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Admixed sexual and facultatively asexual aphid lineages at mating sites

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Abstract

Cyclically parthenogenetic organisms may have facultative asexual counterparts. Such organisms, including aphids, are therefore interesting models for the study of ecological and genetic interactions between lineages differing in reproductive mode. Earlier studies on aphids have revealed major differences in the genetic outcomes of populations that are possibly resulting mostly either from sexual or from asexual reproduction. Besides, notable gene flow between sexual and asexual derivatives has been suspected, which could lead to the emergence of new asexual lineages. The present study examines the interplay between these lineages and is based on analyses of population structure of individuals that may contribute to the pool of sexual reproductive forms in the host alternating aphid *Rhopalosiphum padi*. Using a Bayesian assignment method, we first show that the sexual forms of *R. padi* on mating sites encompass two genetically distinct clusters of individuals in the western part of France. The first cluster included unique genotypes of sexual lineages, while the second cluster included facultatively asexual lineages in numerous copies, the reproductive mode of the two clusters being confirmed by reference clones. Sexual reproductive forms produced by sexual and facultatively asexual lineages are thus admixed at mating sites which gives a large opportunity for the two clusters to mate with each other. Nevertheless, this study also highlights, as previously demonstrated, that the two clusters retained high genetic differentiation. Possible explanations for the inferred limited genetic exchanges are advanced in the discussion, but further dedicated investigations are required to solve this paradox.

Keywords: Bayesian assignment, conspecific coexistence, microsatellite, population genetics, reproductive mode, *Rhopalosiphum padi*

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Introduction

A much debated issue about clonal organisms concerns their long-term persistence. While theory concerning the evolution of sex predicts that asexual lineages should ultimately be eliminated because they are slow at combining favourable mutations and fast at accumulating deleterious ones (Muller 1964; Kondrashov 1988; Hadany & Beker 2003), certain organisms are thought to reproduce for millions of years without sex (Judson & Normark 1996; Mark Welch & Meselson 2000; Butlin 2002). However, there is growing evidence for rare sexual reproduction in species that were previously considered entirely asexual (Maynard Smith *et al*. 1993; Burt *et al*. 1996; Normark 1999; Tibayrenc & Ayala 2002). In addition, some plant or animal species show the coexistence of sexual and facultatively asexual taxa that could exchange genetic material (Kondrashov 1997; Tas & van Dijk 1999; Normark 2003; Simon *et al*. 2003). Gene flow between sexual and asexual lineages has two important consequences: first, genetic diversity may increase in asexual populations through capture of genetic variation from the sexual pool; and second, new asexual lineages could be generated from crosses between sexual and essentially asexual populations, in a contagious manner (Hebert 1981; Delmotte *et al*.

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2001; Simon *et al*. 2003). Experimental evidence of this contagious origin of asexual lineages has been obtained in various organisms with coexisting sexual and asexual taxa (e.g. dandelions, van Dijk *et al*. 1999; planarians, Pongratz *et al*. 1998; Storhas *et al*. 2000; daphnia, Innes & Hebert 1988; aphids, Rispe *et al*. 1999). Despite these observations, few attempts have been made to demonstrate the existence of gene flow between sexual lineages and their asexual relatives *in natura* and to quantify its importance.

Among animals, cyclical parthenogenetic organisms provide an interesting model to study the ecological and genetic interactions between lineages differing by their reproductive mode (including their consequences for genotypic variation and gene flow). Indeed, in these organisms, different levels of investment in sexuality often coexist, which follow from various failures to fulfil the sexual part of this life cycle (Hebert 1987; Moran 1992; Hales *et al*. 1997; Gómez & Carvalho 2000; Simon *et al*. 2002). In cyclical parthenogenetic taxa, such as aphids, cladocerans and rotifers, populations reproduce parthenogenetically for at least part of the year. In these species, parthenogenesis is apomictic, that is, barring mutations, the asexual reproduction is strictly clonal (Hales *et al*. 2002; De Meester *et al*. 2004). In cyclical parthenogenetic (sexual) lineages, asexual multiplication alternates with the production of sexual forms, which mate and lay resting eggs. The resting eggs are more resistant than adults to harsh conditions such as desiccation or low temperatures (Leather 1992; Hairston *et al*. 1995). Conversely, obligately parthenogenetic (asexual) lineages continue to reproduce parthenogenetically all year round. But because they do not produce resistant forms, they play a risky strategy which is only favoured when environmental conditions are continuously favourable (Rispe *et al*. 1998). Many asexual lineages are in fact able to produce some males that could interact with sexual lineages as found in aphids (Blackman 1972; Simon *et al*. 1991) and daphnia (Innes & Hebert 1988). Also, intermediate lineages produce both sexual females and males while sustaining parthenogenetic reproduction (Blackman 1972; Hullé *et al*. 1999; Dedryver *et al*. 2001). These genetically determined strategies often coexist within a species, in proportions influenced by the environmental conditions and local adaptation (Gómez *et al*. 1995; Rispe *et al*. 1998; Simon *et al*. 1999a; Halkett *et al*. 2004).

The population genetic structure of cyclical parthenogenetic aphids is well documented. Much research has quantified the genetic differences between reproductive types, to evaluate the relative impacts of sexual vs. clonal reproduction on genetic diversity and population structure, and to assess the gene flow between sexual and facultatively asexual lineages (Simon *et al*. 1999a; Delmotte *et al*. 2002; Guillemaud *et al*. 2003; Miller *et al*. 2003; Papura *et al*. 2003; Vorburger *et al*. 2003). Most studies have been carried out using microsatellite loci, revealing considerable genetic differentiation between sexual and asexual populations, and suggesting limited gene flow between them (Simon *et al*. 1999a; Delmotte *et al*. 2002; but see Vorburger *et al*. 2003). Notably, asexual populations of aphids usually show significant heterozygote excess, unlike the sexual populations (Sunnucks *et al*. 1997; Simon *et al*. 1999a). This particular feature has been further used as a signature of the asexual reproductive mode, in addition to other genetic indices such as frequent departures from Hardy–Weinberg equilibrium (HWE) and linkage disequilibria, low genetic and genotypic diversity, which are more typical of strictly clonal organisms (Delmotte *et al*. 2002; Papura *et al*. 2003; Vorburger *et al*. 2003).

Among aphids, the bird cherry-oat *Rhopalosiphum padi* (L.) has proven a suitable model in investigating the interplay of lineage reproduction by cyclical and obligate parthenogenesis. *R. padi* is a host-alternating aphid: sexual individuals are produced once a year and have to migrate from Poaceae species (e.g. cereals), where all lineages reproduce parthenogenetically in summer, to a woody host, the bird cherry *Prunus padus*, on which sexual reproduction takes place. This host alternation allows the detection of sexual individuals during period of mating and the collection of sexually-derived progeny. By the end of summer, sexual lineages switch to exclusive production of gynoparae (parthenogenetic morphs that give birth exclusively to sexual females) and males. By contrast, asexual lineages do not host-alternate and reproduce strictly parthenogenetically on herbaceous plants all year. Between these extremes, facultatively asexual lineages can either produce both gynoparae and males in the case of intermediate lineages, or produce only males in the case of male-producing lineages, while sustaining parthenogenetic reproduction. Sexual forms, irrespective of categories of parental lineage, migrate from herbaceous to woody hosts. Asexual and facultatively asexual lineages sustain high levels of parthenogenetic multiplication in autumn and overwinter parthenogenetically on herbaceous plants. Combining biological and molecular approaches has revealed that these reproductive types may coexist locally but display substantial genetic differences (Delmotte *et al*. 2001, 2002, 2003). Although some gene flow is suspected between sexual and facultatively asexual lineages of *R. padi*, there is no demonstration that facultatively asexual lineages actually take part in sexual reproduction events in the field.

The aim of this study was to determine whether *R. padi* lineages that differ in their investment in sexual form production may contribute to the pool of sexual individuals that gather on the bird cherry trees and to what extent. This was achieved by using molecular markers and assignment tests to reveal admixture of sexual forms produced by genetically and biologically distinct reproductive lineages that are otherwise indistinguishable by morphological traits.

Materials and methods

Sample collection

For several years, gynoparae and male *Rhopalosiphum padi* were collected at several mating sites located near Rennes (Brittany) in western France. Each site was delimited by a single *Prunus padus* tree. Sexual morphs were collected for 4 years between 1995 and 2001 (Table 1). Except for 1995, the sampling scheme covered much of the period of colonization of *P. padus* by *R. padi* during its sexual phase (from late September to early November). Depending on the year, two to four trees (spaced at least 5 km apart) were visited (Table 1). Males and gynoparae were easily distinguished with a magnifying glass, and stored separately in 95% ethanol. A population sample was then characterized for morph of its individuals (gynoparae or males), individual tree, and year of collection.

In addition to this sampling, 20 laboratory-reared, reference clones characterized for multilocus genotype and reproductive mode (Delmotte *et al*. 2001) were used to further address the link between reproductive mode and genotypic profiles of the clones (Table 2). All reference clones were sampled in France, most of them being from Brittany.

Table 1 Sampling collection scheme. For each year of collection we reported the number of gynoparae and males collected, the number of prospected sites and the number of loci used for the genetic analysis

	Number of			
Year	Gynoparae	Males	Sites	Loci
1995	88	64	2	6
1998	63	46	2	6
2000	93	87	4	6
2001	230	151	2	7

Microsatellite analysis

DNA from individual aphids was extracted using the 'salting out' protocol in Sunnucks & Hales (1996). Microsatellite analyses were performed at six polymorphic loci isolated from *R. padi* (R5.29, R5.10, R1.35, R2.73; Simon *et al*. 2001) or *Sitobion miscanthi* (S16b, S17b; Wilson *et al*. 2004). An additional *R. padi* locus (R5.50; Simon *et al*. 2001) was used for year 2001 samples (Table 1). S17b and R5.50 are X-linked while the other loci are autosomal (Wilson *et al*. 2004; Prunier-Leterme *et al*. unpublished). For all samples

Table 2 Details of the 20 clones of reference used in this study, including the site and the region where they were collected, the reproductive mode inferred from biological experiments, the allele sizes (according to Delmotte *et al*. 2002) and the assignment coefficient *q* to the 'sexual' cluster

				Locus														
Label	Site	Region	Reproductive mode	S17b		R5.29		R5.10		R2.73		S16b		R1.35		R5.50		Value of *q*
a01	Le Rheu	Brittany	male-producing	162	164	161	171	258	270	266	285	151	153	344	359	307	351	0.07
a12	Talensac	Brittany	male-producing	162	164	161	171	258	274	262	285	151	153	344	356	315	351	0.03
a13*	Tilloy	Picardy	intermediate	162	162	171	181	256	274	270	285	151	153	344	345	305	305	0.01
a27	Rennes	Brittany	male-producing	162	164	171	177	258	260	266	285	151	153	344	353	305	353	0.09
a32	Rennes	Brittany	male-producing	162	162	171	181	258	274	270	270	151	153	344	345	305	305	0.01
a34	Betton	Brittany	Asexual	164	168	171	181	258	274	270	285	151	153	344	359	305	329	0.03
a35*	Breteil	Brittany	male-producing	162	162	171	181	258	262	270	285	151	153	344	346	305	305	0.01
a36	Pacé	Brittany	intermediate	162	162	171	171	256	256	285	285	153	153	344	345	305	305	0.01
a37	Le Rheu	Brittany	intermediate	162	164	173	181	260	274	270	285	151	151	345	358	305	305	0.05
a38	Le Rheu	Brittany	male-producing	162	168	171	177	258	274	270	285	151	153	344	359	305	363	0.02
a33	Rennes	Brittany	male-producing	162	164	171	181	258	274	270	270	153	153	344	359	305	371	0.01
h01	Rennes	Brittany	Sexual	164	164	161	197	260	266	262	262	151	151	346	348	317	325	0.99
h02	Rennes	Brittany	Sexual	162	164	177	203	260	260	262	262	151	151	347	359	317	407	0.98
h03	Rennes	Brittany	Sexual	162	164	173	201	262	262	262	262	151	151	343	348	315	349	0.98
h04	Rennes	Brittany	Sexual	164	164	187	205	260	262	266	266	151	151	346	356	313	339	0.99
h05	Rennes	Brittany	Sexual	162	164	177	195	258	262	262	268	151	151	345	345	325	339	0.96
h08	Colmar	Lorraine	Sexual	164	168	181	199	260	274	262	264	151	151	359	359	297	337	0.91
h10	Colmar	Lorraine	Sexual	164	164	163	165	260	274	262	262	151	151	345	353	331	397	0.95
h12	Colmar	Lorraine	Sexual	162	164	163	203	260	260	262	264	151	151	350	356	297	325	0.99
h15	St Amand	Picardy	Sexual	166	170	173	185	262	266	264	270	151	151	345	348	327	327	0.98

*indicates the two genotypes that have also been sampled in our study (a13 corresponds to genotype 2 and a35 to genotype 1).

except for the year 2001, microsatellite amplifications were carried out following Simon *et al*. (2001), and individuals were analysed by automated capillary electrophoresis following Delmotte *et al*. (2002). For samples collected in 2001, forward primers were fluorescently labelled with IRDye™-700/800 (LI-COR Bioscience). Dyes were assigned to loci in a way that loci with the same dye had nonoverlapping ranges of allele sizes. This allowed simultaneous loading of all loci from a given individual. Microsatellite amplifications were carried out following Caillaud *et al*. (2004). The PCR (polymerase chain reaction) products were separated on 0.2 mm thick 7.5% polyacrylamide sequencing gels (8.4 g urea, 3 mL Long Ranger acrylamide (50%), 1X TBE to 20 mL, 225 µL ammonium persulphate (10%) and 22.5 µL TEMED) run on a LI-COR Global IR² two-dye DNA sequencer. A 65–385 bp sizing standard was run along with samples to determine allele sizes. The resulting images were scored using SAGAGT software (LI-COR Biosciences, version 2.1.4). Allele sizes were standardized among and across the two techniques according to Delmotte *et al*. (2002), loading multilocus profiles from reference clones on each run. The genetic data set is available in the supplementary online material.

Genetic data analysis

Data were checked for misprint and scoring errors using MICRO-CHECKER software (van Oosterhout *et al*. 2004). This software also provided a useful framework to test for null alleles in each locus. In the vast majority of aphid species (including *R. padi*), females are XX while males are X0, i.e. males are genetically identical to their mother, except that one of her X-chromosome is missing (Hales *et al*. 2002). Thus two multilocus male types can be produced by a female heterozygous for X-linked loci. For multilocus genotype analysis, males that shared the same allelic combination at their five autosomal loci, but two alternative suites of alleles at X-linked loci, were considered to belong to the same multilocus genotype. We further checked whether the two alternative male types matched a gynopara multilocus genotype. To perform the genetic analyses available for diploid organisms, we considered the second allele of X-linked loci in males as missing data.

Multilocus genotype analysis. *R. padi* reproduces parthenogenetically for at least part of the year, which could result in the spread of identical multilocus genotypes. However, the insufficient power of molecular markers to discriminate between distinct genotypes could lead to independent instances of a genotype arising by sexual reproduction. To assess the likelihood that copies of multilocus genotypes result from sexual reproduction or clonal spread, we used the MLGSIM program developed by Stenberg *et al*. (2003). This program calculates the probability of observing at least n times a multilocus genotype in a specific sample population, given the observed allele frequencies and assuming HWE and linkage equilibria (here n equals the number of copies of the multilocus genotype). This probability is denoted by *P*sex because it is an estimation of the probability that this observation could result from sexual reproduction (Ivey & Richards 2001). Second, using Monte Carlo simulation method, the program defines the significant threshold for the *P*sex values for the sample size and the allele frequencies of the population (Stenberg *et al*. 2003). The strength of this method is thus identifying which multilocus genotypes are statistically overrepresented, assuming panmixia. Such multilocus genotypes with clonal multicopies are referred to here as 'multicopies' or 'multicopy genotypes'. Other multilocus genotypes that could occur in multiple copies are considered to be distinct genotypes. The genotypic diversity index was thereafter calculated as the ratio of the number of distinct genotypes out of the total number of samples (denoted G/N ratio). The G/N ratio is thus a measure of the level of clonal diversity: its values range from zero (all individuals are clonal multicopies of a single genotype) to 1 (all individuals are distinct genotypes). Clonal reproduction leads to strong deviation from panmixia, and the genetic tests based on this assumption were performed using only one copy per multicopy genotype. Distinction is thus made throughout the text between uses of the data set with or without multicopies.

Bayesian clustering analyses. Prior to the ordination analysis of population structure, we applied a Bayesian approach of genetic mixture analysis (STRUCTURE software), developed by Pritchard *et al*. (2000) and further implemented by Falush *et al*. (2003). This method allows the parameters to be estimated separately from the posterior probability distribution of allele frequencies. The two major parameters are (i) K the number of assumed clusters, characterized by the matrices of allele frequencies at each locus; and (ii) q_i the vector of the proportions of individual i's genome that is derived from each cluster (Pritchard *et al*. 2000; Falush *et al*. 2003). Parameter estimation assumes panmixia, and that each locus is at HWE and independent of the others. Nonetheless, this Bayesian approach is robust to some deviations from these assumptions (Falush *et al*. 2003). Simulations were performed using data sets without multicopies. For all simulations, we assumed uninformative priors of q_i (i.e. we do not force the model with predefined allele frequencies for source clusters). Several test runs were performed to evaluate the convergence time for the simulations. Convergence seemed to occur quite rapidly, and iteration parameters were set to a burning-in period of 60 000 iterations followed by 600 000 iterations. Five independent simulations were performed to test for the consistency of the results.

Table 3 Abundance (percentage of the total number of individuals) and proportion of males of the most frequent multicopy genotypes. For the sake of clarity, only multicopy genotypes sampled more than three times have been detailed. Abundance of multicopy genotypes that are represented by only two or three individuals is summarized in the column 'Rare mcg'. The percentage of multilocus genotypes represented by only one individual (Solitary mlg) was also reported to allow comparison with the most frequent multicopy genotypes. Proportion of males is calculated as the ratio of the number of males over the total number of individuals sampled for each multicopy genotype

	Genotype																Rare	Solitary
	1*	2*	3	4	5	6	7	8	9	10	11	16	17	19	21	25	mcg	mlg
Abundance																		
1995	—	86.2	—	—	—	—	—	—	0.7	—	—	—	—	—	—	—	—	13.2
1998	2.8	39.4	—	—	—	—	1.8	—	—	0.9	—	—	—	0.9	0.9	0.9	1.8	50.5
2000	2.2	28.9	0.6	—	—	0.6	2.8	0.6	—	—	1.7	—	—	2.8	0.6	0.6	1.1	57.8
2001	8.9	15.5	4.2	3.7	1.8	3.1	7.3	1.8	1.0	1.3	5.5	3.7	1.6	2.9	1.8	3.1	3.7	28.9
Mean	5.0	34.7	2.1	1.7	0.9	1.6	4.3	1.0	0.6	0.7	2.9	1.7	0.7	2.1	1.1	1.7	2.2	35.2
Proportion of males	1	0.32	0.25	0.64	0.29	0.83	0.46	0.57	0.50	0.60	0.38	0.57	0.50	0.82	0.29	0		

*indicates the two multicopy genotypes that have previously been sampled in other studies.

Standard population genetic analyses. Because inclusion of clonal multicopies can strongly distort estimates of heterozygosity and other *F*-statistics, population genetic tests were performed without multicopies (Sunnucks *et al*. 1997). Population genetic analysis was performed using GENEPOP version 3.4 (Raymond & Rousset 1995a) implemented on the web (http://wbiomed.curtin.edu.au/genepop/). Allele frequencies and unbiased expected heterozygosity (H_E) were calculated according to Nei (1978). Global linkage disequilibrium between pairs of loci and deviation from HWE expectation (heterozygote excess and deficit) were carried out using the exact tests in GENEPOP (Garnier-Gere & Dillman 1992; Rousset & Raymond 1995). Unbiased estimates of F_{IS} and F_{ST} across loci were calculated according to Weir & Cockerham (1984).

Population differentiation. In order to take account of the spread of clones, which is an important structuring factor of parthenogenetic populations, allele frequencies were estimated using data sets with multicopies. Genic differentiation was computed using Fisher's exact tests in GENEPOP (Raymond & Rousset 1995b), which is the most appropriate method to analyse population differentiation, although it lacks analysis of variance (Balloux & Lugon-Moulin 2002). Combinations of *P*-values across loci were performed according to Fisher's method.

Results

Analysis of multilocus genotype occurrence

Out of the 822 aphids tested, we found 315 distinct six-locus genotypes (G/N = 0.38). For the samples caught in 2001, the addition of a seventh locus (R5.50) did not increase the number of genotypes discriminated. Overall, there were 290 multilocus genotypes represented by only one individual; the remaining 532 individuals sharing only 25 multilocus genotypes. The over-representation of all these multilocus genotypes resulted from clonal amplification according to the highly significant *P*sex values in the MLGSIM analysis. This is in accordance with the well-known high resolving power of microsatellite loci for identifying aphid clones (Sunnucks *et al*. 1997; Haack *et al*. 2000; Delmotte *et al*. 2002). No locus showed evidence of null alleles. Allelic richness ranged from 4 to 40 alleles (mean of 17). Among multicopy genotypes, nearly half of them (12) were sampled several times for 2–4 years (Table 3) but only the two most frequent genotypes (genotype 1 and 2, Table 3) had been detected in previous surveys (Delmotte *et al*. 2002). Multicopy genotype 2 was the most frequent, representing 35% across all samples. It was the only multicopy genotype sampled each year in this survey. Interestingly, while the frequency of genotype 2 showed an abrupt decline from 86% of the aphids sampled in 1995 to 15% in 2001 (Table 3), the frequency of genotype 1 increased during the same period from 0 to 22% of the males sampled. Other multicopy genotypes were much less frequent (Table 3) and mostly found in 2001, probably as a result of a higher sampling effort this year (Table 1).

Bayesian clustering analyses

The assignment tests were first performed using the 7-loci data set without multicopies, i.e. all genotypes sampled in 2001. Results of the inference of *K*, the number of clusters, clearly indicated that the posterior distribution of the allele

Table 4 Inference of the number of cluster(s) (*K*) that best explains the population structure (allele frequencies distribution) of the 7-loci data set (without multicopies). For each tested value of *K*, we reported the log-likelihood of the simulations (i.e. the log-likelihood of observing the posterior distribution of the allele frequencies given the number of clusters) and the probability that the simulated number of cluster(s) matches the data set. Both values were averaged across five simulation runs

K	Log-likelihood	Matching probability
1	–3423	~0
2	–3243	~1
3	–3583	~0
4	–3502	~0
5	–3459	~0

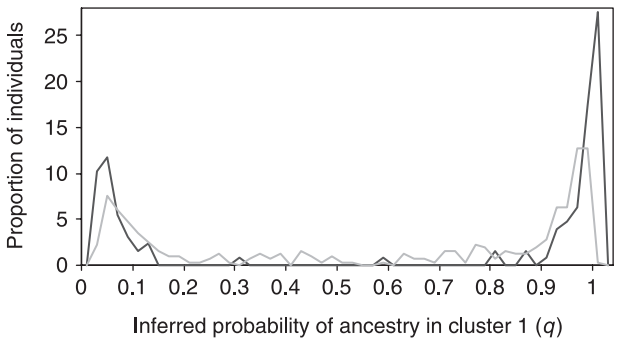

Fig. 1 Distribution of the mean value of *q* (the probability of ancestry in cluster 1) calculated over five simulation runs performed on the seven loci data set without multicopies (126 multilocus genotypes, dark grey) and on the whole data set without multicopies (315 multilocus genotypes, light grey).

frequencies among clusters was best explained when *K* equals 2 (Table 4). The posterior distribution of *q* for the individuals of the 2001 sample was clearly bimodal (Fig. 1, dark grey line), indicating that the two clusters were highly divergent (below). Assuming an arbitrary threshold of $q = 0.9$ for assignment to cluster 1 (0.1 for cluster 2), about 93% of the distinct multilocus genotypes were assigned to one cluster. This assignment coefficient dropped to 70% when using only six loci (without R5.50 — data not shown). A second analysis was performed on the whole data set (six-loci data without multicopies) and gave similar results: the posterior distribution of *q* was still bimodal but flatter (Fig. 1, light grey line), and the assignment coefficient equalled 66%. Including clonal multicopies, 85% of the total number of aphids sampled were assigned to a cluster ($q = 0.9$ threshold).

Putative reproductive mode of the multilocus genotypes

A third assignment analysis was performed using the multilocus genotypes from the year 2001 (seven-loci data without multicopies) and the microsatellite data from the clones of reference (Table 2). Interestingly, the reference clones were clustered unambiguously according to their reproductive mode (Table 2). All sexual (cyclically parthenogenetic) reference lineages were assigned to cluster 1. Conversely, all known facultatively (male-producing and intermediate) or fully asexual (obligately parthenogenetic) lineages were assigned to cluster 2. Considering data from field collections, all multicopy genotypes belonged to cluster 2. Most of them have been sampled in different years, implying they overwinter parthenogenetically and are therefore facultatively asexual clones. Barring genotype 1 and 25, all these multilocus genotypes were sampled at least once as male and gynoparae (Table 3), suggesting they could be intermediate lineages. This is supported for genotype 2, which shared the same multilocus profile as a13, an intermediate clone (Table 2). Although genotype 1 was the second most frequent clone in this survey (representing almost one male out of five in 2001), no gynopara bearing this genotype was found (Table 3). This suggests that genotype 1 is a male-producing lineage. The fact that it shared the same genotype as a35, also male-producer, supports this view (Table 2). Therefore, the good agreement between reproductive mode and multilocus genotype clustering suggests that cluster 1 mostly contains sexual lineages while cluster 2 mostly includes facultatively asexual ones.

Genetic properties of the two clusters of individuals

To investigate the relevance of the clustering results, the genetic characteristics of each cluster were further investigated (Table 5). This and succeeding sections, apply to 85% of the total number of individuals assigned to a cluster ($q < 0.1$ or $q > 0.9$). Of the 822 individuals typed in this study, 576, which share only 88 multilocus genotypes, belonged to the 'facultatively asexual' cluster (G/N = 0.15). By contrast, all multilocus genotypes from the 'sexual' cluster were represented by only one individual across the samples (G/N = 1). The absence of multicopy genotypes in the 'sexual' cluster strongly suggests that the asexual phase between rounds of sexual reproduction is too short for preferential multiplication of a few genotypes (i.e. weak clonal selection), a result in accordance with previous studies on cyclically parthenogenetic organisms. Subsequent analyses (standard population genetic tests) were carried out without multicopies (66% of the multilocus genotypes); i.e. 141 multilocus genotypes for the 'sexual' cluster and 88 multilocus genotypes for 'facultatively asexual' cluster (Table 5). The 'sexual' individuals were at HWE, and F_{IS} across loci was close to zero. Conversely, 'facultatively asexual' multilocus genotypes showed a strong heterozygote excess, associated with large negative values of F_{IS} that fluctuated over loci.

Table 5 Genetic features of the two clusters of individuals revealed by the assignment tests. Population genetic tests were performed using all multilocus genotypes assigned to a cluster ($q < 0.1$ or $q > 0.9$) without clonal multicopies*. G/N: genotypic diversity index, H_O: observed heterozygosity, H_E: expected heterozygosity, L. D. number of pairs of significant linkage disequilibrium

Statistics	Sexual ($n = 141$)	Facultatively asexual ($n = 576$)
No. of distinct genotypes	141	88
G/N ratio	1.000	0.153
mean No. of alleles	11.2	8.0
Mean H_O	0.691	0.761
Mean H_E	0.684	0.657
Heterozygote excess	ns	s
Heterozygote deficit	ns	ns
L. D.	0/15	6/15
No. of private alleles	16	10
F_{IS} per locus		
R5.29	−0.015	−0.176
R5.10	−0.003	−0.100
S16b	−0.007	−0.131
R1.35	−0.031	−0.252
R2.73	0.000	−0.095
S17b	0.050	−0.160
R5.50*	−0.099	−0.318
F_{IS} multilocus	−0.011	−0.160

*The F_{IS} value at the locus R5.50 was estimated using multilocus genotypes sampled in 2001 (without multicopies), i.e. 77 multilocus genotypes for the 'sexual' cluster and 41 multilocus genotypes for 'facultatively asexual' cluster. All global statistics (including the F_{IS} multilocus value) were estimated without taking account of R5.50.

Genetic differentiation between the two clusters of individuals

In addition to the high divergence in the genetic properties of the 'sexual' and 'facultatively asexual' clusters, there was large genetic differentiation between the two: F_{ST} value equalled 0.116 and distributions of allele frequencies across loci differed significantly [Fisher's exact test on the same data set as in previous section without multicopies, $P < 0.0001$ (Raymond & Rousset 1995b)]. One third of the total number of alleles were found in only one of the two clusters and about two thirds (62%) of these alleles were restricted to the 'sexual' cluster. Moreover, detailed examination of population differentiation within year or within site revealed that all pairwise genic differentiation tests between 'sexual' and 'facultatively asexual' individuals (males and gynoparae pooled) were significant [Fisher's exact tests performed with and without multicopies, $P < 0.0001$ (Raymond & Rousset 1995b)]. No specific pattern that could account for gene flow between clusters can thus be assessed. Besides, it is noteworthy that allele frequencies between males and gynoparae did not differ significantly in the 'facultatively asexual' ($P = 0.93$), nor in the 'sexual' cluster ($P = 0.20$).

Relative abundance of the two clusters over sites and years

Considering that two highly divergent clusters of individuals colonized the *P. padus* trees, we wanted to know whether 'facultatively asexual' and 'sexual' individuals were spread over populations and trees or not. In order to answer this question, the frequencies of the individuals belonging to the 'sexual' and 'facultatively asexual' clusters were calculated within each population, defined as all individuals sharing the same sexual morph, collected the same year on the same tree. For these analyses, 85% of the sampled individuals that were assigned to one cluster was used (see previous discussion). All populations, except those collected in 1995, included both 'sexual' and 'facultatively asexual' individuals. In 1995, all individuals were assigned to the 'facultatively asexual' cluster, maybe (i) because they were collected at a single date this year, in contrast the other years (see Materials and methods) and (ii) because of a peculiar population composition in 1995. Focusing on gynoparae, populations showed little differences in the relative proportion of sexuals between sites (except one population in 2000), but large fluctuation between years (Fig. 2a). This observation is supported by population differentiation results: only one site in 2000 (the same that lies apart in Fig. 2) differed significantly from the others ($P < 0.05$, comparison within a year). Results are less clear-cut for males, which exhibited large differences in the relative proportions between sites, especially for year 2000 (Fig. 2b). For both gynoparae and males, all but one pair of samples differed between years (comparison within a same site).

Discussion

Admixture of sexual and facultatively asexual lineages on mating sites

In the present study, we investigated the population genetic structure of a host-alternating aphid during its sexual phase, which occurs between rounds of parthenogenetic generations. Careful examination of genotypic composition with Bayesian clustering analyses revealed that sexual individuals collected on *Prunus padus* belonged to two genetically distinct clusters. These two clusters were further assigned to opposed reproductive modes (i.e. sexual vs. facultatively asexual lineages) based on several lines of evidence. First, reference clones of known reproductive mode were placed correctly into the two multilocus genotype clusters. Second, the two most common multilocus genotypes belonging to the 'facultatively asexual'

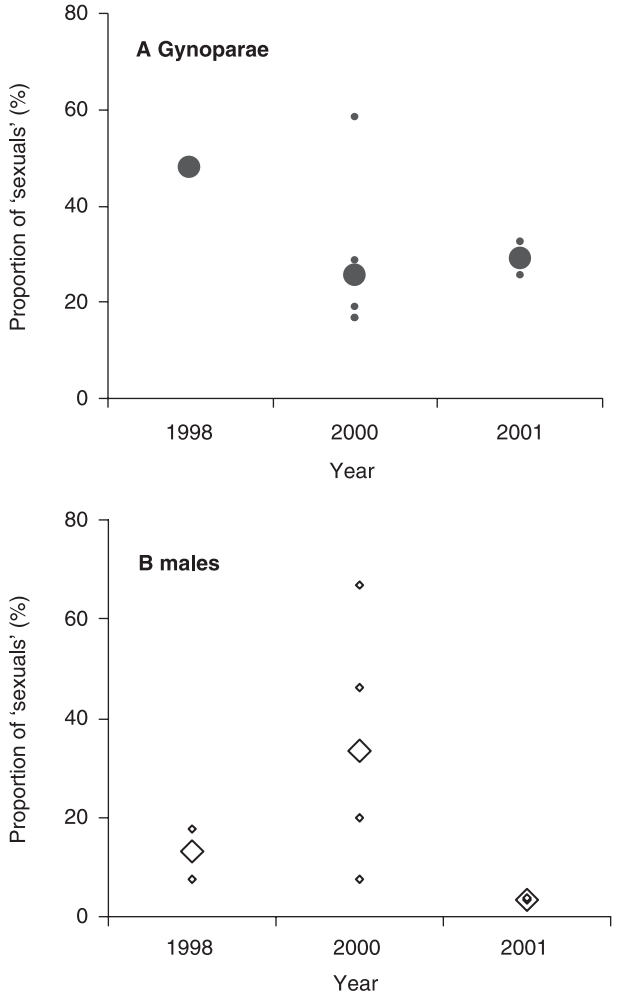

Fig. 2 Relative proportions of 'sexual' individuals among populations (multicopies included) of *R. padi* on *P. padus* sampled across years and sites. The mean proportions of sexual individuals found each year are represented by big dots; single site proportions for a given year are represented by small dots. For gynoparae, all dots are confounded for the year 1998.

cluster have been previously found to overwinter parthenogenetically on cereal fields in different years (Delmotte *et al*. 2002), indicating that these genotypes were able to invest in both sexual and parthenogenetic reproduction at the same time (i.e. they have a mixed reproductive strategy, Rispe & Pierre 1998; Dedryver *et al*. 2001; Halkett *et al*. 2004). Third, the genetic features of the two clusters of individuals gave additional insights into their putative reproductive modes: (i) the 'sexual' cluster encompassed individuals with distinct multilocus genotypes in contrast to the 'facultatively asexual' cluster which included many multicopy genotypes and (ii) HW and linkage disequilibria were absent in the 'sexual' cluster while they were significant in the 'facultatively asexual' one. These characteristics are typically seen when comparing the genetic attributes of sexual and asexual populations in general (e.g. Tibayrenc & Ayala 2002; Balloux *et al*. 2003; Bengtsson 2003) and in aphids in particular (e.g. Delmotte *et al*. 2002; Papura *et al*. 2003; Vorburger *et al*. 2003). All these results clearly indicated that sexual and facultatively asexual lineages of *R. padi* are admixed on *P. padus* at the time of sexual reproduction.

Importance of the intermediate strategy at mating sites

Surprisingly, the 'sexual' cluster encompassed only one fifth of the aphids sampled, while the remaining individuals belonged to the 'facultatively asexual' cluster. This latter cluster seems biologically more heterogeneous than the 'sexual' cluster because it gathered all the reproductive types that do not have a complete shift from clonal to sexual reproduction (i.e. asexual, male-producing and intermediate lineages). However, most of the multicopy genotypes belonging to the 'facultatively asexual' cluster were sampled as gynoparae and males, indicating they were intermediate lineages. Male-producing clones of *R. padi* have already been found to be common among individuals that overwinter parthenogenetically in Brittany (Simon *et al*. 1991; Hullé *et al*. 1999). However, the present study suggests that male-producing clones are uncommon at mating sites, which is in accordance with earlier work based on mitochondrial markers (Martinez-Torres *et al*. 1997). Thus, intermediate lineages, in the conditions of western France, appear to make the major contribution to the pool of sexual reproductive individuals, even more than sexual lineages. The predominance of intermediate lineages is in agreement with theoretical models predicting that this strategy should prevail in areas with an unpredictable winter climate, such as in Brittany, where it pays to invest in both sexes (and to produce frost-resistant eggs), while retaining the ability to produce parthenogenetic individuals (Rispe & Pierre 1998; Halkett *et al*. 2004). In more continental areas with regular harsh winters, the sexual strategy is predicted to perform better (Rispe *et al*. 1998; Rispe & Pierre 1998). These predictions have already been validated in another cereal aphid *Sitobion avenae*, while intermediate lineages predominated in western and northern France (mild or moderately cold winters), they were absent in Romania (cold winters) where sexual lineages prevailed (Dedryver *et al*. 2001; Papura *et al*. 2003). The occurrence of intermediate lineages of *R. padi* has not yet been quantified *in natura*, and this survey is the first demonstration of its abundance and persistence through years.

Between year variation

Changes detected in the genetic composition of sexual forms of *R. padi* through years lead to two a priori exclusive

conclusions. Although genotypes that were found in high copy numbers showed persistence through time, populations were strongly differentiated between years. Multiple samplings of the same multilocus genotype is a common feature of aphid populations (Sunnucks *et al*. 1997; Fenton *et al*. 1998; Fuller *et al*. 1999; Simon *et al*. 1999a; Haack *et al*. 2000; Vorburger *et al*. 2003), some of them being sampled across countries (Llewellyn *et al*. 2003). This is resulting from the high reproductive rate of parthenogenetic reproduction associated with the persistence of parthenogenetic forms during winter (Haack *et al*. 2000; Vorburger *et al*. 2003). As a result, the relative frequencies of such clones fluctuate through time (Haack *et al*. 2000; Llewellyn *et al*. 2003), and this might account for the observed population differentiation between years. However, even after the removal of multicopies, populations still differed significantly among years ($P < 0.001$). Conversely there was no between-year variation when clusters were tested separately ($P = 0.22$ for the 'sexual' cluster and $P = 0.18$ for the 'facultatively asexual' cluster). These differences thus result from interannual fluctuations in the relative proportions of 'sexual' vs. 'facultatively asexual' individuals. Because these two clusters differ in their overwintering strategies, their relative abundance is determined mainly by climate (Rispe *et al*. 1998; Halkett *et al*. 2004). Fluctuation of winter severity between years may influence the local survival of 'facultatively asexual' individuals, leading to population genetic differentiation between years but within a site (Llewellyn *et al*. 2003).

Differences in heterozygosity between sexual and facultatively asexual lineages

A striking difference between sexual and facultatively asexual lineages was found in their respective heterozygosity levels. 'Sexual' populations were at HWE, whereas 'facultatively asexual' populations showed significant heterozygote excess. This discrepancy has already been noted in *R. padi* (Delmotte *et al*. 2002; but here, authors compared a priori sexual and asexual populations based on whether they overwinter as eggs or as parthenogenetic individuals) and was also recorded for other aphids (*S. avenae*: Sunnucks *et al*. 1997; Simon *et al*. 1999a; Papura *et al*. 2003; *Myzus persicae*: Guillemaud *et al*. 2003; Vorburger *et al*. 2003; *Pemphigus bursarius*: Miller *et al*. 2003).

Theoretically, an increase in heterozygosity is expected in strictly clonal organisms as a result of the independent accumulation of mutations on the two sets of the chromosomes that never recombine under strict clonal reproduction (Birky 1996; Mark Welch & Meselson 2000). This mechanism has recently been documented in the ancient asexual Bdelloid rotifers (Mark Welch & Meselson 2000). Mutation accumulation could also apply to aphids that undergo long-term parthenogenesis as demonstrated in both experimental and natural asexual lineages (Downie 2003; Vorburger *et al*. 2003; Wilson *et al*. 2003). In addition, it has been demonstrated that some asexual lineages of *R. padi* had a hybrid origin, which could better account for their heterozygote excess (Delmotte *et al*. 2003).

In a recent study, Balloux *et al*. (2003) analysed the population genetics of simulated populations with various rates of clonal reproduction. The main outcomes of their analyses were that (i) multilocus F_{IS} value only becomes substantially negative at high clonal rate ($c > 0.99$, Fig. 1, Balloux *et al*. 2003); and (ii) for very limited sexual rate, F_{IS} values show large variance between loci (Fig. 2, Balloux *et al*. 2003). Both predictions match the present data set, suggesting that 'facultatively asexuals' mostly reproduce asexually, and that there is a very limited input from sexual reproduction episodes to the 'facultatively asexual' cluster.

Maintenance of genetic differentiation between sexual and facultatively asexual lineages

Nevertheless, an apparent paradox emerges from our study: although sexual and facultatively asexual lineages of *R. padi* were admixed during sexual reproduction and can therefore mate together, there was strong genetic differentiation between the two clusters. Several factors could act to prevent or limit actual gene exchanges. First, sexual forms produced by facultatively asexual lineages may not be functional as it occurs in some asexual taxa that occasionally produce some males (e.g. brine shrimps *Artemia* Browne 1992). Nevertheless, this does not seem to be the case in *R. padi* as (i) gynoparae produced by intermediate clones reared in the laboratory (e.g. clone a13) lay viable offspring (Hullé *et al*. 1999; Bonhomme, personal communication); and (ii) crosses between sexual lineages and facultatively asexual lineages (either intermediate or male-producing) result in viable offspring from new parthenogenetic lineages (Simon *et al*. 1999b). Therefore, it is necessary to invoke other factors preventing gene flow between reproductive types. Such barriers can be influential prior to egg formation (prezygotic barriers of isolation, e.g. mating preferences for the same reproductive type), or thereafter (postzygotic barriers, e.g. lower viability of hybrids), and have both been proven to act on population specialization in another aphid species (Caillaud & Via 2000). However, this hypothesis can not be tested with the present data set, and rather requires dedicated studies such as natural parentage analyses of offspring resulting from the sexual reproduction episode.

Alternatively, genetic differentiation could be maintained despite gene flow between sexual and facultatively asexual lineages, e.g. because of the different turnover of clones. Indeed, sexual lineages have to undergo sexual recombination each year, whereas facultatively asexual individuals (especially those multicopy genotypes that

are very abundant) perpetuate genotypes that, barring mutations, have been 'frozen' since the birth of the parthenogenetic lineage. Assuming those lineages result from sexual reproduction that took place some time ago, the large fluctuations in allele frequencies between years could, at least partially, explain the genetic differentiation between the two clusters.

Conclusion

Based on several lines of evidence, this study demonstrated that lineages that deeply differ in their reproductive mode (sexual vs. facultatively asexual) were admixed at mating sites in western France. Furthermore, facultative asexuals (especially the intermediate) seemed to make the major contribution to the pool of sexual individuals. These individuals could reasonably be suspected to be functional and to lay viable offspring. Consequently, primary conditions for establishing gene flow between these two types of lineages are met. This is the first clear evidence for at least potential genetic interactions between aphid lineages differing in their rate of sexuality in the field. In addition to the large differences in the genetic characteristics between the 'sexual' and the 'facultatively asexual' clusters, this study highlighted the strong genetic differentiation between these two. The next steps would require (i) a careful investigation of the actual genetic contribution of each reproductive type to the offspring following the sexual reproduction event; and (ii) a precise evaluation of the inheritance of the different reproductive modes through a sexual reproductive episode.

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