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MOLECULAR TYPING OF TUNISIAN CLONES OF *Myzus persicae* (HEMIPTERA APHIDIDAE) USING MICROSATELLITE MARKERS

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In order to assess the genetic differentiation among Tunisian clones belonging to the *Myzus persicae* complex *(M. persicae* (Sulzer), *M. antirrhinii* (Macchiati) and *M. nicotianea* Blackman), the molecular technique of microsatellites was used in this study. These markers offer sensitivity and are useful in population genetic studies of parthenogenetic organisms. Here, nine polymorphic microsatellite loci were amplified to distinguish between six parthenogenetic clones belonging to *M. persicae* complex collected from two different Tunisian areas. Interestingly, this technique allowed discrimination between five different genotypic classes among the six clones. Furthermore analysis of genetic relatedness between the genotypic classes revealed that two Tunisian clones did not cluster either in *M. persicae* or in *M. antirrhinii* taxa, whereas, the four other Tunisian clones clustered into the *M. persicae* Sulzer taxa.

Hemiptera, Aphididae, *Myzus persicae* complex, clones, molecular typing, parthenogenesis, microsatellite markers, Tunisie

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U cilju utvrdivanja genetičkih razlika izmedu tuniskih klonova kompleksa *Myzus persicae M. persicae* (Sulzer), *M. antirrhinii* (Macchiati) i *M nicotianea* (Blackman) upotrijebljena je molekularna mikrosatelitska tehnika. Ovi su biljezi vrlo osjetljivi i korisni u takvim istraživanjima partenogenetskih organizama. Umnoženo je devet polimorfnih lokusa mikrosatelita kako bi se razlikovalo šest partenogenetskih

klonova kompleksa *M. persicae* sabranih u dva razlicita područja u Tunisu. Zanimljivo je da je ova tehnika omogućila razlikovanje između pet različitih genotipskih razreda tih šest klonova. Nadalje, analize genetičke srodnosti između genotipskih razreda pokazale su da dva tuniska klona nisu u istoj grupi niti s *M. persicae*, niti s *M. antirrhinii*, dok se četiri druga tuniska klona nalaze unutar vrste *M. persicae* Sulzer.

Hemiptera, Aphididae, *Myzus persicae* complex, klonovi, molekularna tipizacija, parthenogeneza, mikrosatelitski biljezi, Tunis

Introduction

Aphids (Homoptera, Aphididae) are the most frequent insect group for which host-adapted races (biotypes) formation and rapid adaptation to resistant crops have been reported (CAILLAUD et al., 1995; DIXON, 1998). Diploid genome sizes for each aphid species ranged from 0.36 to 1.82 pg. Nested analysis of variants showed that variation in genome sizes between species was significant, as well as between individual of some species. However, there was no significant variation between samples of the same species in different staining runs (FINSTON et al., 1995.). In Tunisia, the great damage in crops caused by aphids and the chemical treatments inefficiency in addition to the appearance of resistant populations to aphicids make the problem of aphids particularly worrying. *Aphis gossypii* (Glover) and the Green Peach-potato Aphid *Myzus persicae* (Sulzer) are the most scaring species on the agronomic level because of their polyphagy (BEN HALIMA & BEN HAMOUDA, 1998).

Systematic and taxonomy of the *M persicae* complex are particularly difficult to study and ambiguous because of the great number of morphological characters required for taxonomic separation and the loss of sexual reproduction in a number of proposed taxa. Three taxa previously ascribed to *M. persicae* have been distinguished as *M. persicae* (Sulzer), *M nicotianea* Blackman and *M. antirrhinii* (Macchiati). Multivariate morphometric analysis has proven its reliability in distinguishing between species when applied to two of the three taxa but unreliable when the third taxa was present in the sample (BLACKMAN & PATERSON, 1986; BLACKMAN, 1987; BLACKMAN & SPENCE, 1992). *M. antirrhinii* is strictly anholocyclic, but *M. persicae* and *M. nicotianea* encompass holocyclic and anholocyclic populations, depending on their geographical origin (BLACKMAN, 1974, 1987; BLACKMAN & PATERSON, 1986; BLACKMAN & SPENCE 1992). In order to examine the aphid genetic diversity, a full range of available genetic markers have been used in the past including allozymes, mitochondrial and nuclear DNA sequence markers, random amplified polymorphic DNA (RAPD) and microsatellite (reviewed in HALES et al., 1997) analysis. In *M. persicae* populations, the allozyme loci were found to be excessively monomorphic (BROOKES & LOXDALE, 1987; LOXDALE et al., 1993; TERRADOT et al., 1999) and showed low heterozygosity, which suggests they could be under selection pressure (DELMOTTE et al., 2002). Allozymes are therefore unsuitable to reflect the actual genetic diversity and structure of aphid populations, which seriously undermines previous conclusions derived using these markers in aphids (reviewed in HALES et al., 1997). The RAPD markers applied on American populations of *M. persicae* showed a lack of intraspecific variation (AL-ABOODI & FFRENCH-CONSTANT, 1995), whereas, using the same technique in a further study, broad variability was detected in Spanish populations (MARTINEZ-TORRES et al., 1997). A more recent RAPD study failed to reveal consistent differences between clones of *M. persicae* and *M. nicotianea* (MARGARITOPOULOS et al., 1998). FENTON et al. (1998) revealed low diversity in Scottish populations of *M. persicae* using analysis of intergenic spacers of the ribosomal DNA.

Recently, microsalellite loci, first described in aphids for *Sitobion miscanthi* (Takahashi), have been described for a number of other aphid species that are major crop pests (SUNNUCKS et al., 1996; FULLER et al., 1999; SIMON et al., 1999a; TERRADOT et al., 1999; JOHNSON et al., 2000; MASSONET et al., 2001; DELMOTTE et al., 2002). These microsatellite loci provide highly polymorphic markers useful for studies on breeding system diversity (JARNE & LAGODA, 1996) to infer the mode of reproduction of a species from detailed analysis of its population genetic structure (AWADALLA & RITLAND, 1997), to resolve clonal diversity in parthenogenetic organisms (SUNNUCKS et al., 1996; FULLER et al., 1999; GOMEZ & CARVALHO, 2000), to understand the evolutionary biology of breeding systems at the intraspecific level (SIMON et al., 1999b) and to discriminate among taxa of the *M. persicae* complex (TERRADOT et al., 1999).

Because of the successful use of microsatellite markers in the molecular discrimination of aphid clones (SUNNUCKS et al., 1996, 1997; SIMON et al., 1999a; WILSON et al., 1999), especially in the *M. persicae* complex (TERRADOT et al., 1999), nine microsatellite markers were used in this study to identify the genotype of six Tunisian *M. persicae* clones. In this work and as a continuation of a previous study, we have compared the genetic pattern of these Tunisian clones with those identified by TERRADOT et al. (1999) according to their taxonomy.

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Materials and methods

Aphid clones

Polymorphism was assessed by sampling six green Tunisian populations of aphids collected from two different plants and areas at the beginning of the growing season (April and May 2002) and putatively identified as *M persicae* according to the identification keys of TAYLOR (1981), LECLANT (1981b) and BLACKMAN & EASTOP (1984). VeBl, VeB3 and VeB7 aphid clones were collected from *Solanum tuberosum* in Tekelsa (North eastern Tunisia) and VeT3, VeT6 and VeT8 aphid clones were collected from *Convolvulus arvensis* in Tunis (Northern Tunisia). Each clone was derived from a single apterous virginiparous female. Subsequently, parthenogenetic cultures of each clone were reared on potato sprouts, in individual one litre plastic boxes kept in the dark, at 20°C (ROBERT et al., 1969). Note that aphids can be stored at -20°C until further analysis.

DNA extraction

Depending on the aphid size, DNA from three or four individuals of each aphid clone was extracted using the "salting out" protocol described by SUNNUCKS & HALES (1996). The DNA was then resuspended in 20-40 mµl of 1x TE buffer (10 mM Tris pH 7.5, 0.1 mM EDTA). DNA extractions were checked and roughly quantified on 0.8% agarose gel electrophoresis and dilutions were made correspondingly in order to obtain a DNA concentration of 5-7 ng/µl.

Microsatellite amplification and analysis

Nine microsatellite loci isolated from three different aphid species were used to characterize six Tunisian aphid clones and to compare their genetic pattern with those described by TERRADOT et al. (1999). Five polymorphic loci were isolated from *M. persicae* (M35, M37, M40, M63, M77; available upon request from A.C.C. WILSON), three from *Sitobion* sp. (Aphididae) (S16b, S17b, S23; SIMON et al., 1999a; WILSON et al., 1999) and the last one from *Rhopalosiphum padi* (Linnaeus) (Aphididae) (R5.10; SIMON et al., 2001). Polymerase chain reaction amplification of microsatellite loci was carried out in 15 µl reaction mixtures consisting of 0.5 U of Taq polymerase (Promega), 1x MgC_2-free reaction buffer, 2.5 mM $MgCl_2$, 400 µM dNTP, 0.2 mM of each primer and 1 µl of the diluted aphid DNA (SIMON et al, 2001). Amplifications were made in a PTC-100 programmable thermal controller (MJ Research, Inc.) using a system of initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 20 s, annealing at 56 °C for 20 s for all loci, and elongation at 72 °C for 30 s, with a final elongation at 72 °C for 2 min. A 1.8% agarose gel electrophoresis

was used to check the good amplification and the concentration of the PCR products. These were diluted to half with a 4x-loading buffer before electrophoresis.

For each sample, about 3 µl (according to DNA concentration) of the diluted PCR product was resolved by an urea denaturating 6% polyacrylamide gel electrophoresis, run for 2 h 30-3 h at 1000 V and 75 W in 0.5x TBE buffer. The gel was then nitrate silver stained as described by BUDOWLE et al. (1991) and optimized as following: the gel was placed for 5 min in a 10% ethanol fixation solution and for 3 min in a 1% nitric acid oxidation solution. It was then rinsed two times for few seconds prior to silver staining (6 mM silver nitrate) at room temperature. Once staining was completed, the gel was rinsed two times for few seconds and placed in two successive baths of sodium carbonate revelation solution (235 mM sodium carbonate, 0.145% formaldehyde). The gel was finally rinsed and dried following 10 min bath in a 10% acetic acid reaction stopping solution. The size of the alleles of each locus was estimated using a sequencing size ladder (sequence of pGEM^R-3Zf(+) vector PROMEGA). An X-OMATRA photograph of the gel was taken as a permanent document.

Analysis of genetic data

In order to investigate the genetic relationships between the six Tunisian clones and the ten genotypic classes characterized by TERRADOT et al. (1999) as belonging to *M. antirrhinii* and *M. persicae* taxa, a matrix of pairwise allele-shared distances (*D*as, CHAKRABORTY & JIN, 1993) between all genotypes was calculated using the microsatellite data, and a neighbour-joining tree (NJ) (SAITOU & NEI, 1987) was constructed. POPULATION v. 1.2.28 software (LANGELLA - CNRS) was used (http://www.pge.cnrs-gif.fr/bioinfo/populations/index.php) to calculate *D*as and to construct neighbour-joining tree. The bootstrap support of nodes (1000 replicates) was calculated for the microsatellite tree.

Results

Overall patterns of microsatellite diversity

Microsatellite polymorphism was detected at seven of the nine tested loci (78%). For the seven polymorphic loci, allelic diversity ranged from two to five alleles per locus with twenty four alleles identified across all nine loci (Table 1). The microsatellite locus M35 showed five alleles, while there were four at M37, three at R5.10, M40 and M63 and two at S16b and S17b, averaging 2.6 alleles per locus. The total number of genotypes discriminated by microsatellite markers was high (n=20 over 6 clones). No multilocus genotype was found in this study (Table 1).

Table 1. Microsatellite genotype variation (allele size in base pairs) at nine loci of six Tunisian clones (underlined names) belonging to M. persicae complex.

Tunisian aphid clones	Microsatellite loci								
	R5.10	S16b	S17b	S23	M35	M37	M40	M63	M77
VeB1	270:272	196:196	168:168	115:115	198:198	155:155	125:137	175:177	138:138
VeB3	270:272	198:198	168:168	115:115	182:186	161:163	125:125	171:177	138:138
VeB7	270:272	196:196	168:168	115:115	198:198	155:155	125:137	175:177	138:138
VeT3	267:270	198:198	170:170	115:115	186:194	155:157	125:127	175:177	138:138
VeT6	270:272	198:198	168:168	115:115	182:184	161:163	125:125	171:177	138:138
VeT8	267:270	198:198	168:168	115:115	182:184	161:163	125:125	171:177	138:138
Allelic diversity	3	2	2	1	5	4	3	3	1
Genotypic diversity	2	2	2	1	4	3	3	2	1

Relatedness of Tunisian clones to Myzus taxa

Microsatellite variation allowed us to distinguish five genotypic classes among the six clones, as VeBl and VeB7 showed an identical genotype (Table 1). In this work, we studied the genetic relatedness of the Tunisian clones to *M. persicae* and *M. antirrhinii* taxa (Table 2 and Fig. 1) previously distinguished by TERRADOT et al. (1999). When compared together, the Tunisian aphid clones shared 0.36 mean allele distance, whereas they shared 0.48 and 0.62 mean allele distances with *M. persicae* and *M. antirrhinii* taxa, respectively (Table 2). Within Tunisian clones, VeB7 and VeT3 were the most distant (0.55). Moreover, VeBl and VeB7 Tunisian aphid clones were as distant from *M. antirrhinii* as from *M. persicae* taxa. Furthermore, VeT3 was the most distant from *M. antirrhinii* (0.83) among the other Tunisian clones. The examination of the NJ tree (Fig. 1) showed that the Tunisian clones collected from the same geographic area do not cluster in separate taxa as VeT3, VeT6 and VeT8 originating from Tunis and VeB3 from Tekelsa belonged to the same *M. persicae* taxa.

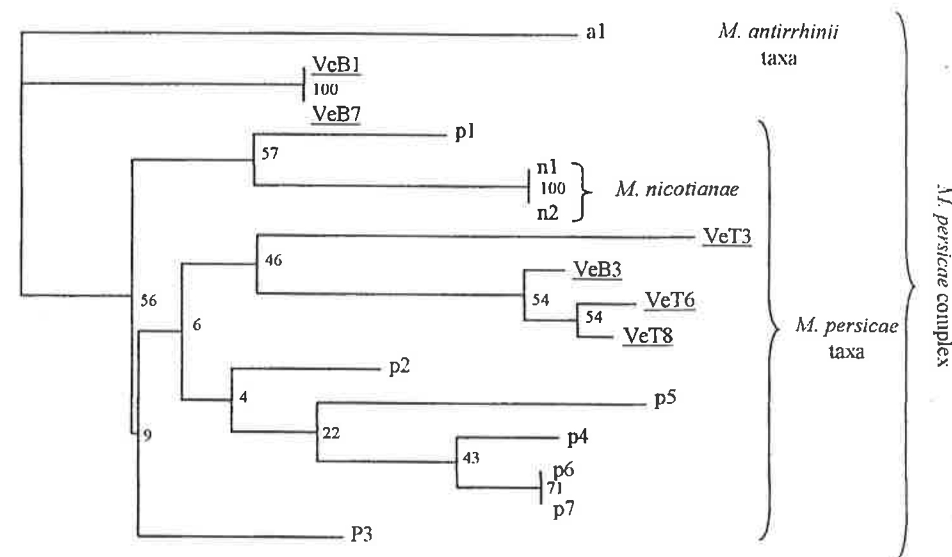

Fig. 1: Neighbour-joining tree (NJ) based on allele shared distance (*Das*) calculated with nine microsatellite loci for six newly typed Tunisian *M. persicae* clones (underlined names) and ten genotypic classes of the *M. persicae* complex identified as belonging to *M. antirrhinii* (al) and *M. persicae taxa* (pl to p7, nl and n2) by TERRADOT et al. (1999)

Discussion

Molecular characterization of the Tunisian *M. persicae* clones made possible the detection of a high number of genotypes (n=20) using microsatellite markers, due to their high polymorphism. These results emphasize the great sensitivity of microsatellites for detecting a high level of aphid genetic variability as previously illustrated (SUNNUCKS et al., 1996, 1997; BOURNOVILLE et al., 2000; HAACK et al., 2000; SIMON et al., 2001; MASSONET et al., 2001, 2002). Surprisingly, some amplified microsatellite loci using primers designed for *Sitobion* sp. and *R. padi* were as polymorphic as those obtained from *M. persicae* (Table 1). In fact, previous studies using microsatellite markers revealed high heterozygosity in asexual lineages of various species of *Sitobion* and *R. padi* aphids (SUNNUCKS et al., 1996; SIMON et al., 1999a; WILSON et al., 1999; DELMOTTE et al., 2002).

Table 2. Matrix of Shared allele distances (Chakraborty & Jin, 1993) of the six newly typed *M. persicae* Tunisian clones (underlined names) and the genotypic classes previously typed by TERRADOT et al. (1999).

	VeB1	VeB3	VeB7	VeT3	VeT6	VeT8	a1	p1	p2	p3	p4	p5	p6	p7	n1	n2
VeB1	0.0000															
VeB3	0.4444	0.0000														
VeB7	0.0000	0.4444	0.0000													
VeT3	0.5000	0.4444	0.5555	0.0000												
VeT6	0.4444	0.0555	0.4444	0.5000	0.0000											
VeT8	0.5000	0.1111	0.5000	0.4444	0.0555	0.0000										
a1	0.5000	0.6111	0.5000	0.8333	0.6111	0.6666	0.0000									
p1	0.4444	0.5000	0.4444	0.4444	0.5555	0.5000	0.6666	0.0000								
p2	0.3888	0.3888	0.3888	0.4444	0.4444	0.3888	0.5555	0.3333	0.0000							
p3	0.3333	0.3333	0.3333	0.5000	0.3888	0.3888	0.5555	0.2777	0.2222	0.0000						
p4	0.5555	0.4444	0.5555	0.5000	0.5000	0.4444	0.5555	0.4444	0.3333	0.3888	0.0000					
p5	0.6666	0.5555	0.6666	0.5000	0.5555	0.5000	0.6666	0.4444	0.2777	0.5000	0.2777	0.0000				
p6	0.5000	0.3888	0.5000	0.5000	0.4444	0.4444	0.5555	0.4444	0.3333	0.2777	0.1111	0.3888	0.0000			
p7	0.5000	0.3888	0.5000	0.5000	0.4444	0.4444	0.5555	0.4444	0.3333	0.2777	0.1111	0.3888	0.0000	0.0000		
n1	0.3888	0.5555	0.3888	0.5555	0.6111	0.5555	0.6111	0.2777	0.3888	0.3888	0.5000	0.5555	0.5000	0.5000	0.0000	
n2	0.3888	0.5555,	0.3888	0.5555,	0.6111	0.5555	0.6111	0.2777	0.3888	0.3888	0.5000	0.5555	0.5000	0.5000	0.0000	0.0000

al: genotypic class enclosing *M. antirrhinii* clones; p1 to p7, n1 and n2: genotypic classes inclosing *M. persicae* clones

The strong genetic differentiation discussed by TERRADOT et al. (1999) between *M. persicae* (pl to p7, nl and n2 genotypic classes) and *M. antirrhinii* (al genotypic class) taxa, was confirmed by our study (Table 2). Furthermore, microsatellites distinguished *M. nicotianae* (genotypic classes n1 and n2) and *M. persicae* (genotypic classes p1 to p7) but also revealed their close relatedness when compared with *M. antirrhinii* (Fig. 1), in agreement with previous studies showing that *M. nicotianae* is essentially a tobacco-adapted form of *M. persicae* making *M. persicae* and *M. antirrhinii* the two major taxa of *M. persicae* complex (BLACKMAN & SPENCE, 1992; MARGARITOPOULOS et al., 1998).

None of the Tunisian clones belonged to the *M. antirrhinii* taxon, as indicated in the NJ tree (Fig. 1), based on allele shared distance between genotypes (Table 2). Genetic divergence between the other classes was small, branches being supported by low bootstrap values (Fig. 1). The Tunisian VeT3 aphid clone has shown to be the most distant from *M. antirrhinii* taxon (genotypic class a1). This result could be correlated to a parallel led study showing that this aphid clone transmits most efficiently the potato leafroll virus (*Luteoviridae*) among all tested Tunisian aphid clones (DJILANI KHOUADJA et al., submitted data). This could confirm that the aphid genotype could influence their virus transmission ability (TERRADOT et al., 1999). The NJ tree showed that Tunisian aphid clones are divided into two groups: *M. persicae* taxa including the VeB3, VeT3, VeT6 and VeT8 clones and another group, as distant from *M. antirrhinii* as from *M. persicae* taxa, including VeBl and VeB7. These two clones could be ascribed to a new distinct taxon, thus, we cannot conclude more for their taxonomy. Thus, additional microsatellite loci will be amplified to confirm the relatedness of these two aphid clones.

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RESEARCH ON THE WESTERN CORN ROOTWORM (*Diabrotica virgifera virgifera* LECONTE, COLEOPTERA: CHRYSOMELIDAE) IN CROATIA (1994-2003)

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Ten years of research work conducted in the Department for Agricultural Zoology of the Agriculture faculty in Zagreb concerning the western corn rootworm (WCR) resulted in 45 scientific and professional papers published in foreign or national publications and meeting proceedings. All of these publications are rewieved in this paper. The development of the WCR in Croatia and Europe confirms the first statement that this insect pest will develop in Europe even faster than in the USA. This paper is intended to support new research on of the WCR, which is underway in many countries of Europe.

***Diabrotica virgifera virgifera*, western corn rootworm, WCR, research, Croatia**

IGRC BARČIĆ, J., BAŽOK, R., MACELJSKI, M., Istraživanje kukuruzne zlatice (*Diabrotica virgifera virgifera* Le Conte) u Hrvatskoj (1994. - 2003). Zavod za poljoprivrednu zoologiju Agronomskog fakulteta Sveučilišta Zagreb, Svetošimunska 25, Hrvatska , E-mail: igrc@agr.hr - Entomol. Croat. 2003. Vol.7. Num. 1-2: 63 - 83.

Desetogodišnja istraživanja znanstvenika Zavoda za poljoprivrednu zoologiju Agronomskog fakulteta u Zagrebu prikazana su u 45 objavljenih znanstvenih i stručnih radova o kukuruznoj zlatici. Radovi su objavljeni u inozemnim i domaćim publikacijama i na skupovima. Svi ti radovi prikazani su u ovom preglednom radu te navedeni u popisu radova. Razvoj kukuruzne zlatice u Europi potvrdio je prvu prognozu da će se štetnik dobro aklimatizirati u Hrvatskoj i Europi i postati još važnijim štetnikom nego što je sada u SAD. Stoga se ovim preglednim radom želi podržati istraživanja koja se sada provode u mnogim europskim zemljama.

***Diabrotica virgifera virgifera*, kukuruzna zlatica, istraživanja, Hrvatska**

Introduction

Western corn rootworm (WCR) was until the last decade of the past century present and a pest solely in North America. In certain areas of North America, especially