

Wing geometry in the populations of *Diaphorina citri* (Hemiptera: Liviidae) in Iran and USA: an evidence for incongruence of molecular and morphometric data

M. Lashkari* and S. Iranmanesh

Department of Biodiversity, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology.
Corresponding author, E-mail: mr.lashkari@gmail.com

Abstract

Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama, is one of the most important citrus pests in the world. In addition to direct feeding damages, it also transmits the greening-disease pathogen. In order to improve understanding of geographic populations of ACP related to the mtCOI Haplotype-1, geometric morphometric was used to compare variations between populations of ACP in Iran and USA (Florida). The ACP populations were collected from the Iranian cities of Jiroft and Chabahar and Florida (USA) in 2014. 150 specimens from five ACP populations (30 adult females per population) were randomly selected and analysed using ANOVA and MANOVA. The results indicate that wing-shape (Wilk's lambda = 0.0910, $F = 5.89$, $P < 0.0001$) and wing-size ($F = 61.41$, $df = 4$, $P < 0.0001$) were different between Iranian and USA populations. This study also showed incongruence between morphometric and molecular data in the studied populations of *D. citri* from Iran and USA. The observed differences are probably due to genetic rather than environmental factors because of 1) significant multivariate differences in shape even after removing size variation and 2) significant correlation between geographic and morphometric distance matrices.

Key words: Citrus greening disease, HLB, intraspecific variation

چکیده

هندسه بال در جمعیت‌های *Diaphorina citri* (Hemiptera: Liviidae) در ایران و آمریکا: دلیلی برای نابرابری داده‌های مولکولی و ریخت‌سنجی

محمدرضا لشکری و سعید ایرانمنش

پسیل آسیایی مرکبات (ACP)، *Diaphorina citri* Kuwayama، یکی از مهم‌ترین آفات مرکبات در دنیا است. پسیل آسیایی مرکبات علاوه بر خسارت مستقیم تغذیه‌ای، عامل بیماری میوه‌سبز مرکبات را نیز منتقل می‌کند. به منظور افزایش دانسته‌های ما درباره جمعیت‌های جغرافیایی پسیل آسیایی مرکبات مرتبط با هاپلوتیپ یک ژن میتوکندریایی COI، روش ریخت‌سنجی هندسی برای مقایسه تنوع بین جمعیت‌های *D. Citri* در ایران و آمریکا (فلوریدا) استفاده شد. جمعیت‌های پسیل آسیایی مرکبات از ایران، از استان‌های کرمان (جیرفت) و سیستان و بلوچستان (چابهار)، و جمعیت‌های آمریکا از ایالت فلوریدا در سال ۲۰۱۴ جمع‌آوری شدند. در مجموع، ۱۵۰ نمونه از پنج جمعیت پسیل آسیایی مرکبات (۳۰ حشره بالغ ماده برای هر جمعیت) به طور تصادفی انتخاب و با آنالیزهای یک و چند متغیره بررسی شدند. نتایج نشان‌دهنده اختلاف معنی‌دار شکل ($F = 61.41$, $P < 0.0001$) و اندازه ($F = 5.89$, $P < 0.0001$) وینگ‌شکل ($F = 61.41$, $P < 0.0001$) وینگ‌سایز (Wilk's lambda = 0.0910, $F = 5.89$, $P < 0.0001$) وینگ‌شکل و وینگ‌سایز بین جمعیت‌های ایرانی و آمریکایی (فلوریدا) بودند. این مطالعه همچنین نابرابری بین داده‌های ریخت‌سنجی (در مطالعه حاضر) و مولکولی (در مطالعات منتشر شده) در جمعیت‌های پسیل آسیایی مرکبات از ایران و آمریکا را نشان داد. به دو دلیل می‌توان این فرضیه را مطرح کرد که تفاوت‌های مشاهده شده ممکن است به‌خاطر فاکتورهای ژنتیکی باشد تا این‌که محیطی (با وجود نابرابری نتایج این مطالعه با مطالعات منتشر شده: ۱) وجود تفاوت معنی‌دار در شکل حتی پس از حذف عامل اندازه؛ ۲) وجود ارتباط معنی‌داری بین ماتریکس شکل و فاصله جغرافیایی.

واژگان کلیدی: بیماری میوه سبز مرکبات، HLB، تنوع درون‌گونه‌ای

Introduction

The existing variations among the populations may originate from differences in their hosts, habitats, landscapes (bottleneck effect), or genetic changes through stochastic events, such as gene flow, genetic drift and mutation or natural selection (Kim & McPherson, 1993; Berlocher & Feder, 2002; Nosil *et al.*, 2012). Moreover, gene flow among populations can be reduced by geographical distance (isolation by distance) (Edelaar *et al.*, 2012). These variations may lead to manifestations of

different forms in local populations, such as biotype and ecotype (Kim & McPherson, 1993).

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is considered as a major citrus pest – it has been identified as the vector of Huanglongbing (HLB) or citrus greening disease (CGD), caused by *Candidatus Liberibacter* spp. (α -Proteobacteria). ACP and HLB have distributed to citriculture regions nearly worldwide (Hall *et al.*, 2013). A worldwide study on its genetic diversity suggested the existence of eight

haplotypes of *D. citri* (Dcit-1 to Dcit-8) based on mitochondrial cytochrome oxidase I (mtCOI) sequences (Boykin *et al.*, 2012). An additional study based on the mtCOI sequences showed that ACP populations from Iran were similar to the populations of India, Saudi Arabia, Brazil, Mexico, and USA (Florida and Texas) (Lashkari *et al.*, 2014). The links between Iran's and USA populations were detected based on the mtCOI sequence (in ACP) and the gene *wsp* sequence data (in the endosymbiont *Wolbachia, wDi*) (Lashkari *et al.*, 2014).

Geometric morphometrics (GM) is an accurate morphometric method which focuses on the geometry of the form rather than linear measurements (Rohlf & Slice, 1990; Bookstein, 1991; Rohlf & Marcus, 1993). This method can show the direct visualization of shape changes (Garnier *et al.*, 2005) that reliably detects subtle shape changes even at intra-specific level (Alibert *et al.*, 2001; Renaud & Millien, 2001; Bertin *et al.*, 2002). It can also make distinct variables (size and shape), and helps assessment of residual allometry.

Studies on morphological differentiation are essential for incorporating both molecular and morphological data that provide better results than a single approach (Hillis & Moritz, 1990) providing a better view of integrated pest management. Therefore, the variations in wing shape and size were examined in the populations from Iran and USA (Florida) using the geometric morphometrics method to assess the wing geometry in ACP populations related to the mtCOI Haplotype-1 (Iran and the USA), and to improve understanding of links between the molecular (Boykin *et al.*, 2012; Lashkari *et al.*, 2014) and morphometric data.

Material and methods

Sampling

Five geographic populations of ACP related to the mtCOI Haplotype-1 (genetic based populations) were collected from Iran (2) and Florida (3) during the spring 2014 (table 1). 30 adult females for each population were randomly selected and examined. Because the mitochondrial DNA is passed along the female line, and it provides a direct comparison with the previous molecular

study (Lashkari *et al.*, 2014), the female adults were selected for this study.

Table 1. Collection sites, codes and number of examined specimens for populations of *Diaphorina citri*.

Country	Province/State	County	Code	n.
IRAN	Sistan & Baluchestan	Chabahar	IR.1	30
	Kerman	Jiroft	IR.2	30
		Palm Beach Gardens, Palm Beach County	FL.1	30
USA	Florida	Port St. Lucie, St. Lucie County	FL.2	30
		USDA ARS colony, Ft. Pierce, St. Lucie County	FL.3	30

Preparing specimens and statistical analysis

The right forewings were mounted in Euparal medium and images captured using a digital camera connected to a stereomicroscope with 40X magnification for all specimens. The images were converted to the TPS format using the tpsUtil program, version 1.46 (Rohlf, 2010b). The coordinates of 11 possible landmarks (type I) were digitized using TPSDIG version 1.40 (Rohlf, 2004) (table 2, fig. 1). The X, Y coordinate data files were opened in the tpsRelw program, version 1.49 (Rohlf, 2010a), and 2D Procrustes superimposition performed on the basis of generalized procrustes analysis (GPA) (Rohlf, 1999; Slice, 2001). The consensus shape was subtracted from all coordinates, and maximal shape variation shown along the first two relative-warp axes (RW1 and RW2) to illustrate ordination of the shape consensus by a relative-warp ordination plot. Subsequently, the centroid size and shape components of the wing were obtained. Each variable data set (weight matrix) was subjected to MANOVA and canonical variate analysis (CVA) (Rohlf *et al.*, 1996). The MANOVA and CVA analyses were performed using SAS statistical program, version 9.1 (SAS Institute, 2003) and NTSYS-pc program, version 2.02 (Rohlf, 1998), respectively. The relationships among the populations were summarized by the UPGMA clustering method using the NTSYS-pc program, version 2.02 (Rohlf, 1998). The correlation coefficient was used as a measure of goodness of fit for the cluster analysis in the NTSYS-pc program. The Mantel test was also performed using the NTSYS-pc program. To find isometry in size variation

between groups, the allometry test was performed using the TpsRegr program (Rohlf, 2009). In the case of rejection, it was examined whether the populations varied in different allometric trajectories. MANCOVAs compared allometric slopes and shape as size held constant. In order to evaluate size among the populations, the centroid size was calculated using tpsRelw, version 1.49 (Rohlf, 2010a), and ANOVA and Tukey pair-wise comparisons were conducted on the whole data set to test centroid size differences, using the SAS statistical program, version 9.1 (SAS Institute, 2003).

Table 2. Description of the landmark positions in the forewing of *Diaphorina citri* (Lashkari *et al.*, 2013).

Landmark	Description
1	Apex of Rs vein in right wing
2	Apex of M_{1+2} vein
3	Apex of M_{3+4} vein
4	Apex of cu_{1a}
5	Apex of cu_{1b}
6	Junction of M_{1+2} and M_{3+4} in the apex of M vein
7	Junction of cu_{1a} and cu_{1b} in the apex of Cu_1 vein
8	In costal break
9	Junction of R and Rs
10	Junction of M and Cu_1
11	Junction of R and $M+Cu_1$ (in the apex of $R + M + Cu_1$)

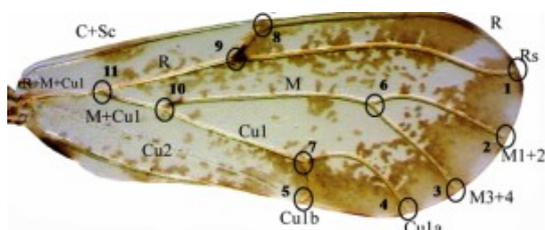


Fig. 1. Nomenclature and landmark positions on the forewing of *Diaphorina citri* (Lashkari *et al.*, 2013).

Results

Size variation

Wing-size variation was significantly different among the populations ($F = 61.41$, $df = 4$, $P < 0.0001$)

(table 3, fig. 2). Pairwise comparisons (HSD post-hoc test) showed that the ACP populations from Iran had smaller wing size. Although the ACP populations from Iran were clearly differentiated from the Florida populations, there was no statistical difference between the two Iranian populations, or among the three populations from Florida. Therefore, based on the size variation, two significantly different groups can be discriminated: 1) Iranian populations (with smaller wing size), and 2) Florida populations (with larger wing size).

Table 3. HSD (post-hoc) test on wing centroid size of *Diaphorina citri* populations. See table 1 for abbreviations.

Population	n.	Mean \pm SE
FL.2	30	1482.33 \pm 5.76 a*
FL.3	30	1473.67 \pm 8.53 a
FL.1	30	1453.33 \pm 8.11 a
IR.2	30	1344.33 \pm 12.25 b
IR.1	30	1338.00 \pm 9.57 b

*Mean within columns followed by the same letter are not statistically different by HSD test ($\alpha = 0.01$).

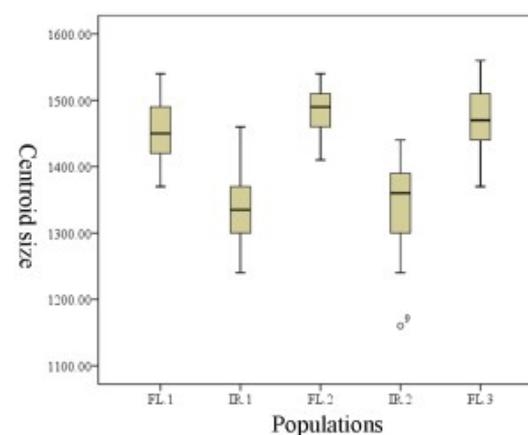


Fig. 2. Comparison of forewing size among the populations of *Diaphorina citri* from Iran and Florida. Black dot: mean and whiskers: standard deviation.

Shape variation

The first two relative warps (RW1 and RW2) showed 28.17% and 12.32% of shape variation among the specimens, respectively. Shape variation along

RW1 illustrated the source of deformation from the consensus configuration (fig. 3). Although at the positive extremes along the RW1 axis (fig. 3a), the marginal landmarks (1-4 and 11) showed a relatively squat shape, at the negative extreme along RW1 (fig. 3c), the marginal landmarks (1-4 and 11) showed more elongated wing shape.

The MANOVAs indicated highly significant wing-shape differences among the populations (Wilk's lambda = 0.0910, $F = 5.89$, $P < 0.0001$). Pair-wise generalized squared distances showed clear wing-shape differentiation between the two main groups of populations as the first one was consistent with the Florida group and the second was consistent with the Iranian group (table 4). A three-dimensional ordination using the mean canonical vector scores (CV1 vs. CV2)

of the populations confirms the separation of the populations (fig. 4).

The Mantel test showed significant correlation between geographic distances and morphological distances at alpha = 0.05 ($R = 0.88792$, $p = 0.0123$), leading to an impact on morphological differentiation.

The regression of shape (partial warps) on the size (centroid size) showed significant allometric growth (Wilks' Lambda = 0.3337, $P = 3.56E-023$). Although allometric slopes did not differ significantly between populations (Wilks' Lambda = 0.580, $P = 0.0626$), shape was significantly different when size was held constant allometrically (Wilks' Lambda = 0.415, $P = 1.007E-006$), indicating different intercept for the studied populations. Therefore, geographic populations have separate allometric directions but are parallel to each other.

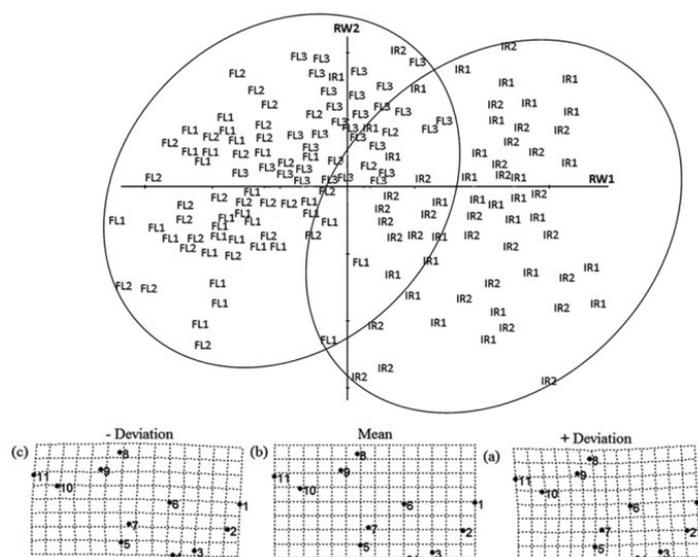


Fig. 3. Upper part: Scatter plot for the RW1 and RW2 on the forewing of *Diaphorina citri*. FL, Florida populations; IR, Iran populations (See table 1 for abbreviations). Lower part: Deformations in shape variables along the RW1 in the forewings of *Diaphorina citri* populations, (a) positive deformation, (b) consensus configuration, and (c) negative deformation.

Table 4. Pair-wise generalized squared distance matrix among the populations of *Diaphorina citri* from Iran and Florida.

Population	IR.2	IR.1	FL.1	FL.2	FL.3
IR.2	0				
IR.1	2.171	0			
FL.1	19.528**	15.157**	0		
FL.2	20.030**	16.654**	1.276	0	
FL.3	13.806**	12.052**	7.002	6.563	0

**P-values significant at the 0.01 level.

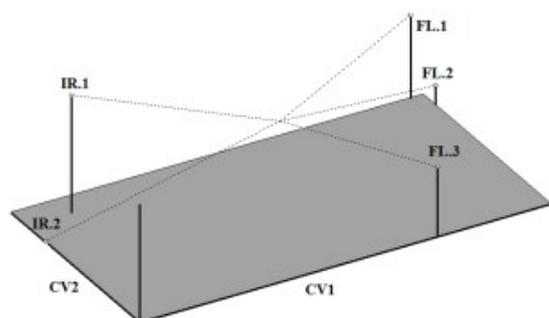


Fig. 4. Canonical variate ordination in the populations of *Diaphorina citri* from Iran and Florida. The vertical axis (not labelled) is the third canonical variate axis. See Table 1 for more description.

Discussion

Multivariate statistical analysis of partial-warp scores showed shape differences among geographic populations. The studied populations of ACP were clustered into two major population groups: Iran and Florida. Although two Iranian populations in the current study (Chabahar and Jiroft) were clustered together, in the previous geometric morphometric study, Lashkari *et al.* (2013) showed different shape and size among Iranian populations, as Sarbaz population (in Sistan & Baluchestan province) differentiated from Kerman populations. It seems that transferring of agricultural materials have led to the occurrence of gene flow among Sarbaz (near to the Pakistan border) and Pakistan *D. citri* populations (Lashkari *et al.*, 2013), also the Sarbaz population was collected from an arid climate region with elevation about 300 m (*above sea level*), whereas the Chabahar population is from a humid climate region with elevation about 7 m (*above sea level*).

Iran populations had a smaller wing size compared with Florida populations, that may develop by the interactions of genetic mechanisms and environmental processes. For example, these localities have different climatic conditions; The Iranian populations belonged to warm desert climate (also known as an arid climate) (Chabahar) and warm semi-arid climate (Kerman-Jiroft), that were characterized by moderate winter and very warm summer, besides cool winter and very warm summers, respectively; whereas, the Florida populations

were collected from a humid subtropical climate, with hot, usually humid summers and mild to cool winters. Although there are a number of climatic and ecological factors that may vary in these regions, one of the most important factors is the effect of temperature on wing size, as wing length has been decreased linearly at higher temperatures in several studies (Nayar, 1969; Partridge *et al.*, 1994; Atkinson, 1995; De Moed *et al.*, 1997; Reiskind & Zarrabi, 2012).

In reviewing the literature, no geometric morphometric data were available on the geographic populations of *D. citri* in Iran and Florida, but molecular data showed that Iran and Florida populations of ACP were mtCOI Haplotype-1, based on a mitochondrial haplotype network (Lashkari *et al.*, 2014). However, our geometric morphometric data contrast previous molecular research, as the Iran and Florida populations were clustered separately. Although, this study showed evidence from incongruence of the morphometric and molecular data in the *D. citri* populations related to the mtCOI Haplotype-1 (Iran and the USA), additional evidence using more populations, genes and morphometric characters needs to be confirmed. In reviewing the literature, when comparisons of molecular and morphological data were made on constructing phylogenies, it was found that these changes may be independent (Hillis & Moritz, 1990). Although data of DNA sequence does have many advantages, but the amount of data is limited by the studied genome size. Studies that incorporate both types of data may provide more appropriate results (Hillis & Moritz, 1990). Another explanation could be the selected molecular markers and sampling conditions used in previous studies. The studies of Boykin *et al.* (2012) and Lashkari *et al.* (2014) were based only on the mitochondrial DNA, with relatively few ACP specimens from each location. Although mtDNA is a very useful marker because of its small size, relatively rapid rate of evolutionary change, and maternal inheritance (Hillis & Moritz, 1990), so additional molecular studies using nuclear DNA markers might be valuable such as microsatellites on the phylogenetic relationships among populations of ACP. Incongruence among the methods

used in intraspecific variation studies has been indicated previously (Bomfim *et al.*, 2011; Gómez-Palacio *et al.*, 2012).

Invasive insects often undergo rapid genetic changes during introduction to new environments (Hernandez *et al.*, 2010). In this study, two arguments lead us to consider that the observed differences between the studied populations are probably due to genetic, rather than environmental factors. Firstly, there were significant multivariate differences in shape, even after removing size variation. Removal of size as a different principle reveals a pattern of variation that would be more related to evolutionary differences rather than environmental factors (Dujardin *et al.*, 1999). These findings are consistent with those of Adams & Funk (1997), Dujardin *et al.* (1999) and Mozaffarian *et al.* (2007), who found shape variation after removing size in populations of *Neochlamisus bebbianae* (Brown, 1943), *Lutzomyia quinquefer* (Dyar, 1929) and *Ectomyelois ceratoniae* (Zeller, 1839), respectively. Secondly, there was a significant correlation between geographic and morphometric distance matrices, suggesting that the amount of morphological divergence could be influenced by the level of geographic isolation, with no need to infer climatic or ecological differences (Dujardin *et al.*, 1999). This result was similar to Lashkari *et al.* (2013), Dujardin *et al.* (1999) and Mozaffarian *et al.* (2007), who found a significant correlation between morphologic and geographic distances in geographical populations of *D. citri*, *Lutzomyia quinquefer* (Dyar) and *E. ceratoniae*, respectively. Lashkari *et al.* (2013) showed differentiation between the populations of Iran and Pakistan.

Thus, it is clear that part of the morphological variation of ACP is developed by evolutionary divergence, which involves interactions of genetic and environmental processes that caused genetic differentiation of populations. These genetic differences can be transferred to the next generations and affect morphology, physiology, behaviour, life-history traits and control methods. The existence of differentiation between populations of *D. citri* might have important

consequences in the insect pest management for their ability to transmit HLB disease agent (Lashkari *et al.*, 2014). The mitochondrial haplotype network that was constructed for *D. citri* suggested an ancestral position of haplotype Dcit-1 (Boykin *et al.*, 2012). The results of this research are useful for detection of the geographical origin of the pest for Iran and Florida. Lashkari *et al.* (2014) showed a link between Iran and Florida and it is estimated that the recent invasion of Iran and Florida has the same origin because of common similarity of *D. citri* specimens in both countries based on the mitochondrial gene cytochrome oxidase I (mtCOI) and the *Wolbachia-diaphorina, wDi*, gene *wsp* sequence analyses. But, these populations might have genetic differentiation. Therefore, it seems differentiations within populations are related to mtCOI haplotype-1. Our understanding about the origin of this pest can be resulted in the identification of effective biological control agents in Iran and Florida. *Tamarix radiata* (Waterston) (Hymenoptera: Eulophidae), one of the most effective biological control agent of ACP has been successfully introduced into some geographical locations, such as Réunion Island, Taiwan, Mauritius, Guadeloupe, Florida, the Philippines and Indonesia (East Java). It significantly reduced the populations of ACP in Réunion Island, but was less effective in Florida (Grafton-Cardwell *et al.*, 2013; Hall *et al.*, 2013). It seems that other haplotypes of *T. radiata* (especially from countries having ACP haplotype 1) might be more effective for Florida. Therefore, it suggests that intraspecific variation of this species could be examined using genetic markers (such as microsatellite markers) and more mtCOI haplotype-1 populations (such as populations from India, Saudi Arabia, Brazil, Mexico and Texas) for further understanding. It would be advantageous for growers in these countries (related to ACP haplotype 1) to compare their control strategies if they have similar populations.

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Wing geometry in the populations of *Diaphorina citri* (Hemiptera: Liviidae) in Iran and USA: an evidence for incongruence of molecular and morphometric data

M. Lashkari* and S. Iranmanesh

Department of Biodiversity, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology.
Corresponding author, E-mail: mr.lashkari@gmail.com

Abstract

Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama, is one of the most important citrus pests in the world. In addition to direct feeding damages, it also transmits the greening-disease pathogen. In order to improve understanding of geographic populations of ACP related to the mtCOI Haplotype-1, geometric morphometric was used to compare variations between populations of ACP in Iran and USA (Florida). The ACP populations were collected from the Iranian cities of Jiroft and Chabahar and Florida (USA) in 2014. 150 specimens from five ACP populations (30 adult females per population) were randomly selected and analysed using ANOVA and MANOVA. The results indicate that wing-shape (Wilk's lambda = 0.0910, $F = 5.89$, $P < 0.0001$) and wing-size ($F = 61.41$, $df = 4$, $P < 0.0001$) were different between Iranian and USA populations. This study also showed incongruence between morphometric and molecular data in the studied populations of *D. citri* from Iran and USA. The observed differences are probably due to genetic rather than environmental factors because of 1) significant multivariate differences in shape even after removing size variation and 2) significant correlation between geographic and morphometric distance matrices.

Key words: Citrus greening disease, HLB, intraspecific variation

چکیده

هندسه بال در جمعیت‌های *Diaphorina citri* (Hemiptera: Liviidae) در ایران و آمریکا: دلیلی برای نابرابری داده‌های مولکولی و ریخت‌سنجی

محمدرضا لشکری و سعید ایرانمنش

پسیل آسیایی مرکبات (ACP)، *Diaphorina citri* Kuwayama، یکی از مهم‌ترین آفات مرکبات در دنیا است. پسیل آسیایی مرکبات علاوه بر خسارت مستقیم تغذیه‌ای، عامل بیماری میوه‌سبز مرکبات را نیز منتقل می‌کند. به منظور افزایش دانسته‌های ما درباره جمعیت‌های جغرافیایی پسیل آسیایی مرکبات مرتبط با هاپلوتیپ یک ژن میتوکندریایی COI، روش ریخت‌سنجی هندسی برای مقایسه تنوع بین جمعیت‌های *D. Citri* در ایران و آمریکا (فلوریدا) استفاده شد. جمعیت‌های پسیل آسیایی مرکبات از ایران، از استان‌های کرمان (جیرفت) و سیستان و بلوچستان (چابهار)، و جمعیت‌های آمریکا از ایالت فلوریدا در سال ۲۰۱۴ جمع‌آوری شدند. در مجموع، ۱۵۰ نمونه از پنج جمعیت پسیل آسیایی مرکبات (۳۰ حشره بالغ ماده برای هر جمعیت) به طور تصادفی انتخاب و با آنالیزهای یک و چند متغیره بررسی شدند. نتایج نشان‌دهنده اختلاف معنی‌دار شکل ($F = 5/89$, $P < 0/0001$) و اندازه ($F = 61/41$, $P < 0/0001$) میان جمعیت‌های مورد مطالعه بود. جمعیت‌های ایران (جیرفت و چابهار) بر پایه شکل و اندازه بال مشابه با یکدیگر، اما متفاوت از جمعیت‌های آمریکا (فلوریدا) بودند. این مطالعه همچنین نابرابری بین داده‌های ریخت‌سنجی (در مطالعه حاضر) و مولکولی (در مطالعات منتشر شده) در جمعیت‌های پسیل آسیایی مرکبات از ایران و آمریکا را نشان داد. به دو دلیل می‌توان این فرضیه را مطرح کرد که تفاوت‌های مشاهده شده ممکن است به‌خاطر فاکتورهای ژنتیکی باشد تا این‌که محیطی (با وجود نابرابری نتایج این مطالعه با مطالعات منتشر شد: ۱) وجود تفاوت معنی‌دار در شکل حتی پس از حذف عامل اندازه؛ ۲) وجود ارتباط معنی‌داری بین ماتریکس شکل و فاصله جغرافیایی.

واژگان کلیدی: بیماری میوه سبز مرکبات، HLB، تنوع درون‌گونه‌ای

Introduction

The existing variations among the populations may originate from differences in their hosts, habitats, landscapes (bottleneck effect), or genetic changes through stochastic events, such as gene flow, genetic drift and mutation or natural selection (Kim & McPherson, 1993; Berlocher & Feder, 2002; Nosil *et al.*, 2012). Moreover, gene flow among populations can be reduced by geographical distance (isolation by distance) (Edelaar *et al.*, 2012). These variations may lead to manifestations of

different forms in local populations, such as biotype and ecotype (Kim & McPherson, 1993).

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is considered as a major citrus pest – it has been identified as the vector of Huanglongbing (HLB) or citrus greening disease (CGD), caused by *Candidatus Liberibacter* spp. (α -Proteobacteria). ACP and HLB have distributed to citriculture regions nearly worldwide (Hall *et al.*, 2013). A worldwide study on its genetic diversity suggested the existence of eight

haplotypes of *D. citri* (Dcit-1 to Dcit-8) based on mitochondrial cytochrome oxidase I (mtCOI) sequences (Boykin *et al.*, 2012). An additional study based on the mtCOI sequences showed that ACP populations from Iran were similar to the populations of India, Saudi Arabia, Brazil, Mexico, and USA (Florida and Texas) (Lashkari *et al.*, 2014). The links between Iran's and USA populations were detected based on the mtCOI sequence (in ACP) and the gene *wsp* sequence data (in the endosymbiont *Wolbachia, wDi*) (Lashkari *et al.*, 2014).

Geometric morphometrics (GM) is an accurate morphometric method which focuses on the geometry of the form rather than linear measurements (Rohlf & Slice, 1990; Bookstein, 1991; Rohlf & Marcus, 1993). This method can show the direct visualization of shape changes (Garnier *et al.*, 2005) that reliably detects subtle shape changes even at intra-specific level (Alibert *et al.*, 2001; Renaud & Millien, 2001; Bertin *et al.*, 2002). It can also make distinct variables (size and shape), and helps assessment of residual allometry.

Studies on morphological differentiation are essential for incorporating both molecular and morphological data that provide better results than a single approach (Hillis & Moritz, 1990) providing a better view of integrated pest management. Therefore, the variations in wing shape and size were examined in the populations from Iran and USA (Florida) using the geometric morphometrics method to assess the wing geometry in ACP populations related to the mtCOI Haplotype-1 (Iran and the USA), and to improve understanding of links between the molecular (Boykin *et al.*, 2012; Lashkari *et al.*, 2014) and morphometric data.

Material and methods

Sampling

Five geographic populations of ACP related to the mtCOI Haplotype-1 (genetic based populations) were collected from Iran (2) and Florida (3) during the spring 2014 (table 1). 30 adult females for each population were randomly selected and examined. Because the mitochondrial DNA is passed along the female line, and it provides a direct comparison with the previous molecular

study (Lashkari *et al.*, 2014), the female adults were selected for this study.

Table 1. Collection sites, codes and number of examined specimens for populations of *Diaphorina citri*.

Country	Province/State	County	Code	n.
IRAN	Sistan & Baluchestan	Chabahar	IR.1	30
	Kerman	Jiroft	IR.2	30
		Palm Beach Gardens, Palm Beach County	FL.1	30
USA	Florida	Port St. Lucie, St. Lucie County	FL.2	30
		USDA ARS colony, Ft. Pierce, St. Lucie County	FL.3	30

Preparing specimens and statistical analysis

The right forewings were mounted in Euparal medium and images captured using a digital camera connected to a stereomicroscope with 40X magnification for all specimens. The images were converted to the TPS format using the tpsUtil program, version 1.46 (Rohlf, 2010b). The coordinates of 11 possible landmarks (type I) were digitized using TPSDIG version 1.40 (Rohlf, 2004) (table 2, fig. 1). The X, Y coordinate data files were opened in the tpsRelw program, version 1.49 (Rohlf, 2010a), and 2D Procrustes superimposition performed on the basis of generalized procrustes analysis (GPA) (Rohlf, 1999; Slice, 2001). The consensus shape was subtracted from all coordinates, and maximal shape variation shown along the first two relative-warp axes (RW1 and RW2) to illustrate ordination of the shape consensus by a relative-warp ordination plot. Subsequently, the centroid size and shape components of the wing were obtained. Each variable data set (weight matrix) was subjected to MANOVA and canonical variate analysis (CVA) (Rohlf *et al.*, 1996). The MANOVA and CVA analyses were performed using SAS statistical program, version 9.1 (SAS Institute, 2003) and NTSYS-pc program, version 2.02 (Rohlf, 1998), respectively. The relationships among the populations were summarized by the UPGMA clustering method using the NTSYS-pc program, version 2.02 (Rohlf, 1998). The correlation coefficient was used as a measure of goodness of fit for the cluster analysis in the NTSYS-pc program. The Mantel test was also performed using the NTSYS-pc program. To find isometry in size variation

between groups, the allometry test was performed using the TpsRegr program (Rohlf, 2009). In the case of rejection, it was examined whether the populations varied in different allometric trajectories. MANCOVAs compared allometric slopes and shape as size held constant. In order to evaluate size among the populations, the centroid size was calculated using tpsRelw, version 1.49 (Rohlf, 2010a), and ANOVA and Tukey pair-wise comparisons were conducted on the whole data set to test centroid size differences, using the SAS statistical program, version 9.1 (SAS Institute, 2003).

Table 2. Description of the landmark positions in the forewing of *Diaphorina citri* (Lashkari *et al.*, 2013).

Landmark	Description
1	Apex of Rs vein in right wing
2	Apex of M ₁₊₂ vein
3	Apex of M ₃₊₄ vein
4	Apex of cu _{1a}
5	Apex of cu _{1b}
6	Junction of M ₁₊₂ and M ₃₊₄ in the apex of M vein
7	Junction of cu _{1a} and cu _{1b} in the apex of Cu ₁ vein
8	In costal break
9	Junction of R and Rs
10	Junction of M and Cu1
11	Junction of R and M+Cu1 (in the apex of R + M + Cu ₁)

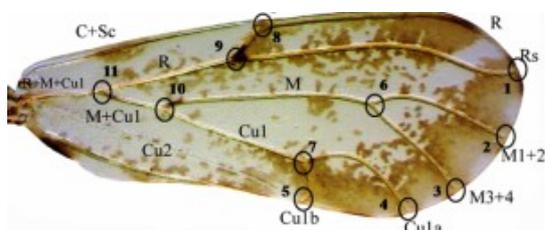


Fig. 1. Nomenclature and landmark positions on the forewing of *Diaphorina citri* (Lashkari *et al.*, 2013).

Results

Size variation

Wing-size variation was significantly different among the populations ($F = 61.41$, $df = 4$, $P < 0.0001$)

(table 3, fig. 2). Pairwise comparisons (HSD post-hoc test) showed that the ACP populations from Iran had smaller wing size. Although the ACP populations from Iran were clearly differentiated from the Florida populations, there was no statistical difference between the two Iranian populations, or among the three populations from Florida. Therefore, based on the size variation, two significantly different groups can be discriminated: 1) Iranian populations (with smaller wing size), and 2) Florida populations (with larger wing size).

Table 3. HSD (post-hoc) test on wing centroid size of *Diaphorina citri* populations. See table 1 for abbreviations.

Population	n.	Mean \pm SE
FL.2	30	1482.33 \pm 5.76 a*
FL.3	30	1473.67 \pm 8.53 a
FL.1	30	1453.33 \pm 8.11 a
IR.2	30	1344.33 \pm 12.25 b
IR.1	30	1338.00 \pm 9.57 b

*Mean within columns followed by the same letter are not statistically different by HSD test ($\alpha = 0.01$).

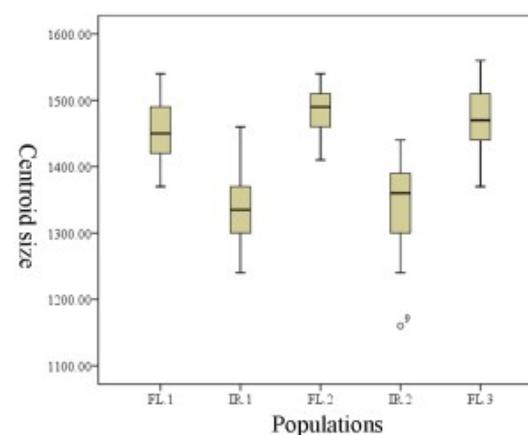


Fig. 2. Comparison of forewing size among the populations of *Diaphorina citri* from Iran and Florida. Black dot: mean and whiskers: standard deviation.

Shape variation

The first two relative warps (RW1 and RW2) showed 28.17% and 12.32% of shape variation among the specimens, respectively. Shape variation along

RW1 illustrated the source of deformation from the consensus configuration (fig. 3). Although at the positive extremes along the RW1 axis (fig. 3a), the marginal landmarks (1-4 and 11) showed a relatively squat shape, at the negative extreme along RW1 (fig. 3c), the marginal landmarks (1-4 and 11) showed more elongated wing shape.

The MANOVAs indicated highly significant wing-shape differences among the populations (Wilk's lambda = 0.0910, $F = 5.89$, $P < 0.0001$). Pair-wise generalized squared distances showed clear wing-shape differentiation between the two main groups of populations as the first one was consistent with the Florida group and the second was consistent with the Iranian group (table 4). A three-dimensional ordination using the mean canonical vector scores (CV1 vs. CV2)

of the populations confirms the separation of the populations (fig. 4).

The Mantel test showed significant correlation between geographic distances and morphological distances at alpha = 0.05 ($R = 0.88792$, $p = 0.0123$), leading to an impact on morphological differentiation.

The regression of shape (partial warps) on the size (centroid size) showed significant allometric growth (Wilks' Lambda = 0.3337, $P = 3.56E-023$). Although allometric slopes did not differ significantly between populations (Wilks' Lambda = 0.580, $P = 0.0626$), shape was significantly different when size was held constant allometrically (Wilks' Lambda = 0.415, $P = 1.007E-006$), indicating different intercept for the studied populations. Therefore, geographic populations have separate allometric directions but are parallel to each other.

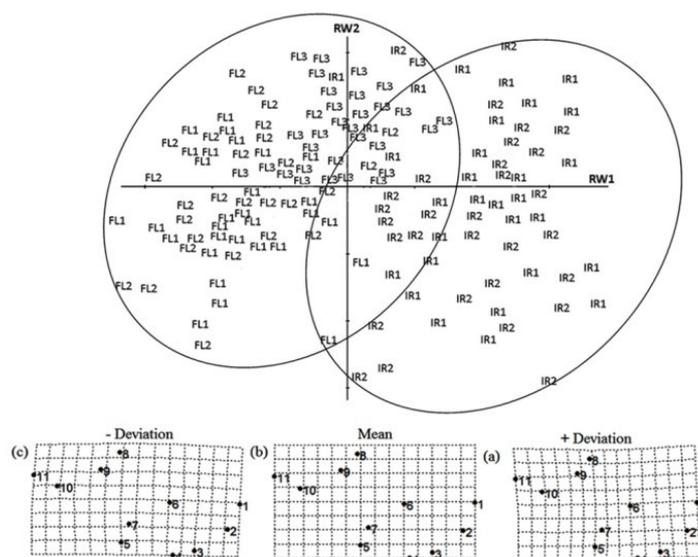


Fig. 3. Upper part: Scatter plot for the RW1 and RW2 on the forewing of *Diaphorina citri*. FL, Florida populations; IR, Iran populations (See table 1 for abbreviations). Lower part: Deformations in shape variables along the RW1 in the forewings of *Diaphorina citri* populations, (a) positive deformation, (b) consensus configuration, and (c) negative deformation.

Table 4. Pair-wise generalized squared distance matrix among the populations of *Diaphorina citri* from Iran and Florida.

Population	IR.2	IR.1	FL.1	FL.2	FL.3
IR.2	0				
IR.1	2.171	0			
FL.1	19.528**	15.157**	0		
FL.2	20.030**	16.654**	1.276	0	
FL.3	13.806**	12.052**	7.002	6.563	0

**P-values significant at the 0.01 level.

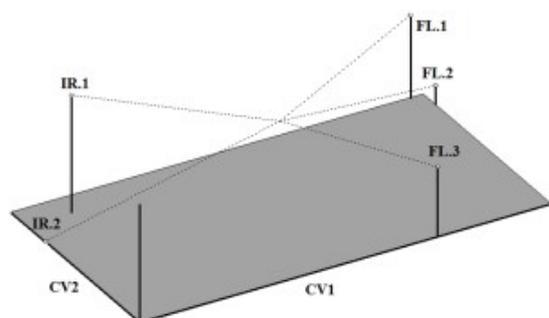


Fig. 4. Canonical variate ordination in the populations of *Diaphorina citri* from Iran and Florida. The vertical axis (not labelled) is the third canonical variate axis. See Table 1 for more description.

Discussion

Multivariate statistical analysis of partial-warp scores showed shape differences among geographic populations. The studied populations of ACP were clustered into two major population groups: Iran and Florida. Although two Iranian populations in the current study (Chabahar and Jiroft) were clustered together, in the previous geometric morphometric study, Lashkari *et al.* (2013) showed different shape and size among Iranian populations, as Sarbaz population (in Sistan & Baluchestan province) differentiated from Kerman populations. It seems that transferring of agricultural materials have led to the occurrence of gene flow among Sarbaz (near to the Pakistan border) and Pakistan *D. citri* populations (Lashkari *et al.*, 2013), also the Sarbaz population was collected from an arid climate region with elevation about 300 m (*above sea level*), whereas the Chabahar population is from a humid climate region with elevation about 7 m (*above sea level*).

Iran populations had a smaller wing size compared with Florida populations, that may develop by the interactions of genetic mechanisms and environmental processes. For example, these localities have different climatic conditions; The Iranian populations belonged to warm desert climate (also known as an arid climate) (Chabahar) and warm semi-arid climate (Kerman-Jiroft), that were characterized by moderate winter and very warm summer, besides cool winter and very warm summers, respectively; whereas, the Florida populations

were collected from a humid subtropical climate, with hot, usually humid summers and mild to cool winters. Although there are a number of climatic and ecological factors that may vary in these regions, one of the most important factors is the effect of temperature on wing size, as wing length has been decreased linearly at higher temperatures in several studies (Nayar, 1969; Partridge *et al.*, 1994; Atkinson, 1995; De Moed *et al.*, 1997; Reiskind & Zarrabi, 2012).

In reviewing the literature, no geometric morphometric data were available on the geographic populations of *D. citri* in Iran and Florida, but molecular data showed that Iran and Florida populations of ACP were mtCOI Haplotype-1, based on a mitochondrial haplotype network (Lashkari *et al.*, 2014). However, our geometric morphometric data contrast previous molecular research, as the Iran and Florida populations were clustered separately. Although, this study showed evidence from incongruence of the morphometric and molecular data in the *D. citri* populations related to the mtCOI Haplotype-1 (Iran and the USA), additional evidence using more populations, genes and morphometric characters needs to be confirmed. In reviewing the literature, when comparisons of molecular and morphological data were made on constructing phylogenies, it was found that these changes may be independent (Hillis & Moritz, 1990). Although data of DNA sequence does have many advantages, but the amount of data is limited by the studied genome size. Studies that incorporate both types of data may provide more appropriate results (Hillis & Moritz, 1990). Another explanation could be the selected molecular markers and sampling conditions used in previous studies. The studies of Boykin *et al.* (2012) and Lashkari *et al.* (2014) were based only on the mitochondrial DNA, with relatively few ACP specimens from each location. Although mtDNA is a very useful marker because of its small size, relatively rapid rate of evolutionary change, and maternal inheritance (Hillis & Moritz, 1990), so additional molecular studies using nuclear DNA markers might be valuable such as microsatellites on the phylogenetic relationships among populations of ACP. Incongruence among the methods

used in intraspecific variation studies has been indicated previously (Bomfim *et al.*, 2011; Gómez-Palacio *et al.*, 2012).

Invasive insects often undergo rapid genetic changes during introduction to new environments (Hernandez *et al.*, 2010). In this study, two arguments lead us to consider that the observed differences between the studied populations are probably due to genetic, rather than environmental factors. Firstly, there were significant multivariate differences in shape, even after removing size variation. Removal of size as a different principle reveals a pattern of variation that would be more related to evolutionary differences rather than environmental factors (Dujardin *et al.*, 1999). These findings are consistent with those of Adams & Funk (1997), Dujardin *et al.* (1999) and Mozaffarian *et al.* (2007), who found shape variation after removing size in populations of *Neochlamisus bebbianae* (Brown, 1943), *Lutzomyia quinquefer* (Dyar, 1929) and *Ectomyelois ceratoniae* (Zeller, 1839), respectively. Secondly, there was a significant correlation between geographic and morphometric distance matrices, suggesting that the amount of morphological divergence could be influenced by the level of geographic isolation, with no need to infer climatic or ecological differences (Dujardin *et al.*, 1999). This result was similar to Lashkari *et al.* (2013), Dujardin *et al.* (1999) and Mozaffarian *et al.* (2007), who found a significant correlation between morphologic and geographic distances in geographical populations of *D. citri*, *Lutzomyia quinquefer* (Dyar) and *E. ceratoniae*, respectively. Lashkari *et al.* (2013) showed differentiation between the populations of Iran and Pakistan.

Thus, it is clear that part of the morphological variation of ACP is developed by evolutionary divergence, which involves interactions of genetic and environmental processes that caused genetic differentiation of populations. These genetic differences can be transferred to the next generations and affect morphology, physiology, behaviour, life-history traits and control methods. The existence of differentiation between populations of *D. citri* might have important

consequences in the insect pest management for their ability to transmit HLB disease agent (Lashkari *et al.*, 2014). The mitochondrial haplotype network that was constructed for *D. citri* suggested an ancestral position of haplotype Dcit-1 (Boykin *et al.*, 2012). The results of this research are useful for detection of the geographical origin of the pest for Iran and Florida. Lashkari *et al.* (2014) showed a link between Iran and Florida and it is estimated that the recent invasion of Iran and Florida has the same origin because of common similarity of *D. citri* specimens in both countries based on the mitochondrial gene cytochrome oxidase I (mtCOI) and the *Wolbachia-diaphorina*, *wDi*, gene *wsp* sequence analyses. But, these populations might have genetic differentiation. Therefore, it seems differentiations within populations are related to mtCOI haplotype-1. Our understanding about the origin of this pest can be resulted in the identification of effective biological control agents in Iran and Florida. *Tamarix radiata* (Waterston) (Hymenoptera: Eulophidae), one of the most effective biological control agent of ACP has been successfully introduced into some geographical locations, such as Réunion Island, Taiwan, Mauritius, Guadeloupe, Florida, the Philippines and Indonesia (East Java). It significantly reduced the populations of ACP in Réunion Island, but was less effective in Florida (Grafton-Cardwell *et al.*, 2013; Hall *et al.*, 2013). It seems that other haplotypes of *T. radiata* (especially from countries having ACP haplotype 1) might be more effective for Florida. Therefore, it suggests that intraspecific variation of this species could be examined using genetic markers (such as microsatellite markers) and more mtCOI haplotype-1 populations (such as populations from India, Saudi Arabia, Brazil, Mexico and Texas) for further understanding. It would be advantageous for growers in these countries (related to ACP haplotype 1) to compare their control strategies if they have similar populations.

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