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ORIGINAL ARTICLE

Genetic structure of populations of *Aphis gossypii* (Hemiptera: Aphididae) on citrus trees in Northern Iran

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Abstract. The cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is a serious pest of citrus in northern Iran, both because of the damaged caused by its feeding and as a vector of several viruses. The genetic structure of populations of *A. gossypii* on citrus trees at eight localities in Iran was surveyed using seven polymorphic microsatellite loci. Of 240 individuals tested, 142 multilocus genotypes (MLGs) were identified. The presence of multicopy genotypes and negative F_{IS} values revealed that the major mode of reproduction in northern Iran is obligate parthenogenesis. The genotypic diversity of populations ranged between 0.24 and 0.93. Considerable genotypic diversity and a high frequency of unique MLGs, confirmed there is some cyclical parthenogenesis in the region. The analysis of molecular variance revealed high intrapopulation and weak interpopulation genetic differentiation (overall F_{ST} = 0.036) among the different populations. The UPGMA dendrogram of eight populations based on Nei's genetic distance indicated two clusters: genotypes from West of Mazandaran and Guilan provinces and those from East of Mazandaran. The same results were also obtained from the STRUCTURE analysis of these populations. This information on the genetic diversity of populations of *A. gossypii* in northern Iran could be useful for improving the Integrated Pest Management of this aphid.

INTRODUCTION

During the last four decades, the use of molecular markers has provided new information about the biology of aphids including their phylogeny, life-cycles, host-plants and geographical dispersal. The greatest contribution of these markers has been in the genetic differentiation among and between populations (Loxdale & Lushai, 2007; Loxdale et al., 2017). Generally, aphids have two modes of reproduction: obligate parthenogenesis and cyclical parthenogenesis with a single sexual generation that produces the over-wintering diapausing eggs (Dixon, 1998). However, some species of aphids have only one overwintering strategy and others overwinter both anholocyclically and holocyclically (Dedryver et al., 1998).

The cotton-melon aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is a cosmopolitan species distributed worldwide (van Emden & Harrington, 2007). It is an important pest of various plant families, such as Rutaceae, Cucurbitaceae, Malvaceae, Solanaceae and Rosaceae (Ebert & Cartwright, 1997; Razmjou et al., 2006). It causes direct damage by feeding on sap and excreting honeydew and also indirect damage by transmitting several pathogenic plant viruses (Bertolini et al., 2000; Wang et al., 2016). There are two major citrus growing areas in Iran: (i) The northern part includes the Mazandaran and Guilan provinces in the Caspian Sea belt with a Mediterranean climate and periodically cold winters. These two provinces produce about 50% of the citrus fruit in Iran; (ii) The provinces in the southern region have tropical and subtropical climates (Bani Hashemian et al., 2013). *A. gossypii* is a serious pest of citrus orchards in northern Iran (Rassoulian et al., 2001), with cotton, cucumber, pumpkin and hibiscus as the other hosts of this aphid (Razmjou et al., 2010; Kheyrollahi et al., 2013). Moreover, it is a vector of *Citrus tristeza virus*, the most important viral pathogen of citrus (Moreno et al., 2008), in the region (Alavi et al., 2000). Citrus yellow vein clearing virus and Citrus vein enation virus were also recently reported in this area (Nouri et al., 2016; Bani Hashemian & Aghajanzadeh, 2017). These pathogens can be transmitted by *A. gossypii* (Vives et al., 2013; Zhou et al., 2015). There are many diverse kinds of natural enemies in the citrus orchards of northern Iran, so little insecticide is used to control aphids in this region (Aghajanzadeh et al., 1997).

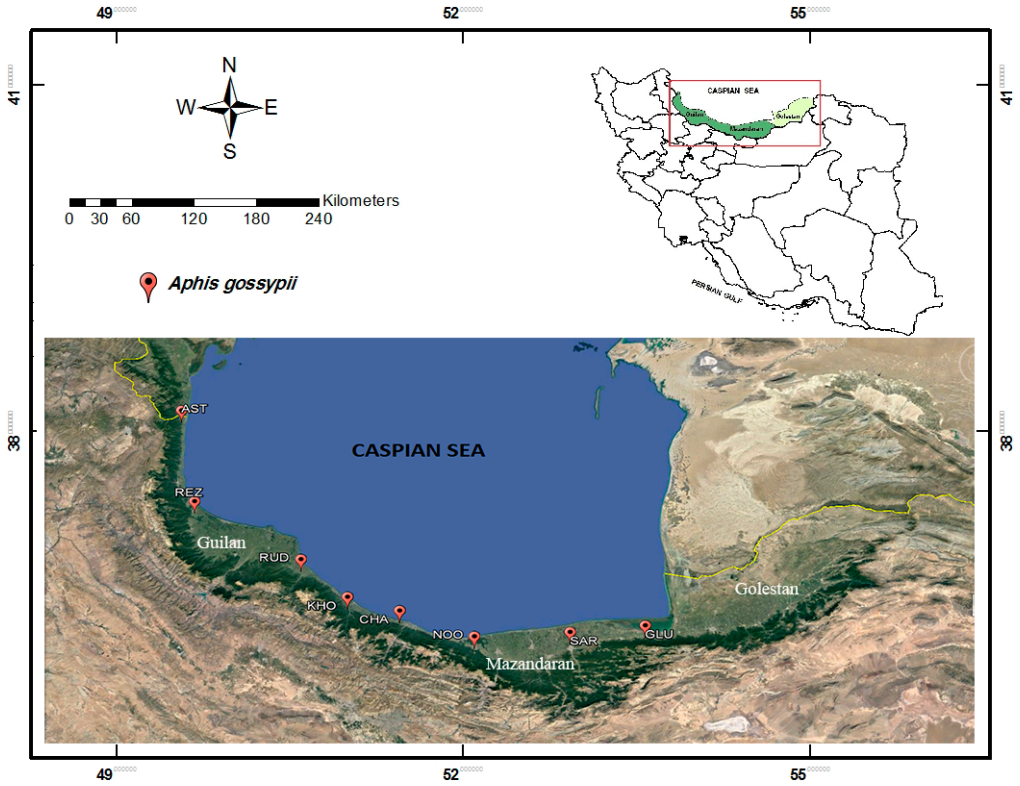

Fig. 1. Maps of Iran (above) and the Caspian Sea region (below) showing the locations where *Aphis gossypii* were collected. Population codes are the same as in Table 1.

The great diversity in the life cycles, host plants and geographical range of aphids makes them excellent models for research into these and many other aspects of their biology (Loxdale & Balog, 2018), including their genetics (Blackman & Eastop, 2007).

The genetic variation of aphid populations can certainly be affected by host plant. The relationship between the genetic diversity of *A. gossypii* and different host plants has been revealed by the use of polymorphic microsatellite markers, which indicate that one of three predominant multilocus genotypes is associated with each of the three plant families, Cucurbitaceae, Solanaceae and Rutaceae (Charaabi et al., 2008; Carletto et al., 2009). Further examples of such host plant-genetic diversity correlations are reported for other species of pest aphid, like the grain aphid, *Sitobion avenae* (F.) on wheat and cocksfoot grass (Sunnucks et al., 1997) and different grasses and cereals. (Lushai et al., 2002).

According to Delmotte et al. (2002), the genetic structure of sexual and asexual populations of the bird cherry-oat aphid, *Rhopalosiphum padi* (L.), based on microsatellite data, reveals a high level of heterozygoty in asexual populations and variations in the life cycle of the black bean aphid, *Aphis fabae* Scopoli, associated with particular climatic conditions (Sandrock et al., 2011). In the case of *A. gossypii* populations in greenhouses in southern France, use of seven microsatellite loci has revealed they are mainly asexual (Fuller et al., 1999).

Genetic differences between aphid populations from different localities is also reported. For example, variation in microsatellite markers is a useful tool for genomic characterization of the soybean aphid, *Aphis glycines* Matsumura and the cabbage aphid, *Brevicoryne brassicae* (L.) from different geographic regions (Ruiz-Montoya et al., 2003; Michel et al., 2009; Jun et al., 2011). In addition, the genetic structure of populations of the Spirea aphid, *Aphis spiraecola* Patch on pear trees in 13 provinces in China has revealed high levels of genetic exchange facilitated by geography and climate (Cao et al., 2012). In other similar studies, molecular markers are used to demonstrate migration and dispersal in several other pest species of aphids (Delmotte et al., 2002; Llewellyn et al., 2003; Lushai & Loxdale, 2004).

In the present study, a suite of polymorphic microsatellite markers was used to study the genetic diversity and genetic structure within and among populations and thereby deduce the life cycle of the *A. gossypii* that infest citrus trees in the north of Iran.

MATERIAL AND METHODS

Sample collection

Aphid samples were collected from different commercial citrus orchards at eight locations in the Guilan and Mazandaran provinces in northern Iran (Fig. 1). Thirty individuals were collected within an area of approximately 10 km^2 at each location in 2015 and 2016 (Table 1). Aphids from a single tree were considered as one sample and only one aphid per sample was analyzed. Aphids were taken from trees that were at least 50 m apart from each other and the distance between any two populations was >50 km. Aphids were stored in micro tubes in 75% ethanol at 20°C prior to DNA extraction.

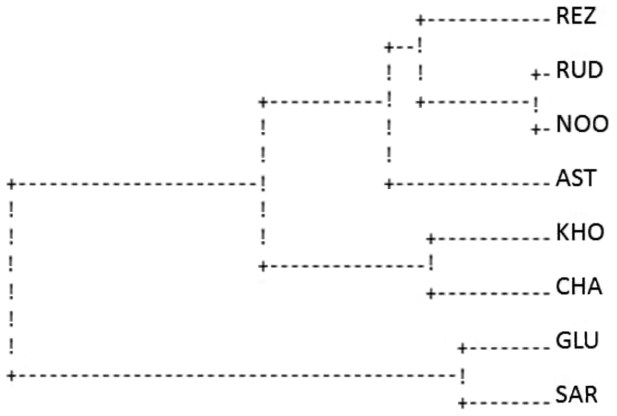

Fig. 2. UPGMA dendrogram based on Nei's genetic distance (1978) showing a clustering according to localities.

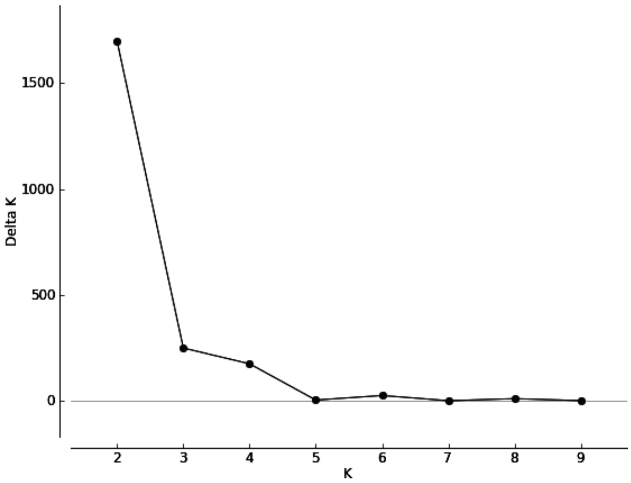

Fig. 3. ΔK plot used to determine most probable *K*.

DNA extraction and microsatellite analysis

DNA was extracted from one aphid using the cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle, 1987). The DNA was diluted 10-fold and stored at –20°C. PCR was performed using 10 µL of the reaction mixture, containing 6 µL of 2× PCR Master Mix (Jena BioScience GmbH, Jena, Germany), 1 µL (10 pM) of each forward and reverse primer (Ago89, Ago66, Ago24, Ago59, Ago53, Ago84, Ago69, Ago126) (Vanlerberghe-Masutti et al., 1999) and 1.5 µL of aphid template DNA (approximately 10 ng), using the following cycling parameters: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, locus-specific annealing temperature 1 min according to Vanlerberghe-Masutti et al. (1999); and extension at 74°C for 30 s, with a final extension at 74°C for 5 min. Products were separated on 6% polyacrylamide urea gel at 75 W constant power (Bio-Rad Laboratories, Hercules, CA, USA). DNA fragments were visualized using ethidum bromide staining and documented using the UV DOC system (EBOX VX5/20, VilberLoumat, France). Allele sizes were determined by comparison with the standard DNA Ladders SM1203 and SM1153 (Fermentase, Waltham, MA, USA).

Data analysis

The GenClone v.2.0 program (Arnaud-Haond & Belkhir, 2007) was used to identify different multilocus genotypes (MLG), calculate genotypic diversity (*R*), defined as $(G-1)/(N-1)$, where *G* is the number of different MLGs and *N* is the number of individuals in a population and P_{sex}, the probability of replicate MLGs being produced by independent sexual events. Calculation of the number of alleles (N_a), the effective number of alleles (N_e), *F*-statistics and Hardy-Weinberg Equilibria (HWE) was done using GENAlEX V 6.5 (Peakall & Smouse, 2012). To analyze deviations from HWE, only a single representative of each multilocus genotype (MLG) was considered. Also, analysis of Molecular Variance (AMOVA) was calculated using this program in order to determine differences within and among populations. A Mantel test of isolation by distance (IBD) with 1,000 permutations was run in GENAlEX. MICRO-CHECKER software (van Oosterhout et al., 2004) was used to test for the possible occurrence of null alleles at the microsatellite loci tested. Genetic distance was calculated and a UPGMA dendrogram depicted using POPGENE 32 (Yeh et al., 1999).

STRUCTURE program version 2.3.4 (Pritchard et al., 2000) was used to infer the structure of the population and estimate the most likely number of distinct population clusters. The number of clusters (*K*) considered was from 1 to 10, and 5 replications with a burn-in length of 500,000 iterations and a Markov chain of 500,000 steps were executed. The most likely number of genetic clusters based on the log probability of the data was calculated using the method of Evanno et al. (2005).

Table 1. Summary of the information and genetic variability revealed by using seven microsatellite loci and *Aphis gossypii* collected from eight populations infesting citrus trees in northern Iran.

Sample collected from	Rezvanshahr - Guilan	Khoramabad - Mazandaran	Chaloss - Mazandaran	Rudsar - Guilan	Noor - Mazandaran	Sari - Mazandaran	Gluogah - Mazandaran	Astara - Guilan
Population code	REZ	KHO	CHA	RUD	NOO	SAR	GLU	AST
Coordinates	37°31´N/ 49°08´E	36°45´N/ 0°49´E	36°40´N/ 51°21´E	37°04´N/ 50°18´E	36°29´N/ 52°07´E	36°36´N/ 53°03´E	36°43´N/ 53°47´E	38°23´N/ 48°50´E
N	30	30	30	30	30	30	30	30
G	13	8	15	16	21	28	17	24
R	0.41	0.24	0.48	0.52	0.69	0.93	0.55	0.79
Repeated genotype	9	6	5	5	5	1	4	5
Significant P_{sex}	5	5	3	4	3	0	2	2
N_a	3.86	2.86	4.00	4.14	4.29	4.71	3.90	4.86
N_e	3.17	2.31	2.61	3.01	3.16	3.18	2.82	3.14
H_o	0.91	0.82	0.93	0.88	0.88	0.85	0.87	0.83
H_e	0.66	0.57	0.63	0.65	0.66	0.67	0.66	0.66
F_{IS}	–0.39	–0.45	–0.50	–0.36	–0.36	–0.29	–0.34	–0.28

N – no. of individuals per pop; *G* – multilocus genotype (clone sensu lato); *R* – clonal diversity; Repeated genotype – number of repeated genotypes per sample; Significant P_{sex} – number of clonal genotypes; N_a – observed no. of alleles per locus; N_e – effective no. of alleles per locus; H_o – observed heterozygosity; H_e – expected heterozygosity; F_{IS} – fixation index.

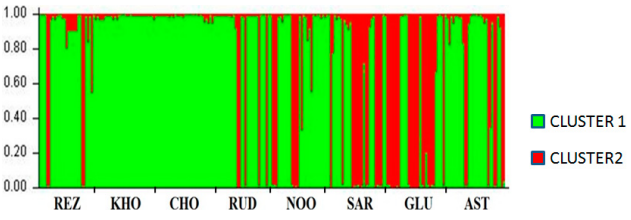

Fig. 4. Cluster analysis of the *Aphis gossypii* samples from northern Iran. Assignment of the multilocus genotypes of the 8 populations to clusters (K = 2). Each multilocus genotype is represented by a vertical bar. Geographic regions in which the populations were located are indicated along the x-axis.

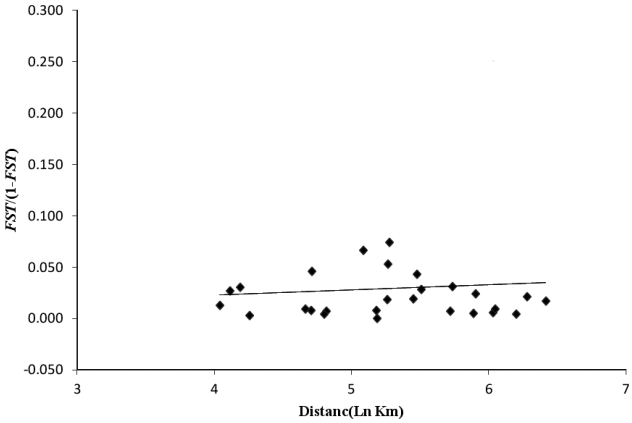

Fig. 5. Isolation-by-distance plot of $F_{ST}/(1-F_{ST})$ plotted against the natural logarithm of geographical distance (km) for all of the 8 geographically separated populations.

RESULTS

Genic and genotypic diversity

Samples were genotyped using seven of the eight microsatellite primer pairs designed earlier by Vanlerberghe-Masutti et al. (1999). Locus Ago24 produced the same band pattern in all individuals and was hence excluded from the calculations. A total of 142 multilocus genotypes (MLGs) were distinguished in the 240 individuals. We refer to these as clones sensu lato (Loxdale, 2008). The number of MLGs ranged from 8 to 28 within each population. The lowest and highest genotypic diversity were recorded for KHO and SAR, respectively (Table 1). In the populations KHO, CHA and REZ, the genotypic diversity was ≤ 0.5.

Highly significant P_{sex} values indicate that most of repeated genotypes in the samples were members of the same clone. However, the number of repeated genotypes were assembled independently by sexual reproduction (Table 1). The observed and effective number of alleles per locus for each population were 2.857–4.857 and 2.310–3.182, respectively (Table 1). The observed (H_o) and expected (H_e) heterozygosity values ranged from 0.82–0.93 and from 0.57–0.67, respectively (Table 1). Comparison of the H_o and H_e values for the 8 populations of *A. gossypii* revealed that H_o values in all populations were greater than their H_e values. The F_{IS} values of –0.28 to –0.50 indicate excess heterozygosity, which is reflected in the negative values for the overall F_{IS} (Table 1).

Genetic differentiation among populations

The AMOVA analyses revealed that 3% of the genetic variation was among populations and 97% within individuals (Table 4). This signifies that the genetic differentiation among populations (overall F_{ST} = 0.036) is less than within populations. Most of the genetic differentiation among the 8 populations of *A. gossypii* were not significant based on the matrix of pairwise F_{ST} values (Table 3). F_{ST} values showed a weak genetic structuring, while only 3 of 28 pairwise comparisons exceeded 0.05 (Table 3).

According to the IBD analysis, there was no significant correlation between linearized F_{ST} and geographic distance (r = 0.072., P = 0.71; Fig. 5) for the 8 populations. The minimum and maximum genetic distances were between the NOO and RUD populations (0.0012), and KHO and SAR populations (0.2031), respectively (Table 2). The UPGMA dendrogram of the 8 populations based on Nei's genetic distance is depicted in Fig. 2. The GLU and SAR populations from East of Mazandaran clustered together and populations from the rest of Mazandaran (NOO, CHA and KHO) and Guilan (RUD, REZ and AST) were in another cluster.

The STRUCTURE analysis of *A. gossypii* populations estimated the log-likelihoods and the number of genetic clusters (K) (Evanno et al., 2005) (Fig. 3). These results indicate that the 8 populations group in two clusters: 97.08% of individuals could be assigned to one of the clusters with more than 80% probability and 2.92% were considered to be admixed with K = 2 (Fig. 4). Most genotypes collected from SAR and GLU were placed in cluster 1, while all genotypes from KHO and CHA, and most of those from the other localities were assigned to cluster 2 (Fig. 4).

Table 2. Nei's unbiased genetic distance measures among eight populations of *Aphis gossypii* collected from citrus trees in northern Iran.

Pop	REZ	KHO	CHA	RUD	NOO	SAR	GLU	AST
REZ	****							
KHO	0.0484	****						
CHA	0.0603	0.0254	****					
RUD	0.0302	0.0779	0.0152	****				
NOO	0.0342	0.1324	0.0731	0.0012	****			
SAR	0.0321	0.2031	0.0164	0.0668	0.0562	****		
GLU	0.0398	0.1683	0.1243	0.0340	0.0220	0.0199	****	
AST	0.0420	0.1046	0.0714	0.0541	0.0215	0.0490	0.0476	****

Table 3. Pairwise F_{ST} values among the eight populations of *Aphis gossypii* collected from citrus trees in northern Iran.

Pop	REZ	KHO	CHA	RUD	NOO	SAR	GLU	AST
REZ	****							
KHO	0.018ns	****						
CHA	0.028ns	0.013ns	****					
RUD	0.007ns	0.030ns	0.004ns	****				
NOO	0.007ns	0.046ns	0.027ns	0.000ns	****			
SAR	0.006ns	0.074**	0.066*	0.024*	0.008ns	****		
GLU	0.004ns	0.053*	0.043*	0.005ns	0.008ns	0.003ns	****	
AST	0.009ns	0.031ns	0.024ns	0.019ns	0.009ns	0.021*	0.017ns	****

* P < 0.05, ** P < 0.01, ns – not significant.

Table 4. Analysis of molecular variance used to compare the genetic variation in populations of *Aphis gossypii*, collected from citrus trees in northern Iran.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P Value
(A) Among geographic populations	7	50.258	0.098	3%	P < 0.001
Within individuals	240	765.000	3.188	97%	P < 0.001
(B) Among 2 clusters inferred by STRUCTURE	1	93.300	0.529	14%	P < 0.001
Within individuals	233	745.000	3.197	86%	P < 0.001

(A) Populations collected from citrus trees; (B) Two clusters according to Bayesian analysis.

DISCUSSION

In the present study, the genetic structure of populations of the aphid, *A. gossypii*, collected from citrus orchards at different localities in the Caspian Sea region of Iran was analyzed. The presence of multicopy genotypes, highly significant P_{sex} values in repeated genotypes and negative F_{IS} values indicate that this aphid reproduces mainly by obligate parthenogenesis in the northern part of Iran (Table 1). *A. gossypii* is an obligate parthenogen in most parts of the world (Blackman & Eastop, 2000), but in some regions like North America, East Asia and China there are populations that are cyclically parthenogenetic (Zhang & Zhong, 1982; Blackman & Eastop, 2007). Previous investigations on the genetic diversity of populations of *A. gossypii* using eight microsatellite loci also indicate asexual reproduction, based on the nonrecombinant genotypes present in the populations (Fuller et al., 1999; Brévault et al., 2008; Charaabi et al., 2008; Chen et al., 2013). The same results are also reported for other species of pest aphids (e.g. Sunnucks et al., 1996; Miller et al., 2003; Vorburger et al., 2003; Vorwerk & Forneck, 2006; Aradottir et al., 2012).

Although we detected a clear genetic signature of clonal reproduction, existence of 142 different multilocus genotypes among the individuals and the number of non-significant P_{sex} values in single population indicate that some of the clones of this aphid in this region reproduced sexually (Table 1). Cyclical parthenogenesis can result in more genotypic diversity in populations of this aphid (Simon et al., 1996; Delmotte et al., 2002; Wilson et al., 2002; Cao et al., 2012). Razmjou et al. (2010) report that samples of *A. gossypii* from cotton in the Caspian Sea region of Iran have a high genotypic diversity and many lineages on this host are cyclically parthenogenetic. A recent study in northern China showed considerable genotypic diversity among *A. gossypii* populations on cotton (Luo et al., 2016). Such studies are very valuable because they can be used to indirectly infer the type of life-cycle of an aphid (Le Trionnaire et al., 2008).

Whether or not aphids reproduce sexually or asexually depends on the winter climate (Rispe & Pierre, 1998). Sandrock et al. (2011) show that life cycle variation in the black bean aphid, *A. fabae*, is related to climate and aphids from areas with mild winters overwinter anholocyclically more than those from areas with cold winters. A mild winter in the Caspian Sea belt enables *A. gossypii* to overwinter parthenogenetically. Nevertheless, the occurrence of periodical cold winters seemingly induces some cyclical parthenogenesis and production of overwintering eggs in this region. When winters are cold, overwintering as sexually produced eggs is a dependable strategy because eggs in diapause are very cold-resistant (James & Luff, 1982; Rispe & Pierre, 1998; Simon et al., 1999).

Application of broad-range insecticides to host plants to control a pest can decrease the number of genotypes. In West and Central Africa, the increased use of insecticides on both cotton and other host plants (okra and roselle) has led to the prevalence of one genotype of *A. gossypii* resistant to different classes of insecticides (Brévault et al., 2011). In the Caspian Sea region, insecticides are rarely used to control citrus aphids, so the presence of many aphid genotypes is expected.

Results of the IBD analysis revealed that geographic distance had no effect on *A. gossypii* population structure. The AMOVA analysis identified a low level of population differentiation (Global F_{ST} = 0.036) among populations of *A. gossypii* from citrus trees in northern Iran. Low genetic differentiation of *A. gossypii* was also indicated by both the UPGMA dendrogram based on Nei's genetic distance and the STRUCTURE analysis. These high intrapopulation and weak interpopulation genetic diversity patterns may reflect significant gene flow among populations. The amount of gene flow based on the F_{ST} values support such a scenario. However, N_m (number of migrants) values varied greatly, ranging from 3.11 to 89.03 (data not shown). Our results are in agreement with the findings that low genetic differentiation confirms high gene flow. Little genetic differentiation among bird cherry-oat aphid (*R. padi*) populations in France, indicate extensive gene flow, at least over short distances (Delmotte et al., 2002). In a study on the peach-potato aphid, *Myzus persicae* (Sulzer) in Victoria, Australia, the low overall F_{ST} value recorded and the widespread occurrence of the two most common genotypes provided evidence that individuals of this aphid move relatively freely in that region (Vorburger et al., 2003).

In northern Iran, citrus orchards are situated along the Caspian Sea belt where there are no geographical barriers so aphids can move easily across this region. As a consequence, the lack of complete genetic separation of populations in this area is a reasonable deduction from our data. Aphids can migrate over long distances (Simon et al., 1999; Llewellyn et al., 2003), although, short-distance movements are generally more important for the distribution of genotypes in aphid populations (Loxdale et al., 1993). The genetic structure of *A. gossypii* collected from six localities in Tunisia confirmed that genetic differentiation among localities was not statistically significant (Charaabi et al., 2008), whilst a study of the genetic structure of sexual French populations of *R. padi* showed little geographical

differentiation among them, confirming the high dispersal ability of this aphid (Delmotte et al., 2002).

Based on the results of the STRUCTURE analysis, the SAR and GLU populations were grouped in one cluster and the other populations in another. The AMOVA also detected significant differences between these two clusters (Table 4). The east of Mazandaran, which includes the SAR and GLU populations has colder winters, warmer summers and less rainfall compared to other parts of the Caspian Sea region (Pirnia et al., 2015). Also, there are alternate hosts for *A. gossypii* there. The aphids on these hosts can also colonize citrus trees. So, the genetic differences between the two clusters may due to the above reasons. Further studies are required to determine the importance of these various factors. According to Cao et al. (2012), differences in climate in different regions should be considered when attempting to understand the population structure of *A. spiraecola*.

In conclusion, there is considerable genotypic diversity and high intrapopulation and weak interpopulation genetic differentiation among geographic populations of *A. gossypii* in northern Iran. Furthermore, *A. gossypii* on citrus, reproduces predominantly by obligate parthenogenesis in northern Iran. As indicated by this study and other studies on different species of aphids, assessment of population genetic diversity can be used in Integrated Pest Management and the findings used to develop more rational and sustainable methods of control. Lastly, based on the information gained during this research, it appears likely that further projects can be developed to study the relationship between the aphid's genotype and virus transmission, such as that done earlier for *M. persicae* by Terrodot et al. (1999).

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