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Host-associated Genetic Differentiation of the Green Citrus Aphid, *Aphis spiraecola* (Hemiptera: Aphididae) in Algeria

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ABSTRACT

The green citrus aphid, *Aphis spiraecola* Patch, is an important pest attacking many plant species, including citrus. We have analyzed the genetic variability among green citrus aphid adults sampled from six citrus cultivars grown in Algeria (an orange, a grapefruit, a lemon and three mandarin cultivars), using the random-amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique. Analysis of molecular variance (AMOVA), based on RAPD markers, indicated a significant difference between the studied samples, correlated to the host plant species, while host cultivar and geographical origin had no significant impact on the genetic diversity. Two-dimensional PCO analysis confirmed AMOVA results, showing the grouping of the different insects into four major clusters according to their host plant species. The Neighbor-Joining dendrogram constructed based on the Euclidian distance grouped the accessions into four main clusters according to their host plant species genotypes, giving insight into the coevolution of insect strains with their corresponding citrus species. In order to investigate any possible relationship between the genetic aggregation of insect genotypes and the leaf morphology in citrus species, we carried out leaf morphological characterization and surveyed the degree of infestation of the studied citrus cultivars. Leaves of grapefruit and orange were the most similar morphologically and the most attacked by aphids, suggesting that genetically close biotypes would be compatible with these two species. Results of this study are a step toward the development of an integrated controlling strategy against *A. spiraecola* in North Africa, taking into account the host specialization that seems to play a key role in shaping the genetic diversity of *A. spiraecola*.

Key words: Citrus, *Aphis spiraecola*, RAPD-PCR, genetic structure, host strains.

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INTRODUCTION

Aphis spiraecola Patch 1914 is a cosmopolitan pest (Barbagallo, Cocuzza, Cravedi, & Komazaki, 2007). It can colonize a very wide range of secondary hosts belonging to more than 20 plant families, including Caprifoliaceae, Asteraceae, Rosaceae, Rubiaceae and Rutaceae (Blackman & Eastop, 2006). Citrus species are the most important hosts for this insect; therefore, another scientific name, *A. citricola* auctt. nec Van der Goot 1912 has been used (Andreev, Rasheva, & Kutinkova, 2009). The primary economic impact of this insect not only arises from foliage distortion, but also from promoting growth of sooty mold and attracting ants which fend off natural enemies of aphids; especially, spring-time attacks are the most deleterious in young citrus orchards (Satar & Uygun, 2008). In addition, it can transmit phytoviruses such as cucumber mosaic virus (CMV), plum pox virus (PPV) and zucchini yellow mosaic virus (ZYMV) (Blackman & Eastop 2007) and may also involve in the transmission of citrus Tristeza virus (CTV) (Yahiaoui, Djelouah, D'Onghia & Catara, 2012).

Hermoso de Mendoza, Arouni, Belliure, Carbonell, & Pérez-Panadés (2006) considered *A. spiraecola* among the most harmful citrus aphids in the Mediterranean region. In Algeria, it is very harmful to citrus fruits (Saharaoui, Benzara, & Doumandji-Mitiche, 2001). Its occurrence in different citriculture regions has been reported by several authors (Benoufella-Kitous, Doumandji, & Hance, 2014; Lebbal & Laamari 2015; Lebbal & Laamari 2016; Ali Arous, Guenaoui, & Djelouah, 2017). It is attacking different citrus species and varieties such as orange (Labdaoui & Guenaoui, 2015), Clementine (Mostefaoui, Allal-Benfekih, Djazouli, Petit, & Saladin, 2014), mandarin (Lebbal, 2017) and lemon (Saharaoui & Hemptinne, 2009). The spreading of *A. spiraecola* has been facilitated by the existence of few effective parasitoids of this aphid (Labdaoui & Guenaoui, 2017). Even though it has been frequently reported, there is a deep lack of studies focusing on the population genetic structure of *A. spiraecola* in Algeria. Therefore, a clear understanding of the potential paths of gene flow could provide useful insights for the management of this pest.

There is a growing awareness of the importance of natural selection in driving population genetic divergence in agricultural pests, and several cases of this differentiation are provided by the existence of genetically distinct host forms in phytophagous insects. The genetic differentiation of insect communities usually occurs through feeding and/or oviposition site choices. These choices are usually driven by the genetic variation in host plants, leading to host-associated differentiation potentially driving insect evolution and speciation (Zytynska et al, 2014). Herbivorous insects are known to feed on a restricted range of plants, and herbivore preference and performance can vary among host plants within a species due to genetically based traits of the plant (e.g. defensive compounds) or among a range of host species. In a natural system, genetic variation within both plant and herbivore communities is a major factor that influences species interactions. Sap-sucking insects, such as aphids, experience an intimate relationship with their host plant and many aphid species exhibit genetic variation in host preference and performance on different host plants (Nikolakakis, Margaritopoulos, & Tsitsipis, 2003). Different aphid genotypes have

also been found to preferentially colonize different host-plant genotypes (Zytynska & Preziosi, 2011). The term 'biotype' can be defined as an intraspecific classification, segregating individuals by a divergent phenotypic response to an ecological variable (Wenger & Michel, 2013). Biotypic differentiation is a common phenomenon in many insect pests, which is also of major concern in identification and deployment of host resistance genes in crop plants (Weng, Perumal, Burd, & Rudd, 2010).

Molecular markers have greatly enhanced our understanding of the genetic diversity and population structure of many insects (Charaabi et al, 2008; Mezghani-Khemakhem, Bouktila, Kharrat, Makni, & Makni, 2012; Kharrat, Mezghani-Khemakhem, Bouktila, Makni, & Makni, 2012; Abdallah, Mezghani-Khemakhem, Bouktila, Makni, & Makni, 2012, 2013; Béji et al, 2013; 2015). The Random-Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) technique (Williams, Kubelik, Livac, Rafalski & Tingey, 1990) has been frequently used in several studies, to evaluate the genetic diversity of some major invasive species, such as the Mediterranean fruit fly, *Ceratitis capitata* Wiedmann (Haymer, He, & McInnis, 1997), the potato whitefly, *Bemisia tabaci* Gennadius (Hasan, 2006) and the date palm root borer, *Oryctes agamemnon* Burmeister (Abdallah et al, 2012). This technique is rapid, simple, and has the advantage that no prior knowledge of the genome is necessary (Babu, Rajesh, Samsudeen, Minoo, Suraby, Anupama, & Ritto, 2014). In addition, it requires only small amounts of DNA (Kumar & Gurusubramanian, 2011).

The study of the specialization of aphids to a botanical species can contribute to a better understanding of the host plant-aphid interaction, and thus to improve the methods of controlling these bio-aggressors. In this study, an attempt has been made to evaluate the genetic variability within individuals of the green citrus aphid from different citrus species and varieties in Algeria, and characterize the eventual relationship between insect infestation and host plant leaf morpho-metric variables.

MATERIALS AND METHODS

Sampling procedure

During the study period, from January to December 2013, *A. spiraecola* insects as well as citrus leaves were sampled from citrus trees planted in the experimental orchard of the "institut technique de l'arboriculture fruitière (ITAF)" (Emjez Edchich, Skikda). This orchard (36° 42' N and 6° 47' E) is located on an altitude of 200 m, and includes an area of 73.12 ha, reserved to fruit trees.

For citrus leaves, 16 young infested leaves were taken from four trees representing each citrus species, at the rate of four leaves / tree, following the four cardinal points. These young leaves were collected at the tips of young shoots and *A. spiraecola* aphids were counted on each leaf. The same sampling technique has been already applied by Fadamiro et al (2008), Yoldas et al (2011), Kamel (2010) and Mostefaoui et al (2014), to study aphid infestation on vegetative organs of crop trees, in the United States, Turkey, Egypt and Algeria, respectively.

For *A. spiraecola* aphids, only one aphid per tree was analyzed and samples were taken from trees that were at least 10 m apart from each other, in order to avoid biases caused by collecting individuals resulting from the same clone. In total, seventeen adults of *A. spiraecola* were kept for the molecular analysis. These specimens were collected from trees of sweet orange (cv. Thomson Navel), grapefruit (cv. Shambar), lemon (cv. Eureka) and mandarin (cv. Commune, cv. Ortanique and cv. Carvalhal). Two additional individuals were used for comparison purposes, which were collected on lemon cultivar (cv. Eureka) grown in the region of Menzel Bouzelfa, in Tunisia (36° 69' N; 10° 67' E). Aphids were stored in micro tubes in 75% ethanol at 20°C prior to DNA extraction. The botanical relationships between citrus species used for sampling as well as the numbers of *A. spiraecola* samples collected, by host species, are shown in figure 1.

DNA extraction and RAPD-PCR analysis

The DNA was extracted from 19 *A. spiraecola* adults using cetyl trimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle, 1987). The DNA extracted from the different samples was suspended in 30 µl of autoclaved distilled water. DNA concentration was quantified by the Qubit apparatus (Qubit® 3.0 Fluorometer, Invitrogen). DNA amplification from each individual insect, was carried out in a total volume of 25 µl, containing the DNA extract, 5 µl of buffer (5x Green Gotaq®) of the polymerization enzyme, 25 mM of $MgCl_2$, 10 mM of each dNTP (dATP, dGTP, dTTP, and dCTP), 10 µM of each primer, 5 U of *Taq* DNA polymerase. The PCR process was carried out in a 2720 thermocycler (Applied Biosystems, Carlsbad, California, USA), programmed as follows: an initial denaturation (3 min at 94 °C.), followed by 35 cycles of amplification (1 min of denaturation at 94 °C, 1 min of annealing at 36 °C, 1 min of extension at 72 °C) and a final extension (7 min at 72 °C).

Initially, a total of 10 oligonucleotides (Operon Technologies, Almeda, CA, USA) were tested as RAPD-PCR primers. These oligonucleotides were OPA09 (5'- GGG TAA CGC C- 3'), OPA12 (5'- TCG GCG ATA G-3'), OPA14 (5'- TCT GTG CTG G-3'), OPA19 (5'- CAA ACG TCG G-3'), OPH11 (5'-CTT CCG CAG T-3'), OPH18 (5'- GAA TCG GCC A- 3 '), OPH20 (5'- GGG AGA CAT C-3'), OPD9 (5'-CTC TGG AGA C-3'), OPD14 (5'-CTT CCC CAA G-3 '), OPD17 (5'-TTT CCC ACG G-3'). On the basis of the number of markers generated by each one, four primers were retained, namely OPA9, OPA12, OPH18 and OPH20. All amplifications were conducted twice to check for their reproducibility. RAPD-PCR products were subjected to electrophoresis on 1.5% agarose gels, in Tris-Borate-EDTA buffer, then visualized under UV light, after staining in 0.5 µg mL ethidium bromide, and photographed using a photo documentation system (Vilber, Marne-la-Vallée, France). A molecular weight marker (1 Kb Ladder, Invitrogen, Carlsbad, California, USA) was used as a standard in electrophoresis.

RAPD data analysis

RAPD-PCR profiles for each aphid sample were identified visually by scoring the presence or absence of all reproducible bands. The finalized fragment data from all four

primers were pooled to define a single binomial phenotype for each of the 19 samples. Analysis of molecular variance (AMOVA) was performed using GenAlEx v. 6.502 (Peakall & Smouse, 2012), with a test of significance by 999 permutations, in order to compare differences among the aphid samples according to the host-plant, host cultivar and geographical origin. Pairwise Euclidian distances between pairs of samples were calculated using PAST 2.17c (Hammer et al, 2001) and subjected to principal coordinates analysis (PCO) and dendrogram construction by the neighbor-joining (NJ) method, to study the variation among *A. spiraecola* populations.

Leaf morphological characterization and infestation assessment

In order to analyze the relationship between the morphology of citrus leaf and infestation by *A. spiraecola*, young leaves sampled as described above, were used to assess 11 leaf descriptors: (a) the length and (b) width of the leaf; (c) the length of the petiole; (d) the presence of wings and (e) their width; (f) green color intensity; (g) emargination; (h) leaf shape in cross section; (i) leaf twisting, (j) blistering and (k) margin undulation. Parallel to these descriptors, we have evaluated the degree of infestation of each citrus species. Infestation rates as well as morphological characters were submitted to analysis of variance (one way ANOVA) with Student-Newman-Keuls test, using SPSS software (version 10).

RESULTS

RAPD polymorphism assessment

All amplification products used in the statistical analysis were reproducible. Each of the four primers used in the RAPD-PCR technique generated a different number of DNA markers according to the genotypes of the analyzed aphids. A wide range of markers, varying in size between 100 bp and 1,500 bp, was obtained, indicating that the amplification involved diverse loci, among which 32 were polymorphic from the 19 aphids analyzed with the four primers. The number of polymorphic markers obtained by each primer, separately, ranged from 2 (OPH-18) to 15 (OPH-20) with an average of 10 markers per primer (Table 1).

Table 1. Total number of randomly amplified polymorphic DNA (RAPD) fragments (N), number (P), percentage (%P) and size range of polymorphic fragments generated by four RAPD primers.

Primer	Nucleotide sequence (5'-3')	N	P	%P	Size range
OPA-09	5'-GGG TAA CGC C-3'	10	8	80.00	100 pb - 1500 pb
OPA-12	5'- TCG GCG ATA G-3'	8	7	88.00	100 pb – 1020 pb
OPH-18	5'- GAA TCG GCC A- 3'	4	2	50.00	150 pb - 1020 pb
OPH-20	5'- GGG AGA CAT C-3'	16	15	94.00	250 pb - 1160 pb
All primers		**38**	**32**	**84.00**	**100 pb – 1500 pb**

Analysis of molecular variance (AMOVA)

AMOVA analysis obtained from the distance matrix enables the partitioning of the overall RAPD variation between defined groups. Three sources of variation were defined according to the geographic origin (Skikda, Algeria vs. Menzel Bouzelfa, Tunisia), the host plant species (Mandarin vs. orange vs. grapefruit vs. lemon), and the host plant cultivars (cv. Eureka vs. cv. Thomson Navel vs. cv. Shambar vs. cv. Commune vs. cv. Ortanique vs. cv. Carvalhal). Results of AMOVA did not indicate any genetic differentiation associated with the geographical areas or host plant cultivars ($p = 0.460$ and 0.2, respectively). However, the host-plant species had a significant effect on the partitioning of the total genetic diversity ($p < 0.05$) (Table 2).

Table 2. AMOVA analysis of the different sources of polymorphism variation.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	p-value
(A) Among Host plant species within individuals	3	29.7	1.23	22%	**0.01 (p < 0.05)**
(B) Among Host plant cultivars within individuals	5	41.98	0.00	25%	0.2 (NS)
(C) Among host plant countries within individuals	1	5.24	1.36	0%	0.460 (NS)

DF: Degrees of freedom.

Factorial analysis

The representation of genetic relatedness was depicted by two-dimensional principal coordinates analysis (PCO) based on Euclidian similarity which revealed diversity among different *A. spiraecola* samples. The first and second coordinates revealed 24.62 % and 17.60 % of the variations in the standardized data set of the 19 samples. The PCO showed significant grouping of the insects that were plotted into four sub-plots, representing each group of *A. spiraecola* with distinctive features (Fig. 2). The groupings were largely in accordance with their host species, irrespective of the origin of samples or the host cultivar. It is noteworthy that insects colonizing mandarin were the most heterogeneous genetically according to PCO axes 1 and 2, and this heterogeneity was not dependent on the mandarin variety. Such result would be, most likely, due to the fact that the host species (mandarin) is a direct genitor of sweet orange and indirect genitor of both grapefruit and lemon (Fig. 1) within the Rutaceae family.

Cluster analysis

The Neighbor-Joining dendrogram showed four clusters corresponding to the four studied citrus species (Fig. 3). Genetic clusters did not show any sub-grouping of the analyzed insects with respect to the host plant cultivars or the geographical location as *A. spiraecola* samples collected on lemon from Tunisia did not cluster distinctly from those collected on the same citrus species from Algeria. This pattern of genetic grouping supports results of PCO, further suggesting that insect genotypes within the same cluster would have co-evolved with the gene pool of the corresponding host citrus species.

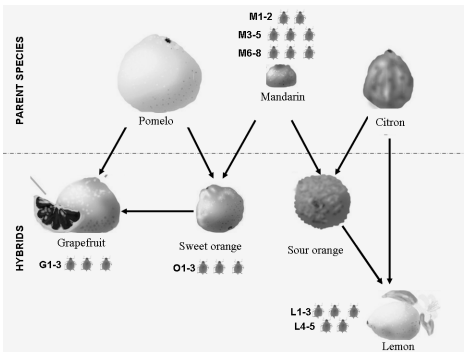

Fig. 1. Botanical relationships, within the family of Rutaceae, between citrus species used for sampling, and numbers of samples of *Aphis spiraecola*, collected by host species or cultivar. M1-2: aphids sampled on mandarin cv. Commune, M3-5: aphids sampled on mandarin cv. Ortanique; M6-8: aphids sampled on mandarin cv. Carvalhal; G1-3: aphids sampled on grapefruit (cv. Shambar); O1-3: aphids sampled on sweet orange (cv. Thomson Navel); L1-3: aphids sampled on lemon (cv. Eureka); L4-5: aphids sampled on lemon (cv. Eureka) from Tunisia.

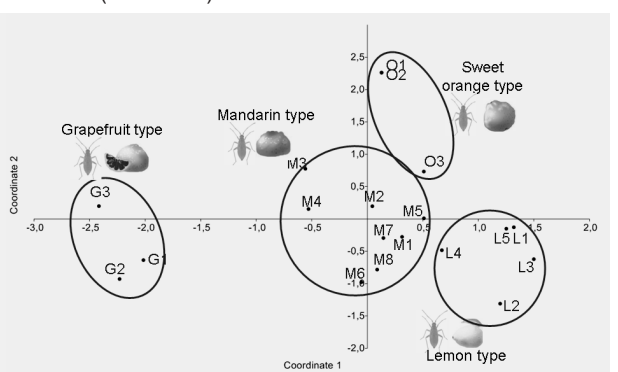

Fig. 2. Bidimensional principal coordinates analysis (PCO) scatter plot showing the pattern of diversity among 19 *Aphis spiraecola* samples collected on four citrus species and six cultivars, based on 32 polymorphic RAPD markers (populations are designated according to their labels in fig. 1).

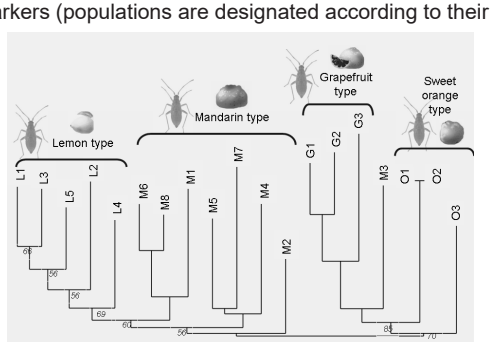

Fig. 3. Neighbor-Joining dendrogram illustrating pattern of genetic diversity of 19 *Aphis spiraecola* samples collected on four citrus species, based on 32 RAPD markers (populations are designated according to their labels in fig.1).

Morphological characterization of citrus species

Morphological markers correspond to the visually scoring of qualitative traits as well as measurements of quantitative traits that are influenced by plant biology. The selected accessions were characterized phenotypically in this study by comparing the leaf characteristics. The ANOVA analysis revealed a significant difference concerning the quantitative traits between the four species examined (p=0, p=0, p=0.009 and p=0.023, for the length of petioles, width of petiole wings, width of leaves and length of leaves, respectively) and for the leaf infestation degree (p=0.026). Sweet orange and grapefruit appeared to be relatively close morphologically to each other (similar dimensions of the leaf, similar length of the petiole, dark color of the leaf, presence of wings on petiole, a weakly concave leaf, and a weakly undulated leaf margin), and were the most heavily infested species (Table 3).

Table 3. Leaf morphological characteristics and degree of leaf infestation by *Aphis spiraecola*, in four citrus species.

Descriptors		Sweet orange	Grapefruit	Lemon	Mandarin
Leaf morphological characteristics	a. Mean length of leaves (cm)	8.7 [a]	9.27 [a]	7.52 [a]	5.61 [a]
	b. Mean width of leaves (cm)	4.47 [ab]	5.77 [a]	3.89 [ab]	2.60 [b]
	c. Mean length of petioles (cm)	1.07 [b]	1.82 [a]	0.60 [c]	0.81 [bc]
	d. Presence of petiole wings	+	+	-	-
	e. Mean width of petiole wings (cm)	0.30 [b]	1.05 [a]	0 [c]	0 [c]
	f. Leaf color	Dark green	Dark green	Light green	Dark green
	g. Emargination	+	+	+	+
	h. Leaf shape in cross section	Weakly concave	Weakly concave	Straight	Straight
	i. Twisting of leaf	Weak	Weak	Intermediate	Strong
	j. Blistering of leaf	+	+	-	-
	k. Undulation of leaf margin	Weak	Weak	Intermediate	Weak
Leaf infestation	Mean number of aphids / leaf in April 2015	95.37 [a]	50.75 [ab]	29.50 [b]	26.98 [b]

DISCUSSION

Analysis of the genetic diversity of *A. spireacola*, collected from different citrus species and varieties from Skikda (Algeria) and Menzel Bouzelfa (Tunisia), was performed based on 32 polymorphic DNA markers. Analysis of molecular variance (AMOVA), factorial analysis (PCO) and cluster analysis (NJ) did not show any significant difference among adult samples concerning their geographical origin (Algeria vs. Tunisia). This result may be related to the ability of winged aphids to move. In fact, the migration phenomenon has been well documented in several aphid species such as *Sitobion avenae* (Liewellyn, Loxdale, Harrington, Clark & Sunnucks, 2004) and *Elatobium abietinum* (Halldórsson et al, 2004). Several authors (Zitoudi, Margaritopoulos, Mamuris, & Tsitsipis, 2001; Halldórsson et al, 2004; Figueroa et al,

2005; Béji et al, 2013; Jun, Michel, Wenger, Kang, & Rouf Mian, 2013) mentioned that the region has no significant effect on genetic variability in many aphid species. Further, some populations of Italian peach-potato aphid, *Myzus persicae* were reported to have similar insecticide resistance profiles, independent of geographical origin (Monti et al, 2016). In addition, Callejas, Beitia, Gobbi, Velasco, & Ochando (2005) have shown that populations of whitefly *Bemisia tabaci* collected from several Canary Islands are of the same biotype.

Our results suggest that genetic distance between *A. spiraecola* individuals is rather shaped by the citrus species from which they were collected. This fact is probably due to the selection pressure exerted by the nutritional requirements of each genotype of this aphid, which can only be assured by well-defined plant species. For example, Auclair & Boisvert (1980) reported significant differences in vitamin requirements in two biotypes of *Acyrthosiphon pisum* Harris. Similarly, the results of Caillaud & Via (2000) suggested that the major determinant of host specialization for pea aphids is the behavioral acceptance of a plant rather than the toxicity of the food source. In the case of *Aphis gossypii,* as previously reported by Carletto et al (2009), specialization occurred on three very different plant families, Cucurbitaceae, Solanaceae and Malvaceae, which exhibit a different phenology and probably a great variability in their sap compounds that might act as selective factors for host acceptance by aphids. This specialization process may be seasonal for some aphids such as *Aphis fabae* Scopoli (Powell & Hardie, 2000), while the pea aphid complex illustrates how insect biotypes blend into species by gradual reduction of gene flow (Peccoud et al, 2009).

Mezghani-Khemakhem et al, (2012) reported that the genetic variation of *A. spiraecola* populations, in Tunisia, was governed exclusively by the host plant species. Such a genetic variation strongly depending on the host plant has also been reported in *Sitobion avenae* F. aphids (Lushai, Markovitch, & Loxdale 2002), *Rhopalosiphum maidis* (Blackman, Halbert, & Carroll, 1990), *Aphis gossypii* (Charaabi et al, 2008), *Acyrthosiphon pisum* (Peccoud et al, 2009) and *Myzus persicae* (Rubiano-Rodríguez et al, 2014).

The genotypes of *A. spiraecola* analyzed in this study, showed no differences depending on the cultivars of mandarin. Apparently, the three cultivars used are genetically similar, leading to an offer of similar suitability and thus hosting the same aphid biotypes. Several authors have demonstrated genetic similarities between mandarin varieties (Uzun, Yesiloglu, Aka-Kacar, Tuzcu, & Gulsen, 2009; Youseif et al, 2014). Besides, some mandarin species, such as willow leaf mandarin, *Citrus deliciosa* Ten., were shown to have a low genotypic variability (Herrero et al, 1996).

The genetic analysis allowed classifying the individuals of *A. spiraecola* colonizing orange and grapefruit in close genetic groups. These two species of citrus are very similar. Grapefruit is the result of hybridization between orange and pummelo (*Citrus maxima*) (Luro, Gatto, Costantino, & Pailly, 2011), while the lemon tree is relatively distant (Youseif, El-Halwagi, Sayed, & El-Itriby, 2014). We have found that these two species were similar to each other with respect to the leaf morphological traits and were the most infested, suggesting that they are attacked by well adapted biotypes.

Recognition of a plant as a suitable host and subsequent feeding initiation by an aphid depends on a complex interaction between aphid and plant traits (Züst & Agrawal, 2016). Plants possess a great repertoire of traits (e.g. color, shape, odour, etc.) that insects could utilize to make oviposition decisions (Carrasco, Larsson, & Anderson, 2015). For instance, leaf color intensity (related to the photosynthesis ability) is important for attracting several fly and aphid species (Bernays & Chapman, 1994). In our case, it seems that large dimensions of grapefruit and orange leaves, compared with mandarin and lemon, were responsible for the attraction of winged *A. spiraecola*, providing a sufficient nutrition for proliferation.

In conclusion, our study revealed evidence for patterns of genetic structuring among *A. spiraecola* populations related to their host plants, suggesting a possible host specialization that should be taken into account in the development of integrated controlling strategies that should be varied, following the disrupted gene flow between populations colonizing different citrus species. Our results provide baseline information for monitoring the genetic structure of *A. spiraecola* in Algeria. The conclusions herein presented will need to be analyzed in depth, and extended to a larger geographical zone, based on larger sample sizes and combining several molecular markers.

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