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Incidence of insecticide resistance alleles in sexually-reproducing populations of the peach–potato aphid *Myzus persicae* (Hemiptera: Aphididae) from southern France

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Abstract

Intensive chemical treatments have led to the development of a number of insecticide resistance mechanisms in the peach–potato aphid *Myzus persicae* (Sulzer). Some of these mechanisms are known to be associated with negative pleiotropic effects (resistance costs). Molecular and biochemical methods were used to determine the genotypes or phenotypes associated with four insecticide resistance mechanisms in single aphids from sexually-reproducing populations in southern France. The mechanisms considered were E4 and FE4 carboxylesterase overproduction, modified acetycholinesterase, and *kdr* and *rdl* resistance-associated mutations. A new method for determining individual *kdr* genotypes is presented. Almost all resistant individuals overproduced FE4 carboxylesterase, whereas modified acetylcholinesterase was rare. Both the *kdr* and *rdl* resistance mutations were present at high frequencies in French sexually-reproducing populations. The frequencies of insecticide resistance genes were compared before and after sexual reproduction in one peach orchard at Avignon to evaluate the potential impact of selection on the persistence of resistance alleles in the over-wintering phase. The frequencies of the *kdr* and *rdl* mutations varied significantly between autumn and spring sampling periods. The frequency of the *kdr* mutation increased, probably due to pyrethroid treatments at the end of the winter. Conversely, the frequency of the *rdl* mutation decreased significantly during winter, probably because of a fitness cost associated with this mutation.

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Introduction

The intensive use of chemical insecticides against the peach–potato aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) since the 1950s has led to the development of various insecticide resistance mechanisms in the field (review in Devonshire *et al.*, 1999). These mechanisms have been studied in detail (Blackman *et al.*, 1977; Devonshire & Sawicki, 1979; Field *et al.*, 1988; Moores *et al.*, 1994b; Martinez-Torres *et al.*, 1999), and the description of their molecular and biochemical bases has made it possible to develop a set of efficient molecular and biochemical tools for field surveys (Field *et al.*, 1997, 1999; Field & Foster, 2002; Foster *et al.*, 2002). The first insecticide resistance mechanism to be discovered in *M. persicae* was the overproduction of detoxifying E4 or FE4 carboxylesterases. This mechanism is known to confer resistance to organophosphates (OPs) and carbamate and low resistance to pyrethroid insecticides. It results from amplification of the structural gene (Field *et al.*, 1988). Both types of enzyme may be overproduced to different extents, resulting in various levels of insecticide resistance. Aphids are therefore classified as S (susceptible), R1 (moderately resistant), R2 (highly resistant) or R3 (extremely resistant) (Field *et al.*, 1988).

Another resistance mechanism involves changes in the structure of acetylcholinesterase (AChE), the target of OPs and carbamates. In *M. persicae*, two alleles may be present at the acetylcholinesterase locus: *Ace.S*, which encodes an AChE susceptible to the action of insecticides, and *Ace.R*, which encodes an AChE conferring high-level resistance specifically to the di-methylcarbamates, pirimicarb and triazamate (Moores *et al.*, 1994b). Mutations in the structural genes of two additional targets were recently discovered in *M. persicae* and shown to be associated with insecticide resistance. The first involved the *para*-like gene, which encodes a voltage-gated sodium channel that acts as the target of DDT and pyrethroids (Martinez-Torres *et al.*, 1999) and the second involved mutations in the GABA receptor subunit gene *Rdl* (Anthony *et al.*, 1998), the target site for cyclodiene insecticides.

Molecular and biochemical methods were therefore used to investigate the current status of these four resistance genes in natural sexually-reproducing populations of *M. persicae* in southern France. During the course of the year, sexually-reproducing aphids complete a cycle involving several asexual generations on herbaceous host plants and a single phase of sexual reproduction on peach trees (Blackman, 1972). The sexual phase of *M. persicae* is the ideal part of the life cycle for studying variability in insecticide resistance genes. Sampling in this phase maximizes the genotypic variability sampled (Wilson *et al.*, 2002) and overcomes the problem of analysing the same clone many times when working on parthenogenetic aphids (e.g. Fenton *et al.*, 1998).

In addition to sampling issues, the sexual phase of the aphid life cycle may have a major impact on insecticide resistance dynamics in several ways. Firstly, genetic recombination occurs in sexually-reproducing aphids and leads to changes in multilocus genotype frequencies relative to those in asexual aphids. Secondly, high levels of migration occur before and after sexual reproduction, potentially homogenizing genotype and gene frequencies. Thirdly, it is often assumed that sexual reproduction results in more rapid evolution than strict parthenogenesis (Maynard Smith, 1998). This effect may favour the selection of resistance alleles with lower fitness costs. Finally, strong selective forces are at work during the period of sexual activity, and mortality rates may be high (Ward *et al.*, 1998; Doherty & Hales, 2002), leading to a possible strong resistance cost during this period. Conversely, it should be noted that low temperature selection against resistance genes (Foster *et al.*, 1997) in winter is less likely to be detected in sexually reproducing populations of *M. persicae*, as the sexually-produced eggs are the only form that can resist frost in cold regions.

Materials and methods

Aphids

Adult *M. persicae* aphids were sampled in six peach tree orchards in southern France (fig. 1, table 1). The sampled

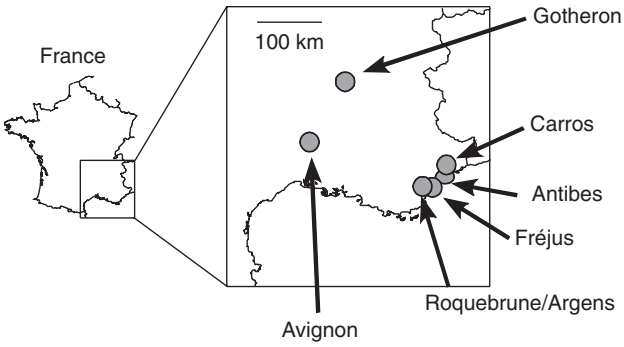

Fig. 1. Geographical locations of the *Myzus persicae* sampling sites used in this study.

Table 1. Characteristics of the samples of *Myzus persicae* from peach.

Location	Region	Longitude	Latitude	Date	*n*
Fréjus	Alpes-Maritimes	6°44′E	43°26′N	3 November 2000	6
Roquebrune/Argens	Alpes-Maritimes	6°38′E	43°26′N	3 November 2000	23
Antibes	Alpes-Maritimes	7°07′E	43°34′N	7, 15 November 2000	5
Carros	Alpes-Maritimes	7°11′E	43°47′N	5 November 2000	16
				27–28 March 2002	17
Gotheron	Drôme	4°57′E	44°58′N	25 February 2002	9
Avignon	Vaucluse	4°48′E	43°56′N	14 November 2000	87
				22 March 2001	36
				2 April 2002	6

n is the number of aphids analysed in the genetic study. See text for details.

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insects were frozen and stored at –80°C. Aphids from an orchard in Avignon were sampled in autumn 2000 and spring 2001. This orchard was made of two groups of trees (group A and group B) of about 50 trees each. The distance between two contiguous trees in each group was about 2–3 m. The distance between the two groups of trees was about 20 m. In autumn, one to five aphids were sampled per tree on different branches. Fifty nine aphids were sampled from group A and 28 from group B. In spring, nine aphids were sampled from group A, with at most one aphid sampled per tree, and 27 aphids were evenly collected on different branches of two trees of group B. The autumn samples corresponded to the sexual males, sexual females (oviparae) and to the parthenogenetic generation giving birth to those females (gynoparae). Spring samples were assumed to be fundatrices developing from sexually-produced eggs and their progeny.

During the winter and spring, the following insecticides were used in the Avignon peach orchard: tau-fluvalinate (pyrethroid) and imidacloprid (neonicotinoid). In addition, during the previous years, this orchard was treated with fenitrothion, methidation, and heptenophos (OP); esfenvalerate, bifenthrin, and deltamethrin (pyrethroid); and pirimicarb (carbamate). No insecticides were used in the orchards at Frejus, Roquebrune/Argens, Gotheron, Antibes and Carros during the period considered.

Five clones with known resistance phenotypes were used to calibrate resistance assays:

1. SUS, an insecticide-susceptible clone derived from US1L, described by Devonshire & Sawicki (1979).

2. COL, which is moderately resistant to OPs and pyrethroid insecticides, collected on oilseed rape near Paris, France, in 1999 (Delorme *et al*., 1999). This clone was classified as R1–R2 and displayed no acetylcholinesterase modification (Delorme *et al*., 1999).

3. LYON, a clone highly resistant to OPs classified as R3 according to the method of Devonshire *et al*. (1992), collected in 1997 near Lyon in France.

4. AV, a clone collected from a peach tree in Avignon in summer 1997 (Sauge *et al*., 1998).

5. BIA, a clone collected from a glasshouse near Nice in 2001 in which the following insecticides failed to control resident *M. persicae* aphids: dichlorvos, parathion-ethyl, endosulfan and pymetrozine.

Resistance gene determination

Single aphids were homogenized in 100 µl of 20 mM phosphate buffer pH 6.5, 0.1% Triton X-100 (PB-triton) on ice. One third of the homogenate was used for DNA extraction for PCR identification of esterase alleles and *kdr* and *rdl* genotypes. Genomic DNA was isolated as described by Sunnucks & Hales (1996). Briefly, the homogenate was incubated for 1 h at 55°C with 30 µg of proteinase K in 270 µl of TNES buffer (50 mM Tris pH 7.5, 400 mM NaCl, 20 mM EDTA, 0.5% SDS). Proteins were precipitated by adding 85 µl of 5 mM NaCl. Genomic DNA was isolated from the supernatant by ethanol precipitation and was resuspended in 50 µl H_2O. The rest of the homogenate was centrifuged for 10 min at 10,000 *g*. The supernatant was carefully transferred to a new 1.5 ml tube and stored at –80°C for subsequent biochemical analysis.

Esterase overproduction

The amplified genes encoding carboxylesterases E4 and FE4 are different enough to be distinguished by an restriction fragment length polymorphism (RFLP) procedure (Field *et al*., 1996). Therefore the presence of E4 or FE4 genes in individual aphids was investigated according to a PCR–RFLP method described by Field *et al*. (1996). New polymerase chain reaction (PCR) primers were designed based on the E4 and FE4 esterase gene sequences reported in Field *et al*. (1996), with the aim of improving PCR yield. The primers used were: Est3N 5′-AAATCATATTCCCGGGTTC-3′ and Est4p 5′-TGAGTAATCTTAGTGAACCTGA-3′. The PCR products were digested with *Hin*dIII (specific for FE4 alleles) or *Spe*I (specific for E4 alleles). Individuals were classified as FE4, E4, E4/FE4 or S aphids.

The level to which enzymes were overproduced in individual aphids was estimated by means of a microtitre plate assay, as described by Devonshire *et al*. (1992), using 50 µl of aphid homogenate. This total esterase assay was used to discriminate between three classes of aphid: those displaying no esterase overproduction, those with moderate or high resistance (R1 and R2) and those with extremely high levels of resistance (R3), following the nomenclature of Devonshire & Field (1991) and used in Devonshire *et al*. (1992). SUS (susceptible), COL (R1-R2) and LYON (R3) were used as the reference clones when classifying individual aphids.

Ace *genotype determination*

A modified version of the fluorometric method described by Moores & Devonshire (2000), using CPM (7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin, Molecular Probes, Eugene, USA), was used to determine the genotype at the acetylcholinesterase (AChE) locus, *Ace*. This protocol was designed to function with as little as one tenth of a single aphid. Only the two alleles described by Moores *et al*. (1994a,b) were considered: *Ace.S* (the allele encoding the susceptible AChE) and *Ace.R* (the allele encoding the modified, resistant AChE). The method described by Moores *et al*. (2000) was first applied to a pool of BIA aphids; this clone was found to be heterozygous *Ace.R*/*Ace.S*. BIA and SUS were then used as AChE reference clones in subsequent analyses.

Assays were performed at 20–22°C, with 1 mM substrate. Ninety microlitres of a ten-fold dilution in PB-triton of individual aphid homogenates were introduced into two adjacent wells of a microtitre plate. Ten microlitres of water or 100 µM pirimicarb were added to each well and incubated the plate at room temperature for 5 min. Then 100 µl of the developing solution (0.03 % (w/v) acetythiocholine iodide and 100 µM CPM in 0.01 M PB, pH 6.5) were added to each well. The microtitre plate was then read in a spectrofluorimeter (Fluorolite 1000, Dynatech Laboratories Inc, Chantilly, Virginia), once per minute for 15 min. The excitation and emission wavelengths were 390 and 450 nm respectively. Residual activity was then estimated by comparing wells with and without inhibitor. Ranges of residual activity of 0–15%, 65–80% and 95–100% corresponded respectively to the *Ace.S*/*Ace.S*, *Ace.R*/*Ace.S*, and *Ace.R*/*Ace.R* genotypes.

Kdr *determination*

Martinez-Torres *et al*. (1999) showed that pyrethroid resistance was correlated with the *kdr* mutation. This mutation consists of the substitution of a T (*kdr* allele) for the

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C (*kds* allele) present in position 322 of the partial cDNA sequence of the *para*-like gene obtained from *M. persicae*. This mutation corresponds to the *kdr*-type Leu1014 mutation in domain IIS6 of the *para*-type sodium channel gene originally described in *Musca domestica* Linnaeus (Diptera: Muscidae) (Williamson *et al.*, 1996). Polymerase chain reaction amplification of specific alleles (PASA) (Sommer *et al.*, 1992) was used to determine individual genotypes (*kds*/*kds*, *kdr*/*kds*, and *kdr*/*kdr*) at this position within the *para*-like gene. Genomic DNA from the SUS, COL and AV clones was used to obtain the partial sequence of the *para*-like gene. Analysis of the sequences of the SUS, COL and AV clones gave the corresponding *kds*/*kds*, *kdr*/*kds*, and *kdr*/*kdr*, genotypes. These clones were used as references in the PASA test. Four primers were designed from these sequences for specific amplification of the susceptible and resistant alleles (containing (resistant) or not containing (susceptible) the *kdr* mutation). Primers Mp889 (5′-ATCTGGCATAAGTCTAA-GAG-3′) and Mp103 (5′-GTCATGGGTATGCAGTTATTTG -3′) were designed to amplify a ~900 bp fragment encompassing the *kdr* mutation. Primer Sd2 (5′-CTACT-GTTGTCATTGGTAACC-3′) was designed to amplify specifically the *kds* allele, in combination with Mp889. Primer Rv2 (5′-ATAGTACTTATACATACCACGAA-3′) was designed to amplify specifically the *kdr* allele, in combination with Mp103. The positions of the external primers Mp889 and Mp103 were chosen so as to produce fragments of different sizes, facilitating discrimination between the *kdr* (~300 bp) and *kds* (~600 bp) alleles. Polymerase chain reaction on single aphids were performed in a total volume of 25 µl, with 2 µl of a 1:10 dilution of DNA extract, 1 µM of each of the four primers, 200 µM of each dNTP, 1.5 mM $MgCl_2$ and 1.25 units of *Taq* polymerase (Q.BIO Gene, Carlsbad, CA) in 1 × enzyme buffer, as supplied by the manufacturer. The reactions were heated at 94°C for 2 min and were then subjected to 30 cycles of 94°C for 30 s, 61°C for 45 s and 72°C for 30 s. Aphids homozygous for the *kdr* mutation gave two fragments at 300 and 900 bp, those homozygous for the *kds* allele gave two fragments at 600 and 900 bp and heterozygous aphids gave all three fragments. The 900 bp fragment served as a control fragment, present in all cases.

Rdl *determination*

The *Rdl* genotype was determined with the single strand conformation polymorphism (SSCP) method described by Anthony *et al*. (1998). The GABA receptor gene seems to have been duplicated in *M. persicae*. One of the genes, *Rdl*1, is involved in insecticide resistance and has two known alleles: *sdl*1 (encoding an alanine in position 302), and *rdl*1 (encoding a glycine in position 302). The second gene, *Rdl*2, does not appear to be associated with resistance and has two alleles, *rdl*2 and *rdl*′2 (each encoding a serine residue in position 302). Polymerase chain reaction with the primers F1 (5′-CGATCCATGGGCTACTAT-3′) and R1 (5′-ACGGTG-GTRACACCCAA-3′) resulted in the specific amplification of *Rdl*1 (i.e. the *sdl*1 and *rdl*1 alleles), using an annealing temperature of 50°C and a concentration of 1 mM $MgCl_2$. Plasmids (TA cloning vector, Invitrogen) containing the four known *Rdl* GABA receptor subunit alleles – *sdl*1, *rdl*1, *rdl*2 and *rdl*′2 (Anthony *et al.*, 1998) – were used as controls. Following the PCR amplification of *Rdl*1, 4 µl of the denatured products were run on a 10% acrylamide gel (ratio 49/1 of acrylamide/bisacrylamide) at 6 W and 4°C for 3 h. The gels were silver stained and dried or photographed. As only one of the two *Rdl* genes was considered, the *sdl*1 and *rdl*1 alleles will be referred to as *sdl* and *rdl*, respectively. Individual genotypes at the *Rdl*1 locus were thus classified as *rdl*/*rdl*, *rdl*/*sdl* and *sdl*/*sdl*.

Statistical analysis

Linkage disequilibrium between loci and departure from Hardy-Weinberg equilibrium at each locus were tested with the exact test procedures implemented by GENEPOP (ver. 3.3) software (Raymond & Rousset, 1995). The homogeneity of genotypic and allelic frequency between samples was assessed by means of the exact tests supplied by this program. The overall significance of several independent tests was determined according to Fisher's method (Sokal & Rohlf, 1995, pp. 794–797). Sequential Bonferroni correction was applied for non-orthogonal (pairwise differentiation and linkage disequilibrium) comparisons (Sokal & Rohlf, 1995, pp. 241–242).

Results

Insecticide resistance genes in the field

The frequencies of esterase phenotypes and insecticide resistance genes in the sexually-reproducing populations of *M. persicae* are given in table 2. No linkage disequilibrium was detected between pairs of loci within each sample or

Table 2. Insecticide resistance phenotype (esterase) and genotype frequencies in sexual samples of *Myzus persicae* in 2000, 2001 and 2002.

Sample		Esterase (phenotype)				*Ace*				*para*-like gene				GABA receptor gene			
Date	Location	S	R1–R2	R3	*n*	SS	RS	RR	*n*	kds/kds	kdr/kds	kdr/kdr	*n*	sdl/sdl	rdl/sdl	rdl/rdl	*n*
Autumn 2000	Fréjus	0	0	1	6	–	–	–	–	0	0.67	0.33	6	0	1	0	6
	Roq./Ar.	0	0.22	0.78	23	–	–	–	–	0.13	0.57	0.3	23	0.48	0.52	0	23
	Carros	0.06	0.06	0.88	16	–	–	–	–	0	0.69	0.31	16	0.69	0.25	0.06	16
	Antibes	0	0	1	5	–	–	–	–	0	0.8	0.2	5	0.8	0.2	0	5
	Avignon	0	0.09	0.91	86	0.98	0.02	0	57	0.17	0.53	0.3	81	0.77	0.22	0.01	86
Spring 2001	Avignon	0	0.08	0.92	36	1	0	0	36	0	0.53	0.47	36	0.94	0.06	0	36
Spring 2002	Avignon	0	0.17	0.83	6	1	0	0	6	0	0.83	0.17	6	0.67	0.33	0	6
	Gotheron	0	0.44	0.56	9	0.89	0	0.11	9	0	0.44	0.56	9	0.89	0.11	0	9
	Carros	0	0.53	0.47	17	0.71	0.29	0	17	0	0.53	0.47	17	0.63	0.31	0.06	16

The number of individuals analysed (*n*) is indicated for each locus. Esterase allele frequencies are not shown because all individuals had the FE4 allele except three females caught in Carros and Avignon in 2000 (see text for details).

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across all populations ($P > 0.05$). No significant differences in genotype or gene frequencies were found between sites within periods.

Esterase overproduction

Regardless of sampling period or site, all individuals of *M. persicae* displayed the FE4 allele, except for one female caught in Carros in autumn 2000 that overproduced the E4 esterase, and two individuals for which the esterase gene could not be amplified by PCR. In 2000–2001, 90% of the aphids were R3 and 10% were R1–R2. In 2002, 56% of the aphids were R3 and 44% were R1–R2. The COL and LYON clones displayed the FE4 esterase allele. Both BIA and AV clones were classified R3 and displayed the FE4 allele.

Modified acetylcholinesterase

It was not possible to determine *Ace* genotype for all 204 individuals, because 79 of these individuals were analysed using a method not reliable for single aphids (not shown). The modified acetylcholinesterase described by Moores *et al*. (1994b) was rare in the remaining 125 aphids analysed. Only one heterozygous *Ace.R*/*Ace.S* individual was identified in 2000 and 2001, among the 93 individuals analysed. In 2002, five heterozygous and one homozygous *Ace.R*/*Ace.R* aphids were identified among 32 individuals. The Carros sample collected in 2002 was polymorphic for the *Ace* locus and genotype frequencies did not significantly deviate from Hardy-Weinberg equilibrium ($P > 0.5$). All the reference clones were *Ace.S*/*Ace.S*, except for clone BIA, which was used as an *Ace.R*/*Ace.S* control.

kdr

The reliability of the PASA procedure was assessed by comparing the results obtained using this method with the nucleotide sequence of 17 individuals (not shown). No differences in genotype determination at the *kdr* position were found between the two methods. The PASA procedure was then used to determine the *kdr* genotype of 199 individuals. An example is shown in fig. 2.

The *kdr* mutation was present at high frequencies in all samples. The frequency of the *kdr* allele was 62% in 2000–2001 (72% in 2002), with 10% (0% in 2002) of the individuals being *kds*/*kds*, 56% (56% in 2002) *kdr*/*kds*, and 34% (44% in 2002) *kdr*/*kdr*. Frequencies were at Hardy-Weinberg equilibrium both at and between sites ($P > 0.05$). No differentiation was found between the two groups of trees of the autumn 2001 sample at Avignon (Fisher's exact test, $P > 0.05$). The reference clones SUS, COL and AV were *kds*/*kds*, *kdr*/*kds*, and *kdr*/*kdr*, respectively; LYON was *kdr*/*kds* and BIA was *kds*/*kds*.

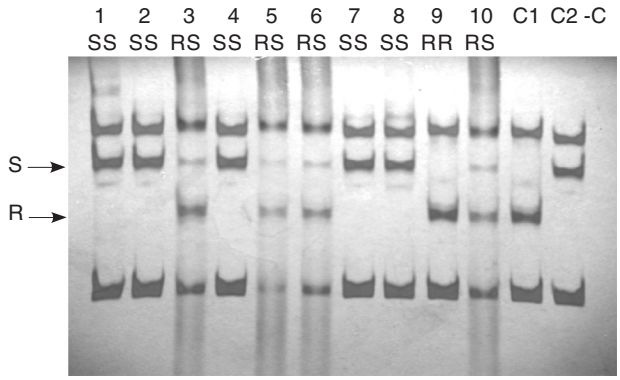

Fig. 3. Single strand conformation polymorphism for the determination of *Rdl* genotypes of *Myzus persicae*. The profiles obtained from ten individuals are shown, together with those for two reference plasmids, C1 and C2 corresponding to the *rdl* and *sdl* alleles of the *Rdl*1 locus, respectively (see text for details). A negative control (–C), corresponding to PCR without a DNA template is also shown. RR, RS and SS correspond to the *rdl*/*rdl*, *rdl*/*sdl*, and *sdl*/*sdl* genotypes, respectively. S and R indicate the *sdl* and *rdl* allele-specific bands, respectively.

Rdl

With the SSCP procedure, 203 of the 204 individuals were genotyped unequivocally. Using the PCR conditions described by Anthony *et al*. (1998), the gene involved in resistance (*Rdl*1) was specifically amplified whereas the other gene, *Rdl*2, was not. The migration patterns obtained from individual aphids matched those obtained with plasmids, suggesting that the alleles identified in our samples were identical to the *sdl* and *rdl* alleles described by Anthony *et al*. (1998) (fig. 3). The *rdl* allele was moderately frequent in the samples (14%); only three aphids homozygous for *rdl* were identified and there were 52 heterozygous individuals. Hardy-Weinberg equilibrium was rejected neither across, nor within sites ($P > 0.05$). No differentiation was found between the two groups of trees of the autumn 2001 sample at Avignon (Fisher's exact test, $P >$

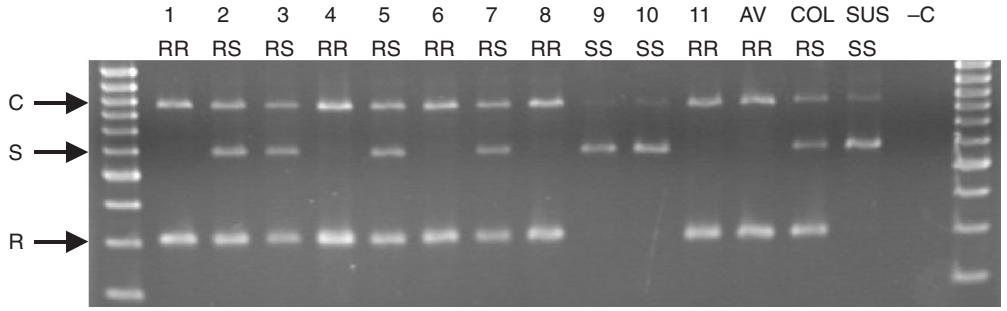

Fig. 2. Polymerase chain reaction (PCR) amplification of specific alleles (PASA) of the *para*-like gene of *Myzus persicae* permitting the discrimination of *kdr* genotypes at this locus. The profiles obtained from 11 individuals are shown together with *kdr*/*kdr* (AV), *kdr*/*kds* (COL), *kds*/*kds* (SUS) reference clones. A negative control (-C), a PCR product without DNA template, is also shown. RR, RS and SS abbreviations are used for *kdr*/*kdr*, *kdr*/*kds*, and *kds*/*kds* genotypes, respectively. C, S and R refer to the control band, *kds* and *kdr* allele-specific bands, respectively. The first and last wells contain a 100 bp DNA ladder, and the first apparent band is 200 bp in size.

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0.05). The SUS, LYON and COL clones were all *sdl*/*sdl* and the BIA and AV clones were both heterozygous *rdl*/*sdl*.

Temporal variation in the frequency of resistance alleles

Our temporal analysis was limited to comparisons between samples of *M. persicae* collected in autumn 2000 and spring 2001 in the same orchard at Avignon. This restriction was imposed for all the genes considered, due to limited sample sizes.

In the Avignon orchard, the proportions of esterase activity classes (S, R1-R2 and R3) were similar before and after sexual reproduction (Fisher's exact test, $P > 0.05$), with about 90% R3 and no S individuals in autumn 2000 and spring 2001. No differences were found between the two samples in the frequency of the *Ace.R* allele (Fisher's exact test, $P > 0.05$). The frequencies of the various genotypes at the *para*-like gene locus differed significantly between autumn 2000 and spring 2001 in the samples from Avignon (table 2; Fisher's exact test, $P = 0.007$, SEM = 0.0001), with *kds*/*kds* individuals totally absent from the spring sample. Finally, the genic and genotypic frequencies at the *Rdl*1 locus differed significantly between the two time intervals (table 2; Fisher's exact test, $P = 0.03$, SEM = 0.0013 and $P = 0.019$, SEM = 0.0016, respectively). Between autumn 2000 and spring 2001, the frequency of heterozygous aphids decreased from 22% to 6% and that of homozygous *sdl*/*sdl* aphids increased from to 77% to 94%.

Discussion

Incidence of resistance genes in sexually-reproducing populations of M. persicae *from southern France*

Prevalence of FE4 in sexually-reproducing populations of M. persicae

Almost all the aphids in the samples studied produced the FE4 carboxylesterase whereas only one orchard studied (Avignon) was treated with insecticides (including OPs, carbamates and pyrethroids) during the period considered. Until recently, all aphids caught on *Prunus persica* (L.) Batsch (Rosaceae) overproduced this enzyme whereas those collected on secondary hosts produced mostly E4 (e.g. Blackman *et al.*, 1999). Recent surveys of esterase overproduction in *M. persicae* collected from secondary hosts suggested that esterase FE4 is gradually replacing E4 in England (Field & Foster, 2002; Foster *et al.*, 2002).

The frequency of the R3 class of esterase-based resistance decreased between 2000–2001 and 2002. This result must be interpreted with care because the samples analysed in 2002 were small and no details of insecticide treatments over this period were available. However Field & Foster (2002) also reported a shift towards lower levels of FE4 overproduction, with 1/16 of FE4 aphids caught in 1997 being R1 and 30/33 being R1 in 2000. The combination of a resistance cost, associated directly or indirectly with esterase gene amplification, and the decrease in organophosphate insecticide use have been suggested to account for this (Field & Foster, 2002; Foster *et al.*, 2002).

The modified acetylcholinesterase is rare

The modified AChE described by Moores *et al.* (1994b) was rare in samples from peach in France over the period of the study, with a mean frequency of about 3% across all sites. Genotype analysis revealed only one aphid homozygous for the altered acetycholinesterase among 125 individuals genotyped. No correlation was found between the frequency of this resistance mechanism and insecticide treatments. Pirimicarb was frequently used in the orchard of Avignon where the frequency of the altered AChE was zero or low, and no insecticides were used in the other orchards.

This resistance mechanism was first detected in 1990 in Greece (Moores *et al.*, 1994b), and then in Japan and northern Europe. It was observed in the UK in 1996, where more than a third of the clones analysed had modified AChE (Foster *et al.*, 1998). Its frequency then rapidly decreased in the UK (Foster *et al.*, 2002). One possible reason for this sudden decline (Foster *et al.*, 2002) involves a link between the altered AChE and other costly genes that may be perpetuated through asexual reproduction. The frequency of the modified AChE was low in sexually-reproducing aphids. The situation may correspond to a transient state rather than a true equilibrium because the resistant form of AChE probably appeared recently: it was first described less than 15 sexual generations ago (Moores *et al.*, 1994b).

Another possible explanation for the very low frequency of modified AChE in natural populations collected from peach trees in southern France is that this resistance mechanism is directly associated with a heavy fitness cost. A resistance cost associated with production of a modified acetylcholinesterase has already been observed in other insect species (e.g. Zhu *et al.*, 1996; Chevillon *et al.*, 1999). In the common mosquito, *Culex pipiens* Linnaeus (Diptera: Culicidae), the fitness cost due to this mechanism in natural populations has been estimated at 11–50%, depending on the life cycle stage (Lenormand *et al.*, 1999).

The possibility of a direct or indirect fitness cost in *M. persicae* cannot be ruled out, but it is also possible that there are other resistant alleles of the acetylcholinesterase gene. The existence of other modified forms of AChE, not detected by the technique used here and conferring a selective advantage, would account for the low incidence of the known resistance allele.

The rdl *mutation is present in French sexually-reproducing populations of* M. persicae

The *rdl* mutation is known to confer cyclodiene resistance in a wide range of insect species (ffrench-Constant, 1999). In *M. persicae*, the presence of the *rdl* mutation and resistance to endosulfan are significantly correlated (Anthony *et al.*, 1998), suggesting that this mutation is involved in cyclodiene resistance. Single strand conformation polymorphism procedure showed that the *rdl* mutation present in French samples was indistinguishable from that found in the United States (Anthony *et al.*, 1998). This finding requires confirmation by sequencing, but it suggests that a single mutation event, followed by migration, may be responsible for the presence of this resistance allele in both the US and Europe. Our findings also suggest that cyclodiene resistance is common in sexually-reproducing populations from southern France: about 25% of the sampled individuals were heterozygous and 1.5% were homozygous for *rdl*. Cyclodiene insecticides are not commonly used in France but they may serve as alternative insecticides in cases of treatment failure (e.g. endosulfan on peach trees). No cyclodiene insecticides were used in the orchards studied during the period considered. However, the resulting selection pressure would be sufficient to maintain the *rdl* mutation at a moderate frequency in the field. As no other studies have investigated the frequency of this resistance

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mechanism in Europe, no conclusion can be drawn as to whether the frequency of *rdl* is increasing or decreasing in natural populations.

The kdr mutation is frequent in sexually-reproducing populations of M. persicae

The association of the *kdr* mutation with DDT and pyrethroid resistance in *M. persicae* was demonstrated only a few years ago (Martinez-Torres *et al*., 1999). However, the *kdr* mutation may have been present in natural populations of this species for some time. Previous surveys of resistance genes in this species (Field *et al*., 1997; Field & Foster, 2002; Foster, 2000; Foster *et al*., 2002) revealed the frequency of the *kdr* phenotype (as determined by a DDT diagnostic assay) to be apparently high in Europe, the USA and Japan. Our genotypic analysis confirmed that this mutation is common in southern France, with more than half of the aphids studied being *kdr*/*kds* and a third, *kdr*/*kdr*. It is noteworthy however that only one orchard studied (Avignon) was treated with insecticides (including pyrethroids) during the period considered. As reported in recent studies of UK samples (Field & Foster, 2002; Foster *et al*., 2002), the statistical relationship between the *kdr* phenotype (including both *kdr*/*kds* and *kdr*/*kdr* genotypes) and the overproduction of esterase E4 (Field *et al*., 1997) is not apparent in the French samples. Until recently, the *kdr* mutation was generally found in combination with E4 in strictly parthenogenetic aphids (Field *et al*., 1997). Asexual lineages can produce males that participate in sexual reproduction (these lineages are therefore called androcyclic). It is therefore possible for genes to be transmitted from asexual to sexually-reproducing *M. persicae* (Blackman, 1972). A cross between androcyclic males and sexual females may result in the production of new androcyclic lineages (Blackman, 1972). Thus, it is also possible for genes to be transmitted from the sexually-reproducing to the asexual pool. This may have allowed: (i) the transfer of the *kdr* mutation from E4- to FE4-producing aphids, thereby destroying the linkage disequilibrium between *kdr* and E4; and (ii) the transfer of the FE4 esterase to asexual populations of *M. persicae*. Positive selective effects of *kdr* versus *kds* and of FE4 versus E4 alleles (see above) may have favoured these transfers.

Variation of resistance gene frequencies during winter and selective hypotheses

The frequencies of resistance genes in aphids collected from the peach tree orchard at Avignon in autumn and spring were compared, with a view to determining the effects of sexual reproduction or adverse winter climatic conditions on those frequencies.

During the winter and spring, the following insecticides were used in the Avignon peach orchard: tau-fluvalinate (pyrethroid) and imidacloprid (neonicotinoid). An increase in the frequency of the *kdr* allele following sexual reproduction and the maintenance of about 90% R3 aphids before and after sexual reproduction were observed in Avignon. The increase in *kdr* mutation frequency corresponded to a decrease in *kds*/*kds* genotype frequency and an increase in the frequency of *kdr*/*kdr* homozygotes. These results are not surprising as the *kdr* mutation confers resistance to pyrethroid insecticides in *M. persicae* (Martinez-Torres *et al*., 1999). Moreover, whereas esterase overproduction confers a low level of resistance to pyrethroid insecticides, the combination of both resistance mechanisms confers a very high level of resistance to this class of insecticide (Martinez-Torres *et al*., 1999).

The frequency of the rdl *mutation decreased during winter*

A decrease in the frequency of the *rdl*/*sdl* genotype and an increase in the frequency of the *sdl*/*sdl* genotype in *M. persicae* were observed between autumn 2000 and spring 2001 (i.e. following sexual reproduction) in Avignon. It is possible that there is a fitness cost associated directly or indirectly with the *rdl* mutation in the GABA receptor gene of *M. persicae* during winter (but see below). This putative cost may be co-dominant or dominant because heterozygous aphids were severely affected. A fitness cost associated with resistance to cyclodiene insecticides has been detected in other species. In the blowfly *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae), a fitness cost associated with the *Rdl* locus has been demonstrated (McKenzie, 1990; McKenzie & Yen, 1995). For instance, McKenzie (1990) found a dominant cost associated with resistant phenotypes in overwintering populations of *L. cuprina*, with *rdl*/*rdl* and *rdl*/*sdl* flies displaying an increased rate of mortality.

If there is a fitness cost, then the subsequent reduction in the cost at the *Rdl* locus seen in *L. cuprina* (Clarke & McKenzie, 1987; McKenzie & Game, 1987) and *C. pipiens* for other resistance genes (Guillemaud *et al*., 1998; Lenormand *et al*., 1998) is not apparent in *M. persicae*. No data are available concerning the date of appearance of the *rdl* mutation in natural populations of *M. persicae*, so it is difficult to make inferences about the long-term temporal dynamics of this mutation. However, sexual reproduction occurs only once per year in sexually-reproducing aphids and this may result in a much slower rate of evolution than is observed in species that have many sexual generations per year.

The apparently selective effect on *Rdl* genotypes may also result from selection at other loci linked to the GABA receptor gene (transient state of linkage, as for the altered acetylcholinesterase, see above). Indeed, the *rdl* allele may be physically associated with alleles at other loci that were selected against by other selective factors. Insecticide treatments (pyrethroids and neonicotinoids) applied before collection of the spring sample may be responsible for such selection. No significant resistance to imidacloprid has yet been described in *M. persicae*; it therefore seems unlikely that this treatment has a selective effect on *rdl*. Moreover, no linkage disequilibrium was found between the GABA receptor and *para*-like genes or with esterase overproduction, so pyrethroid treatments may not have been the cause of variations in the frequency of *rdl*.

Alternatively, sampling bias may be put forward to account for variation in the frequency of the *rdl* mutation in the GABA receptor gene during the winter. Spatial heterogeneity of the sampling may account for genetic heterogeneity, however in spring no heterogeneity in the frequency of the *rdl* mutation was found between the two groups of trees at Avignon. More detailed studies are needed to clarify this point.

Knowledge of the genotypes underlying resistance phenotypes can yield valuable information, including estimation of the frequency of resistance alleles in the field, the dominance of the resistance effects of these alleles and the dominance of their putative pleiotropic effects. Such estimations are essential for describing the dynamics and evolution of insecticide resistance in natural populations (e.g. Foster *et al*., 2002). Such observations could be used to

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inform resistance managers about the effectiveness of their ongoing control regimes and to make predictions and recommendations concerning the management of future pest outbreaks.

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