length (AL), forewing length (WL) and Rs vein length of forewing (RL) contributed most to the variation found among the four populations. These results were similar to a previous study conducted by Garcia-Perez et al. (2013). They indicated that wing length, wing width and body

Kommentar [DB11]: There may have been different cultivars involved. Not clear from the information in material and methods.

Kommentar [DB12]: What does this mean?

length were the main variables contributing to discrimination of populations of *D. citri* on various host plants in Mexico. A comparison of the female ACP body size from the populations in the current study with those from different countries (Mexico, ~~Reunion~~Réunion, Venezuela~~,~~ and India) indicated that the populations from Iran and Florida were most similar to those from India (body length 2.4 mm; forewing length 2.17 mm), while the Pakistan populations stood alone while being shorter than the others (García-Pérez et al., 2013; Étienne et al., 2001; Fonseca et al., 2007; Mathur, 1975; Chhetry et al. 2012).

There are three pieces of evidence that suggest the invasion of ACP into Iran and the USA (Florida) originated from southwestern Asia, particularly India: 1- Southwestern Asia, i.e. India, has been suggested as the origin of ACP based on plant host origins and historical information (Hall, 2008). 2- The mitochondrial haplotype network for *D. citri* suggests a basal and thus ancestral position for Dcit-1 haplotype (Boykin et al., 2012). Boykin et al. (2012) showed that the Indian, USA, Saudi Arabian, Brazilian and Mexican populations of ACP belong to the mtCOI Haplotype-1 group. 3- Some size similarities between Iran, USA and India (Mathur, 1975; Chhetry et al., 2012). However, additional studies based on the morphological and other molecular markers such as microsatellite on the phylogenetic relationships among worldwide ACP populations are needed to confirm our hypothesis. Boykin et al. (2007) developed twelve polymorphic microsatellite markers for ACP ~~and~~ which should be explored on a global scale.

Kommentar [DB13]: These studies are not really comparable.

The differentiation of populations may originate from one of the following events: insect migration, a new host or a new habitat or both of them, landscape changes (bottleneck effect), and genetic changes by stochastic events, such as gene flow, genetic drift and mutation or natural selection (Kim & McPheron, 1993; Berlocher & Feder, 2002). These variations may be changes

Kommentar [DB14]: There are considerable morphologicla and behavioral differences in some psyllids between generations from different seasons.

in morphology, physiology, behavior~~,~~ and life history traits, and subsequently would lead to the manifestation of the different taxonomic status of local populations such as biotype and ecotype (Kim & McPheron, 1993).

We conclude that *D. citri* populations related to the mtCOI haplotypes-1 (Iran and Florida) and 6 (Pakistan) have distinct morphometric characters based on multivariate analysis of morphological data. Future ACP studies are needed to confirm the relationship found here between the mtCOI haplotypes and morphology.

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Table 1 Morphological traits used for morphometric analysis of populations of *Diaphorina citri*.

Character No.	Acronym	Character
1	BL	Body length
2	HW	Head width
3	VW	Vertex width
4	VL	Vertex length
5	GL	Genal process length
6	GW	Genal process width
7	AL	Antenna length
8	WL	Forewing length
9	WW	Forewing width
10	RL	Rs vein length of forewing
11	RC	Length of the line connecting apices of vein Rs and Cu1a of forewing
12	a	Length of the line connecting the base and apex of vein M1+2 of forewing
13	b	Length of the line connecting the base and apex of vein M3+4 of forewing
14	c	Length of the line connecting apices of veins M1+2 and M3+4of forewing
15	d	Length of the line connecting apices of vein Cu1a and Cu1b of forewing
16	e	Length of widest perpendicular distance to d in cell cu1
17	ML	Metatibial length
18	FP	Female proctiger length
19	CL	Circumanal ring length
20	SL	Female subgenital plate length

Kommentar [DB15]: How was this measured?

Kommentar [DB16]: What about male terminalia?

Table 2 Size (MM±SE) of the 20 morphological traits in the populations of Asian citrus psyllid, *Diaphorina citri* from Iran, Florida and Pakistan.

Variable [a]	Population					
	Iran-Chabahar	Iran-Jiroft	Florida- USDA ARS colony	Florida- Palm Beach County	Florida- St. Lucie County	Pakistan
BL	2.477±0.029 a[b]	2.459±0.029 a	2.49±0.018 a	2.557±0.009 a	2.535±0.013 a	1.980±0.035 b
HW	0.575±0.002 a	0.569±0.002 a	0.558±0.005 a	0.573±0.003 a	0.567±0.004 a	0.573±0.006 a
VW	0.375±0.003 a	0.372±0.003 ab	0.359±0.003 b	0.376±0.003 a	0.370±0.003 ab	0.364±0.004 ab
VL	0.136±0.002 a	0.132±0.002 a	0.136±0.003 a	0.141±0.002 a	0.139±0.003 a	0.137±0.004 a
GL	0.122±0.003 a	0.121±0.003 a	0.124±0.003 a	0.132±0.002 a	0.128±0.003 a	0.122±0.004 a
GW	0.101±0.002 a	0.095±0.002 a	0.097±0.002 a	0.103±0.002 a	0.102±0.002 a	0.094±0.003 a
AL	0.447±0.006 a	0.441±0.006 a	0.436±0.005 a	0.447±0.003 a	0.442±0.004 a	0.403±0.003 b
WL	1.990±0.014 c	2.091±0.012 b	2.151±0.011 ab	2.172±0.009 a	2.164±0.010 a	2.090±0.017 b
WW	0.869±0.007 b	0.910±0.006 a	0.909±0.004 a	0.923±0.003 a	0.914±0.004 a	0.912±0.006 a
RL	1.135±0.010 c	1.222±0.007 ab	1.246±0.008 ab	1.261±0.006 a	1.250±0.007 a	1.206±0.012 b
RC	0.728±0.008 b	0.760±0.007 a	0.762±0.005 a	0.775±0.005 a	0.770±0.005 a	0.775±0.007 a
a	0.555±0.005 a	0.592±0.005 a	0.579±0.004 a	0.768±0.179 a	0.587±0.003 a	0.571±0.006 a
b	0.490±0.005 b	0.520±0.004 a	0.499±0.003 b	0.508±0.001 ab	0.504±0.003 ab	0.503±0.005 ab
c	0.319±0.004 b	0.336±0.005 ab	0.350±0.004a	0.355±0.002 a	0.353±0.003 a	0.337±0.006 ab
d	0.409±0.004 c	0.423±0.005 cb	0.440±0.003 ab	0.446±0.002 a	0.445±0.003a	0.426±0.003 cb
e	0.259±0.002 b	0.260±0.003 ab	0.259±0.002 ab	0.266±0.002 ab	0.264±0.002 ab	0.271±0.003 a
ML	0.563±.005 a	0.557±0.005 ab	0.540±0.003 b	0.556±0.003 ab	0.548±0.004 ab	0.539±0.004 b
FP	0.511±0.007 a	0.509±0.007 a	0.479±0.004 b	0.495±0.004 ab	0.489±0.004 ab	0.450±0.007 c
CL	0.133±0.001 ab	0.130±0.001 b	0.133±0.003 ab	0.141±0.002 a	0.138±0.003 ab	0.116±0.002 c
SL	0.423±0.005 a	0.421±0.005 a	0.409±0.004 a	0.418±0.003 a	0.414±0.003 a	0.386±0.005 b

[a] See Table 1 for abbreviations.

[b] Means with the same letter within each variable are statistically equal (Tukey, $P \leq 0.01$).

Table 3 Mahalanobis distances among populations of *Diaphorina citri* from Iran, Florida and Pakistan (the below diagonal).

Population	Iran-Chabahar	Iran-Jiroft	Florida- USDA ARS colony	Florida- Palm Beach County	Florida- St. Lucie County	Pakistan
Iran-Chabahar	0					
Iran-Jiroft	7.486	0				
Florida- USDA ARS colony	18.277	9.264	0			
Florida- Palm Beach County	16.603	8.999	2.644	0		
Florida- St. Lucie County	15.511	8.526	1.127	0.720	0	
Pakistan	24.478	23.119	32.071	36.756	33.397	0

Table 4 Standardized coefficients for canonical variables on the first (CVA1) and second (CVA2) canonical axes.

Variable [a]	CVA1	CVA2
BL	0.853	0.390
HW	-0.127	0.147
VW	0.068	0.261
VL	0.048	-0.079
GL	0.151	-0.107
GW	0.200	0.091
AL	0.453	0.356
WL	0.427	-0.660
WW	0.151	0.498
RL	0.417	-0.616
RC	0.039	-0.462
a	0.093	-0.046
b	0.057	-0.094
c	0.317	-0.446
d	0.390	-0.518
e	-0.156	-0.263
ML	0.083	0.398
FP	0.307	0.572
CL	0.576	0.160
SL	0.340	0.408
Eigenvalues	4.4741	1.7065
Proportion	0.6515	0.2485

[a] See Table 1 for abbreviations.

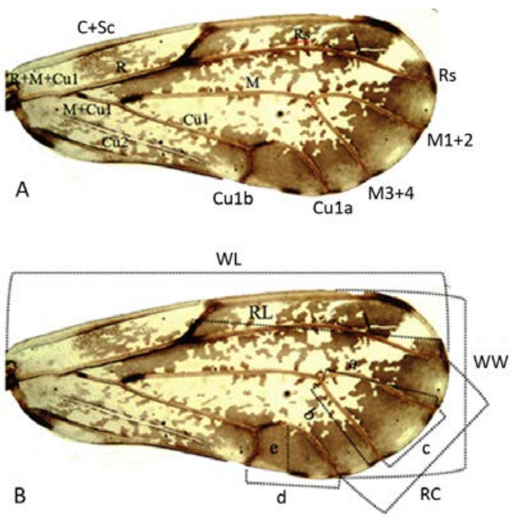

Figure 1 A- ~~Wing~~ Forewing vein terminology based on Hodkinson and White (1979), and B- lines indicating measurements based on Mifsud and Burckhardt (2002) in the right forewing of *Diaphorina citri*. See Table 1 for abbreviations.

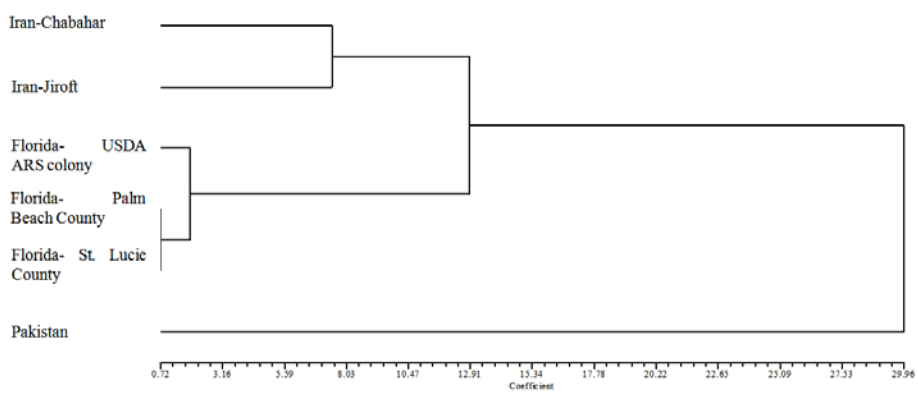

Figure 2 Dendrogram plotted by UPGMA method based on squared Euclidean distance of *Diaphorina citri* populations.