Does insecticide resistance alone account for the low genetic variability of asexually reproducing populations of the peach-potato aphid *Myzus persicae*?

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The typical life cycle of aphids includes several parthenogenetic generations and a single sexual generation (cyclical parthenogenesis), but some species or populations are totally asexual (obligate parthenogenesis). Genetic variability is generally low in these asexually reproducing populations, that is, few genotypes are spread over large geographic areas. Both genetic drift and natural selection are often invoked to account for this low genetic variability. The peach-potato aphid, *Myzus persicae*, which encompasses both cyclical and obligate parthenogens, has developed several insecticide resistance mechanisms as a consequence of intense insecticide use since the 1950s. We collected asexually reproducing *M. persicae* from oilseed rape and examined genetic variability at eight microsatellite loci and three insecticide resistance genes to determine whether their

genetic structure was driven by drift and/or selection. We identified only 16 multilocus microsatellite genotypes among 255 individuals. One clone, which combined two insecticide resistance mechanisms, was frequently detected in all populations whatever their location over a large geographical area (the northern half of France). These unexpected findings suggest that drift is not the unique cause of this low variability. Instead, the intensification of both insecticide treatments and oilseed rape cultivation may have favored a few genotypes. Thus, we propose that selective pressures resulting from human activities have considerably modified the genetic structure of *M. persicae* populations in northern France in a relatively short period of time.

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Introduction

Like many aphid species, the peach-potato aphid *Myzus persicae* shows an extreme variation in reproductive modes, from cyclical to obligate parthenogenesis (Blackman, 1972; Vorburger *et al.*, 2003). The annual life cycle of *M. persicae* includes cyclical parthenogenesis for several generations on herbaceous plants and one sexual generation on peach trees during the winter. By contrast, obligate parthenogenetic aphids have lost the ability to reproduce sexually and remain on herbaceous plants all year long, although they still retain the ability to produce some males (Blackman, 1972).

By using hypervariable microsatellite markers or ITS fingerprints, several studies have shown that asexually reproducing populations of *M. persicae* sampled on herbaceous plants display a low genotypic variability, both within and among populations at a regional scale (Fenton *et al.*, 1998; Terradot *et al.*, 1999; Guillemaud *et al.*,

2003b; Vorburger *et al.*, 2003). These analyses of European and Australian populations also revealed that some clonal genotypes could be repeatedly found in geographically distant (more than 100 km apart) populations.

What could be the causes of this low genetic variability? Does it result from selective factors favoring a few genotypes or from stochastic factors randomly acting on all genotypes during the asexual phase of the life cycle. Both drift and directional selection, in combination with clonal reproduction, could explain the coexistence of the limited number of clonal genotypes within populations (Balloux *et al.*, 2003; Bengtsson, 2003). However, the low variability observed at a larger scale (within and among populations) is probably not the result of drift alone, because, being a random process, it would result in different genotypes becoming frequent in different populations (Wright, 1969; Bengtsson, 2003). Recent anthropogenic selection, such as insecticide treatment, is likely to explain the low genetic variability observed at a large scale. In insects, pesticide resistance has often evolved from the recent appearance and spread of single (or a few) adaptive genes, which are selected for over large geographical areas (e.g. Guillemaud *et al.*, 1996; Daborn *et al.*, 2002).

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M. persicae has evolved at least four insecticide resistance mechanisms in the field: (1) The overproduction of detoxifying esterases, either E4 or FE4, and conferring resistance to organophosphates (OP) and pyrethroids. (2) The modification of the acetylcholinesterase (AChE), the target of OPs and carbamates, providing resistance against dimethyl carbamate insecticides (Moore and Devonshire, 2000). (3) The *kdr* mutation of the voltage-gated sodium channel (Martinez-Torres *et al.*, 1999), which is the target of pyrethroids. (4) The *rdl* mutation of the GABA receptor (Anthony *et al.*, 1998), which is the target of cyclodiene insecticides.

The recent acquisition of insecticide resistance genes selected for in large geographical areas, combined with the absence or rarity of sexual recombination, could have led to the low genetic variability observed in *M. persicae* at a wide geographical scale. The comparison of populations from a large geographic area can be used to test this last hypothesis. Moreover, the analysis of the genetic variability of the putatively selected genes would directly address the selective hypothesis.

We adopted this approach by studying the genetic variability of French populations of *M. persicae* collected on oilseed rape (*Brassica napus*, known as canola in the USA) using eight microsatellite markers and three insecticide resistance genes.

Materials and methods

Aphid samples

A total of 22 batches of 13–54 wingless parthenogenetic females of *M. persicae* were collected from oilseed rape in France between 19 October and 16 November 2001 (see Table 1 and Figure 1). The sampling period corresponds to the beginning of the oilseed rape cropping season in France. Only one aphid per plant was collected to avoid repeated sampling of aphids from the same colony, and

Figure 1 Geographic locations of the *M. persicae* sampling sites. Boxed names are sites from which samples were used in the microsatellite analysis. When more than one field was sampled per locality, the first number in brackets corresponds to the number of fields sampled and the second number refers to the number of samples that were used in the microsatellite study.

between 13 and 54 plants were randomly chosen in each field. The aphids were stored at -80°C until analysis. In three locations, samples were collected in October 2001 from both insecticide-treated and untreated fields. In each case, the insecticide was alphamethrin, a pyrethroid insecticide (see Table 2 for details).

Six reference clones described in Guillemaud *et al.* (2003a) were also analyzed: SUS, an insecticide-susceptible clone derived from US1L (Devonshire and Sawicki, 1979); COL and LYON, R2 and R3 clones, respectively, which both overproduce the FE4 esterase (aphids are classified as either S (susceptible), R1, R2 or R3, depending on the levels of esterase overproduction and resistance (Devonshire and Field, 1991)), and are both *kdr/kds* and *Ace.S/Ace.S* (see below for resistance mechanism determination); AV, an R3-FE4, *kdr/kdr* and *Ace.S/Ace.S* clone; BIA, an R3-FE4, *kds/kds* and *Ace.R/Ace.S* clone; and Mp37/6, an R2-FE4, *kdr/kdr* and *Ace.R/Ace.R* clone.

Resistance gene characterization

For each of the 625 aphids sampled, three insecticide resistance mechanisms were analyzed: the class of esterase overproduction and the corresponding esterase allele, the presence of the *kdr* mutation and the modified AChE. The *rdl* mutation was not considered because cyclodiene insecticides are not commonly used against aphids in Europe.

Single aphids were used both for DNA analysis and biochemical assays as described in Guillemaud *et al.* (2003a). 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 (Sunnucks and Hales, 1996). 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 until biochemical analysis.

Table 1 Characteristics of the samples of *Myzus persicae*

Pop	Location	Date	Longitude	Latitude	n
Pa	Payns	16/11/01	03°59'	48°23'	13
Ba	Bazanville	07/11/01	−00°35'	49°18'	33
Su_1	Surgère	19/10/01	−00°45'	46°07'	39
Su_2	Surgère	22/10/01	−00°45'		36
Su_3	Surgère	22/10/01	−00°45'		60
Su_4	Surgère	24/10/01	−00°45'		30
Su_5	Surgère	24/10/01	−00°45'		18
Su_6	Surgère	25/10/01	−00°45'		28
Su_7	Surgère	25/10/01	−00°45'		21
Su_8	Surgère	25/10/01	−00°45'		13
Su_9	Surgère	30/10/01	−00°45'		25
Ch_1	Chaux	29/10/01	04°54'	47°08'	34
Ch_2	Chaux	29/10/01	04°54'		30
Ch_3	Chaux	29/10/01	04°54'		25
Ch_4	Chaux	29/10/01	04°54'		19
Co	Comblanchien	11/11/01	04°55'	47°06'	35
Fr	Franxault	11/11/01	05°17'	47°03'	29
Le	Lesdains	24/10/01	03°16'	50°06'	23
SH	St Hilaire	24/10/01	03°25'	50°11'	19
SG_1	St Germain/Eaulnes	07/11/01	01°30'	49°46'	21
SG_2	St Germain/Eaulnes	07/11/01	01°30'		27
Gr	Grignon	23/10/01	01°55'	48°51'	47

Pop is the name of the sample, and *n* is the number of sampled aphids. See text for details.

Table 2 Insecticide resistance phenotype (esterase) and genotypic frequencies (to two decimal places) in samples of *M. persicae* in 2000

Pop	T	kdr				Esterase overproduction				Combination of esterase alleles					AChE			
		n	ss	rs	rr	n	ss	R1-R2	R3	n	E4	FE4	E4/FE4	s	n	ss	rs	rr
Pa		13	0	1	0	12	0	0.83	0.17	13	0	1	0	0	13	1	0	0
Ba		33	0.06	0.94	0	33	0	0.58	0.42	31	0.06	0.94	0	0	33	0.97	0.03	0
Su_1		39	0	1	0	39	0	0.97	0.03	37	0	1	0	0	39	1	0	0
Su_2		35	0.29	0.66	0.06	36	0	1	0	36	0.03	0.97	0	0	36	0.97	0.03	0
Su_3		60	0	0.95	0.05	30	0	0.93	0.07	57	0	1	0	0	60	0.97	0.03	0
Su_4		30	0	1	0	29	0	0.97	0.03	30	0	1	0	0	28	1	0	0
Su_5		18	0.11	0.89	0	18	0	1	0	18	0	1	0	0	18	1	0	0
Su_6		27	0	0.96	0.04	28	0	0.71	0.29	28	0	1	0	0	27	1	0	0
Su_7		20	0.05	0.95	0	21	0	0.81	0.19	21	0.05	0.95	0	0	21	1	0	0
Su_8		10	0	1	0	13	0	1	0	10	0	0.9	0	0.1	13	1	0	0
Su_9		22	0.23	0.73	0.05	25	0	0.76	0.24	20	0.05	0.95	0	0	25	1	0	0
Co		35	0.20	0.80	0	35	0	0.63	0.37	35	0.03	0.97	0	0	34	1	0	0
Fr		26	0.08	0.92	0	29	0	0.52	0.48	27	0	1	0	0	29	1	0	0
Le	nt	22	0.18	0.73	0.09	23	0	0.96	0.04	20	0	1	0	0	23	0.91	0.09	0
SH	t	18	0.11	0.89	0	19	0	0.26	0.74	19	0	1	0	0	19	0.89	0.11	0
Ch_1	t	34	0	0.97	0.03	32	0	1	0	33	0	1	0	0	34	1	0	0
Ch_2	nt	30	0.03	0.93	0.03	30	0	1	0	30	0	1	0	0	29	1	0	0
Ch_3	nt	22	0	0.91	0.09	25	0	0.88	0.12	25	0	1	0	0	25	1	0	0
Ch_4	t	18	0	1	0	19	0	1	0	19	0	1	0	0	19	1	0	0
SG_1	nt	20	0.1	0.9	0	0	—	—	—	21	0.05	0.8	0.1	0.05	21	1	0	0
SG_2	t	23	0.39	0.61	0	24	0	1	0	23	0.3	0.7	0	0	25	1	0	0
Gr		47	0.11	0.89	0	47	0	1	0	46	0.09	0.91	0	0	47	1	0	0
Total		602	0.09	0.89	0.02	567	0	0.85	0.15	599	0.03	0.97	0	0	618	0.99	0.01	0

For each resistance mechanism, the number of individuals analyzed, *n*, is given. The column 'T' indicates if the fields were treated (t) or not (nt) with pyrethroid insecticides before sampling.

Esterase overproduction: The presence of the E4 and FE4 alleles at esterase genes in individual aphids was determined using a modified version (Guillemaud *et al.*, 2003a) of the PCR-RFLP method described by Field *et al.* (1996). Genotypes could not be determined using this method because the esterase loci can be located at several different chromosomal sites (Blackman *et al.*, 1995).

The level of E4 and/or FE4 esterase overproduction was estimated for each aphid by using the microplate total-esterase assay described by Devonshire *et al.* (1992). This method can distinguish between three classes of aphids: susceptible, R1/R2 and R3 (Devonshire and Field, 1991).

Ace genotype determination: A fluorometric method was used to determine the genotype at the AChE locus, *Ace* (Moore and Devonshire, 2000; Guillemaud *et al.*, 2003a). Only the two alleles described by Moore *et al.* (1994) were considered: *Ace.S* (the allele encoding the susceptible AChE) and *Ace.R* (the allele encoding the modified, resistant AChE). Each individual was classified as either *Ace.S/Ace.S*, *Ace.R/Ace.S* or *Ace.R/Ace.R*.

Kdr determination: Pyrethroid resistance is correlated with the presence of the *kdr* mutation, that is, the presence of a T (*kdr* allele) instead of C (*kds* allele) at position 322 of the partial cDNA sequence of the *para*-like gene of *M. persicae* (Martinez-Torres *et al.*, 1999). A PCR amplification of specific alleles (PASA) procedure (Sommer *et al.*, 1992) was used to determine individual genotypes (*kds/kds*, *kdr/kds* and *kdr/kdr*) at this position as described by Guillemaud *et al.* (2003a). In the

following, the *para*-like gene will be referred to as *kdr* gene.

Microsatellite analysis

Nine of the 22 samples were subjected to microsatellite analysis to investigate the genotypic variability of *M. persicae* collected on oilseed rape over the geographic range considered for the insecticide resistance genes study: Le, Ba, Co, Fr, Pa, Su_1, Su_2, Su_3 and SG_2 (see Figure 1). Each aphid was genotyped using eight microsatellite loci: five (Myz25, M37, M40, M55 and M62) were isolated from *M. persicae* and three (S16b, S17b and S23) from *Sitobion miscanthi* (Takahashi) (Wilson *et al.*, 2004).

The microsatellites were amplified according to Sloane *et al.* (2001) using the PMS1 PCR program. Microsatellite products were then loaded onto an 8% nondenaturing polyacrylamide gel and visualized by ethidium bromide staining as described by Vanlerberghe-Masutti *et al.* (1999). Each individual was described by its multilocus genotype (MLG): the allelic combination at the eight microsatellite loci. The observed MLGs were then carefully scored by using an automatic sequencer (ABI Prism 3100, Applied Biosystem) and reference DNAs from *P. Sunnucks* (La Trobe University, Australia).

Analysis of genetic data

Genetic variability: Allele frequencies, number of alleles and heterozygosity were calculated using the GENEPOP (ver. 3.3) software (Raymond and Rousset, 1995b). Hardy-Weinberg (H-W) equilibrium and linkage equilibrium are not expected if the samples display a clonal structure because of repeated parthenogenetic generations. We therefore tested for these equilibria to

evaluate the relative importance of the successive parthenogenetic generations and of the putative sexual reproduction in shaping the population genetic structure. Linkage disequilibrium (LD) between loci within each population and departure from H-W equilibrium at each locus were tested using the pseudo-exact test procedures in GENEPOP 3.3. Note that the LD test does not assume HW equilibrium. We built a neighbor-joining tree based on allele shared distance (DAS) using Population (version 1.2.23 from <http://www.cnrs-gif.fr/pge>) to investigate relationships between MLGs.

Between-sample analysis: Differences in the frequencies of insecticide resistance mechanisms between populations were analyzed by testing for genic and genotypic differentiation (pseudo-exact test described in Raymond and Rousset, 1995a).

To take into account departures from H-W and linkage equilibria, the following statistical treatments were applied to MLGs. The spatial genetic structure revealed by the microsatellites was analyzed by estimating F_{ST} parameters (Weir and Cockerham, 1984) and by testing for MLG frequencies homogeneity using the Struc program in the GENEPOP 3.3 package. The use of F_{ST} to study a species with a mixture of cyclical and obligate parthenogens may be problematic. Indeed, F_{ST} can be hard to interpret in asexually reproducing populations where it does not necessarily reflect migration/drift equilibrium because of selection at loci which have not been scored. However, we chose this genetic distance parameter to enable comparisons with other published population genetics studies on aphids (eg Delmotte *et al.*, 2002; Guillemaud *et al.*, 2003b). The Isolde program from the GENEPOP package was used to test for an MLG isolation by distance model. This program performs Mantel tests of independence between the matrix of genetic distance and that of geographic distance.

General statistical methods: Most of the statistical genetics analyses described here were performed on the whole data set because (1) we had no *a priori* information about the reproductive mode of *M. persicae* populations (ie cyclical *vs* obligate parthenogenesis) and (2) very few MLGs were found to consider only one individual per MLG (and per population for the geographic structure analysis). The sequential Bonferroni correction was applied for nonorthogonal tests, that is, pairwise differentiation and LD tests (Sokal and Rohlf, 1995, pp 241–242). In each sample, this correction was also applied to the several (one per locus) H-W equilibrium tests performed (Sokal and Rohlf, 1995, pp 241–242). Overall significance of several independent tests was calculated using Fisher's method (Sokal and Rohlf, 1995, pp 794–797).

Results

Frequency of insecticide resistance genes in the field

The frequencies of the insecticide resistance phenotypes and genotypes are given in Table 2. A significant association was detected between genotypes at the *kdr* gene and esterase alleles across all populations (combination of the probabilities by the Fisher's method, $P < 0.001$) and within Gr and SG samples ($P < 10^{-4}$,

$SE < 10^{-4}$ for each test). This corresponded to an association between the E4 elevated esterase and the *kds/kds* genotype.

The FE4 allele was present in more than 95% of the aphids sampled from all sites. On the contrary, the allele encoding the E4 esterase was very rare (about 3% of the aphids). Two individuals displayed both overproduced esterase alleles and two aphids had alleles that did not correspond to either E4 or FE4 according to the PCR-RFLP method. These two aphids were classified as R1/R2. About 85% of the individuals were classified as R1/R2 and 15% as R3.

Unexpectedly, the modified acetylcholinesterase described by Moores *et al.* (1994) was absent from most samples. Only eight aphids were found to be heterozygous for this insensitive AChE. In the polymorphic samples and when the tests could be performed, H-W equilibrium was not rejected ($P > 0.05$ in each case).

Among the 625 aphids analyzed, 23 could not be genotyped for the *kdr* mutation by the PASA method because no PCR products were obtained. In all, 89% of the individuals were *kdr/kds* and 2% were *kdr/kdr*. Most samples (except Le, Su_2, Su_9 and SG_2) displayed significant heterozygous excess, with F_{IS} estimates ranging from -1 to -0.37 .

When considering the three different resistance mechanisms, 13 classes of aphids were found. One type was particularly frequent: 76% of the aphids were *kdr/kds*, *Ace.S/Ace.S*, R1-R2 and had the elevated esterase FE4. In all, 12% were *kdr/kds*, *Ace.S/Ace.S*, R3 and FE4. All other types of aphids were found at a low frequency. In total, 90% of the aphids analyzed had both the *kdr* mutation and an elevated esterase.

Resistance–gene differentiation between populations

Differentiation between populations was tested using the genetic information obtained from insecticide resistance genes. Significant differences in the frequencies of esterase overproduction classes and of *kdr* genotypes were found among populations ($P < 10^{-5}$ for both resistance mechanisms). We then compared samples treated (SH, Ch_1, Ch_4 and SG_2) or not (Le, Ch_2, Ch_3 and SG_1) with pyrethroid insecticides. Significant differentiation was found in the frequency of esterase overproduction categories between the treated SH sample and the three untreated samples for which data were available (Le, Ch_2 and Ch_3, $P < 2 \times 10^{-4}$ for each comparison). In each case, the insecticide-treated SH sample contained more R3 and less R1/R2 individuals than the samples from the untreated fields (see Table 2). Genotypic differentiation at the *kdr* gene was also found between the treated SG_2 sample and the untreated populations Ch_2 and Ch_3 ($P = 1.5 \times 10^{-3}$, $SE = 4 \times 10^{-4}$; and $P = 4 \times 10^{-4}$, $SE = 10^{-4}$, respectively). However, the treated sample contained more susceptible *kds/kds* individuals (39%) than the untreated ones (less than 3%, see Table 2). When considering only groups of populations that were treated or untreated and that were sampled from the same location (SG_1 and SG_2, Le and SH and the four Ch samples), no differentiation was found for any of the resistance mechanisms except for the esterase overproduction class between Le and SH.

Within-sample ‘neutral’ genetic diversity

Samples from nine fields (Su_1, Su_2, Su_3, Le, SG_2, Ba, Co, Fr and Pa) were genotyped at the eight microsatellite loci. The genetic markers proved to be polymorphic, with 2–8 alleles per locus. The genotypic diversity was globally low, with three (loci S23 and S16b) to eight (loci Myz25, M37, M40 and M62) genotypes per locus.

A total of 21 pairs of loci out of 28 showed significant LD over all populations ($P < 10^{-4}$ for each significant comparison). The H–W equilibrium was often rejected (Table 4), frequently due to heterozygote excess (ie negative F_{IS} estimates).

Only 16 MLGs were detected among the 255 individuals (Table 3). One frequent MLG, referred to as G1 hereafter, was found in 86% of the aphids. The other genotypes were rare and the most frequent of them had a frequency of only 4.6%. Most of these rare MLGs corresponded to single-copy MLGs (0.4% in Table 3). It is noteworthy that the occurrence of heterozygote deficiency or excess at a locus depends on whether G1 was heterozygote or homozygote at the locus considered. The neighbor-joining tree based on DAS distances (Figure 2) revealed that, with the exception of G14, which differs from G1 by only two alleles, none of the detected MLGs were closely related to G1, as at least five alleles differentiated G1 from the other genotypes.

When considering only one individual per MLG, the H–W equilibrium was rejected for loci Myz25, M55 and M62 ($P < 0.015$, $SE < 0.02$), with F_{IS} estimates ranging from 0.17 to 0.69 for significant deviations. No significant LD was detected within these 16 individuals ($P > 0.05$, $SE < 10^{-2}$ for each pair of loci).

‘Neutral’ genetic differentiation between samples

The spatial genetic structure was analyzed by testing the homogeneity of MLG frequencies between samples at various spatial scales. An indication of the genetic differentiation between samples or groups of samples was given by F_{ST} estimates.

Samples of eastern France (Co, Fr and Pa) showed no significant MLG differentiation ($P > 0.05$; $F_{ST} = 0.008$), and were therefore lumped together in the following analyses of spatial structure. Among western samples, a significant and moderate differentiation ($P < 10^{-4}$, $SE < 10^{-4}$, $F_{ST} = 0.04$) was found and was due to the Su2 sample (Table 5) in which 22% of the aphids displayed the G2 MLG (Table 3). The three samples in northern France were significantly heterogeneous overall

($P < 10^{-4}$, $SE < 10^{-4}$, $F_{ST} = 0.05$), and in each pair-wise comparison (Table 5).

The seven remaining samples or group of samples were significantly but moderately heterogeneous for their MLG composition ($P < 10^{-5}$, $SE < 10^{-5}$; $F_{ST} = 0.032$). The same differentiation pattern was obtained when considering the frequency of G1 only (not shown). After correction for nonorthogonal tests, no differentiation was found between eastern and western samples (Table 5). No pattern of isolation by distance was observed between the samples, that is, no significant correlation was found between the matrices of genetic distance (F_{ST} , $F_{ST}/(1-F_{ST})$ or DAS) and of log geographic distance (Mantel test with 10 000 permutations, $P > 0.05$; see Rousset (2001) for details).

Resistance genes and microsatellite combinations

We next looked at the correspondence between the microsatellite genotypes and the resistance genes (Table 6). Esterase overproduction phenotypes were not

Figure 2 Neighbor-joining tree of the 16 multilocus microsatellite genotypes detected in *M. persicae* sampled from oilseed rape fields of northern France based on the allele shared distance. With eight loci, a ‘one allele’ distance is $1/16 = 0.0625$.

Table 3 Distribution of the microsatellite multilocus genotypes (in % to one decimal place) among the samples of *Myzus persicae* (see Table 5 for details of the genotypes)

Pop	n	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16
Su_1	39	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Su_3	30	96.7	—	—	3.3	—	—	—	—	—	—	—	—	—	—	—	—
Su_2	36	69.4	22.2	—	—	—	—	2.8	2.8	—	2.8	—	—	—	—	—	—
Le	22	72.7	—	—	4.5	4.5	4.5	—	—	4.5	—	—	—	4.5	—	—	4.5
SG_2	24	62.5	—	37.5	—	—	—	—	—	—	—	—	—	—	—	—	—
Ba	33	93.9	—	6.1	—	—	—	—	—	—	—	—	—	—	—	—	—
Co	30	80	10	—	—	—	—	—	—	—	—	3.3	3.3	—	3.3	—	—
Fr	28	96.4	—	—	—	—	—	—	—	—	—	—	—	—	—	3.6	—
Pa	13	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total	255	85.9	4.3	4.3	0.8	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4

Table 4 Within sample variability of *Myzus persicae* inferred from the microsatellite analysis

Sample	n	Myz25			M37			M40			S16b			S17b			S23			M55			M62		
		n _{all}	f	P	n _{all}	f	P	n _{all}	f	P	n _{all}	f	P	n _{all}	f	P	n _{all}	f	P	n _{all}	f	P	n _{all}	f	P
Su_1	39	1	—	—	2	-1	<10 ⁻⁴	1	—	—	1	—	—	1	—	—	1	—	—	2	-1	<10 ⁻⁴	2	-1	<10 ⁻⁴
Su_3	30	3	0.5	0.02	3	-0.94	<10 ⁻⁴	2	0	1	2	0	1	2	0	1	1	—	—	2	-0.93	<10 ⁻⁴	2	-0.93	<10 ⁻⁴
Su_2	36	4	-0.03	0.31	4	-0.63	<10 ⁻⁴	3	-0.11	1	2	-0.13	1	2	-0.11	1	2	0	1	4	-0.55	<10 ⁻⁴	4	0.01	<10 ⁻⁴
Le	23	6	0.51	7.10 ⁻⁴	5	-0.57	4.10 ⁻³	4	0.45	4.10 ⁻³	2	0.34	0.22	3	-0.07	1	1	—	—	3	-0.53	9.10 ⁻³	4	-0.15	<10 ⁻⁴
SG_2	27	3	0.32	<10 ⁻⁴	2	-1	<10 ⁻⁴	2	-0.21	0.55	1	—	—	1	—	—	1	—	—	3	-0.61	<10 ⁻⁴	2	-0.44	0.05
Ba	33	3	0.49	7.10 ⁻⁴	2	-1	<10 ⁻⁴	2	-0.02	1	1	—	—	1	—	—	1	—	—	3	-0.89	<10 ⁻⁴	2	-0.88	<10 ⁻⁴
Co	30	3	0.16	0.07	5	-0.66	<10 ⁻⁴	4	0.82	0.13	2	0.36	0.16	2	-0.06	1	2	0	1	3	-0.56	<10 ⁻⁴	5	-0.40	<10 ⁻⁴
Fr	28	2	0	1	3	-0.93	<10 ⁻⁴	2	0	1	2	0	1	2	0	1	1	—	—	3	-0.93	<10 ⁻⁴	3	-0.80	<10 ⁻⁴
Pa	13	1	—	—	2	-1	8.10 ⁻⁴	1	—	—	1	—	—	1	—	—	1	—	—	2	-1	8.10 ⁻⁴	2	-1	8.10 ⁻⁴

n is the number of aphids analyzed per sample, *n*_{all} is the number of alleles per locus and per sample, *f* is the estimate of Wright's *F*_{IS} parameter and *P* is the *P*-value of the Hardy-Weinberg equilibrium test. Bold type indicates that tests are significant after the sequential Bonferroni correction.

considered because of the possibility of phenotypic variation within genotypes. One category of individual predominated (83.5% of aphids). This group had the G1 genotype, was heterozygous for the *kdr* mutation, homozygous for the susceptible form of the AChE and displayed the overproduced esterase FE4. All the other combinations were very rare, with frequencies ranging from 0.4 to 4.6%. Most of the rare combinations (13 out of 20) corresponded to *kds/kds* aphids, but they accounted for no more than 13% of the total. Apart from the G1 aphids, only four genotypes (and four individuals) had the *kdr* mutation.

No strict associations could be established between the different kinds of markers because (a) no significant linkage could be detected between microsatellites and insecticide resistance markers when only one individual per MLG (20 individuals) was considered, (b) each resistance mechanism was found in different MLGs and (c) some resistance genes showed variation within microsatellite MLGs (Table 6). Indeed, four different insecticide resistance gene associations were found within G1, comprising all *kdr* genotypes and both the *Ace.S/Ace.S* and *Ace.R/Ace.S* genotypes.

Discussion

Putative reproductive modes of *M. persicae* genotypes

It has been shown that cyclical and obligate parthenogenetic populations of *M. persicae* can coexist at large but also at local geographic scales (Vorburger *et al.*, 2003). We have no direct evidence of whether our sampled aphids come from lineages that are capable of sexuality each year (cyclical parthenogens) or not (obligately parthenogens). Here, *M. persicae* individuals were sampled in the mid-autumn on herbaceous hosts while they were reproducing parthenogenetically. Most cyclical parthenogens have already switched back at this time of the year to winter hosts, the peach trees, to accomplish sexual reproduction. It is thus probable that our samples contained partially or entirely asexual genotypes.

The analysis revealed frequent H-W and linkage disequilibria, that a particular MLG was frequent within populations and had a ubiquitous distribution among geographically distant populations. Can sexual reproduction in the preceding winter account for this peculiar genetic structure? A founder event in which a few sexually produced aphids establish in each field, followed by clonal reproduction, might account for the within-population genetic structure. However, when considering the ubiquitous distribution of G1 among population, it would then be necessary to suppose that G1 appeared independently by recombination at least 9 times in nine distant populations.

Wilson *et al.* (2002) and Guillemaud *et al.* (2003b) showed that sexual *M. persicae* aphids harbor a large microsatellite variability suggesting that the likelihood of obtaining many individuals with the same MLG by sexual recombination in a population is minute. However, it is worth noting that selection could notably increase this likelihood by rapidly increasing the frequency of certain clones during the parthenogenetic phase of their life cycle. Alternatively, we could suppose that a single individual with the G1 MLG was produced by sexual reproduction or by mutation from an obli-

Table 5 Analysis of genetic population differentiation in *M. persicae* based on the microsatellite analysis

	Eastern France			Western France			Northern France		
	Co	Fr	Pa	Su_1	Su_3	Su_2	Le	SG_2	Ba
Co	.	0.01	0.01	0.01	0.00	0.03	0.02	0.07	0.00
Fr	0.10	.	0						
Pa	0.41	1	.						
Su_1		0.712	.	.	0.00	0.06	0.05	0.10	0
Su_3		0.677	0.438	0.438	.	0.05	0.03	0.08	0
Su_2		0.004	<10 ⁻³	<10 ⁻³	0.003	.	0	0.07	0.05
Le		0.018	0.001	0.001	0.063	0.007	.	0.05	0.03
SG_2		<10 ⁻³	<10 ⁻³	<10 ⁻³	<10 ⁻³	<10 ⁻³	0.002	.	0.06
Ba		0.340	0.206	0.206	0.358	0.002	0.023	0.006	.

F_{ST} estimators for each pair of populations considered are given above the diagonal. Type I error probabilities of absence of MLG differentiation between populations are given below the diagonal. Bold characters indicated tests that were significant after the Bonferroni correction.

Table 6 Global frequencies of the different types of individuals observed combining the microsatellite data and the insecticide resistance genotypes and phenotypes

Gi	Myz25	M37	M40	S16b	S17b	S23	M62	M55	kdr	AChE	Est. allele	freq
G1	119/119	155/163	125/125	196/196	168/168	115/115	131/135	119/127	rs	ss	FE4	83.5
—									rr	ss	FE4	0.4
—									rs	rs	FE4	1.3
—									ss	ss	FE4	0.4
G2	119/121	157/163	125/135	196/198	158/168	115/115	127/127	121/127	ss	ss	FE4	4.6
G3	121/123	155/163	125/135	196/196	168/168	115/115	131/131	119/125	ss	ss	E4	3.8
G4	121/123	144/163	125/135	196/198	168/170	115/115	131/131	127/127	rs	ss	FE4	0.4
—									rr	ss	FE4	0.4
G5	119/119	157/163	125/127	196/196	168/168	115/115	131/133	121/121	ss	ss	FE4	0.4
G6	119/119	157/163	125/127	196/196	168/168	115/115	131/131	121/121	ss	ss	FE4	0.4
G7	121/123	152/161	131/135	198/198	158/168	115/119	131/137	119/127	ss	ss	E4	0.4
G8	142/142	159/163	114/135	196/198	168/170	115/115	127/127	127/127	ss	ss	FE4	0.4
G9	115/144	163/163	125/125	198/198	168/168	115/115	127/127	127/127	ss	ss	m	0.4
G10	119/144	163/163	125/125	196/198	168/168	115/115	127/127	127/127	ss	ss	FE4	0.4
G11	121/126	154/163	125/129	196/196	168/168	115/117	125/125	137/137	ss	ss	E4	0.4
G12	119/119	157/163	129/129	196/196	168/170	115/115	129/129	127/127	ss	rs	FE4	0.4
G13	121/127	155/157	129/135	196/196	168/168	115/115	127/131	127/127	ss	ss	FE4	0.4
G14	119/119	155/163	125/125	196/196	168/168	115/115	125/125	119/127	rr	ss	FE4	0.4
G15	119/121	163/163	125/135	196/198	164/168	115/115	129/129	119/129	rr	rs	FE4	0.4
G16	119/121	157/163	125/135	196/198	164/168	115/115	127/127	121/127	ss	ss	FE4	0.4

'Gi' are the microsatellite multilocus genotypes (MLGs). The names of the MLGs and their microsatellite genotypes are omitted when they are the same than in the line above. The column 'Est. allele' refers to the alleles of the elevated esterases. 'freq' is the frequency of the type of individual in % to one decimal place. Only the 237 individuals without missing data or with missing data ('m' for G9) that do not influence the classification are shown (out of a total of 255 individuals).

gately parthenogenetic clone. This event could then have been followed by several years of selection and migration between populations without sexual recombination.

Under this hypothesis, G1 aphids should be obligately or partially asexual, that is, each winter at least part of the G1 lineage would be expected to reproduce by continuous parthenogenesis. On the other hand, the 15 rare MLGs detected in oilseed rape fields are not close relatives of G1, suggesting that none of the rare genotypes observed in this study have been recently derived from G1 by mutation. Although continuous parthenogenesis seems most likely to explain the frequency and geographic distribution of G1 individuals, the rare MLGs could have been sexually produced during the preceding winter.

Biological characterization of reproductive modes of various *M. persicae* genotypes would help evaluate the

alternative hypotheses. To this end, the G1 and other genotypes could be subjected to conditions that induce sexual reproduction (ie low temperature and shortened day length).

Insecticide resistance variation within and between *M. persicae* genotypes

We found that genetic variation in insecticide resistance genotypes was present within microsatellite MLGs and that the same insecticide resistance mechanisms were present in different MLGs. We propose several hypotheses that could explain these unexpected results.

- (1) Sexual recombination. The same resistance mutations in different MLGs could be readily explained if those genotypes have been generated by sexual reproduction. Recombination could also lead to a

polymorphism at resistance genes within MLGs provided that multiple individuals with the same microsatellite genotypes were produced sexually. We have already argued that the probability of such outcomes is extremely small but could be higher because of the effects of selection.

- (2) Cryptic meiotic or mitotic recombination. Different combinations of insecticide resistance mechanisms in individuals of the same clonal lineage could be the consequence of recombination localized in regions of the genome containing the insecticide resistance genes. Localized crossing over between homologues, or sister chromatid exchange, has previously been proposed to explain the distribution of rDNA arrays on X chromosomes in *M. persicae* (Hales *et al.*, 2002). A careful analysis of insecticide resistance inheritance within parthenogenetic lines could allow this hypothesis to be tested.
- (3) Polymorphism due to recent mutations. Resistance mutations at insecticide resistance genes could have occurred very recently, resulting in a transient resistance polymorphism within clonal MLGs. During this transition, both susceptible and resistant individuals would coexist within a MLG before the fixation of resistant genotypes. A study of temporal stability over several years is necessary to test this hypothesis. Also, mutations at microsatellite loci may explain the presence of the same insecticide resistance combination among different MLGs. Indeed, with more than 300 generations since the use of pyrethroids and organophosphate insecticides, a large number of mutations at the microsatellite loci could have occurred within insecticide resistant clonal lineages.
- (4) Multiple origins of insecticide resistance. Resistance mutations at each resistance gene could have appeared independently in different clonal MLGs. This could have been achieved by independent origins of the same mutation or by different mutations leading to the same resistant phenotype (homoplasy for the AChE for which the test is phenotypic). Multiple independent origins of the same insecticide resistance mutation within a species have already been reported in several cases (eg Andreev *et al.*, 1999). The analysis of nucleotide variability surrounding the different insecticide resistance mutations should allow discrimination between single and multiple origins of each resistance mechanism.

Selection vs genetic drift

Only 16 microsatellite MLGs were detected among 255 *M. persicae* individuals collected from oilseed rape fields over a large geographic range in France (about 500 km × 500 km). This confirms the tendency for low genotypic variability in parthenogenetic populations of aphids, as observed in previous studies on *M. persicae* (Terradot *et al.*, 1999; Guillemaud *et al.*, 2003b; Vorburger *et al.*, 2003) and on other species (eg Fuller *et al.*, 1999; Simon *et al.*, 1999; Delmotte *et al.*, 2002; Papura *et al.*, 2003).

However, the variability found in our study is far lower than that found in three previous studies that dealt with clonal populations of *M. persicae* collected at the regional scale. Fenton *et al.* (1998) found 80 different

genotypes among 276 aphids collected from potato and brassicas in 1995 in Scotland (in their study, the ratio of the number of genotypes over the number of individuals is $G=0.29$). An analysis of microsatellite diversity in samples collected from four aerial suction traps located in France in 2000 revealed higher variability, with 100 different MLGs among 174 flying individuals ($G=0.57$) (Guillemaud *et al.*, 2003b). In Victoria, Australia Vorburger *et al.* (2003), detected 73 different MLGs among 167 individuals ($G=0.44$) sampled on herbaceous hosts in 2002. We found only one frequent (G1) and 15 rare MLGs ($G=0.06$). G1 was frequent in all the populations analyzed, whatever their location over a large geographic area (northern half of France), which is similar in size to the studies cited above. None of the frequent MLGs that were detected among flying aphids caught in aerial suction traps in France in 2000 (Guillemaud *et al.*, 2003b) nor the frequent p6 MLG observed by Terradot *et al.* (1999) among French and Spanish samples of *M. persicae* were the same as G1.

Founder events initiated by a few aphids followed by clonal reproduction could account for the very low genetic variability within populations. However, the ubiquitous distribution of G1 suggests that the samples considered in our study suffered a large reduction of genetic diversity due to a deterministic rather than a stochastic factor. A substantial migration could also homogenize the genotypic composition of the populations but it could not account for both the high frequency of G1 in oilseed rape fields and its apparent absence on other plants or in suction traps. In other words, we propose that genetic drift (or migration-drift balance) and parthenogenetic reproduction alone cannot account for the low variability observed. Large-scale selection probably accounts for this result.

Does insecticide selection account for the low genetic variability of *M. persicae* populations?

In France, oilseed rape fields are commonly treated with pyrethroid insecticides (Delorme *et al.*, 1999); therefore, insecticide selection could be one of the primary causes of the reduced genetic variability. The combination of an elevated esterase (E4 or FE4) and the *kdr* mutation, even in the heterozygous state, confers very high levels of resistance (Foster and Devonshire, 1999; Martinez-Torres *et al.*, 1999; Foster *et al.*, 2002).

In the samples analyzed here, the *kdr* mutation was frequently associated with elevated esterases (90% of the aphids) and these resistance mechanisms were combined in the most frequent microsatellite MLG, G1. Thus, these data support the hypothesis of an insecticide-induced selective sweep that reduced genetic variability in French populations of *M. persicae* feeding on oilseed rape.

However, the comparison of insecticide resistance mechanism frequencies between untreated fields and those treated with pyrethroids did not give clear-cut results. Indeed, most differences were not significant and some unexpected differences were detected. More *kds/kds* individuals were found in a treated population (SG_2) than in two untreated fields. It is noteworthy that all *kds/kds* individuals found in the treated sample SG_2 had the second most frequent microsatellite MLG (G3), whereas the *kdr/kds* individuals had the genotype G1. It is therefore possible that the G3 genotype had a selective

advantage over G1 (ie an undetected insecticide resistance mechanism). The super-*kdr* mutation of the *para*-like gene, which confers resistance to pyrethroids in several insect species (Martinez-Torres *et al*, 1997), has been recently discovered in *M. persicae* (M. Williamson, IACR, Rothamsted, UK, 2003, personal communication), and is a plausible candidate.

Multiple selection acting on populations of *M. persicae*

The hypothesis of an insecticide-induced selective sweep is tempting. However, a few rare MLGs were found in association with both the *kdr* mutation and an overproduced esterase. These rare genotypes and the frequent insecticide-resistant G1 were probably selectively equivalent in the face of the pesticide treatments used in these fields. Moreover, Guillemaud *et al* (2003a) found numerous (about 50) different MLGs that had the *kdr* mutation and the overproduced esterase FE4 in sexually reproducing populations of *M. persicae* in France. These previous results, together with the present study, imply that insecticide selection alone cannot fully explain the observed genetic structure, in particular the high frequency of G1 in all populations (unless G1 possesses an additional undetected resistance gene, but see below).

A possible scenario involves two or more different selective pressures. The additional selective factor(s) may have favored one (or a few) particular genotype(s) in all oilseed rape fields sampled in 2001, but not the flying aphids caught in 2000 (Guillemaud *et al*, 2003b) nor aphids sampled on other plants in various years (Terradot *et al*, 1999) in the same region. We therefore propose that the additional selective pressure is a consequence of the agricultural practices associated with the oilseed rape cultivation and may include the nature of host plant itself. Host plant adaptation strongly affects the ecology and the genetic structure of many aphid species such as the cotton aphid *Aphis gossypii* (Glover) (Vanlerberghe-Masutti and Chavigny, 1998; Fuller *et al*, 1999) and the pea aphid *Acyrtosiphon pisum* (Via *et al*, 2000). Host plant specialization of adapted genotypes is theoretically possible in *M. persicae*; however, clear evidence of host-based genetic differentiation is lacking (Fenton *et al* (1998), but see Margaritopoulos *et al* (1998)). This evidence is not direct, as none of these studies considered aphids sampled from oilseed rape.

The alternative explanation for the predominance of G1 is that it possesses an additional undetected insecticide resistance gene. Although not impossible, this hypothesis seems unlikely because it could not explain why G1 was not detected among aphids caught outside oilseed rape fields (Terradot *et al*, 1999; Guillemaud *et al*, 2003b).

The present study suggests that human-induced selective pressures probably shape the genetic structure of *M. persicae* populations living on oilseed rape. Additional information is needed to evaluate fully the hypothesis that this selection involves both insecticide treatment and additional factors. In particular, the stability of the genetic structure over several years would provide strong support. In addition, genetic comparison of aphid populations growing on oilseed rape and those on other crops would help to ascertain the existence of host-adapted genotypes or host races.

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