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ROMANIAN AGRICULTURAL RESEARCH, NO. 30, 2013
Print ISSN 1222-4227; Online ISSN 2067-5720

GENETIC STRUCTURE OF *APHIS FABAE* SCOPOLI (HEMIPTERA, APHIDIDAE) IN TUNISIA, INFERRED FROM RAPD MARKERS

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ABSTRACT

***Aphis fabae* is a common polyphagous aphid represented by a complex of heteroecious holocyclic subspecies, using a wide range of secondary host plant species. In this study, we used Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) to assess the genetic diversity and analyse the genetic structure of three subspecies from this complex, namely *A. f. fabae, A. f. solanella and A. f. cirsiiacanthoidis*, collected from 4 locations in the North and South of Tunisia. Using 108 aphid samples, a total of 96 polymorphic markers and 81 RAPD phenotypes were revealed, showing variation between subspecies, locations and primers. The presence of overlapping RAPD phenotypes was detected between subspecies, locations and samples within each location. The genetic structure was analysed, based on molecular variance (AMOVA), genetic distances (Fst), multidimensional scaling (MDS) and cluster analysis (UPGMA). AMOVA revealed that diversity among locations within each subspecies was the most significant diversification factor (58.68% of total diversity). In addition; Fst, MDS and UPGMA indicated that the distinction between the 6 studied *A. fabae* populations was related to both subspecies and location. These results strongly suggest that a conjugated effect of subspecies and region governs the genetic differentiation of the *A. fabae* complex in Tunisia. Both factors should therefore be taken into account for the implementation of a controlling strategy.**

Key words: *Aphis fabae*, genetic diversity, subspecies, location, RAPD-PCR.

INTRODUCTION

Aphis fabae Scopoli, commonly called black bean aphid, is a major constraint of faba bean (*Vicia faba* L.) production, causing a destructive damage throughout the world. In addition to direct plant injury, aphid infestation damages faba bean due to honeydew excretion on the leaves, which interferes with physiological processes in the host plants (Hurej and Van Der Werf, 1993). Furthermore, this aphid vectors the bean leaf rolling virus (BLRV) and the faba bean necrotic yellows virus (FBNYV) (Katul et al., 1993; Najar et al., 2000; Blackman and Eastop, 2007). *A. fabae* is holocylic, reproducing sexually on its primary host, the European spindle-tree *Euonymus europaea* L., and asexually on the secondary hosts. Nevertheless, in regions with warm climate such as the Mediterranean basin, the Middle East and several warm regions in Africa and America; it keeps reproducing exclusively through parthenogenesis (Blackman and Eastop, 2007). *Aphis fabae* is represented by a complex of at least five subspecies using the same primary host, but differing by the secondary host plant species (Jörg, 1995). In Tunisia, *A. fabae* species complex causes severe yield losses on faba culture.

Received 16 August 2012; accepted 22 January 2013. First online: 9 February 2013. DII 2067-5720 RAR-215

Bouhachem-Boukhris (2002) reported three subspecies (*A. f. fabae, A. f. solanella* and *A. f. cirsiiacanthoidis*) of the *A. fabae* complex, being at the origin of losses.

In recent years, several highly sensitive molecular markers have been developed that reveal the genetic structure of aphid natural populations (Fenton et al., 1998; Loxdale and Lushai, 1998; Sloane et al., 2001). One of the most successful techniques is the Randomly-Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR), which has been applied to reveal genetic variation in several aphid species, including *Sitobion avenae* F. (De Barro et al., 1995), *Aphis gossypii* Glover (Vanlerberghe-Masutti and Chavigny, 1998), *Schizaphis graminum* Rondani (Lopes-Da-Silva et Vieira, 2004), *Rhopalosiphum padi* L. (Bulman et al., 2005), *Metopolophium dirhodum* Walker (Lopes-Da-Silva and Vieira, 2007), *Macrosiphum euphorbiae* Thomas (Raboudi et al., 2011) and *Aphis spiraecola* Patch (Mezghani-Khemakhem et al., 2012). In these species, the partitioning of RAPD variability into different levels of hierarchy, such as within and between populations, has been a useful tool in assessing genetic diversity and demonstrated that the rate of genetic diversity in aphid species was subjected to several pressures, such as the geographical origin (Lopez-da-Silva and Vieira, 2007; Nicol et al., 1997), year of collection (Nicol et al., 1997, De Barro et al., 1995; Dedryver et al., 2008) and/or host plant species (Charaabi et al., 2008; Mezghani-Khemakhem et al., 2012).

From a practical point of view, studies on the genetic structure of insect pests, especially those using molecular tools, could provide valuable information for optimising crop protection programs. The present study aims to evaluating the amount and pattern of genetic diversity of the major *A. fabae* subspecies in Tunisia, on the basis of data inferred from RAPD-PCR.

MATERIAL AND METHODS

Aphid sampling

Samples of *A. f. fabae, A. f. solanella* and *A. f. cirsiiacanthoidis* were collected, from December 2008 to February 2010, respectively on *V. faba, Solanum nigrum* L. and *Cynara scolymus* L., from four sites in Tunisia, three in the North and one in the south (Figure 1, Table 1). Aphids were collected by hand, then placed in ethanol (96%) and stored at -20°C. Samples showing fungal infections and/or hymenopteran parasitism were not included.

Figure 1. Map of Tunisia, showing the geographical location of the studied sites (●)

Table 1. *Aphis fabae* sampling data

Aphis fabae subsp.	Sampling site	Geographical domain	Host plant species	Sampling date	Sample code	Sample size
A. f. fabae	Douala	North	*Vicia faba*	19/12/2008	FDO	18
	Brij	North	*Vicia faba*	09/01/2009	FBR	18
	Bizerte	North	*Vicia faba*	06/02/2009	FBI	18
A. f. solanella	Douala	North	*Solanum nigrum*	04/02/2010	SDO	18
	Gabès	South	*Solanum nigrum*	02/02/2009	SGA	18
A. f. cirsiiacanthoidis	Bizerte	North	*Cynara scolymus*	01/04/2009	CBI	18

DNA Extraction and RAPD-PCR

Genomic DNA was extracted separately from 108 *A. fabae* samples, using the Doyle and Doyle (1987) procedure. RAPD-PCR reactions were performed in 25 µl volume in a mixture containing 2.5 mM $MgCl_2$, 1x buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0), 0.1 mM of each dNTP, 0.1 µM of each primer, 50ng of DNA and 1U of Taq DNA polymerase (Promega, USA). The amplification process was conducted in a Thermal cycler 2720 (Applied Biosystem, USA). For each amplification process, an initial heat denaturation of DNA at 94°C for 3 min was performed, followed by 35 cycles consisting of 1 min at 94°C, 1 min at 36°C, and 1 min at 72°C. A final incubation for 7 min at 72°C was performed. The amplification products were analysed on 1.5% agarose gel in Tris-borate-EDTA buffer, stained in ethidium bromide and visualized under UV light. A 100bp ladder (Invitrogen) was used as molecular size standard. Five RAPD-PCR primers (Operon Technologies) were used:

OPD-01 (5'-ACCGCGAAGG-3'),
OPH-02 (5'-TCGGACGTGA-3'),
OPH-03 (5'-AGACGTCCAC-3'),
OPH-04 (5'-CTGCATCGTG-3') and
OPH-06 (5'-ACGCATCGCA-3').

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 five primers were pooled to define a single binomial phenotype for each of the 108 samples. An analysis of the molecular variance (AMOVA) was performed using ARLEQUIN version 3.0 (Excoffier et al., 2005) to compare differences between and within the aphid samples grouped according to the subspecies. In addition, pairwise genetic distances (Fst) between pairs of populations were calculated using ARLEQUIN version 3.0 (Excoffier et al., 2005) and subjected to multidimensional scaling (MDS, Shepard, 1974) in PC SAS (SAS Institute, Cary, North Carolina, USA) to study the variation between populations. A Mantel test was conducted using the SPSS program 14.0 (SPSS Inc., 2005), to compare genetic and geographic distances. In order to illustrate genetic relationships between the 108 *A. fabae* samples, the genetic distance matrix was submitted to cluster analysis using the Unweighted Paired Group Method for the Arithmetic Average (UPGMA) (Sneath and Sokal, 1973). This analysis was performed using the program NEIGHBOR of the PHYLIP software package version 3.68c (Felsenstein, 2008).

RESULTS

Polymorphic RAPD markers

All amplification products obtained were reproducible. For the 108 *Aphis fabae* individuals, 96 DNA profiles were obtained with the five primers used. Product sizes ranged from 100 to 2000 bp.

The percentage of polymorphic RAPD fragments generated by each primer alone varied from 100%, with primers OPD-01, OPH-02, OPH-03 and OPH-06, to 93.33 % with primer OPD-04, with an average of 98.66% per primer (Table 2). By studying polymorphism of each subspecies, it could be revealed that *A. f. fabae* was the most polymorphic (%P = 95), followed by *A. f. solanella* (%P = 93.33), whereas *A. f. cirsiiacanthoidis* was characterized by the lowest rate of polymorphism (%P = 36.83).

RAPD phenotypes

Eighty-one (81) different RAPD phenotypes have been identified within 108 aphids sampled, with a remarkable variability in number for each subspecies. *A. f. fabae, A. f. solanella* and *A. f. cirsiiacanthoidis* have revealed 74, 57 and 13 RAPD phenotypes, respectively. For each subspecies there was variability with respect to primers and/or locations.

In fact, for the three populations of *A. f. fabae*, we have attested the highest phenotypic diversity in the population of Douala with 31 different phenotypes. For *A. f. solanella*, the population collected from Douala has

generated 46 RAPD phenotypes and this phenotypic diversity was reduced to 11 in the population collected from Gabès. Overlapping RAPD phenotypes were detected between populations of the same subspecies, as well as between different subspecies.

Table 2. Total number of RAPD fragments (N), number (P) and percentage (%P) of polymorphic fragments generated by primer, location and subspecies

		N	P	%P
Primers	OPD-01	22	22	100
	OPH-02	19	19	100
	OPH-03	17	17	100
	OPH-04	19	18	93.33
	OPH-06	19	19	100
	Mean			98.66
Sites	Bizerte	78	36	46.15%
	Douala	115	102	88.66%
	Brij	55	46	83.63%
	Gabès	29	6	20.68%
	Mean			59.78
Subspecies	*A. f. fabae*	80	76	95%
	A. f. solanella	60	56	93.33%
	A. f. cirsiiacanthoidis	38	14	36.84%
	Mean			75.05

Molecular Variance (AMOVA)

To investigate the contribution of subspecies, regions as well as their conjugated effect to the total genetic diversity, we performed a hierarchical AMOVA (Table 3). Results revealed that the diversity among regions within each subspecies represented the major part (58.68%) of the total diversity, followed by the diversity between regions (26.70%) and the diversity between subspecies (14.62%).

Table 3. AMOVA results for *A. fabae* RAPD data

Source of variation	Sum of squares	% total variation
Among subspecies *(A. f. fabae) vs. (A. f. solanella)* vs. (*A. f. cirsiiacanthoidis)*	953.537	14.62
Among regions (Bizerte) vs. (Douala) vs. (Brij) vs. (Gabès)	861.333	26.70
Among regions within subspecies	985.759	58.68

Genetic distances (Fst)

Pairwise population comparison was performed by calculating the Fst values and performing a test of significance of these values using a non-parametric permutation approach (Excoffier et al., 2005). Table 4 shows the matrix of pairwise Fst values for the 6 studied populations belonging to 3 subspecies.

The genetic distances Fst between pairs of populations were significant. Thus, it is possible to consider that each population was clearly differentiated. The highest genetic distance (0.905) was observed between SGA and CBI populations, while the lowest (0.469) was between SDO and FDO. No correlation was revealed, using the Mantel test, between physical and genetic distance spaces.

In fact, in some cases, populations originating from the same region (e.g. CBI and FBI) were more genetically distant (0.85983) than were ones collected from far-located regions (e.g. SGA and SDO, 0.67528).

Table 4. Matrix of pairwise Fst between 6 *Aphis fabae* populations based on 96 Random Amplified Polymorphic DNA (RAPD) markers

	CBI	SGA	SDO	FBI	FBR	FDO
CBI	0.00000					
SGA	**0.90580***	0.00000				
SDO	0.71849*	0.67528*	0.00000			
FBI	0.85983*	0.83063*	0.62512*	0.00000		
FBR	0.83084*	0.79044*	0.61313*	0.73927*	0.00000	
FDO	0.74189*	0.66057*	**0.46937***	0.63193*	0.60349*	0.00000

Fst value are all significant (*) at the P<0.05 level
The highest and lowest genetic distances are indicated in bold.

Multidimensional scaling (MDS)

Multidimensional scaling (MDS) detected a clear distinction between the *A. fabae* populations (Figure 2). The differentiation of populations did not reflect a grouping, in perfect correlation with subspecies or geographic origin. Concerning regions, SDO and FDO populations, that are close in geographic space, were also close in genetic space. This was not the case of CBI and FBI, collected from the same site, but comparatively more distant genetically. Concerning the subspecies criterion, we noted that SGA and SDO, both belonging to the *A. fabae* subsp. *solanella*, were genetically distant across dimension 1. Within each subspecies, there was a genetic dispersal of populations collected from different regions, suggesting a strong conjugated effect of subspecies and region, as pointed out by the AMOVA analysis.

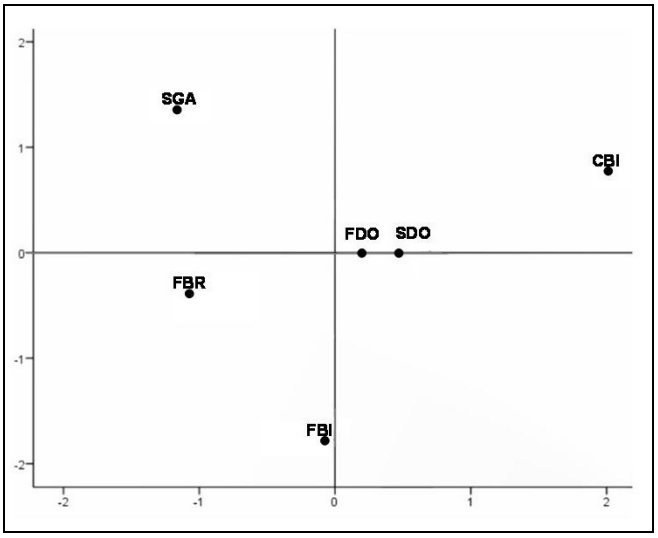

Figure 2. Multidimensional scaling (MDS) scatter plot showing patterns of diversity among 6 *Aphis fabae* populations belonging to 3 subspecies and 4 locations, based on 96 RAPD markers
(Populations are designated according to their label from table 1.)

Cluster analysis (UPGMA)

The UPGMA dendrogram showed 6 clusters corresponding to the 6 studied populations differing simultaneously by the subspecies and the site of sampling (Figure 3). Although the *A. fabae* subspecies was an effective criterion in clustering, samples belonging to the same subspecies but differing by the geographic location did not cluster together. In the same way, genetic clusters did not show a geographic gradient (North vs. South) as populations from the North of Tunisia (Bizerte, Douala and Brij) did not cluster distinctly from the unique southern population (Gabès). This pattern of genetic grouping supports a combined effect of subspecies and geographic origin, as inferred from MDS.

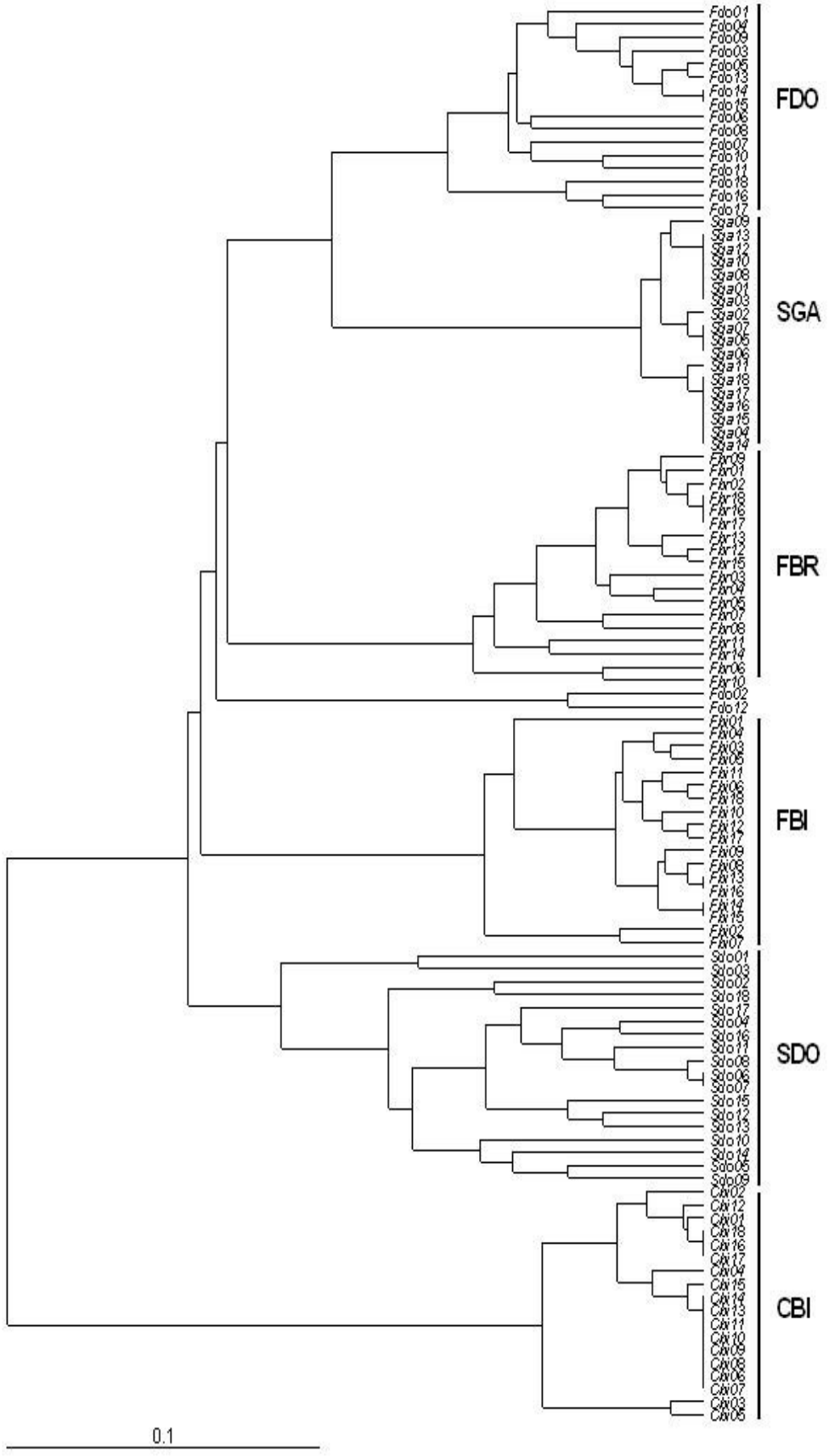

Figure 3. UPGMA dendrogram showing pattern of genetic diversity of 108 *Aphis fabae* samples belonging to 3 subspecies and 4 locations, based on 96 RAPD markers
(Populations are designated according to their label from table 1.)

DISCUSSION

In the present study, a high level of polymorphism was detected for *A. fabae* subsp. *fabae* and *A. fabae* subsp. *solanella* (P= 95% and P = 93.33%, respectively), whereas *A. fabae* subsp. *cirsiiacanthoidis* was characterized by a lower level (P = 36.83%). This spectacular decrease in polymorphism might be due to the date of sampling which was in the month of April, when the clonal reproduction is enhanced. This hypothesis is supported by the number of generated phenotypes, which was also comparatively low (13), in *A. fabae cirsiiacanthoidis*.

In Tunisia the primary host of *A. fabae*, *Euonymus europaea*, is absent, which means that the sexual reproduction is very improbable. Consequently, members of the complex of *A. fabae* most likely overwinter on uncultivated plants. Therefore, the high level of diversity detected, in *A. fabae* subsp. *fabae* and *A. fabae* subsp. *solanella* here, could be attributed either to (i): non sexual factors, such as mutations (Delmotte et al., 2002) or (ii): sexual episodes on uncultivated species, as it was reported in *S. avenae* by Vialette et al. (2005). Such uncultivated species would potentially play the role of refuge, providing a reservoir of genetic diversity that could enhance adaptation of the insect to environmental stresses, particularly in response to modifications of agricultural practices.

The genetic structure of *A. fabae* populations, inferred from Fst and MDS did not show direct correlation with the geographical origin, as populations originating from the same location could be more genetically distant than those collected from geographically far-located regions. Thus, the geographical origin, considered alone, seems not to be the most determining factor in the specializing pattern of *A. fabae* complex, in Tunisia. Similarly, the subspecies of *A. fabae* did not appear to be the unique factor discriminating *A. fabae* genotypes, as high genetic distances were revealed between populations belonging to the same subspecies.

AMOVA showed that the "among regions within subspecies" parameter was the source having the highest contribution to total variation (58.68%). This fact was confirmed by the UPGMA analysis, which demonstrated that both the subspecies and the sampling location were crucial, in the clustering pattern. Several studies on aphid species (Lushai et al., 2002; Charaabi et al., 2008; Peccoud et al., 2009; Mezghani-Khemakhem et al., 2012) tend to indicate that the host plant species/cultivar was, in most cases, the key factor for genetic differentiation among sympatric aphid populations. However, genetic structuring in relation with geographical scale has also been reported, such as in *S. avenae* (De Barro et al., 1995; Dedryver et al., 2008), *Myzus persicae* Sulzer (Guillemaut et al., 2003), *M. dirhodum* (Nicol et al., 1997). This fact is probably linked to abiotic (mainly climatic) selection pressure exerted on aphid populations.

CONCLUSIONS

Results obtained in this study strongly suggest that asexual populations of *A. fabae*, in Tunisia, could exhibit considerable genetic variation, although variants were not distributed according to an exclusively geographic or taxonomic pattern. The large genetic pool of the pest, along with the eventual availability of refuge host species in the surrounding landscape, should be considered as key parameters for the implementation of an efficient management program against the *A. fabae* complex.

Acknowledgements

The authors gratefully recognize funding support from the Tunisian Ministry of Higher Education and Scientific Research.

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