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Genetic architecture of sexual and asexual populations of the aphid *Rhopalosiphum padi* based on allozyme and microsatellite markers

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Abstract

Cyclical parthenogens, including aphids, are attractive models for comparing the genetic outcomes of sexual and asexual reproduction, which determine their respective evolutionary advantages. In this study, we examined how reproductive mode shapes genetic structure of sexual (cyclically parthenogenetic) and asexual (obligately parthenogenetic) populations of the aphid *Rhopalosiphum padi* by comparing microsatellite and allozyme data sets. Allozymes showed little polymorphism, confirming earlier studies with these markers. In contrast, microsatellite loci were highly polymorphic and showed patterns very discordant from allozyme loci. In particular, microsatellites revealed strong heterozygote excess in asexual populations, whereas allozymes showed heterozygote deficits. Various hypotheses are explored that could account for the conflicting results of these two types of genetic markers. A strong differentiation between reproductive modes was found with both types of markers. Microsatellites indicated that sexual populations have high allelic polymorphism and heterozygote deficits (possibly because of population subdivision, inbreeding or selection). Little geographical differentiation was found among sexual populations confirming the large dispersal ability of this aphid. In contrast, asexual populations showed less allelic polymorphism but high heterozygosity at most loci. Two alternative hypotheses are proposed to explain this heterozygosity excess: allele sequence divergence during long-term asexuality or hybrid origin of asexual lineages. Clonal diversity of asexual lineages of *R. padi* was substantial suggesting that they could have frozen genetic diversity from the pool of sexual lineages. Several widespread asexual genotypes were found to persist through time, as already seen in other aphid species, a feature seemingly consistent with the general-purpose genotype hypothesis.

Keywords: allozyme, asexuality, cyclical parthenogenesis, evolution of sex, microsatellite, reproductive system

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Introduction

Despite the two-fold cost of sex, the vast majority of multicellular eukaryotic species reproduce sexually (Maynard Smith 1971; Williams 1975; Bell 1982). Many theories have been proposed to account for the paradoxical evolutionary success of sexual reproduction in the living world (Kondrashov 1993; Barton & Charlesworth 1998; West *et al*. 1999). Experimental tests of these theories employ biological models whose reproductive system and genetic structure is well known (Sunnucks *et al*. 1997; Butlin *et al*. 1999). Cyclical parthenogens (e.g. aphids, cladocerans), which alternate several parthenogenetic generations with one sexual generation, are especially useful because they frequently experience transitions to complete asexuality (Hebert 1987; Moran 1992; Dufresne & Hebert 1994; Hales *et al*. 1997). This leads to conspecific cyclically and obligate parthenogenetic lineages (hereafter, sexual and asexual lineages, respectively). However, few attempts have been made to study the genetic consequences of sexual and asexual reproductive modes which determine their respective evolutionary advantages.

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In fact, many theoretical models of the maintenance of sex assume that through recombination, sexual reproduction generates a greater genetic diversity than asexuality, a premise that has rarely been investigated properly. Moreover, most studies that compare the genetic structure of sexual and asexual populations have been limited by the low level of allozyme polymorphism (Innes *et al*. 1986; Hebert *et al*. 1988; Ward *et al*. 1994; Hales *et al*. 1997). Furthermore, no general trend between heterozygosity and reproductive mode has been found: asexual populations show either lower heterozygosity than sexual populations at most allozyme loci (aphids) or higher (fixed) heterozygosity (cladocerans).

In aphids, the recent application of microsatellite markers has, first, allowed the detection of a high genetic diversity, whereas allozymes showed little variation (Sunnucks *et al*. 1997; Simon *et al*. 1999a; Wilson *et al*. 1999). Second, microsatellites have started to reveal patterns of genetic structure that contradict conclusions derived from allozyme studies. In particular, they seem to show higher heterozygosity in asexual than sexual populations, whereas allozymes showed the opposite (Simon *et al*. 1999a). This renders necessary a comparison of allozyme and microsatellite data in aphids in order to understand the causes of such discrepancies. This was the first aim of this study.

Among aphids, *Rhopalosiphum padi* has been the subject of extensive biological and molecular studies that have allowed the characterization of two major reproductive lineages: (i) sexual (cyclically parthenogenetic) lineages that alternate sexual and asexual reproduction and complete their annual life cycle on two different hosts (spring and summer asexual generations on cereals are followed by a single annual sexual generation in autumn on *Prunus padus*, with production of cold resistant eggs); (ii) asexual (obligately parthenogenetic) lineages that live year-round on cereals although most of them have retained the ability to produce males in autumn (Simon *et al*. 1991).

In a recent study, we demonstrated, using phylogenetic inference, that asexual lineages have appeared many times and by several mechanisms such as mutation from originally sexual lineages and gene flow mediated by males produced by asexual lineages (Delmotte *et al*. 2001a). These dynamics of emergence of asexual lineages potentially increase clonal diversity in asexual populations. The second aim of this study was to evaluate whether asexual populations could indeed be as genetically diverse as sexual populations (nullifying the theoretical advantages of recombination) and to gain more insight into the genotypic features of asexual lineages that could influence their evolutionary fate.

Until now, genetic studies on *R. padi* have been considerably constrained by the low polymorphism of the markers used (allozymes or mitochondrial DNA; Martinez-Torres *et al*. 1996; Simon *et al*. 1996a). Therefore, we recently developed microsatellite markers that are hypervariable in this species (Simon *et al*. 2001; Delmotte *et al*. 2002b). In this study, we compared microsatellite data on sexual and asexual French populations of the aphid *R. padi* with allozyme data previously acquired on different individuals of the same populations to further our understanding of how reproductive mode shapes the genetic structure of populations.

Materials and methods

Sample collection

In early spring, asexual and sexual lineages are clearly separated in space, being on cereals and *Prunus padus*, respectively (Simon *et al*. 1996a). Asexual lineages were sampled on cereal plants separated by at least 1 m in order to minimize the collection of aphids from the same clonal lineage. Sexual lineages were collected on *P. padus* just after hatching from eggs, thus ensuring that they did not undergo asexual reproduction.

Six asexual populations and eight sexual populations were sampled in spring 1995 across France (Fig. 1). In order to assess the persistence of asexual lineages through time, we also collected individuals on cereals in the Rennes area (western France) in December 1999. All samples were stored at –70 °C for analysis.

Microsatellite analysis

DNA from individual aphids was extracted using the 'salting-out' protocol described by Sunnucks & Hales

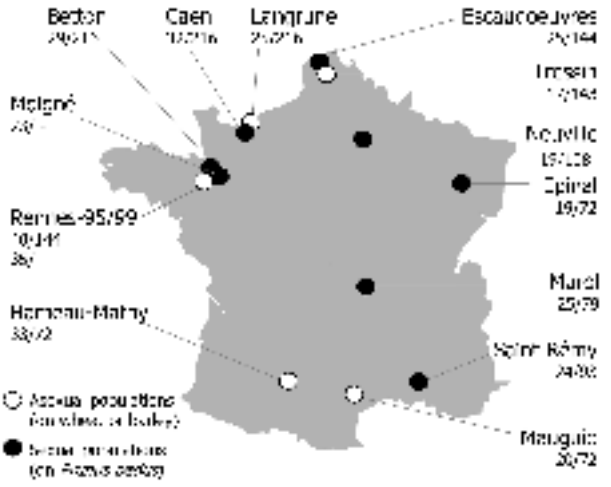

Fig. 1 Collection sites of French sexual and asexual populations of *Rhopalosiphum padi*. Sample sizes are given below for both markers: microsatellite/allozyme.

(1996) and resuspended in 1× TE buffer (10 mM Tris pH 7.5, 0.1 mM EDTA) to a final DNA concentration of 5–7 ng/μL.

Microsatellite analyses were performed at seven polymorphic loci isolated either from *Rhopalosiphum padi* (*R5.10*, *R2.73*, *R1.35*, *R6.3*, *R5.29*; Simon *et al*. 2001) or *Sitobion miscanthi* (*S17b*, *S16b*; Wilson *et al*. 1999). Microsatellite amplifications were carried out following Simon *et al*. (2001). Four loci (*R5.10*, *R2.73*, *R1.35*, *R6.3*) were analysed by automated capillary electrophoresis following Delmotte *et al*. (2002b), and three loci (*R5.29*, *S16b*, *S17b*) were analysed by electrophoresis in polyacrylamide gel as described in Simon *et al*. (2001).

Allozyme analysis

We reanalysed a subset of the allozyme data obtained by Simon & Le Gallic (1998). It is important to clarify here that allozyme data had been obtained from different individuals than the ones used in our microsatellite survey, but from the same populations (Fig. 1). Variation was studied at four polymorphic enzymes: aminoaspartate transferase (AAT, EC 2.6.1.1), phosphoglucomutase (PGM, EC 5.4.2.2), sorbitol dehydrogenase (SDH, EC 1.1.1.44) and peptidase (PEP, EC 3.4.11-13).

Genetic data analysis

Because clonal amplification of genotypes can influence data interpretation (Sunnucks *et al*. 1997), analyses were performed on microsatellite data with one representative of each genotype (i.e. clonal copies removed). Comparisons between allozyme and microsatellite data sets were made with all individuals because allozymes revealed too little polymorphism to perform the tests with one copy per genotype.

The resolving power of microsatellite markers was investigated by examining the relationship between the number of genotypes identified (i.e. number of individuals/number of different genotypes) and the number and type of combined loci as in Rossi *et al*. (1998).

Allele frequencies and unbiased heterozygosity (Nei 1978) were calculated for each sample. Before testing for deviation from Hardy–Weinberg equilibrium (HWE), genotypic proportions and global linkage disequilibrium between pairs of loci were tested using the exact tests in GENEPOP Version 3.1b (Raymond & Rousset 1995). Deviations from HW expectations and unbiased estimates of F_{IS} (f) (Weir & Cockerham 1984) were estimated using FSTAT Version 2.9.1 (Goudet 1995) permuting alleles 1000 times among individuals. Sequential Bonferroni corrections were used to compute table-wide significance levels for simultaneous statistical tests (Rice 1989).

Population structure inferred from nuclear loci was assessed by calculating F_{ST} (θ) (Weir & Cockerham 1984) between populations and tested by randomising multilocus genotypes between each pair of samples using FSTAT. Pairwise F_{ST} were computed using GENEPOP. These F-statistics were then tested for significance by performing 5000 permutations of alleles or genotypes in FSTAT. Hierarchical analysis of molecular variance (AMOVA, Excoffier *et al*. 1992) including (i) population, (ii) populations within reproductive mode, and (iii) reproductive mode was performed using ARLEQUIN Version 2.000 (Schneider *et al*. 2000).

Comparisons of genotypic frequencies among asexual populations and genic differentiation between sexual and asexual populations were carried out using Fisher's exact tests using SAS (SAS Institute Inc. 1988). Clonal diversity was calculated as the number of genotypes/number of individuals in asexual populations.

For sexual populations, isolation by distance (IBD) was tested as suggested by Rousset (1997). The Mantel test for significance of the correlation between geographical and genetic matrices was performed by GENEPOP on both allozyme and microsatellite data. In addition, we constructed a multivariate Mantel correlogram following the technique of Oden & Sokal (1986) on microsatellites only, because of the low genetic variation detected by allozymes. A normalized Mantel statistic was obtained for each distance class by combining a binary matrix describing the geographical relationships between sexual populations and corresponding Cavalli-Sforza and Edwards' (CSE) (Cavalli-Sforza & Edwards 1967) genetic distance matrices (e.g. Arnaud *et al*. 1999). The significance of each normalized Mantel statistic was tested using 10 000 permutations in NTSYS Version 2.02i (Rohlf 1997).

To investigate relationships within sexual and asexual populations, respectively, we performed a comparison of the distributions of pairwise genetic distances among their multilocus genotypes, using the allele shared distance (D_{AS}, Chakraborty & Jin 1993) which counts the number of different alleles between multilocus genotypes. All genetic distances were calculated using POPULATION Version 1.2.01 (http://www.cnrs-gif.fr/pge/bioinfo).

A neighbour-joining (NJ) tree based on CSE distance between sexual and asexual populations was built, and bootstrap values were computed over 2000 replications using POPULATION. The chord distance of CSE was chosen because it produces the most reliable topologies with microsatellite loci (Takezaki & Nei 1996).

To study the value of microsatellite and allozyme markers to assign genotypes to their reproductive mode, we used a Bayesian assignment method based on individual genotypes and population allele frequencies. This method was computed using the program GENECLASS (Cornuet *et al*. 1999).

Table 1 Comparison of allelic diversity, genotypic diversity and *F*-statistics at microsatellite and allozyme markers in sexual and asexual populations of *Rhopalosiphum padi*. Statistics are performed on the full data set for both types of markers

Populations	*N*	No. of multilocus genotypes	No. of alleles/Total no. of alleles per locus							Mean (SE) no. of alleles per locus	F_{IS} across pop	F_{ST} across pop
Microsatellites			*S16b*	*S17b*	*R5.29*	*R6.3*	*R5.10*	*R2.73*	*R1.35*			
Asexual	180	83	3/4	5/8	8/25	4/5	9/14	7/9	10/16	6.57 ± 0.276	−0.499*	0.0603*
Sexual	204	204	4/4	8/8	25/25	5/5	13/14	9/9	15/16	11.3 ± 0.311	0.182*	0.0323*
Allozymes			AAT	PGM	SDH	PEP						
Asexual	755	14	2/2	3/4	2/4	2/3				1.54 ± 0.118	0.339*	0.169*
Sexual	962	29	2/2	4/4	4/4	3/3				2.21 ± 0.0845	0.020*	0.0264*

N, sample size. *Multilocus F_{IS} and F_{ST} are significantly different from 0 at $P < 0.001$.

Results

Overall diversity

Allelic diversity at the seven microsatellite loci ranged from 4 to 25 alleles per locus with 81 alleles identified across all 7 loci. Most microsatellite loci, with the exception of *R6.3* and *R2.73*, displayed continuous allele sizes separated by one or two nucleotides (Fig. 2). The size distributions of alleles at loci *R6.3* and *R2.73* were strongly disjunct. Allozyme markers were less polymorphic with only 2–4 alleles per locus and a total of 13 alleles identified across the 4 loci. The asexual populations possessed a subset of the alleles present in sexual populations with the exception of one rare allele at microsatellite locus *R5.10* (Table 1).

Population structure revealed with allozymes

The number of multilocus allozyme genotypes was very low in both sexual and asexual populations (29 in 962 sexual individuals and 14 in 755 asexual individuals) (Table 1). Eight multilocus allozyme genotypes were common to both sexual and asexual populations and represented > 85% of the total number of individuals. However, genic and genotypic differentiation between sexual and asexual populations was highly significant with allozymes ($P < 0.001$; Table 1). Assignment efficiency with four loci was low: 62% of individuals were correctly assigned to their reproductive mode.

In sexual populations no linkage disequilibrium (LD) was found between the pairs of allozyme loci that were testable (SDH, PEP, PGM). We found low but significant overall F_{IS} (0.02, $P < 0.01$) and F_{ST} (0.0264, $P < 0.001$) values indicating that there was some genetic structure within and among sexual populations, respectively (Table 1). Asexual populations were not testable for LD and showed significant heterozygote deficits within populations ($F_{IS} = 0.339$, $P < 0.001$) and geographical differentiation ($F_{ST} = 0.169$, $P < 0.001$).

Resolving power of microsatellite loci

The total number of multilocus genotypes detected with microsatellites was high ($N = 287$) because of their higher level of polymorphism. With microsatellites, no genotype was shared by sexual and asexual populations. In addition, each sexual lineage possessed a unique multilocus genotype. In sexual populations, five microsatellite loci were sufficient to identify 94–100% of the genotypes in the sample depending on the combination of loci (Fig. 3). This indicates that sexual genotypes were very different from each other. In asexual populations, one half (54%) of individuals were copies of genotypes and the number of genotypes did not increase significantly with sampling effort ($R = 0.68$, $P = 0.093$, $N = 83$). The mean number of asexual genotypes increased slowly with the number of loci. However, a subset of asexual individuals ($N = 12$) belonging to the same seven-loci genotype were further analysed at five additional microsatellite loci and still shared exactly the same genotype (data not shown) suggesting that genotypic diversity was almost resolved with the first seven loci. The number of alleles in asexual populations was not very different with different combinations of a given number of loci: consequently, the proportion of asexual genotypes discriminated did not vary very much.

Within-population microsatellite variation

No pairwise LD was found for sexual populations, so independent assortment was assumed in such populations. By contrast, asexual populations had 12/21 pairs of loci that were in LD ($P < 0.001$) after Bonferroni correction for multiple tests.

All sexual populations showed significant heterozygote deficit (data not shown). Consequently, F_{IS} values across sexual populations were all positive and highly significant at most loci (Table 2). Deviations from HWE at loci isolated from *Sitobion miscanthi* (*S16b*, *S17b*) were not significant

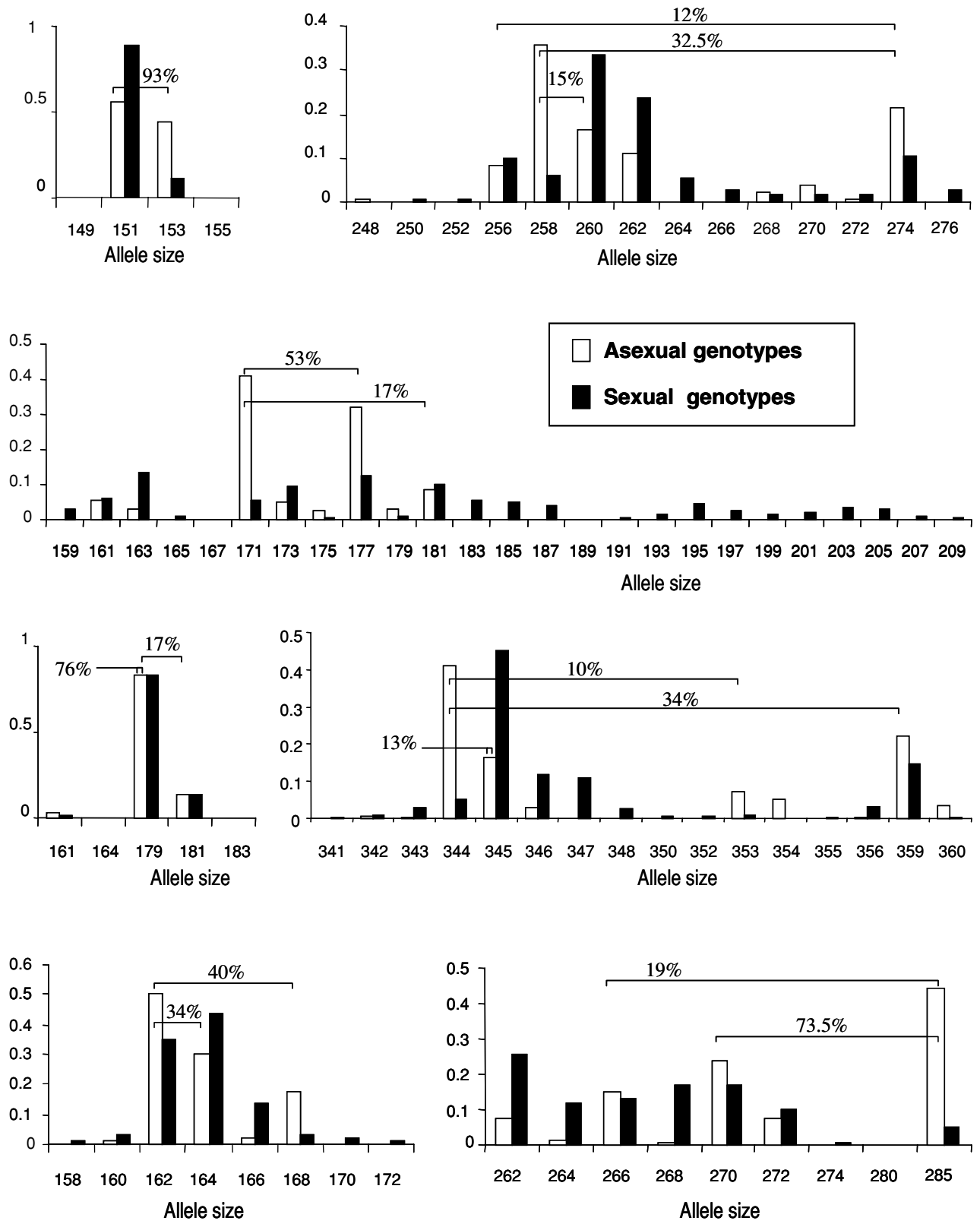

Fig. 2 Allelic frequencies of sexual and asexual genotypes across populations of *Rhopalosiphum padi* after removing copies of genotypes. The percentage above the lines indicates the most frequent (>10%) diploid associations of alleles in asexual genotypes.

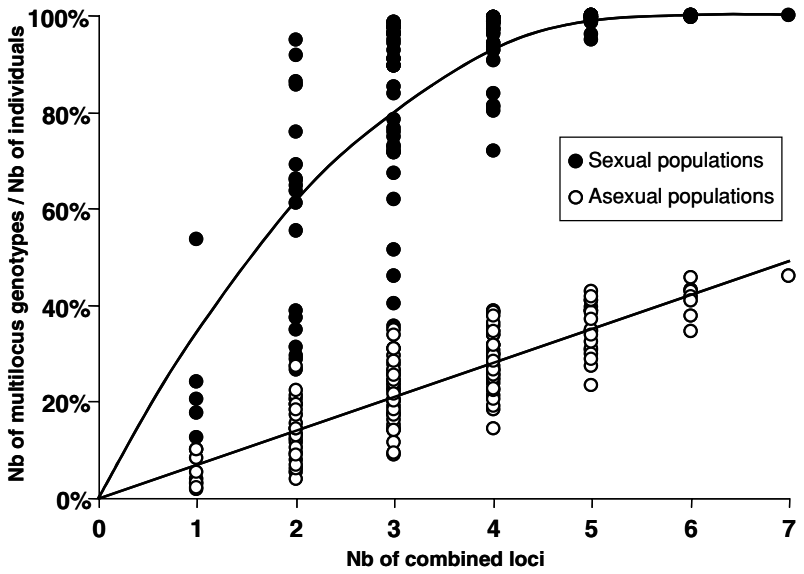

Fig. 3 Number of genotypes discriminated (number of multilocus genotypes/number of individuals sampled) as a function of the number of loci combined to discriminate genotypes in sexual and asexual populations of *Rhopalosiphum padi*.

because these loci showed a lower polymorphism than those isolated from *Rhopalosiphum padi* (Table 1).

Although random mating was not expected, HWE was tested in asexual populations to quantify the magnitude of the deviation from HW proportions. We found a strong excess of heterozygotes in all asexual populations ($H_O = 0.813$; $H_E = 0.600$). Moreover, 83% of the asexual genotypes were heterozygous at least at five of seven loci. The most frequent asexual genotypes were heterozygous at every locus but *R6.3* (Fig. 2).

Between-population microsatellite differentiation

Genotypic frequencies were significantly different among asexual populations ($P < 0.001$, Fisher exact test, Table 2). Asexual populations were partitioned into southern populations (Mauguio and Hameau-Mathy) and northern populations (all others). Many genotypes (35–75%) were restricted to a single population (Fig. 4). The remaining individuals from asexual populations were copies of eight repeated genotypes that were present in at least two populations. Among these common genotypes, 'genotype 3' was frequent in all asexual populations and persisted over time (found in both 1995 and 1999) (Fig. 4).

We found significant overall differentiation among sexual populations of *R. padi* (Table 2). Although the matrix of pairwise $F_{ST}/(1 - F_{ST})$ estimates was not significantly correlated with the matrix of geographical distances (Mantel test, $P > 0.05$ for the two classes of markers), we found a pattern of isolation by long distance using the normalized Mantel statistic on microsatellites. At short distances, a significantly positive association was found between the matrices of genetic (CSE) and geographical distances. At higher distances (> 350 km), negative correlations were found, which were only significant at 1000 km. The lack of significance at intermediate geographical distances was likely to be due to small sample sizes.

Table 2 Comparison of the genetic structure of sexual and asexual populations of *Rhopalosiphum padi* revealed by microsatellite markers. Statistics are performed on the data set after removing individuals with identical multilocus genotype (Materials and methods)

Statistics	Locus	Asexual	Sexual
Mean H_O		0.813	0.509
Mean H_E		0.600	0.642
Heterozygote deficit		s	s
Heterozygote excess			ns
F_{IS} per locus across pop	*S16b*	−0.894*	−0.0475
	S17b	−0.340*	0.0110
	R6.3	−0.130	0.308*
	R5.29	−0.469*	0.038*
	R5.10	−0.211*	0.294*
	R2.73	0.431*	0.213*
	R1.35	−0.0481	0.376*
F_{IS} multilocus across pop		−0.355*	0.182*
F_{ST} across pop	*S16b*	0.00164	0.0567*
	S17b	0.0326*	0.0281
	R6.3	0.0232*	0.0282*
	R5.29	0.0570*	0.0392*
	R5.10	0.0235*	0.0243*
	R2.73	0.00900*	0.0204*
	R1.35	0.00648	0.0353*
F_{ST} multilocus across pop		0.0223*	0.0323*
F-test over genotypic freq. between pop		s	—

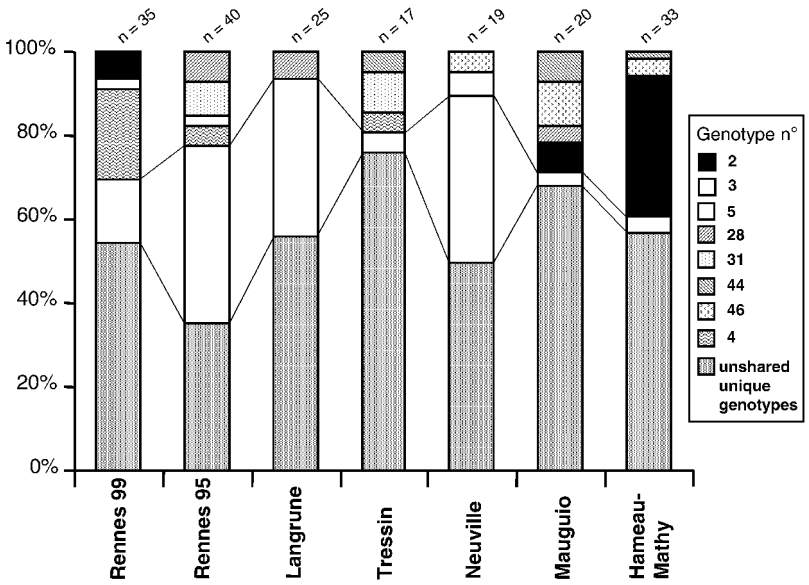

Fig. 4 Multilocus genotypic frequencies in asexual populations of *Rhopalosiphum padi* based on microsatellite markers.

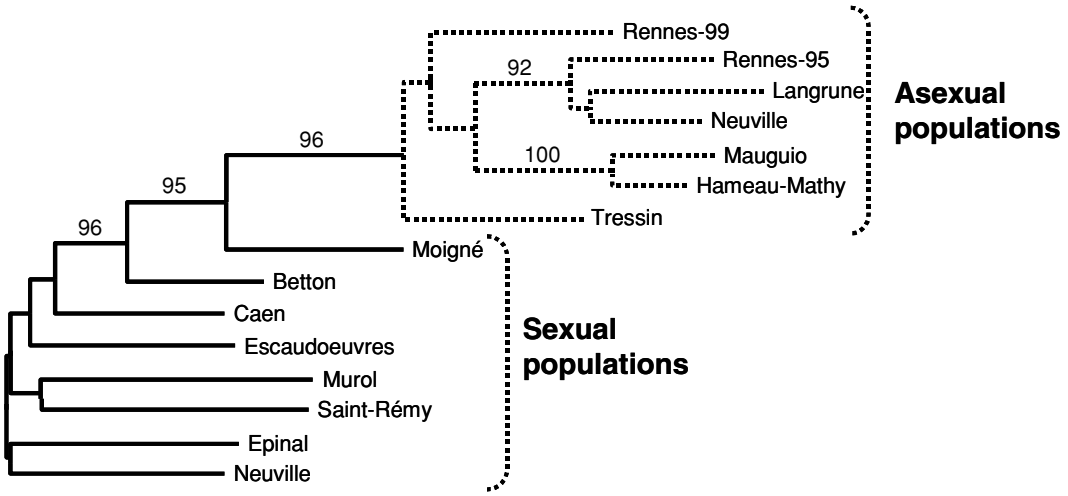

Fig. 5 NJ clustering from the allele shared distance (D_{AS}) matrix calculated among seven asexual populations (dashed lines) and eight sexual populations (solid lines) of *Rhopalosiphum padi* based on seven microsatellite loci. Numbers above branches are bootstrap percentages (1000 resamplings).

Microsatellite differentiation between sexual and asexual populations

Between sexual and asexual populations, the multilocus F_{ST} ($F_{ST} = 0.144$) and genic differentiation for all loci but *R6.3* were highly significant ($P < 0.001$). This is illustrated by the well-supported population tree that splits sexual and asexual populations (Fig. 5) and by the correct assignment of 95% of the multilocus genotypes to their sexual or asexual population. The hierarchical AMOVA performed at each locus gave two results (Table 3): first, most of the variance was contained within populations; second, loci *S16b*, *R2.73* and *R1.35* explained a high percentage (> 13%) of the among groups (sexual/asexual) variance suggesting an association with reproductive mode (physical linkage or stochastic phenomena when asexual lineages arose).

Relationships among sexual and asexual genotypes

Mean pairwise allele shared distance (± SE) between genotypes was significantly greater among sexual than asexual populations (0.596 ± 0.001 and 0.382 ± 0.003; $P < 0.001$, respectively) (Fig. 6). This result is partly explained by the lower genotypic variability found in asexual populations. It also indicates that asexual genotypes differ from each other by fewer alleles than sexual ones.

Table 3 Subdivision of variance in populations of *Rhopalosiphum padi* based on *F*-statistics estimated from microsatellite data. d.f. degree of freedom

		% total variance							
		Microsatellite loci							
Source of variation	d.f.	*S16b*	*S17b*	*R5.29*	*R6.3*	*R5.10*	*R2.73*	*R.135*	All loci
Among groups (sexual/asexual)	1	25.50	5.93	10.42	−0.381	9.16	13.12	13.73	11.35
Among pop within groups	13	1.49	2.82	3.7	4.38	2.54	1.90	2.89	2.75
Within pop	621	73.01	91.25	85.88	96.00	88.30	84.98	83.38	85.9

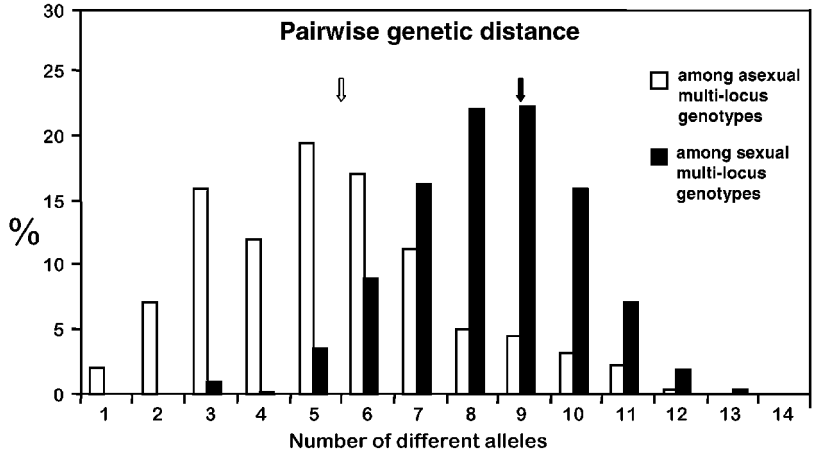

Fig. 6 Distribution of pairwise genetic distances (D_{AS}) calculated between asexual genotypes and between sexual genotypes of *Rhopalosiphum padi* based on seven microsatellite loci. Open and filled arrows indicate mean genetic distance for asexual multilocus genotypes and for sexual multilocus genotypes, respectively.

Discussion

Comparison of microsatellites and allozymes

Remarkably, five microsatellite loci were sufficient to fully resolve the genotypic diversity of populations of *Rhopalosiphum padi*. Whereas microsatellites allowed discrimination of all genotypes in sexual populations, allozymes only discriminated 3% of individuals. This is in agreement with previous studies on aphids that found low genetic variation at most allozyme loci (reviewed in Hales *et al*. 1997) and also confirms the great sensitivity of microsatellites for resolving clonal diversity in parthenogenetic organisms (Sunnucks *et al*. 1996; Fuller *et al*. 1999; Gomez & Carvalho 2000). No linkage disequilibrium was found in sexual populations among both microsatellites and allozymes. In contrast, highly significant associations between loci were found in asexual populations with both types of markers. These are the classical outcomes of, respectively, sexual and asexual reproduction. In sexual populations, both F_{ST} and F_{IS} estimates calculated with both types of markers were broadly in agreement and significantly positive. In contrast, in asexual populations, we found discordant patterns with the two types of markers. Although allozymes showed significant heterozygote deficit, microsatellites revealed strong heterozygote excess. In asexual populations of aphids, this disproportionate allozyme homozygosity appears to be a general phenomenon (Loxdale *et al*. 1985; Loxdale & Brookes 1990; Simon & Hebert 1995; Simon *et al*. 1996a). A similar pattern has been found at the sequence level, for a protein-coding gene (*EF1α*), which revealed unexpectedly low levels of allele divergence in the Tramini, a tribe that might have undergone long-term asexuality (Normark 1999). We discuss now several forces that could increase homozygosity.

It has been suggested that intense clonal selection in asexual populations could lead to homozygosity of favourable alleles at protein-coding loci (Simon & Hebert 1995). Because we sampled sexual populations right after sexual reproduction, before any clonal selection could take place, we do not expect a similar effect in these populations. The hypothesis of selection for homozygosity predicts a decrease in heterozygosity over the course of the year in sexual populations: it should be highest right after sexual reproduction, decreasing during the following parthenogenetic generations. Other hypotheses could account for a decreased heterozygosity in allozymes in asexual populations. For example, dissociations and fusions of chromosomes have been well documented in asexual lineages of aphids (Blackman & Spence 1996; Blackman *et al*. 2000). This could result in changes in gene expression, for instance the loss of expression of one of the two homologous alleles

(Suomalainen *et al*. 1976). Also, increased homozygosity may result from gene conversion as suggested for the Tramini (Normark 1999). In our case, however, this does not seem to readily explain the pattern of comparatively lower heterozygosity in allozymes, because gene conversion might equally affect allozymes and microsatellites.

Genetic differentiation between sexual and asexual populations of aphids

With about twice as many alleles and genotypes in sexual than asexual populations, we confirm that populations undergoing sexual reproduction had more genetic variability than asexual populations. Although higher genotypic variability in sexual populations is the logical outcome of recombination and allele shuffling, the higher allelic diversity in sexual populations remains to be explained. Two processes could account for this pattern. First, we have shown in a previous study that asexual lineages of *R. padi* are derived from sexual ancestors (Delmotte *et al*. 2001a). This is illustrated here by the fact that allozyme and microsatellite alleles found in asexual populations are a subset of those present in sexual populations. Although we also demonstrated that there have been recurrent origins of asexuality in this aphid (Delmotte *et al*. 2001), it seems that the dynamics of emergence of asexual lineages is slow enough to limit the allelic diversity of asexual populations. Second, whereas alleles of asexual populations are only generated by slippage errors during replication, allelic diversity in sexual populations could also be enhanced by recombination although this has not been fully demonstrated yet (Hancock 1999).

Significant genetic differentiation ($P < 0.001$) was found between sexual and asexual populations with multilocus F_{ST} estimates ranging from 0.103 for allozymes to 0.144 for microsatellites. In addition, the reproductive mode of 95% of individuals was correctly assigned with microsatellites. This indicates a limited exchange of genes between reproductive modes. It has been proposed that gene flow between sexual and asexual populations of *R. padi* was an important source of new asexual lineages. This was supported by two lines of evidence. First, most asexual lineages produce a significant number of males (5–30%) at the time of sexual reproduction (Simon *et al*. 1991). Second, these males are able to fertilize females of sexual lineages, some of their offspring giving rise to new asexual lineages (Rispe *et al*. 1999; Delmotte *et al*. 2001a). Our results show, however, that crosses between sexual and asexual lineages must be very rare in nature for such a level of differentiation to be maintained. Males of asexual lineages may be less efficient than males of sexual lineages at localizing and mating with sexual females. Alternatively, asexual lineages generated by gene flow may be counter-selected. Indeed, it has been shown that the viability of F_1 hybrids between well-differentiated aphid lineages can be strongly reduced (Via *et al*. 2000).

Why did asexual populations show microsatellite heterozygote excess?

In large sexually reproducing populations, theory predicts that neutral sequence divergence between two alleles of a locus will be low because of recombination and large population size that prevent genetic drift. In contrast, allele sequence divergence within individual genomes tends to increase rapidly in asexual lineages and if asexual reproduction continues for a long time, these sequence differences will become specific to each lineage (Birky 1996). Provided that descendants of formerly allelic sequences are not lost by deletion or gene conversion, heterozygosity should therefore increase with the age of asexual lineages. Testing this prediction constitutes a method to uncover evidence of ancient asexuality (a test originally conceived by Meselson and discussed by Birky 1996 and Judson & Normark 1996). This molecular test was successfully applied to the class Bdelloidea of the rotifers, providing further evidence that this group has evolved without sexual reproduction for 35 Myr (Mark Welch & Meselson 2000). In aphids, this method was also applied to the putative ancient asexual tribe of Tramini (Normark 1999). Surprisingly, Tramini showed very low sequence heterozygosity indicating that recombination or other homogenizing processes may have occurred. However, regular meiosis could be excluded by the finding of rapid karyotype evolution in this tribe (Normark 1999; Blackman *et al*. 2000).

In contrast, previous studies using microsatellite markers revealed high heterozygosity in asexual lineages of various species of *Sitobion* aphids (Sunnucks *et al*. 1996; Simon *et al*. 1999a; Wilson *et al*. 1999). Similarly, we found here that asexual genotypes of *R. padi* have high levels of heterozygosity, thus suggesting, in line with the above predictions, a possible ancient loss of sexuality in both *Sitobion* and *R. padi*. The long-term persistence of asexual lineages of *R. padi* is also supported by mitochondrial phylogenetic analysis which suggests that most asexual lineages could have evolved separately for ≈ 400 000 years (Simon *et al*. 1996b; Delmotte *et al*. 2001a). However, alternative hypotheses could also explain the high level of heterozygosity in asexual populations. For example, hybridization events between two closely related species could create highly heterozygous asexual lineages, with very divergent mitochondrial haplotypes. Interspecific hybridization is not rare in aphids (e.g. Müller 1985; Guldemond 1990; Sunnucks *et al*. 1997; Rakauskas 1999) but has not been reported to date in *R. padi* (Simon *et al*. 1999b). The application of the 'Meselson' test would be valuable to

discriminate between the two hypotheses, ancient asexuality or hybrid origin of asexuality.

Why did sexual populations show microsatellite heterozygote deficit?

The heterozygote deficits observed in sexual populations with microsatellites contrast with previous allozyme studies on *R. padi* (Simon & Hebert 1995; Simon *et al*. 1996a; Simon & Le Gallic 1998) that found no deviation from HWE. Here we examine four explanations for this pattern:

1 *Null alleles*. This hypothesis is unlikely because first, no null homozygotes were observed at any loci in our samples and second, null alleles were searched for but not found among the progeny of many crosses obtained in the laboratory.
2 *Selection*. Differences in survival of over-wintering eggs could produce HW deviations (Dedryver *et al*. 1998). However, clonal selection could not operate because sexual populations were sampled right after eggs hatched, before aphids started reproducing.
3 *Inbreeding*. Several factors such as population size, host-plant distribution, differences in habitat choice or mating behaviour can set the stage for inbreeding by favouring crosses between related clones or between individuals of the same clone (Hebert *et al*. 1991; Caillaud & Via 2000).
4 *Allochronic isolation*. Heterozygote deficit could also result from a Wahlund effect within sexual populations due to differences in the timing of production of sexuals between aphid lineages (Austin *et al*. 1996). Discriminating between these different explanations will require further fine-scale analyses of the genetic structure of mating populations on the winter host *Prunus padus* and better knowledge of the mating behaviour of *R. padi*.

Genetic differentiation and dispersal

Sexual populations. Little genetic differentiation was found among sexual populations (with microsatellites and allozymes) suggesting extensive gene flow at least at short distances. These results are consistent with previous allozyme studies on *R. padi* (Loxdale & Brookes 1990; Simon & Hebert 1995; Simon *et al*. 1996a; Simon & Le Gallic 1998) and with microsatellite studies on *Sitobion avenae*, another cereal aphid, at both small and large geographical scales (Sunnucks *et al*. 1997; Simon *et al*. 1999a; Haack *et al*. 2000). The broad distribution of cereal crops, together with the large dispersal ability of cereal aphids, may contribute to this pattern. When leaving their summer hosts, sexual lineages of *R. padi* have to migrate to a sparsely distributed winter host (*P. padus*) to achieve sexual reproduction resulting in the mixing of individuals from distant subpopulations (Loxdale & Brookes 1990). This is likely to result in greater gene flow between sexual populations of *R. padi* compared with nonhost-alternating cereal aphids such as *S. avenae*.

Common asexual genotypes. The presence of a few asexual lineages that are spread over large geographical areas and present on several host plants appears to be a feature common to many aphid species (e.g. Sunnucks *et al*. 1996; Fenton *et al*. 1998; Fuller *et al*. 1999; Wilson *et al*. 1999; Haack *et al*. 2000). In this study too, several widespread asexual genotypes of *R. padi* were found throughout France. One of these asexual genotypes was present in all asexual populations in 1995 and persisted in 1999. This could be a validation of the general-purpose-genotype hypothesis (Lynch 1984) that predicts the selection of a few, broadly adapted, asexual genotypes in fluctuating environments (such as crop rotations).

Rare asexual genotypes. In addition to these predominant asexual genotypes, we detected rare genotypes that were restricted to single populations and were responsible for the strong differentiation among asexual populations (Fig. 4). Furthermore, no persistence through time was observed for these genotypes, suggesting either a rapid turn-over or biases in our sampling procedure. Clonal diversity reached 46% in asexual lineages of *R. padi*. This relatively high level of clonal diversity may result from: (i) mutations within existing asexual lineages; or (ii) repeated emergence of asexual lineages from the pool of sexual lineages (Hebert 1987). According to the 'Frozen Niche Variation Model' (Vrijenhoek 1984), asexual lineages of *R. padi* freeze genetic diversity from sexual lineages either by spontaneous loss of sexuality or gene flow mediated by males produced by asexual lineages (Delmotte *et al*. 2001a).

Conclusions

The comparison of allozymes and microsatellites showed that microsatellites not only modified genotypic resolution and parameter values, but also the power of tests assessing within and between population structure. Moreover, as opposed to microsatellites, allozymes showed low polymorphism and also low heterozygosity, which suggests they could be under selection. Allozymes are therefore unsuited to reflect the actual genetic diversity and structure of aphid populations, which seriously undermines previous conclusions derived using these markers in aphids (reviewed in Hales *et al*. 1997).

An important result of this study is that sexual populations are more genetically diverse than asexual populations, indicating that the dynamics of emergence of asexual lineages in this species is slow enough to limit the allelic diversity of asexual populations. Sexual lineages could benefit

from greater genetic diversity, possibly resulting in better adaptability or weaker competitive interactions compared with asexual lineages. Furthermore our most intriguing result concerns the genetic architecture of asexual populations that showed surprisingly high levels of heterozygosity at microsatellite loci. High heterozygosity in asexual populations of *Rhopalosiphum padi* could result from ancient asexuality, but could also have other origins (for instance interspecific hybridization events). The next step will require a careful investigation of the age and mode of origin of asexual lineages of *R. padi* based on molecular divergence of both nuclear and mitochondrial markers.

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This study forms part of the PhD research of François Delmotte, which addresses the origin and maintenance of asexual lineages in the aphid *Rhopalosiphum padi* using population genetic and molecular phylogenetic approaches. J.-C. Simon and C. Rispe are population biologists interested in evolutionary biology of aphids with special interest in breeding system evolution, dispersal strategies, and the coevolution of aphids with their symbionts and host plants. In this team, N. Leterme is in charge of molecular techniques and J.P. Gauthier develops programs for analysing genetic data.