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Temporal and spatial incidence of alleles conferring knockdown resistance to pyrethroids in the peach–potato aphid, *Myzus persicae* (Hemiptera: Aphididae), and their association with other insecticide resistance mechanisms

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Abstract

The peach–potato aphid, *Myzus persicae* (sulzer), is an important arable pest species throughout the world. Extensive use of insecticides has led to the selection of resistance to most chemical classes including organochlorines, organophosphates, carbamates and pyrethroids. Resistance to pyrethroids is often the result of mutations in the *para*-type sodium channel protein (knockdown resistance or *kdr*). In *M. persicae*, knockdown resistance is associated with two amino-acid substitutions, L1014F (*kdr*) and M918T (*super-kdr*). In this study, the temporal and spatial distributions of these mutations, diagnosed using an allelic discriminating polymerase chain reaction assay, were investigated alongside other resistance mechanisms (modified acetylcholinesterase (MACE) and elevated carboxylesterases). Samples were collected from the UK, mainland Europe, Zimbabwe and south-eastern Australia. The *kdr* mutation and elevated carboxylesterases were widely distributed and recorded from nearly every country. MACE and *super-kdr* were widespread in Europe but absent from Australian samples. The detection of a strongly significant heterozygote excess for both *kdr* and *super-kdr* throughout implies strong selection against individuals homozygous for these resistance mutations. The pattern of distribution found in the UK seemed to indicate strong selection against the *super-kdr* (but not the *kdr*) mutation in any genotype, in the absence of insecticide pressure. There was a significant association (linkage disequilibrium) between different resistance mechanisms, which was probably promoted by a lack of recombination due to parthenogenesis.

Keywords: *Myzus persicae*, kdr, insecticide resistance, MACE

Introduction

The control of peach–potato aphids, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), is achieved primarily by the application of insecticides, often with multiple applications each year. This has led to the evolution of insecticide resistance conferred by three genetically-independent mechanisms (Devonshire *et al*., 1998). The first of these to be characterized was the over-production of one of two closely related carboxylesterases (E4 and FE4). Depending on the amount of carboxylesterase produced, individuals are classified into one of four somewhat arbitrary

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categories: S (susceptible), R_1 (moderately resistant), R_2 (highly resistant) or R_3 (extremely resistant) (Devonshire *et al.*, 1986). This mechanism confers strong resistance to organophosphates and lower resistance to carbamates and pyrethroids.

Two types of target-site insensitivity conferring insecticide resistance have also been found in *M. persicae*. Individuals with modified acetylcholinesterase (MACE) show high levels of resistance to dimethyl carbamates such as pirimicarb and triazamate (Moores *et al.*, 1994). The MACE phenotype has recently been shown to be associated with a single amino acid substitution (serine to phenylalanine, S431F) within the active site of the enzyme (Nabeshima *et al.*, 2003; Andrews *et al.*, 2004). Two mutations in a voltage-gated sodium channel protein (*kdr* and *super-kdr*), conferring knockdown resistance to pyrethroids and DDT, have also been identified in *M. persicae* (Martinez-Torres *et al.*, 1999; Eleftherianos *et al.*, 2002). The two mutations are L1014F (*kdr*) and M918T (*super-kdr*), based on the housefly para sequences (Embl acc: X96668).

Resistance to pyrethroids in *Myzus persicae* has been recorded throughout Europe (Field *et al.*, 1997; Foster *et al.*, 1998; Field & Foster, 2002; Mazzoni & Cravedi, 2002; Guillemaud *et al.*, 2003a; Nauen & Elbert, 2003; Anstead *et al.*, 2004; Fenton *et al.*, 2005; Zamoum *et al.*, 2005), and in the USA, Japan and Chile (Field *et al.*, 1997; Fuentes-Contreras *et al.*, 2004). Due to the low number of samples in most of these studies, there are little meaningful data available on the frequency of resistance (with the exception of Zamoum *et al.* (2005)). Australian populations have been found to contain the 1,3 autosomal translocation associated with elevated E4 esterase (Wilson *et al.*, 2002), indicating this resistance mechanism is probably present. Prior to this study, only a single clone (2169G), which was collected in October 1997 from Lincolnshire, had been found with the M918T mutation (in conjunction with L1014F) (Eleftherianos *et al.*, 2002).

In the present study, we use a novel allelic discriminating polymerase chain reaction (PCR) assay (Anstead *et al.*, 2004) to investigate the temporal and spatial incidence of *kdr* alleles in samples collected from either field sites or 12.2 m suction traps in the UK, mainland Europe, Zimbabwe and south-eastern Australia. The new assay enables higher-throughput screening for *kdr* mutations than was previously possible and has also enabled an examination of associations between *kdr* genotypes and the MACE and overproduced esterase mechanisms.

Materials and methods

Aphid samples

Samples from field crops were obtained from a variety of sources in Europe, Zimbabwe and the state of Victoria in Australia. Aphids from Europe and Africa were either shipped alive on plant material or in 95% ethanol. The ethanol preserved specimens could only be tested for *kdr* mutations as the MACE and carboxylesterase tests need active enzymes that are denatured by storage in alcohol. Samples from Australia were shipped frozen in 'solution 21' (25% glycerol, 0.5% Triton X-100, 100 mM KCl, 20 mM Tris (hydroxymethyl) aminomethane, 1 mM oxytetracycline, 10 μM $CuSO_4$) (Tatchell *et al.*, 1988), preserving enzyme activity and DNA. Aphids were also obtained from Rothamsted's

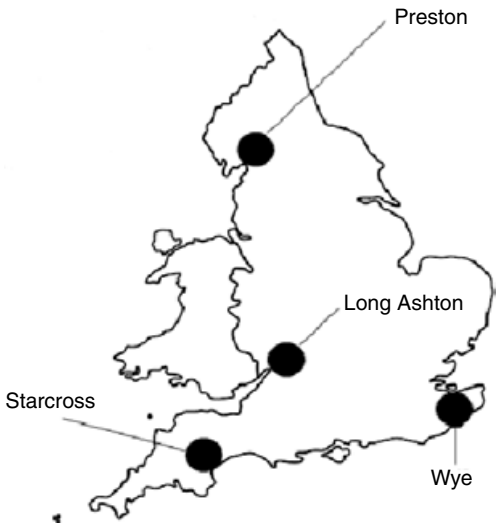

Fig. 1. The locations of 12.2 m suction traps used for the collection of *Myzus persicae* from the United Kingdom in 2002/3.

UK-wide network of suction traps (Woiwod & Harrington, 1994), and these were always shipped in alcohol. The four UK traps used were Preston (53°51′16″, 2°45′47″), Long Ashton (51°25′35″, 2°40′2″), Starcross (50°37′44″, 3°27′13″) and Wye (51°11′50″, 0°56′21″). The trap locations are shown in fig. 1. The Long Ashton trap was discontinued at the end of 2002, so collections were made from Preston in 2003. Aphids were collected from the three UK suction traps daily; up to two aphids from each day of collection were tested for *kdr* and *super-kdr* from each of the traps. A further 12 individuals were obtained in Sweden from a suction trap based on the design of the Rothamsted traps. European sample site locations are shown in fig. 2. All the Australian samples were collected from the state of Victoria, and these field locations are shown in fig. 3.

Insecticide resistance

The *kdr* and *super-kdr* mutations were diagnosed using a rapid allelic discrimination PCR assay (Anstead *et al.*, 2004). During PCR amplification of the resistance allele, two Taqman MBG probes, labelled with different flourophores, bind to the resistant and susceptible allele. The ratio between the fluorescence at the appropriate wavelengths allows the genotype of the resistance allele to be determined. Aphids were assigned genotypes using the following codes: SRSR, heterozygous at both sites; RRSS, homozygous resistant at *kdr*, homozygous susceptible at *super-kdr;* SRSS, heterozygous at *kdr*, susceptible at *super-kdr*; SSSS, homozygous susceptible at both sites; RRRR, homozygous resistant at both sites. MACE was diagnosed using a kinetic enzyme inhibition assay (Moores *et al.*, 1994), and carboxylesterase levels were determined by immunoassay (Devonshire *et al.*, 1986). For genotypic testing, single aphids were homogenized in 50 μl of PBS/Tween (phosphate buffer, 0.02 M, pH 7.0 containing 0.05 v/v Tween 20) in the wells of a microtitre plate using a multihomogenizer (ffrenchConstant & Devonshire, 1987). One μl was used for carboxylesterase

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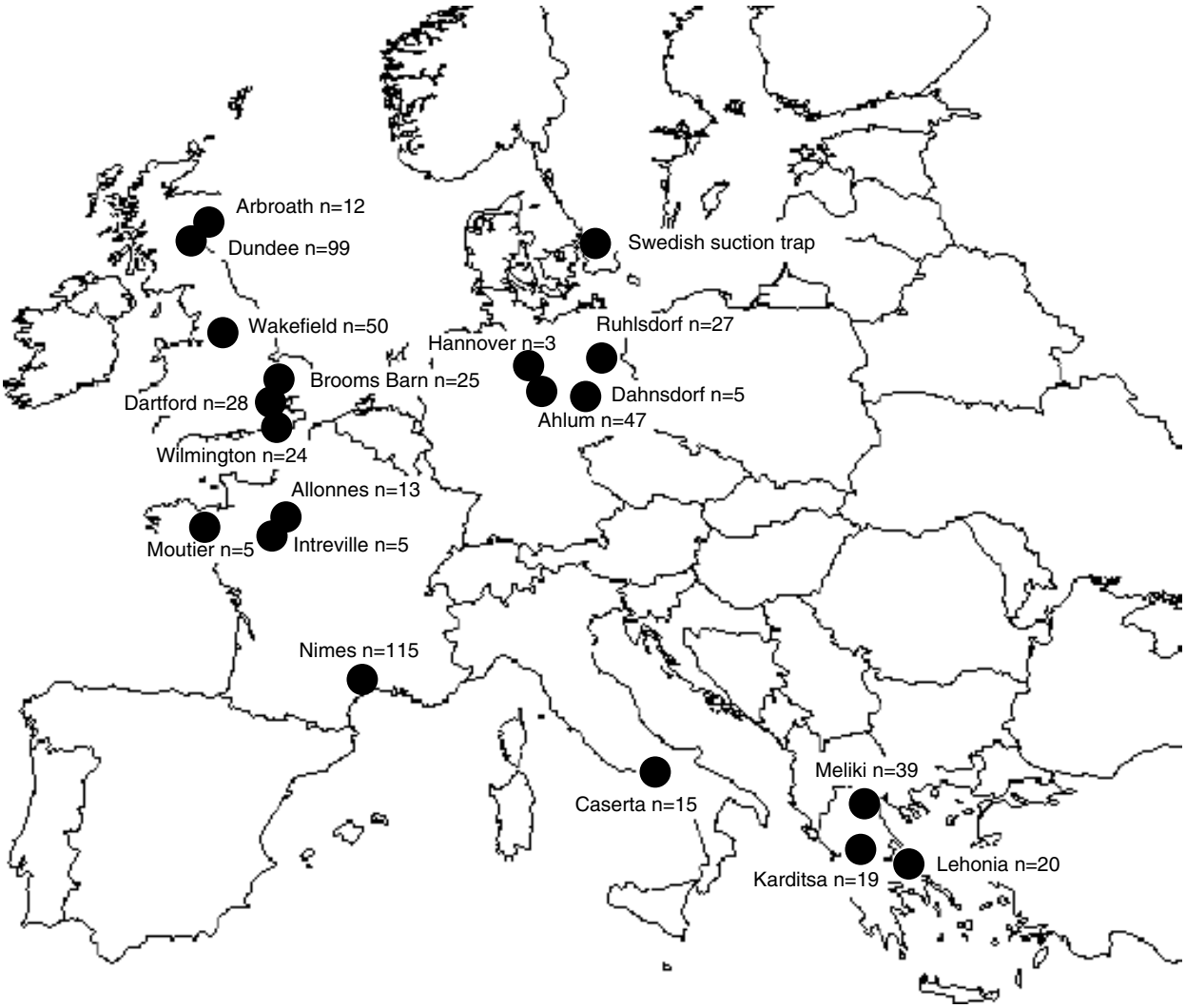

Fig. 2. The location of European *Myzus persicae* collection field sites and the Swedish suction trap 2001–2003. The number of individuals collected is indicated.

testing, 5 µl was removed for the extraction of genomic DNA for diagnosing *kdr* and *super-kdr* and the remainder was used to test the MACE phenotype (either susceptible or resistant, the latter including aphids both heterozygous and homozygous for the MACE mutation.

Statistical analysis

Deviations from Hardy-Weinberg equilibrium at the *kdr* and *super-kdr* mutation sites were tested for in-field collected samples when more than ten aphids were available. Associations between elevated carboxylesterase, *kdr*, *super-kdr* and MACE were analysed using standard t-tests and analysis of variance (unbalanced design) (ANOVA). Linkage disequilibrium between the two *kdr* mutations and MACE was calculated using DIS (available from http://www.ucl.ac.uk/taxome/jim/bin/software.html) (Dasmahapatra *et al*., 2002), as were deviations from Hardy-Weinberg equilibrium. Data were pooled by country and year to increase the sample size for linkage disequilibrium calculations. The French and Australian samples were omitted from the linkage disequilibrium analysis as neither MACE nor *super-kdr* was found in these areas.

Results

Overall geographical distribution of resistance

A summary of the distribution of resistance mechanisms is shown in table 1. Elevated carboxylesterase was the most widespread resistance mechanism and was present in all samples. The *kdr* mutation was also widely found, only being absent from the Zimbabwean sample. MACE and the *super-kdr* mutation were both absent from samples from Australia and Zimbabwe. There were considerable differences between the frequencies of resistance mechanisms in the samples, probably due to different insecticide selection pressures. *Kdr* was present at frequencies between 0.19 and 0.66 in all but two of the samples; it was absent from aphids

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Table 1. Geographical incidence of resistance mechanisms and genotypes of *Myzus persicae*.

Country	*n*	Resistance frequency						
		Knockdown resistance		Carboxylesterase				MACE
		kdr	*super-kdr*	S	R1	R2	R3	
2001								
UK	96	0.26	0.19	0.45	0.15	0.07	0.33	0.32
France	8	0.66	0.66	0.17	0.17	0.33	0.33	0.13
2002								
Australia	36	0.36	0	0.64	0.28	0.08	0	0
France	23	0.44	0	0.22	0.78	0	0	0
Germany	83	0.19	0	0.10	0.63	0.25	0.04	0.05
Greece	71	0.41	0.22	0	0	0.34	0.66	0.84
Italy	15	0.5	0.3	0	0	0.67	0.33	0.53
Scotland	12	0.42	0.05	0	0.5	0.47	0.03	0.38
UK trap[a]	53	0.42	0.06					
Sweden trap[a]	12	1	0					
2003								
Zimbabwe[a]	15	0	0					
France[a]	85	0.42	0.33					
Germany	14	0.43	0	0	0.36	0.57	0.07	0
UK trap[a]	154	0.28	0.06					
UK	146	0.39	0.03	0.04	0.45	0.44	0.06	0.39

[a] Samples collected and subsequently stored in alcohol could not be tested for carboxylesterase levels or for MACE.
Carboxylesterase levels are divided into the four standard categories of resistance. *Kdr* and *super-kdr* are shown as allele frequencies. MACE is shown as the frequency of the MACE phenotype, i.e. homozygotes and heterozygotes combined.

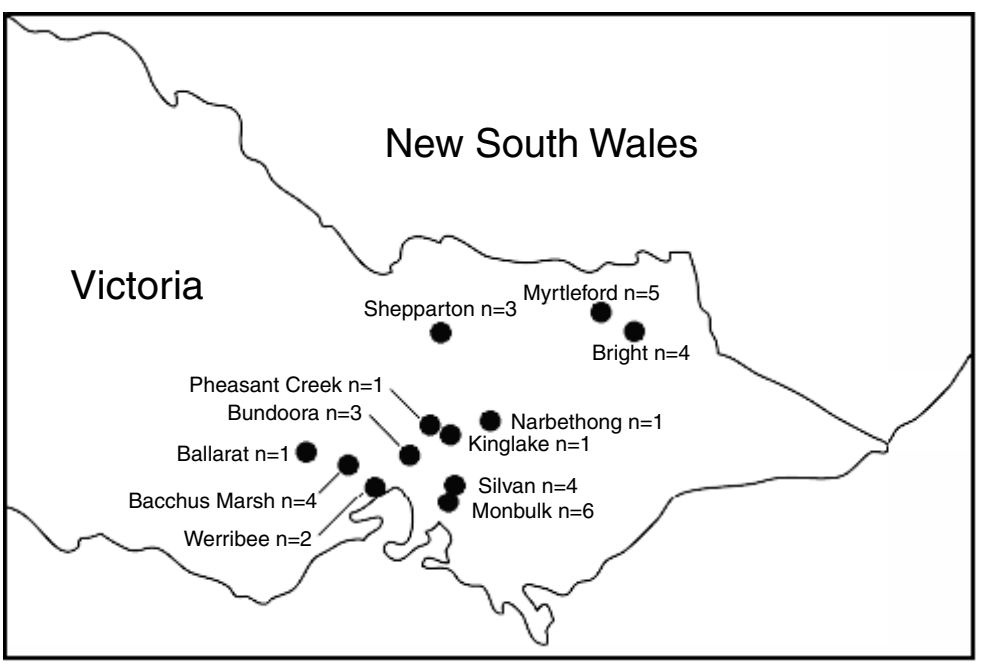

Fig. 3. The location of collection sites for *Myzus persicae* in the state of Victoria, Australia 2002, and the number of clonal lineages tested.

from Zimbabwe and was present, as a homozygote, in all the aphids from the Swedish suction trap. *Super-kdr* was present at much lower frequencies and was absent from a number of samples including some that were relatively large (e.g. samples collected from Germany in 2002 and 2003). In some samples, especially those from Greece and Italy, selection seems to have completely removed the lower carboxylesterase categories and all aphids collected scored as R2 or R3.

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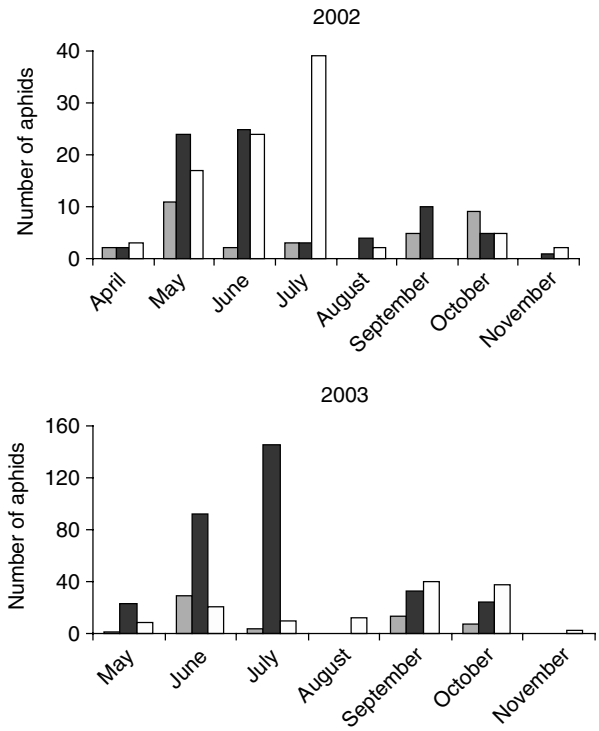

Fig. 4. Total number of *Myzus persicae* collected from three UK suction traps in 2002 (▨, Long Ashton; ■, Wye; □, Starcross) and 2003 (▨, Preston; ■, Wye; □, Starcross).

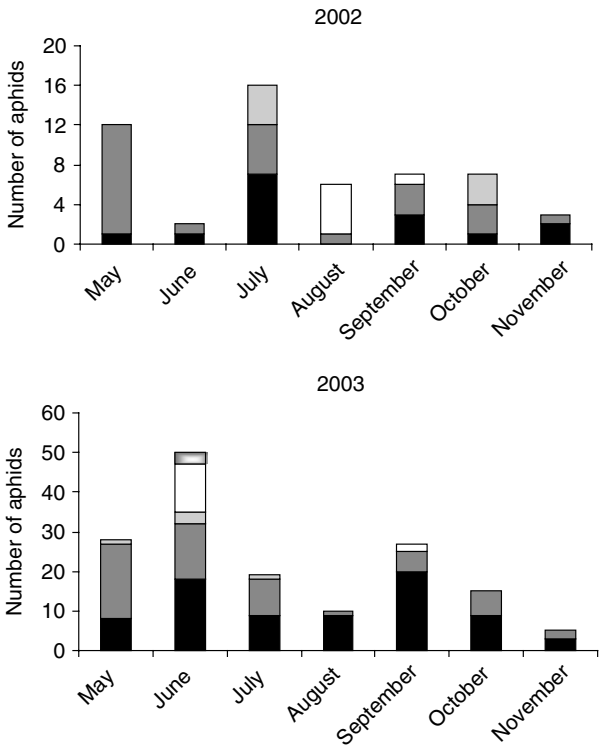

Fig. 5. Knockdown resistance genotypes of *Myzus persicae* collected from three UK suction traps (combined) in 2002 (□, SRSR; ▨, RRSS; ■, SRSS; ■, SSSS) and 2003 (▨, RRRR; □, SRSR; ▨, RRSS; ■, SRSS; ■, SSSS). See text for genotype annotation.

UK suction trap catches in 2002 and 2003

Numbers of *M. persicae* caught in the three suction traps in 2002 and 2003 shown a bi-modal distribution typical of *M. persicae* in the UK (fig. 4) where two major flights per season occur (Karley *et al*., 2004). This pattern was generally apparent in the data from each trap (fig. 4), although the timing and magnitude of the flights varied between traps and years. In 2002, the highest total number of aphids was collected from Starcross (92), then Wye (74) and Long Ashton (32). In 2003 higher total numbers were collected from Wye (318) than Preston (131) and Starcross (54). Overall aphid numbers were higher in 2003 and this was reflected at each trap.

Figure 5 shows the incidence of knockdown resistance detected in the combined trap catches for 2002 and 2003. In both 2002 and 2003 the *kdr* mutation was present during every month of *M. persicae* collection, although the frequency varied considerably; ranging from 5% (August 2003) to 65% (October 2002) (fig. 5). In 2003 it was found during every month at every trap, with the exception of two months at the Preston trap when only a single aphid was recorded. The *kdr* mutation was found most commonly as a heterozygote without the *super-kdr* mutation (SRSS). In contrast the *super-kdr* mutation was only detected during August and September 2002 and June and September 2003. In June 2003 when the *super-kdr* mutation was at a frequency of 0.15, aphids with this mutation were caught at all three traps. However in September 2003, *super-kdr* aphids were only caught at Preston. The two most common genotypes were SSSS (no resistance mutations) and SRSS (heterozygous for *kdr*, homozygous susceptible for the *super-kdr* mutation). No individual from field or suction trap sample was found with the *super-kdr* mutation alone. It was always accompanied by the *kdr* mutation.

Hardy-Weinberg equilibrium

Deviations from Hardy-Weinberg equilibrium were calculated from samples collected from single fields and from the UK trap samples in 2002 and 2003 (pooled for the whole year) (table 2). The sample sites ranged from Scottish samples where, it is believed, no sexual recombination occurs (due to the absence of peach), to Greek peach samples, which were spring-collected, when the aphids had just gone through a cycle of sexual reproduction (*M. persicae* undergoes sexual reproduction exclusively on peach trees). All but one set of samples showed a heterozygous excess, although the level of statistical significance varied.

Associations between mechanisms

To improve statistical power when calculating associations between resistance, the continuously distributed carboxylesterase immunoassay values were used (Devonshire *et al*., 1986) instead of the usual four categories of carboxylesterase level (S, R1, R2, R3). Figure 6 shows the mean carboxylesterase levels for aphids collected in 2002 and 2003 with, and without, the MACE mutation. Aphids containing the MACE mutation had mean carboxylesterase

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Table 2. Tests for Hardy-Weinberg equilibrium of knockdown resistance mutations in field samples of *Myzus persicae*. The frequency of each resistant genotype is also shown.

Country	Year	Location	Crop	*n*	Knockdown resistance genotype					F	
					SSSS	SRSS	RRSS	SRSR	RRRR	*kdr*	*super-kdr*
UK	2001	Dundee	Mixed	99	0.52	0.12	0.01	0.35	0	−0.275**	−0.215**
Greece	2002	Karditsa	Tobacco	19	0.37	0.32	0	0.32	0	−0.462*	−0.188
Greece	2002	Meliki	Tobacco	20	0.2	0.35	0	0.45	0	−0.637**	−0.286
Italy	2002	Caserta	Peach	15	0	0.4	0	0.6	0	−1.000***	−0.429*
Greece	2002	Lehonia	Peach	20	0.1	0.2	0.1	0.6	0	−0.653**	−0.546**
Greece	2002	Meliki	Peach	19	0.11	0.37	0.11	0.42	0	−0.478*	−0.226
France	2002	Allonnes	Potato	13	0.08	0.92	0	0	0	−0.868***	–
Germany	2002	Ruhlsdorf	Potato	27	0.67	0.33	0	0	0	−0.238	–
Scotland	2002	Arbroath	Brassica	12	0	1	0	0	0	−1.000***	–
Germany	2002	Ahlum	OSR	47	0.64	0.36	0	0	0	−0.221*	–
UK traps	2002		Trap	53	0.26	0.48	0.13	0.12	0	−0.280*	−0.059
France	2003	Nimes	Peach	115	0.16	0.16	0.03	0.64	0.01	−0.621***	−0.454***
England	2003	Wakefield	Sprouts	50	0.04	0.82	0	0.14	0	−0.923***	−0.075
England	2003	Wilmington	Cabbage	24	0.60	0.40	0	0	0	−0.243	–
UK traps	2003		Trap	154	0.49	0.37	0.03	0.09	0.01	−0.196**	0.123

A negative value for F represents an excess of heterozygotes * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

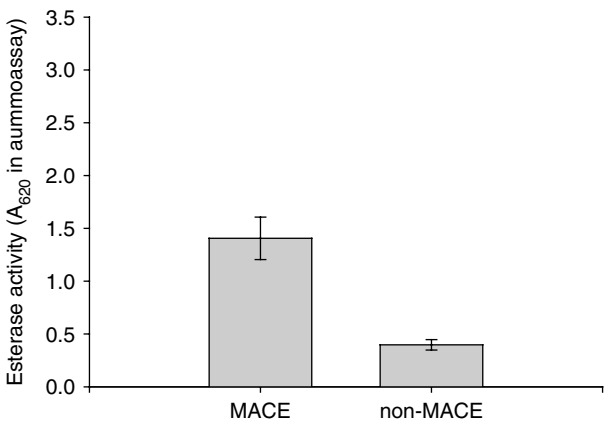

Fig. 6. The relationship between carboxylesterase level and the presence of the MACE mutation in samples of *Myzus persicae*. Error bars indicate 95% confidence of the mean (MACE, $n = 129$; non-MACE, $n = 161$).

levels significantly higher (equivalent to R2) than those without it (equivalent to R1) ($t = 9.7$, $P < 0.001$).

Mean carboxylesterase levels for *M. persicae* collected in 2002 and 2003 with different *kdr* genotypes are shown in fig. 7. Based on ANOVA, SSSS, SRSS and RRSS, aphids had significantly lower carboxylesterase levels than SRSR. SSSS and SRSS aphids had significantly lower carboxylesterase levels than RRSS aphids. There was no significant difference in carboxylesterase levels between SSSS and SRSS aphids. SRSR aphids had a mean carboxylesterase activity equivalent to R3, RRSS to R2 and SSSS/SRSS to R1.

In samples for which data were available on *kdr*, *super-kdr* and MACE showed that the *kdr* and *super-kdr* mutations were in very strong linkage disequilibrium, as expected because of the very tight linkage between these two sites in the same gene (table 3). There was also significant linkage disequilibrium between MACE and *kdr* and MACE and *super-kdr* in the UK, but no significant linkage disequilibrium between MACE and *kdr* in Germany (where *super-kdr* was absent). In Greece and Italy, there was no significant linkage disequilibrium between MACE and *kdr* or *super-kdr*.

Discussion

The availability of a high throughput method for screening knockdown resistance genotypes in *M. persicae* has enabled the most comprehensive analysis to date of the incidence of alleles responsible for this mechanism. The inclusion of suction trap samples provides an interesting contrast to those from field crops, which are more likely to reflect localized events including insecticide treatment regimes. Research has shown that populations caught in suction traps are representative of a large area (Taylor, 1979). Observations of *M. persicae* abundance from individual traps were found to be correlated over distances up to approximately 700 km (Cocu *et al.*, 2005), indicating the population sampled should be representative of the population as a whole over these distances.

Host-alternating aphids, such as *M. persicae*, typically undergo two migratory events during a season, one in spring as aphids move from their primary to their secondary host and one in the autumn when they return (Taylor, 1979; Weisser, 2000). This pattern was seen in *M. persicae* caught in 2002 and 2003. However, in both years a single trap (Starcross in 2002 and Wye in 2003) showed a peak in July that was later than expected for a spring migration event. This peak was likely to have been due to the production of alates on secondary hosts. Aphid alates on secondary hosts are normally produced in response to over-crowding or reduced host-quality (Muller *et al.*, 2001).

The *kdr* mutation (L1014F) was present in all the countries sampled with the exception of Zimbabwe. L1014F has now been found in *M. persicae* from every continent where this species is known to occur. Elevated carboxylesterases were also common in all the countries sampled, in some cases to the exclusion of susceptible aphids. MACE and *super-kdr* were both found throughout Europe but were not found in samples from Australia and Zimbabwe. However, the Zimbabwean sample was a small one from a single field, so

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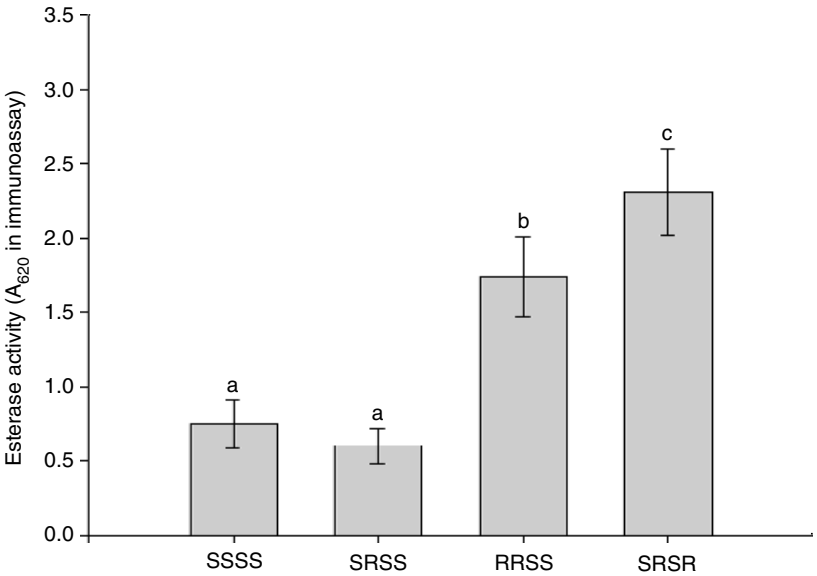

Fig. 7. The relationship between *kdr* genotype and carboxylesterase levels in *Myzus persicae*, error bars indicate 95% confidence of the mean (SSSS, $n = 86$; SRSS, $n = 148$; RRSS, $n = 8$; SRSR, $n = 44$). Bars denoted by different letters differ significantly in mean carboxylesterase levels based on ANOVA ($P < 0.05$, 3 df, LSD = 0.4, $F < 0.001$).

Table 3. Correlation coefficients for linkage target-site resistance mutations in *Myzus Persicae*.

Country	*n*	*Kdr/super-kdr* R	*Kdr*/MACE R	*Super-kdr*/MACE R
UK 2001	99	+0.81***	+0.46***	+0.41***
UK 2003	146	+0.21*	+0.41**	+0.13*
Germany	83	–	+0.40	–
Greece	71	+0.64***	−0.10	−0.11
Italy	15	–	–	+0.39

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

no conclusions can be drawn about the status of *kdr*, *super-kdr* or MACE from this country. The samples from Australia were collected from a number of different sites and hosts and represented the most common clones found in the state of Victoria (Vorburger *et al*., 2003). Some of these samples had also been subject to intense selection with pyrethroids and carbamates. If the *super-kdr* mutation and/or MACE were present in Victoria, it should have been present in these samples.

There was a strong contrast in the temporal patterns of *kdr* and *super-kdr* mutations in the UK suction trap catches. The *kdr* mutation was present throughout the trapping season, whereas the *super-kdr* mutation appeared only in samples from August and September 2002 and from June and September 2003. Numbers tested were too low to disclose clear trends in the occurrence of this mutation, but a plausible explanation is that numbers build up under selection by pyrethroids but then decline in the absence of insecticide pressure (although this was not directly tested). This would imply that *super-kdr* in *M. persicae* confers a greater fitness cost than *kdr* in the absence of insecticide selection. Various side-effects of resistance on the biology of *M. persicae* have been postulated or demonstrated to influence fitness, including reduced overwintering success and varying response to environmental cues (Foster *et al*., 2003a,b).

Both the *kdr* and *super-kdr* mutations were in heterozygous excess; and in most cases, deviation from Hardy-Weinberg expectation was significant. This heterozygous excess is most likely to indicate selection against individuals homozygous for *kdr* and *super-kdr*. Homozygous susceptible *M. persicae* are most likely selected against by the application of insecticides as *kdr* gives some resistance to pyrethroids even when present as a heterozygote in *M. persicae* (Foster *et al*., 2002). Individuals heterozygous at both mutations are highly resistant and unaffected by high doses of pyrethroids (Eleftherianos *et al*., 2002). Homozygous resistant individuals are rare for *kdr* and very rare for *super-kdr*. This is almost certainly due to fitness costs associated with resistance, either as a direct result of the resistance or because deleterious recessive mutations are associated with the resistance mutation. There are known fitness costs associated with the *kdr* mutation in *M. persicae*. The presence of *kdr* is associated with a decreased response to alarm pheromone, which could render them more vulnerable to predation or parasitism (Foster *et al*., 2003c). Both susceptible and heterozygous individuals show a significantly decreased response when compared to homozygous resistant individuals, and susceptible individuals show a significantly decreased response when compared to heterozygous individuals. Heterozygote deficit in microsatellites seems to be the general rule in *M. persicae* populations studied to date (Wilson *et al*., 2002; Fenton *et al*., 2003; Guillemaud *et al*., 2003b). Homozygous excess has also been found in French *Sitobion avenae* (Fabricius) populations (Simon *et al*., 1999) although heterozygous excess has been found in *Rhopalosiphum padi* (Linnaeus) (Delmotte *et al*., 2002) and various *Sitobion* species (Sunnucks *et al*., 1996; Wilson *et al*., 1999). As these loci are in non-coding regions, selection probably would not have caused these deviations. Asexuality coupled

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with strong clonal selection, so that a few clones are highly over-represented, is instead likely to cause deviations from Hardy-Weinberg equilibrium for all polymorphic loci (including microsatellites) via hitch-hiking. This effect could result in heterozygote deficit or excess and would be expected to differ strongly among loci. The data for microsatellites generally shows this pattern (e.g. Zamoum *et al*., 2005). Therefore, while the strong heterozygote excesses at *kdr* and *super-kdr* are consistent with selection against homozygotes, they may also be caused by over-representation of certain clones that just happen to be heterozygous at resistance loci. Nonetheless, because of the consistent strong heterozygote deficit across all populations, including known sexual populations, selection is the most likely explanation.

During the sampling, only some of the possible genotypic combinations of the two mutations conferring *kdr* resistance were detected. The *super-kdr* mutation was only ever found in conjunction with *kdr*. The most likely explanations for this are either that *super-kdr* arose in one or more alleles already containing the *kdr* mutation (Anstead *et al*., 2005) and little or no recombination has occurred between the mutations, or that the presence of the *super-kdr* mutation alone is highly disadvantageous (see Morin *et al*., 2002). In either case, only three alleles would be expected to predominate: fully susceptible, resistant at *kdr* but susceptible at *super-kdr*, and resistant at both sites. These three alleles form six possible genotypes, of which only five were found. No individuals were found that were homozygous resistant for *kdr* and heterozygous for *super-kdr* (RRSR). The likely frequency, assuming Hardy-Weinberg equilibrium, of this genotype can be calculated from the *kdr*/*super-kdr* gamete frequencies (*kdr* resistant, *super-kdr* susceptible = 0.206; *kdr* resistant, *super-kdr* resistant = 0.127) from all sites combined. This means approximately 5% of the individuals ($0.206 * 0.127 * 2 = 0.0522$) should be RRSR. One problem with determining if the absence of this genotype is significant is that there is no information available on the clonal diversity within these samples. However, the relationship between the number of individuals and the number of clonal lineages has been explored in other studies. If these samples follow the same pattern as those in Fenton *et al*. (1998), for instance, there would be approximately 200 separate clonal lineages. The chance of failing to detect one RRSR individual from 200 randomly selected clonal lineages, if they were present at 5% of the population, is $(1-0.05)^{200}$ which is < 0.005.

As expected, there was strong linkage disequilibrium shown between *kdr* and *super-kdr*. These are two mutations of the same gene and, as such, are strongly physically linked. It is also not surprising to see an association between the levels of carboxylesterase and the *kdr* and *super-kdr* resistance mutations since both give resistance to pyrethroids. Elevated levels of carboxylesterase sequester and detoxify the pyrethroid, and the *kdr* and *super-kdr* reduce the sensitivity of the voltage-gated sodium channel (Devonshire *et al*., 1998). Both should be selected for by insecticide pressure and, in asexual populations, would be likely to stay associated. The association between esterase and MACE is less pronounced. MACE aphids have an average R2 esterase level and non-MACE have R1. The association between MACE and esterase and its state of linkage disequilibrium with the two sodium channel mutations is likely to be related to asexual reproduction. If a population of asexually reproducing, clones are sprayed with a pyrethroid and then a carbamate; only those with both sets of resistance will survive leading to linkage disequilibrium within the population. Some popular insecticides are mixtures of carbamates and pyrethroids and select for mechanisms simultaneously. In southern Europe, where *M. persicae* can reproduce sexually, the linkage between MACE and *kdr*/*super-kdr* breaks down, presumably as a consequence of recombination.

In summary, insecticide resistance remains a large problem in *M. persicae*, with all three mechanisms widespread throughout Europe. This is likely to present control difficulties, especially where all three resistance mechanisms are present in individual aphids. This was shown to have been the case in Scotland, where two clones possessing all three resistance mechanisms were responsible for a number of insecticide control failures in 2001 (Fenton *et al*., 2005). A recent study in northern France also showed the presence of a predominating microsatellite genotype, which combined the *kdr* and elevated esterase mechanisms; although in this case, it did not have the MACE mutation and its *super-kdr* status was unknown (Zamoum *et al*., 2005). However, the apparent selective disadvantage for *kdr*, as a homozygote, and to *super-kdr*, in any form in the absence of insecticide pressure, should reduce the frequency of resistance between seasons. More work is needed on this subject. The *super-kdr* mutation, in particular, seems to be associated with a high fitness cost in the absence of insecticide selection. As yet it is not clear if this fitness cost is caused by the mutation itself or associated deleterious mutations. If it is caused by associated mutations, we might expect this cost to diminish over time as sexual recombination 'frees' it from these mutations, and this could lead to a major outbreak of highly resistant strains.

One weakness of studies utilizing suction trap material is that it is almost impossible to relate samples to insecticide application data, as suction traps are representative of populations over very large heterogeneous areas. This reveals a need for detailed field studies relating the frequency of resistant genotypes over time to the application of insecticides at the field level. This kind of study could answer questions about the fitness of different resistance genotypes in the presence and absence of insecticide applications and under different abiotic conditions.

There is also intriguing interaction between selection on the one hand and life-cycle on the other. Sexual reproduction produces a large number of different genotypes, both for insecticide resistance and other genes (e.g. for adaptation to different climatic conditions). Asexual reproduction then fixes these combinations temporarily, and the most successful clones increase massively. This combination of a single sexual cycle followed by numerous asexual cycles is very successful in adaptation to the sudden strong selection pressures of insecticide application. The sexual stage ensures the production of new combinations of genes, e.g. a clone with three resistance mechanisms, and the asexual generations ensure the most successful combinations are maintained.

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