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PHYLOGENETIC EVIDENCE FOR HYBRID ORIGINS OF ASEXUAL LINEAGES IN AN APHID SPECIES

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Abstract.—Understanding the mode of origin of asexuality is central to ongoing debates concerning the evolution and maintenance of sexual reproduction in eukaryotes. This is because it has profound consequences for patterns of genetic diversity and ecological adaptability of asexual lineages, hence on the outcome of competition with sexual relatives both in short and longer terms. Among the possible routes to asexuality, hybridization is a very common mechanism in animals and plants. Aphids present frequent transitions from their ancestral reproductive mode (cyclical parthenogenesis) to permanent asexuality, but the mode of origin of asexual lineages is generally not known because it has never been thoroughly investigated with appropriate molecular tools. *Rhopalosiphum padi* is an aphid species with coexisting sexual (cyclically parthenogenetic) and asexual (obligately parthenogenetic) lineages that are genetically distinct. Previous studies have shown that asexual lineages of *R. padi* are heterozygous at most nuclear loci, suggesting either that they have undergone long-term asexuality (under which heterozygosity tends to increase) or that they have hybrid origins. To discriminate between these alternatives, we conducted an extensive molecular survey combining the sequence analysis of alleles of two nuclear DNA markers and mitochondrial DNA haplotypes in sexual and asexual lineages of *R. padi*. Both nuclear and cytoplasmic markers clearly showed that many asexual lineages have hybrid origins, the first such demonstration in aphids. Our results also indicated that asexuals result from multiple events of hybridization between *R. padi* and an unknown sibling species, and are of recent origin (contradicting previous estimates that asexual *R. padi* lineages were of moderate longevity). This study constitutes another example that putatively ancient asexual lineages are actually of much more recent origin than previously thought. It also presents a robust approach for testing whether hybrid origin of asexuality is also a common phenomenon in aphids.

Key words.—Allele sequence divergence, aphids, evolution of sex, hybridization, parthenogenesis, *Rhopalosiphum padi*.

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Sexual reproduction is the dominant mode of reproduction in multicellular life. Asexual reproduction is generally regarded as an evolutionary dead end, owing to deleterious mutation accumulation and because low variability is expected to lead to low evolutionary potential (e.g., Hughes 1989). Most asexuals indeed occupy phylogenetically recent positions. Only two ancient asexual groups that would represent clear exceptions are known: darwinulid ostracods and bdelloid rotifers. But even these have been controversial (Judson and Normark 1996; Little and Hebert 1996; Schön et al. 1998; Mark Welch and Meselson 2000). Few other lineages apparently persist for long periods with little or no sex. Nonetheless, asexual reproduction has clear short-term evolutionary advantages over sexual reproduction, including major demographic advantages, the ability to colonize with only one female, and the possibility for amplification of coadapted gene combinations (in aphids, see Hales et al. 1997, Simon et al. 2002).

Given the apparently overwhelming immediate advantages of asexual reproduction, it remains obscure how sex persists in the short term. Insights may arise by understanding the speed at which asexual lines suffer the deleterious consequences of asexuality. For example, lineage selection is more likely to favor sexuals if asexuals experience costs rapidly (Nunney 1989), and it is possible that asexuals experience deleterious effects so quickly that their short term demographic advantages are overwhelmed (e.g., Dunbrack et al. 1995). Costs of asexuality may be realized very quickly under ''Kondrashov's hatchet,'' a model in which deleterious mutations have strong synergistic effects (Kondrashov 1988). In fact, the mechanisms that generate asexual lineages in natural populations play a key role in determining the balance between costs and benefits of asexuality. For example if parthenogenesis were rare and sufficiently difficult to evolve, the long-term benefits of sexuality may be more likely to be realized (Nunney 1989). In addition, different modes of origin of asexuality are expected to have different effects on long-term evolutionary potential of asexual clones because mode has important consequences for levels of genetic diversity, ecological adaptability, and the outcome of competition with sexual relatives (Howard and Lively 1994, 1998; Butlin et al. 1999; Law and Crespi 2002). Indeed, if genetic systems maintaining sexuality are labile, asexual lineages will emerge repeatedly, leading to a pool of diverse, polyphyletic asexual lineages. Under these circumstances, the advantage of sex should be reduced both under soft (competitive) selection (e.g., Tangled Bank hypothesis) and under frequency–dependant selection (e.g., Red Queen hypothesis), which require only genotypic diversity rather than sex and recom-

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bination. It is notable that most asexual taxa are polyphyletic at the species or the genus level; examples include fishes, snails, ostracods, cladocerans, and insects (Hebert et al. 1989; Normark 1996; Vrijenhoek 1998; Johnson and Bragg 1999; Schön et al. 2001; Law and Crespi 2002).

Cyclically parthenogenetic organisms including aphids present frequent transitions to permanent asexuality, and these events represent valuable biological cases for investigating evolution of sex (Normark 1999; Simon et al. 2002). Asexuality is a derived character in aphids and reversion to sexuality is unlikely (Moran 1992). Moreover, transitions to asexuality in aphids are not confounded with changes in ploidy levels as in many other groups of organisms. Despite the potential of aphid models in this regard and the importance of understanding the frequency and mode of transitions to asexuality, little is known about the mode of origin of asexual aphids. In contrast to many parthenogenetic taxa, asexual aphids are not known to be formed by hybridization (Simon et al. 1996; Hales et al. 1997), but this issue has never been investigated in aphids with appropriate molecular genetic tools. In particular, allozymes are notoriously invariant in the group (Hales et al. 1997), whereas these markers have been central in detecting parthenogenesis by hybridization in groups such as cladocera (Hebert et al. 1993; Taylor and Hebert 1993) or in snails (Johnson and Bragg 1999). Interestingly, on at least one of the rare occasions when hybridization was detected using DNA molecular markers in aphids (*Sitobion avenae* and *S. fragariae*), hybridization had not been detected by variable allozymes even though the pure parent lineages are differentiated at several loci (De Barro et al. 1995; Sunnucks et al. 1997). Given that allozymes can apparently be under strong selection for homozygosity in parthenogenetic aphid lineages (Hales et al. 1997; Delmotte et al. 2002), it may be that these markers are not appropriate for seeking evidence of a hybrid origin of asexual aphids.

Among aphids, *Rhopalosiphum padi* has been the subject of extensive biological and molecular studies that have allowed the characterization of two major reproductive lineages: (1) 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 Poaceae are followed by a single annual sexual generation in autumn on *Prunus padus*, with production of cold resistant eggs); and (2) asexual (obligately parthenogenetic) lineages that live year-round on Poaceae although most of them retain the ability to produce males in autumn (Simon et al. 1991). Previous studies based on nuclear (allozyme and microsatellite) and mitochondrial DNA (cytochrome *b*) markers have revealed two important aspects of the evolution of reproductive modes in *R. padi* (Simon et al. 1999a; Delmotte et al. 2001). First, they have shown that asexual lineages of this aphid were polyphyletic: microsatellite and mitochondrial data revealed at least three independent origins of asexuality in *R. padi*. Second, they have revealed that there are two types of asexual lineages, some with a nuclear and mitochondrial profile similar to sexual ones (e.g., they have the same mtDNA, haplotype II—denoted hII) and some with a genetic profile very distinct from sexual ones (e.g., they have a very distinct mtDNA, haplotype I—denoted hI). We have demonstrated that the first type of asexual lineages could result from two mechanisms: first, a complete spontaneous loss of sex such as would occur through mutations along the pathways leading to production of sexual forms and successful meiosis. Second, these asexual lineages could result from ''contagious'' transmission of alleles causing parthenogenesis, arising when males produced by essentially asexual lineages mate with females of sexual lineages, leading to sexual lineages that have inherited genes of asexuality (Delmotte et al. 2001). However, the mode of origin of the vast majority of hI asexual lineages could not be elucidated with the available molecular data. Our main focus in this study was to analyze the phylogenetic relationships between sexual lineages, hII asexual lineages and hI asexual lineages, with the purpose of uncovering the mechanism(s) leading to hI asexual lineages and in the process examining whether asexual lineages of *R. padi* are of ancient origin. Previous genetic surveys have shown that asexual lineages of *R. padi* display high levels of heterozygosity in neutral nuclear genetic markers (Simon et al. 1999a, Delmotte et al. 2001, 2002). Two alternative hypotheses could account for this pattern. On one hand, asexual aphids could have undergone long-term asexuality, which can lead to accumulation of neutral heterozygosity in aphids (Simon et al. 1999b; Wilson et al. 1999; Delmotte et al. 2002). On the other hand, high heterozygosity in asexual aphids is readily explained if they result from hybridization between differentiated lineages. If proven for *R. padi*, it would represent the first demonstration of hybridization leading to the loss of sex in aphids.

With the aim of discriminating between these two hypotheses, we conducted a test of molecular divergence of nuclear and mitochondrial markers. The test consisted of a comparison of allelic divergence within and among sexual and asexual taxa. If asexual lineages are old, we expect each allele at most neutral loci in an asexual lineage to have its nearest relative in another asexual lineage (Birky 1996, Mark Welch and Meselson 2000). Moreover, alleles and mitochondrial haplotypes in the sexual lineages from which asexual clones are derived might also be very divergent from those in the asexual lineages. In contrast, when hybridization leads to asexuality, hybrid asexual lineages will inherit half of their nuclear alleles from each of two separate lineages capable of producing sexual forms, and will inherit cytoplasmically transmitted organelle and symbiont haplotypes from the maternal lineage (the aphid obligatory endosymbiont *Buchnera* being strictly maternally inherited, Moran and Baumann 1994). Here, we report the results of an extended phylogenetic survey of sexual and asexual lineages of an aphid species based on sequence of two nuclear markers and a mitochondrial marker (cytochrome *b*). Our results strongly support the contention that hybridization is the major mode of origin of asexual lineages in the aphid *R. padi*.

Materials and Methods

Aphid Samples

We used a subset of samples of *R. padi* ($n = 950$) analyzed in three earlier molecular studies (Martinez-Torres et al. 1997; Simon et al. 1999a; Delmotte et al. 2001). Aphid sampling was done in the early spring when sexual and asexual

TABLE 1. Pooled samples (n = 950) of three earlier studies (Martinez-Torres et al. 1997; Simon et al. 1999a; Delmotte et al. 2001) on *R. padi* allowed the discrimination between several main genetic variants in sexual and asexual linages based on nuclear (SCAR) and mitochondrial (cyt. *b*) markers and the estimation of their frequencies. The putative origin of hI and hII lineages is given following previous conclusions of Delmotte et al. (2001). A subset of these samples (n = 314) have been typed at seven microsatellite loci and gave very close estimates of the relative abundances of these genotypes excluding clonal amplification (Delmotte et al. 2002).

mtDNA	SCAR	% abundance within life cycle	Representative lineages	Putative origin of lineages
Asexual lineages				
hI	192/201	79.3	150, 168, 325, 358	Unknown (ancient loss of sex or hybridization)
hII	192/201	13.3	1, 2, 35, 171, 188	Gene flow between sexual and asexual lineages
hII	192/192	7.4	271, 327, Ker	Spontaneous loss of sex
Sexual lineages				
hI	192/201	0	—	—
hII	192/201	4.5	B10, F15	Gene flow between sexual and asexual lineages
hII	192/192	95.5	N10, N27, C17, E30, M8, P1	Typical sexual lineages of *R. padi*

lineages of this aphid are clearly separated on different host plants: asexual lineages were sampled on Poaceae (mainly cereals) while sexual lineages were collected on *P. padus* after hatching from eggs and before the onset of parthenogenetic reproduction. The same previous studies allowed the discrimination between several main genetic variants in sexual and asexual lineages based on nuclear (SCAR) and mitochondrial (cyt. *b*) markers (Table 1 and see below). Among these, we chose 20 French lineages representative of the main types of sexual and asexual lineages of *R. padi*. To this, we added three *R. padi* clones collected in Kenya, La Réunion island, and China (Tables 1, 2) for which the reproductive mode is unknown.

Putative Origins of Asexual Lineages

A few asexual lineages (7.4%) have a genetic profile at nuclear (based on the SCAR and several microsatellite loci) and mitochondrial genomes identical to sexual lineages, which was interpreted as spontaneous, recent loss of the sex-

TABLE 2. Name, population of origin, collection date, mitochondrial haplotype (mtDNA), SCAR, and R5.29 allele sizes for 12 asexual lineages (collected on cereals) and eight sexual lineages (collected on *P. padus*) of *R. padi* collected across France and three outgroups collected on cereals outside France. An asterisk indicates that mtDNA has been sequenced or that nuclear alleles have been cloned and sequenced (the remaining alleles have been studied by SSCP; see Table 3); ‡ indicates lineages that were first studied and presented in Delmotte et al. (2002); † in Simon et al. (1999a), and all others in Delmotte et al. (2001).

Name	Population	Date DD/MM/YY	mtDNA	SCAR		R5.29	
Asexual lineages							
150‡	Neuville	13/4/95	I	192*	201	170*	176
168‡	Neuville	14/4/95	I*	192	201*	170*	176
328‡	Tressin	11/4/95	I*	192*	201*	160*	170*
358‡	Mauguio	6/5/95	I	192	201*	170	176
1†	Rennes	5/5/94	II	192*	201*	—	—
2†	Rennes	5/5/94	II	192*	201*	—	—
35‡	Rennes	3/4/95	II	192	201	170*	180*
171‡	Neuville	15/4/95	II	192	201	170*	180*
188‡	Neuville	16/4/95	II*	192	201*	170*	180*
271‡	Rennes	3/4/95	II	192*	192*	162*	176*
327‡	Tressin	12/4/95	II	192	192	172*	176*
Ker	Kerguelen	1998	II*	192	192	172	176
Sexual lineages							
B10‡	Betton	4/4/95	II	192	201*	170*	186*
F15‡	Escaud.	11/4/95	II	192*	201*	170*	180*
C17‡	Caen	1/4/95	II	192*	192*	166*	186*
E30‡	Epinal	18/4/95	II	192*	192*	164*	176*
M8‡	Moigné	3/4/95	II*	192*	192*	172*	180
N10‡	Neuville	12/4/95	II*	192*	192	176*	186*
N27‡	Neuville	13/4/95	II	192*	192	172*	186*
P1†	Rennes	5/5/95	II	192*	192*	—	—
Outgroups							
Kenya	Kenya	9/11/95	I*	201*	201*	170*	170*
La Réunion	La Réunion	1/3/99	I	201*	201*	170	170
China	China	1/8/98	I*	201*	201*	166	174

ual phase in typical sexual lineages (Delmotte et al. 2001) (Table 1). A second type of asexual lineages (13.3%) have a typically sexual mtDNA haplotype (hII) but differ from sexual lineages at several nuclear loci. We interpreted this as the result of gene flow between typical sexual lineages and asexual lineages that produced males (Simon et al. 1999a; Delmotte et al. 2001). But the third and commonest type of asexual lineages (79.3%) are characterized by high heterozygosity and possess a very distinct mtDNA haplotype (hI). Their mode of origin has remained uncertain until the present study; they could result either from ancient loss of sex or by hybridization between two differentiated taxa (Delmotte et al. 2001, 2002).

DNA Extraction and Microsatellite Analysis

The DNA of individuals was extracted by ''salting-out'' (details given in Sunnucks and Hales 1996), and resuspended in Tris-EDTA buffer (10mM Tris pH 7.5, 0.1 mM EDTA) to obtain a final DNA concentration of about 5–7 ng/μl.

All 23 lineages were typed for 10 microsatellite loci: S16b, S17b, R6.3, R5.29, R5.10, R2.73, R1.35, R3.171, R5.138, R5.50 (details of ''S'' loci, cloned from the aphid *Sitobion miscanthi*, are currently unpublished but available from the authors; details of ''R'' loci from *Rhopalosiphum padi* are given in Simon et al. 2001).

Amplification of Nuclear Markers

Two anonymous nuclear markers likely to be noncoding regions were used for sequence analysis:

Flanking region of microsatellite R5.29.—A 711 base pair (bp) fragment containing the pure microsatellite locus R5.29 $(GA)_n$ (hereafter called R5.29-FR) from the 23 *R. padi* DNA clones were amplified by PCR using *Taq* DNA polymerase (Promega) and the R5.29-b-F and R5.29-rf-R oligonucleotide primers. Cycling was performed in a PCR Express thermocycler (Hybaid, Inc.) with a regime of initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C, annealing at 56°C, and elongation at 72°C, each for 50 sec, and a final extension at 72°C for 4 min.

SCAR (Sequence Characterized Amplified Region).—A 739-bp region of the nuclear genome was amplified and part of this product was digested with *Dra*I and separated in agarose gel following Simon et al. (1999a). This analysis revealed genotypes that are strongly predictive of the reproductive mode (192/192 for cyclical parthenogenesis and 192/201 for obligate parthenogenesis, Tables 1 and 2). The remaining part of the PCR product was used for cloning.

Cloning and Sequencing of Nuclear Markers

PCR products of SCAR and R5.29-FR were purified by gel electrophoresis on 1.2% agarose gel in 0.5 × Tris boric-acid EDTA with ethidium bromide (0.5 μg/ml). The bands were cut out with a sterile razor blade, and DNA purified using CONCERTTM Gel Extraction System (Life Technologies, Carlsbad, CA). About 10 nanogram of each PCR product were ligated into 5 nanogram of pGEM-T Easy vector (Promega, Madison, WI) and transformed into *E.coli* XL1-Blue MRF' competent cells (Stratagene, La Jolla, CA). Transformed cells were plated onto LB agar/ampicillin (50 μg/ml) blue-white color-selection plates. To recover the two alleles of each individual, 9 to 16 white colonies were picked from each plate and inoculated into 4 ml of LB / ampicillin (80ug/ml) medium, grown over night at 37°C with agitation. Minipreparations were done using CONCERTTM rapid plasmid purification system (Life Technologies). Positive clones of R5.29-FR were tested for amplification of the R5.29 locus, and alleles selected for sequencing on the basis of size by running them on polyacrylamide gels (Simon et al. 2001) silver stained following Budowle et al. (1991). Positive clones of SCAR were tested for amplification and alleles selected for sequencing on the basis of their restriction profile after digestion by *Dra*I. Finally, positive clones containing each of the two alleles from each individual were sequenced using Big-Dye in an automatic sequencer (ABI PRISM 310, Applied Biosystems, Foster City, CA).

SSCP of Nuclear Markers

To estimate the frequencies of the commoner alleles (revealed by sequencing) in sexual and asexual populations, PCR products of the nuclear markers (SCAR and R5.29-FR) of 40 asexual and 32 sexual lineages (including the lineages for which we failed to clone nuclear markers, Table 2) were scored using SSCP (single stranded conformation polymorphism, review in Sunnucks et al. 2000). For R5.29-FR, primers were designed to amplify a 260-bp polymorphic region in the flanking region of the R5.29 locus. Reaction mixtures and DNA amplifications were as for microsatellite loci. For the SCAR marker, PCR products (739 bp) were obtained following Simon et al. (1999a). Each PCR product was then diluted by half with standard formamide loading buffer for electrophoresis. Samples were run in a 10% Acrylamide/Bisacrylamide (w/w,49:1) gel, for 4h at 1500V, 40 W in 0.5× TBE. The gel was silver stained following Budowle et al. (1991).

Mitochondrial DNA

The mitochondrial DNA (mtDNA) haplotype of sexual and asexual lineages was characterized following Simon et al. (1996). Briefly, a segment of the mitochondrial genome containing the ND1 gene and portions of the 16S and the cytochrome *b* genes were amplified by PCR and cut with restriction enzymes that discriminate between the two main groups of mtDNA haplotypes (hI and hII) encountered in *R. padi* (Simon et al. 1996; Martinez-Torres et al. 1996, 1997). In addition, 1006-bp cytochrome *b* PCR products of four sexual (with hII mtDNA haploptypes) and four asexual lineages (with either hII or hI) of *R. padi* as well as three outgroups (*Rhopalosiphum maidis*, *R. insertum*, and *R. cerasifoliae*) were sequenced with an ABI 310 following the Applied Biosystems protocol.

Data Analysis

Microsatellite analysis.—To investigate the relationships between sexual and asexual lineages, a matrix of pairwise allele shared distance (D_{AS}, Chakraborty and Jin 1993) between all lineages (but 1, 2, P1) was calculated using ten

microsatellite loci and a Neighbor-Joining (NJ; Saitou and Nei 1987) tree was constructed using Population version 1.2.01 (http://www.cnrs-gif.fr/pge/bioinfo). Bootstrap support for nodes (1000 replicates) was calculated for the microsatellite tree.

Sequence alignment.—Clones were sequenced in both directions and the results for the two strands were aligned and checked using Sequencer version 3.0 (Gene Codes Corporation, Ann Arbor, MI). Sequences were aligned by eye using BioEdit (Hall 1999). Because overall divergences were low, these alignments were largely unambiguous. For the R5.29-FR, the microsatellite core repeat of each allele was excluded from the data set to perform the phylogenetic analysis on the flanking region only. The microsatellite information (core repeat and number of repetitions) was later reported on the tree.

Tree reconstruction.—Pairwise matrices genetic distances were calculated using Tamura-Nei (1993) distance, which takes into account excess transitions, unequal nucleotide frequencies, and variation of substitution rate among sites. Reconstruction of neighbor-joining trees and bootstrapping with 1000 replicates were conducted with MEGA version 2 (Kumar et al. 2000). Likelihood analyses were conducted with TREEPUZZLE version 4.0.2, a program that implements quartet puzzling (Strimmer and von Haeseler 1996). This fast tree search algorithm allows analysis of large data sets and assigns estimations of support to each internal branch. In the maximum likelihood analyses, base frequencies were set to empirical levels, substitution rates were variable among sites according to a gamma distribution, and the Tamura-Nei model of substitution was selected. Trees of nuclear sequences (SCAR and R5.29-FR) were rooted using China, La Réunion, and Kenya alleles as outgroups and cytochome *b* tree using other species of the genus *Rhopalosiphum* (*R. maidis*, *R. insertum*, *R. cerasifoliae*) as outgroups.

Results

Microsatellite Data

Asexual lineages possessed only a subset of alleles present in sexual lineages but showed high heterozygote excess (H_O = 0.813 ± 0.260; Delmotte et al. 2002). In fact, the most frequent allele at each locus in asexual lineages (''asexual'' alleles, i.e., those that most strongly characterize asexual lineages viz S16b^{151}, S17b^{162}, R5.29^{170}, R5.10^{258}, R2.73^{285}, and R1.35^{344}) are seen in heterozygous state in almost every asexual aphid bearing hI type mtDNA. By contrast, these alleles were significantly rarer in sexual lineages (Table 3). The ''asexual'' alleles were homozygous in Kenya and La Réunion lineages. Furthermore, the hI and hII asexual lineages are different from each other at nuclear markers: hII asexual lineages have significantly different allele frequencies at several loci (e.g., S17b^{164}, R5.29^{174}, R1.35^{344}; Table 3). In fact, they constitute a more heterogeneous group comprising genotypes that resemble hI lineages and others resembling sexual lineages (Delmotte et al. 2001).

The unrooted microsatellite tree yielded two well-supported groups (Fig. 1): the ''sexual group'' encompassed most sexual lineages (C17, E30, M8, N10, N27) and comprised all lineages with hII mtDNA and SCAR 192/192. In-

TABLE 3. Frequencies at common microsatellite alleles (19 alleles, six loci) for asexual (hI, n = 68; hII, n = 22) and sexual (n = 224) lineages of *R. padi*. Allelic frequencies at the SCAR marker were estimated from a subset of 72 lineages by SSCP: hI-asexual (n = 26), hII-asexual (n = 14), sexual (n = 32). Fisher exact tests were done for each allele (Ho = no difference between sexual and asexual-hI or asexual-hI frequencies). *** $P < 0.001$, ** $0.01 < P < 0.001$, * $0.05 < P < 0.01$, ns $P > 0.05$; — indicates the exact test could not be performed. Genotypes of outgroup lineages (China, La Réunion, and Kenya) are given for the same markers. Alleles in bold characterize hI asexual lineages.

		Microsatellite loci																SCAR		
		S16b		S17b			R5.29			R5.10			R2.73		R1.35					
	mt DNA	149	**151**	**162**	164	168	**170**	174	176	180	**258**	260	262	274	262	270	**285**	**344**	345	359	192	**201a**	**201b**
Asexual	hI	0.51	**0.49**	**0.51**	0.25	0.22	**0.48**	0	0.31	0.09	**0.41**	0.12	0.05	0.27	0	0.28	**0.50**	**0.43**	0.15	0.23	0.5	**0.5**	**0**
	hII	0.71	**0.26**	**0.57**	0.31	0.10	**0.20**	0.07	0.26	0.1	**0.15**	0.12	0.23	0.18	0.26	0.24	**0.26**	**0.17**	0.38	0.21	0.72	**0**	**0.28**
Sexual	hII	0.87	**0.12**	**0.36**	0.43	0.03	**0.07**	0.03	0.12	0.11	**0.10**	0.33	0.23	0.12	0.27	0.17	**0.05**	**0.05**	0.44	0.15	0.963	**0**	**0.047**
Exact Test																							
hI-asexual/sexual		***	***	***	***	***	***	ns	***	ns	***	***	***	***	***	***	**	***	***	**	***	***	ns
hII-asexual/sexual		**	**	**	ns	ns	**	**	**	ns	ns	ns	ns	ns	**	ns	*	**	ns	ns	***	—	**
China	hI	153/153			158/160			166/174			**258/258**					266/282			347/352			**201b/201b**	
La Réunion	hI	**151/151**		**162/162**			**170/170**				256/**258**					**285/285**			**344/344**			**201a/201b**	
Kenya	hI	**151/151**		**162/162**			**170/170**				**258**/260					**285/285**			**344/344**			**201a/201b**	

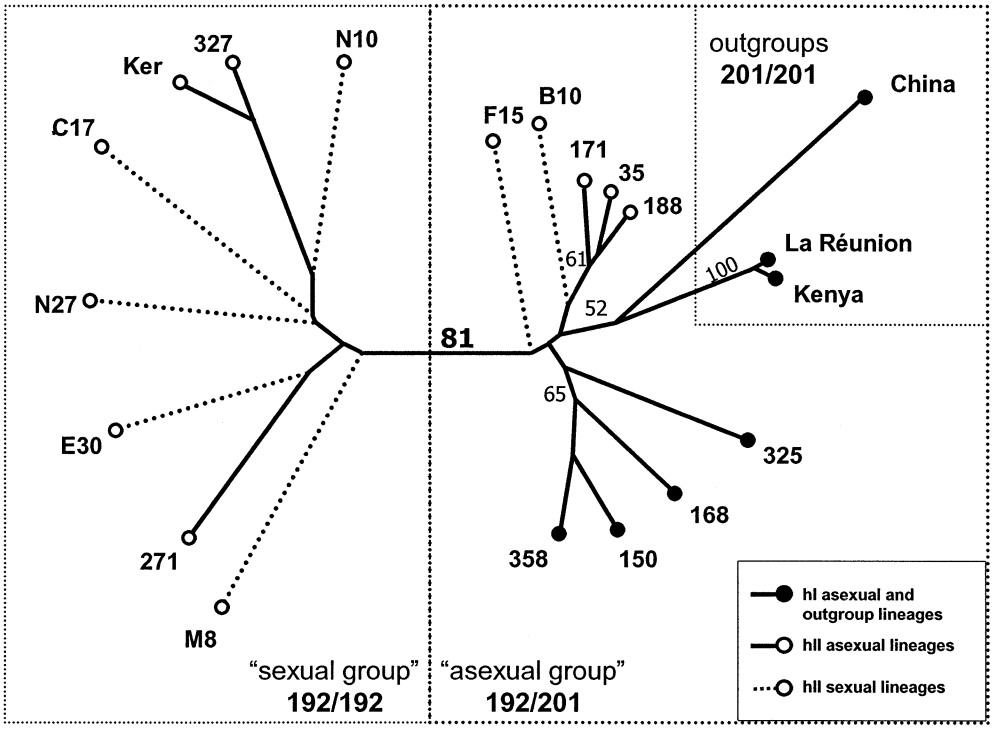

FIG. 1. Unrooted neighbor-joining tree based on allele shared distance (D_{AS}) calculated with 10 microsatellite loci for seven sexually produced lineages (dashed lines) and seven asexual lineages plus three outgroup lineages (solid lines) of the aphid *R. padi* (see Table 2). Mitochondrial variation scored as two different haplotypes (full circle for hI, empty circles for hII) was reported on the tree. The numbers at the nodes are bootstrap percentages >50% of microsatellite loci (1000 replicates). The tree yielded two well supported groups: the sexual group including all lineages being 192/192 at SCAR locus which was mainly constituted of sexually-produced lineages, and the asexual group including all lineages being 192/201 and 201/201 at SCAR locus which was mainly constituted of asexual lineages.

terestingly, it also included three hII asexual lineages (271, 327, Ker) that may have arisen by a spontaneous and recent loss of sex, which would explain their high genetic relatedness to sexual lineages (Delmotte et al. 2001). The ''asexual group'' encompassed most asexual lineages and comprised all lineages that are 192/201 or 201/201 at the SCAR locus. This includes all lineages with hI mtDNA (150, 168, 325, 358, China, Kenya, La Réunion) and several hII asexual lineages (35, 171, 188). This group also included two atypical sexual lineages (B10, F15).

Analysis of Nuclear and Mitochondrial DNA Sequences

R5.29-FR.—A total of 30 alleles of the flanking region of the microsatellite locus R5.29 were sequenced over 711 bp. Seventy-five variable sites comprising 16 parsimony-informative sites were found. The ML tree showed two very well-supported monophyletic groups of alleles (S for sexual and A for asexual): molecular divergence between the two groups of alleles reached 2% (Fig. 2). Genetic distance was three times larger between than within groups, and within-group variation was higher in the group of S alleles than in the group of A alleles (0.0074 ± 1.10^{-3}, 0.000018 ± 5.10^{-4}, respectively, Fig. 2). The lengths of the microsatellite arrays of S alleles ranged from 160 to 186 bp. In stark contrast, the A alleles all have the same pure $(AC)_{13}$ core repeat (170 bp) with one exception: 188–2 probably derived by point mutation from $(AC)_{13}$.

The typical sexual lineages (N10, E30, M8, M27, C17—those with hII mtDNA and the SCAR 192/192 genetic profile, representing 95% of sexual lineages; Table 1), all had both their alleles in the S group. The atypical sexual lineages (B10, F15—with hII mtDNA and the SCAR 192/201 genetic profile representing 5% of sexual lineages; Table 1), had one allele in the S group and the other allele in the A group (Fig. 2). Asexual lineages (271, 327) with the closest genetic profile to sexual lineages (hII mtDNA and SCAR 192/192) had both their alleles in the S group. Asexual lineages with SCAR 192/201 (with hII mtDNA as 35, 171, 188, or with hI mtDNA as 325, 168, 150) had one allele in the S group and the other allele in the A group; only the A allele was sequenced for 168 and 150. Notably, Kenya (the only outgroup studied for this marker) has SCAR 201/201 and was the only lineage to have both alleles in the A group.

To investigate the difference in mutation accumulation of S alleles with respect to reproductive mode, maximum-likelihood branch length of quartet puzzling tree of sexual and asexual lineages were compared. There was no significant difference in branch length between S alleles of sexual and asexual lineages (*t*-test, $P = 0.48$) indicating that asexual lineages did not accumulate mutations at a greater rate than did sexual lineages.

SCAR.—A total of 31 alleles of the SCAR marker were sequenced over 739 bp, revealing 53 variable sites among which 12 were parsimony informative. Phylogenetic rela-

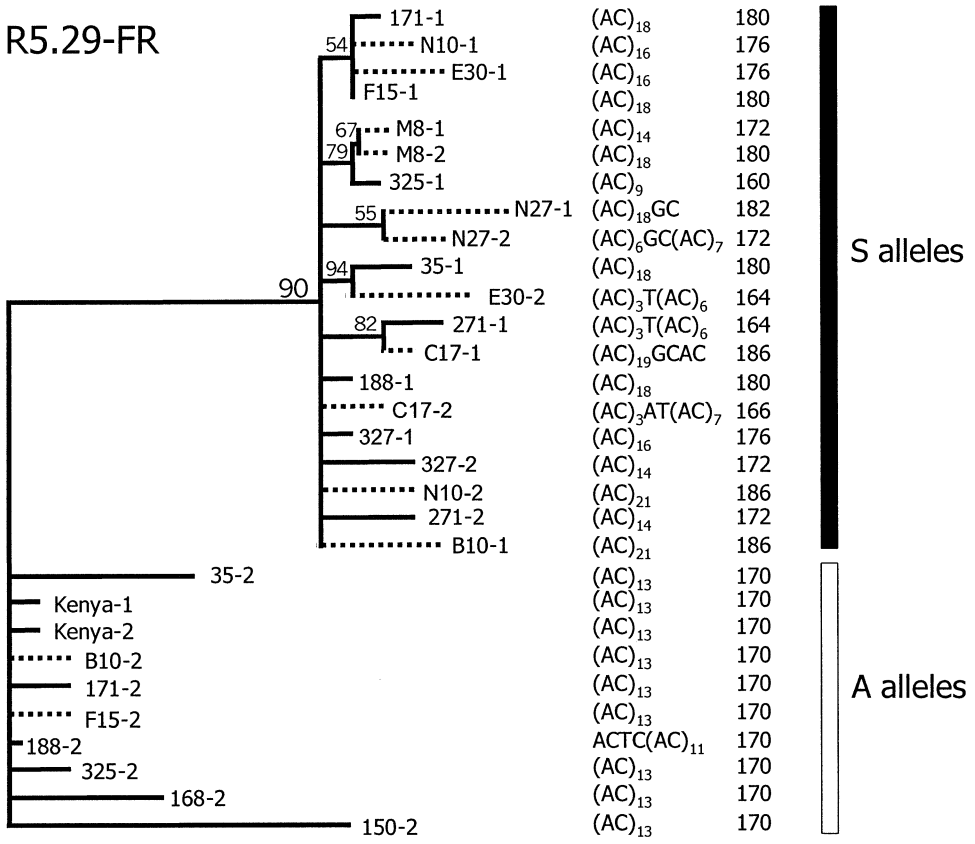

FIG. 2. Maximum-likelihood allele tree for R5.29-FR nuclear marker. Solid lines represent alleles of asexual lineages and dashed lines alleles of sexual lineages.

tionships among alleles showed three monophyletic groups of alleles with strong support (≥98; Fig. 3): all 192 alleles fell in a first group, while 201 alleles were divided into two groups, 201a and 201b. Divergence between allele groups 192 and 201(a, b) reached 1.2%—at least ten times higher than within-group variability. The typical sexual lineages (with hII mtDNA and SCAR 192/192) all had both their alleles in the 192 group (E30, P1, M8; Fig. 3). Such sexual lineages, for which only one allele was sequenced, also fell in that group (N10 and N27). The atypical sexual lineages (with hII mtDNA and SCAR 192/201b) had one allele in the 192 group and the other allele in the 201b group (F15)—the only sequenced allele of B10 falling in the 201b group. As seen for R5.29-FR above, there was no significant difference in branch lengths between sexual and asexual SCAR 192 alleles (*t*-test, $P = 0.28$), again indicating no difference in mutation accumulation between the two types of lineages.

Asexual lineages with the genetic profile closest to sexual lineages (with hII mtDNA and SCAR 192/192) had both their SCAR alleles in the 192 group (271). Asexual lineages with hII mtDNA but with SCAR 192/201 had one allele in the 192 group and the other allele in the 201b group (1,2)—the only sequenced allele of 188 also falling in the 201b group. Asexual lineages with hI mtDNA and SCAR 192/201 had one allele in the 192 group and the other allele in the 201a group (325); only the 201 allele was sequenced for 358 and 168 and only the 192 allele for 150. The outgroups contrasted with all other lineages, because the Kenya and La Réunion lineages had one allele in the 201a group and the other in the 201b group, while the China lineage had both alleles in the 201b group.

In summary, allele sequence divergence shows a similar pattern for both nuclear markers (R5.29-FR, SCAR): all sexual lineages (except the atypical B10, F15) have their two alleles in a monophyletic group of alleles (S/S-192/192), whereas all asexual lineages (but 271, 327) have one allele grouped among alleles of sexual lineages and another allele in a very divergent monophyletic group (S/A-192/201). Interestingly, allele 201b was restricted to hII asexual lineages, while 201a was restricted to hI asexual lineages (Table 3). This pattern was confirmed by SSCP for additional French sexual ($n = 32$) and asexual ($n = 40$) lineages of *R. padi*, including lineages for which a unique allele was sequenced. Finally, both alleles of outgroup lineages (Kenya, La Réunion, China) fell exclusively among the group of asexual alleles (A/A-201a/201b, except China A/A-201b/201b).

Cytochrome b.—Comparison of outgroup and ingroup sequences of the cytochome *b* fragment (1006 bp) revealed 101 variable sites of which 44 were parsimony informative (4.4% of total sites). Among *R. padi* sequences, 20 variable sites allowed the discrimination of six haplotypes over eight sequenced samples. Variation at third positions accounted for 85% ($n = 17$) of the differences, whereas first and second positions accounted respectively for 10% ($n = 2$) and 5% (*n*

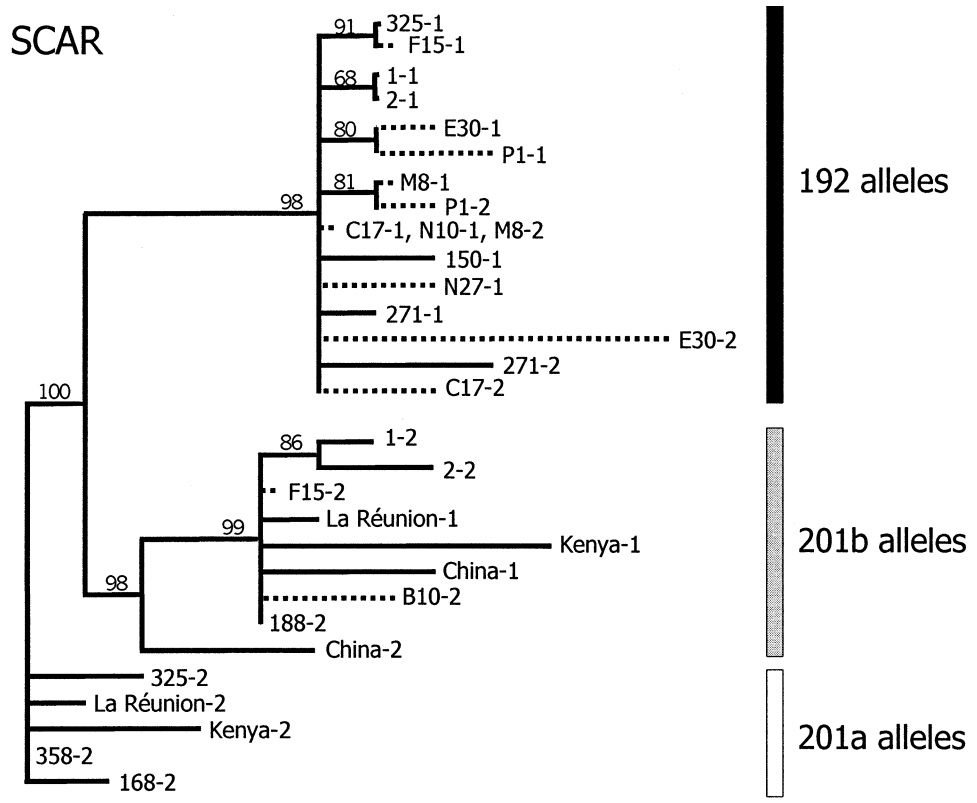

FIG. 3. Maximum-likelihood allele tree for SCAR nuclear marker. Solid lines represent alleles of asexual lineages and dashed lines alleles of sexual lineages.

= 1) of differences. Regression of the number of transitions and transversions at third position against sequence divergence did not show phylogenetic saturation. Maximum-likelihood (ML) phylogenetic analysis revealed a monophyletic origin for each of haplotype group I and II (node supported by a bootstrap value of 100) and strong sequence divergence (1.8%) between the two haplotype groups (Fig. 4), a result also found with neighbor joining (data not shown). The divergence between haplotypes I and II is due to 17 synonymous substitutions and one non-synonymous substitution that corresponds to the replacement of a tryptophan residue (hI and *R. maidis*) with methionine (hII). Mean (±SE) genetic distance variation (Tamura and Nei 1993) within haplotype groups did not exceed 4.10^{-4} ($\pm 1.10^{-5}$) although it reached

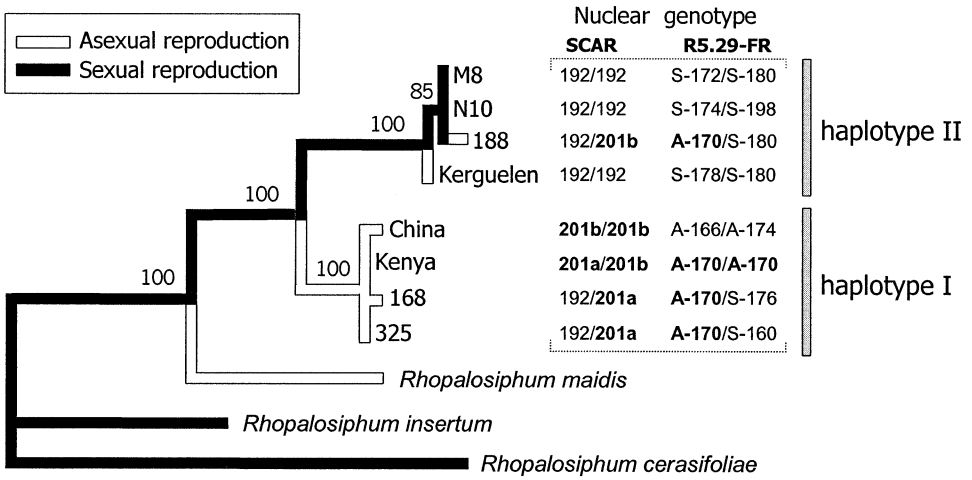

FIG. 4. Rooted maximum-likelihood tree of 10 mitochondrial haplotypes (1006 bp of cytochrome *b*). Support for the internal branches of the rooted quartet puzzling tree topology is given as a percentage. Changes in reproductive mode are mapped over the M-L tree assuming that sexual reproduction is ancestral and that reversion to sexuality is impossible. Nuclear genotype is given for the two sequenced markers: SCAR and R5.29-FR. Alleles in bold characterize hybrid asexual lineages and outgroups.

0.019 ($\pm 6.10^{-3}$) between the two groups, equivalent to 52% of the genetic distance between *R. padi* and *R. maidis*. Using the average rate of sequence divergence of mtDNA coding region in insects of 2% per million years (De Salle et al. 1987), time of divergence between haplotype I and II is aproximately 0.9 million year. Moreover, intraspecific phylogenetic congruence among mitochondrial (cyt. *b*) and endosymbiotic (GroEL from the aphid obligatory endosymbiont *Buchnera*) genomes was found and confirmed the rate of divergence between the two mitochondrial haplotypes (data not shown, Delmotte et al., in unpubl. data). Finally, nuclear genotypes of lineages were reported on the ML tree (Fig. 4) and showed that asexual lineages 168, 325 and 188 received one allele from sexual lineages of *R. padi* and one allele from a parent like the outgroups (Kenya, China).

Comparison of marker divergence.—Average A+T content was high in cytochrome *b* (78.7%) and in nuclear markers (76.9% and 69.8% for SCAR and R5.29-FR). As in many nuclear genes, transition/transversion ratios (Ts/Tv) were less than 2, with maxima of 1.1 and 1.8 in SCAR and R5.29-FR, respectively. However, the Ts/Tv ratio was much higher in cytochrome *b* (6.4). The Ts/Tv ratio in the mitochondrial marker also showed a typical variable distance-dependant pattern, for example, it decreased to 3.5 when outgroups were included (Nei and Kumar 2000). We estimated mean sequence divergence between groups of nuclear alleles for R5.29-FR and SCAR, respectively 2% and 1.2%. The mtDNA marker is expected to evolve faster than the nuclear ones, which holds for SCAR. However, R5.29-FR seems to evolve faster than expected, perhaps because flanking regions of microsatellites tend to have elevated rates of evolution (Brohede and Ellegreen 1999).

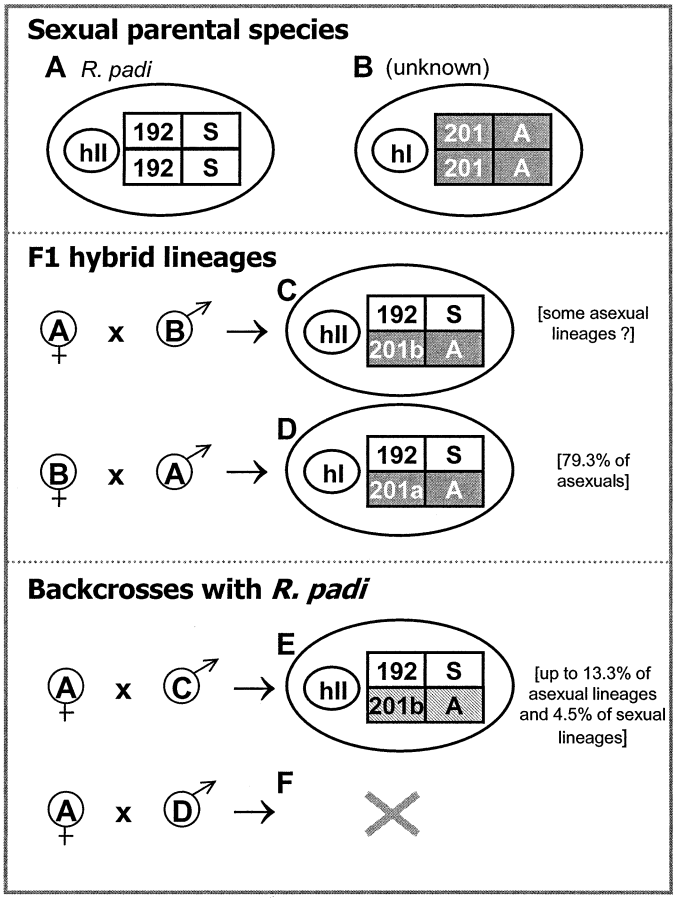

FIG. 5. Hypothetical hybridization events involving two sexual parent species (A, B) explaining the origin of hybrid asexual lineages of *R. padi* found in France (C, D, E). Mitochondrial DNA is symbolized by circles in which cytochrome *b* haplotypes (hI, hII) information is reported. Nuclear genome is symbolized by squares in which SCAR alleles (192, 201) and microsatellite alleles (A, S) are reported. The frequency of each type of French lineage is given in brackets (see Table 1). Backcrosses (E) give rise to lineages with a majority of sexual alleles (3/4: 1/4). The last type of backcrosses (F) does not seem to occur in France because hII-192/201a have never been sampled.

DISCUSSION

Evidence for Hybrid Origins of Asexual Lineages

The main result of this work concerns the mode of origin of the bulk of asexual lineages (those with mtDNA haplotype I) of *R. padi*. Our phylogenetic data discard the possibility of an ancient origin and demonstrate that they have been produced by interspecific hybridization as supported by the following four lines of evidence. First, asexual lineages show strong heterozygote excess at microsatellite loci while sexual lineages show heterozygote deficit (Delmotte et al. 2002). Second, French asexual lineages appear to have a composite genome since they have one allele at most microsatellite loci that is specific to, and homozygous in, outgroup lineages, whereas the other allele is common in sexual and asexual lineages. Third, phylogenetic analysis of allele divergence at two nuclear markers showed that asexual lineages have one allele in common with outgroup lineages, whereas the other allele is indistinguishable from alleles in sexual lineages. Fourth, mtDNA revealed the presence of two highly divergent haplotype groups (showing >50% of an interspecific divergence in the genus *Rhopalosiphum*), each displaying very low within-group variation. Altogether, these points strongly suggest that hybridization has occurred between two well-differentiated taxa.

Interspecific hybridization could therefore account for the formation of hI, but also of some hII asexual lineages (Fig. 5): the cross involving females of *R. padi* bearing hII-192/192 and males of an unknown species bearing hI-201/201 would result in the formation of hII asexual lineages, while the reciprocal cross would generate hI asexual lineages. It is intriguing that the two types of asexual hybrids (hI-192/201 and hII-192/201) have a rather differentiated allele at the SCAR locus (201a and 201b, respectively). This divergence could be explained by several episodes of hybridization involving 201a/201a and 201b/201b parents from the unknown species. These different hybridization events would not have occurred in the same direction, accounting for the link between the mtDNA haplotype and the type of 201 allele. Alternatively, the parent of the unknown species could all have been heterozygous (201a/201b). In this case, some combinations are missing (hI-192/201b and hII-192/201a) possibly due to inviability. Indeed, hybridization that disrupts natural combinations of nuclear and mitochondrial genes can generate a syndrome of nuclear-cytoplasmic incompatibility that

might severely affect the fitness of some F1 hybrids (Edmands and Burton 1999). This hypothesis could be further investigated in *R. padi* by laboratory crosses experiments when parental species have been confidently identified.

Alternative Routes to Asexuality

As discussed above, hII-192/201b asexual lineages can be generated either by direct hybridization between *R. padi* and a differentiated hI parent or by later backcrosses of F_1 hybrids with *R. padi* (type ''C'' and ''E,'' Fig. 5). If these lineages result from a recent hybridization event, their hI parent should be either 201b/201b or 201a/201b: but because this hI parent has never been found in France among the many lineages genotyped in earlier studies, we think that the vast majority of hII-192/201b asexual lineages are unlikely to be the direct result of interspecific hybridization. This implies that most of hII-192/201b asexual lineages are the product of backcrosses. This second hypothesis is also supported by the presence of 4.5% of sexual lineages with introgressed asexual alleles (e.g., B10, F15), strongly suggesting that these backcrosses still presently occur in France or nearby (Delmotte et al. 2001). Thus, we propose that interspecific hybridization outside France may be responsible for the existence of some hII asexual lineages (type ''C'') that are presently seen in the extant French populations and continuously produce new hII-192/201b asexual lineages by backcrosses with sexual lineages (type ''E''). Finally, a minority of hII asexual lineages (7.4%) have genetic profiles identical to sexual lineages at both nuclear alleles and mitochondrial haplotypes (e.g., 271, 327). The most plausible explanation for the genotypic features of these asexual lineages is a recent loss of sex by spontaneous mutation at the gene controlling the production of sexual phenotypic forms.

In conclusion, in addition to indicating asexuality by hybridization in an aphid, this phylogenetic study confirms in the same species existence of the two other modes of origin of asexuality: backcrosses of males from essentially asexual lineages with females of sexual lineages, and loss of sex by spontaneous mutation (Delmotte et al. 2001).

Frequency and Directionality of Hybridization

Our data strongly indicate that hybrid *R. padi* have resulted from multiple hybridization events. The core repeat of microsatellite associated with S alleles of asexual lineages are of very different sizes ranging from 160 to 180 bp, whereas it was monomorphic for A alleles (170). The high representation of this allele size suggests that relatively few parental lines donated these alleles, and/or that the mutation rate at this microsatellite motif is lower than the rate of substitution in the flanking region and/or that evolutionary convergence to this size class has been common (Orti et al. 1997). In any case, the difference in size variation between S and A alleles suggests that multiple events of hybridization involving more hII than hI parental lines have occurred.

Another intriguing pattern is that hI hybrid lineages are much more numerous than hII 192/201 lineages (Table 1). This therefore suggests a strong directionality of hybridization, the ''C'' type of hybrid crosses being much less frequent than the ''D'' type (Fig. 5). First, hybridization could have been unidirectional, generating only hI hybrids. In that case, all hII asexual lineages would be produced by backcrosses. Unidirectional hybridization with later backcrossing of the hybrids occurs in some species (Arnold et al. 1999; Wirtz 1999), and could potentially explain the disparity in frequency of hI and hII hybrids. However, as noted above, hI and hII asexual lineages do not have the same SCAR 201 allele, which invalidates recurrent backcrosses between hI asexual (type ''D'') and *R. padi* (Fig. 5). A second hypothesis is asymmetric hybridization resulting from female choice, known to be a major prezygotic mechanism. For many animal species, the cross of two species is often more difficult to achieve in one direction than the other (for aphids see Rakauskas 2000). Difference in discrimination intensity of the females of the two species is likely responsible for this pattern as it has been shown to occur in the aphid *Acyrthosiphum pisum* (Via 1999; Via et al. 2000). Consequently, it has been proposed that hybrid matings are usually between the females of a rare species and the males of a common species, but rarely vice versa (sexual selection hypothesis; Wirtz 1999). Indeed, rare females accept allospecific males more readily than allospecific males are accepted by females. If the hI parent is rare in the hybrid zone then we expect more hI than hII asexual hybrid lineages. Finally, postmating barriers could also account for the unequal frequency of asexual hybrids: for instance, lower viability of one cross over the other has been found in *R. padi* (90% against 10%; J.-C. Simon, unpubl. data).

Parental Species

Geographical separation or adaptation to two different primary hosts (genus *Prunus*) on which sexual reproduction occurs could account for the differentiation of two taxa. In France, sexual lineages of *R. padi* are likely candidates as parental species of the French asexual hybrids. These sexual lineages seem to have contributed to the hybrid pool several microsatellite alleles at each locus, consistent with their being abundant when hybridization occurred. In contrast, the second parental lineage contributed few microsatellite alleles as revealed by the homogeneity in the core repeat of R5.29 locus, which may be caused by the rarity and/or low diversity of this lineage. Since rare taxa are more likely to go extinct, there is a chance that the second sexual parental species has done so. The potential candidate for the second parental line is/was genetically closed to the outgroup lineages (i.e., Kenya, La Réunion and China). To identify this second parent, we need to further investigate phylogeography of the genus *Rhopalosiphum*, in particular in Asia where some *Rhopalosiphum* species are endemic (Remaudière and Naumann-Etienne 1991).

Reproductive Mode of Hybrids

Although all unisexual lineages of vertebrates have been shown to originate by recent hybridization between two well-differentiated bisexual taxa (Avise et al. 1992), evidence for hybrid origins of diploid asexual insect lineages is not so overwhelming, and in particular, until now asexual aphids have been considered to arise by mechanisms other than hybridization (see Introduction). Nonetheless, hybridization

events leading to polyploid asexuals have been reported in parthenogenetic *Warramaba* grasshoppers (Honeycutt and Wilkinson 1989), *Gymnopais* black flies (Rothfels 1989), *Otiorhynchus scaber* and *Aramigus* weevil species complexes (Tomiuk and Loeschcke 1992; Normark 1996), gynogenetic *Muellerianella* leafhoppers (Drosopoulos 1976), and gynogenetic as well as hybridogenetic *Bacillus* stick insects (Mantovani et al. 2001). In fact, most asexual insect taxa that have been carefully examined have revealed hybrid origins. In aphids, there are complexes of closely related forms that may be able to hybridize and which share a host on which sexual reproduction occurs. Although natural hybridization may be reduced by mate recognition systems, it has been documented in *Aphis fabae*, *Cryptomyzus*, and *Sitobion* (Thieme and Dixon 1996; Guldemond and Dixon 1994; Sunnucks et al. 1997). Laboratory crossing experiments between *Aphis grossulariae* and *A. triglochinis* showed that established hybrid clones expressed normal parthenogenetic reproduction but that they developed very few sexual forms (Rakauskas 1999, 2000). In the same way, we may interpret the small male production in most asexual lineages of *R. padi* as a relic of sexual reproduction lost following hybridization (Simon et al. 1991; Delmotte et al. 2001). The chance of a hybrid founding a unisexual population is determined by a balance of genetic factors affecting the disruption of meiosis and the remainder of the developmental program. In vertebrates this results in infrequent transition to asexuality (Dowling and Secor 1997). In contrast, aphid asexual lineages all derive from sexual lineages that already are able to undergo parthenogenetic reproduction as part of their life cycle. Perhaps as a consequence, partial transitions to asexuality are frequent in aphids (about 37% of Aphididae species display both sexual and asexual lineages).

Recent Origin of Asexual Hybrids

Several lines of evidence indicated that hybrid lineages detected in *R. padi* are of recent origin. First, S alleles (R5.29-FR) and 192 alleles (SCAR) of asexual lineages are not distinguishable from alleles in sexual lineages, indicating that these alleles have not had time to differentiate. Second, the very low divergence found within mtDNA haplotype groups and within allele groups of nuclear markers suggests that hybrids have arisen recently. An ancient origin of asexual lineages could not account for the consistency of $(AC)_{13}$ core repeat found in nuclear alleles of asexuals, given that sexual alleles occur with larger and smaller arrays. Thus, we must revise the conclusions of Martinez-Torres et al. (1996) and Simon et al. (1996), wherein it was suggested that hI asexual lineages could be of moderately ancient origin in this aphid (0.4–1.4 MY). A similar molecular test has already been applied to the putatively ancient asexual aphid tribe Tramini, in which sexual reproduction was unknown. Tramini revealed low levels of intraspecific sequence divergence along with very low levels of sequence heterozygosity at the nuclear marker studied, suggesting that recombination episodes have occurred (Normark 1999). Blackman et al. (2000) showed that this very low genetic diversity contrasted with the high intraspecific karyotype diversity suggesting rapid chromosomal evolution in this tribe. Although one cannot discount that Tramini are made of ancient asexual species that have undergone gene conversion, this result suggests that asexual lineages of this tribe are recently derived from sexual ones. In any case, our study constitutes another example that putatively ancient asexual lineages are actually of much more recent origin than previously thought (Judson and Normark 1996).

Conclusions

The most significant finding of this phylogenetic survey is the first strong evidence for any aphid that hybridization is the major source of asexual lineages. By applying a molecular test, we demonstrated that the divergence between asexual and sexual lineages and the high level of heterozygosity found in asexual lineages were not due to an ancient origin as previously suggested (Martinez-Torres et al. 1996; Simon et al. 1996), but to repeated and recent hybridization events between *R. padi* and an unknown related species. Whether hybrid origin of permanent asexuality is a general phenomenon in aphids could be evaluated by employing the same method to other species. It is the second time that asexual aphids proved to be of much more recent origin than previously thought (Normark 1999), a finding in agreement with the general prediction that asexual lineages are evolutionary doomed in the longer term. Hybridization as a major source of asexuality should result in substantial genetic variation in asexual lineages, and could cause large differentiation between sexual and asexual lineages that could be translated into phenotypic differences. For example, asexual lineages of hybrid origin could benefit from heterosis and out-perform their sexual parental lineages (although the reverse is possible with highly diverged parents, Arnold et al. 1999). In support of possible heterosis, asexual hybrid lineages of *R. padi* seem to be ecologically successful because they have been found at high frequency over large geographic areas, and may persist through time (Delmotte et al. 2002). This is seemingly consistent with the general-purpose genotype hypothesis (Baker 1965; Lynch 1984), suggesting that some broad-niche fit asexual genotypes have been sorted out by selection. At a broader level, because of the presence of 50% foreign genes, the set of hybrid asexual lineages could occupy a somewhat different niche than that of sexual lineages and not systematically compete in the same environment (Vrijenhoek 1998). This could increase the chances of coexistence between the two reproductive modes.

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