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Crosses between isolates of *Phytophthora infestans* from potato and tomato and characterization of F_1 and F_2 progeny for phenotypic and molecular markers

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Nineteen crosses were carried out *in vitro* between seven and nine isolates of *Phytophthora infestans* originating from potato and tomato, respectively. Oospores were produced abundantly in all but two crosses, but oospore germination was generally low (a few per cent) and depended on the combination of parental isolates. The highest fertility in F_1 progeny was observed when at least one parental isolate originated from tomato, and the lowest in crosses of isolates from potato; half the crosses were not fertile. Forty-three F_1 and 51 F_2 single-oospore progeny of a selected cross, along with the 16 field isolates, were analysed phenotypically and with molecular markers. Phenotypic characterization included mating type; sensitivity to the phenylamide fungicide metalaxyl-M; specific virulence on potato R-gene differentials; and aggressiveness (infected leaf area) on potato and tomato leaf discs (host preference). Isolates and progeny were also assessed for mitochondrial DNA haplotype with RFLP–PCR (restriction fragment length polymorphism–polymerase chain reaction), and characterized with AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeats, microsatellites). Parental isolates were sensitive and resistant to metalaxyl-M, whereas all F_1 were intermediate phenotypes. In the F_2 progeny, the majority of isolates (43 of 51) were intermediate in sensitivity and four each were sensitive and resistant to metalaxyl-M, respectively. In both F_1 and F_2 progeny, four isolates emerged through selfing. The A1 : A2 ratio was 25 : 18 in F_1 and 24 : 21 (plus six self-fertiles) among the F_2 progeny. Many F_1 progeny isolates were highly aggressive on both hosts, but 15 and 23% of isolates preferred tomato and potato, respectively. Among F_2 progeny, few highly aggressive isolates were recorded and a quarter of isolates lost pathogenicity almost completely. Isolates preferring tomato increased, and those preferring potato significantly decreased in the F_2 progeny. Inheritance of mitochondrial haplotype in F_1 progeny was uniparental and mostly (25 of 27) from one parent only. Six and four different SSR genotypes were identified in F_1 and F_2 progeny, respectively, of which two were identical to the parents. The two microsatellite loci, 4B and 4G, segregated in the ratios 15 : 22 : 2 and 22 : 17 in F_1 and 24 : 17 and 26 : 15 in F_2 progeny, respectively, while the majority of AFLP markers segregated in either a 1 : 0, 1 : 1, 3 : 1 or 1 : 2: 1 ratio. There was no obvious association between AFLP and SSR genotypes, nor between genotypic and phenotypic traits.

Keywords: AFLP, genotypes, microsatellites, potato/tomato late blight, segregation, sexual crosses

Introduction

The population structure of *Phytophthora infestans*, the causal agent of late blight of potato and tomato, has undergone major changes over the past 20 years. In Europe the 'old' populations were displaced by 'new' populations through long-distance migration from central Mexico (Niederhauser, 1991; Spielman *et al*., 1991; Fry *et al*., 1993). Since then, local processes including sexual recombination and selection for higher fitness and aggressiveness have resulted in an increase of race diversity in the population (Drenth *et al*., 1994; Legard *et al*., 1995; Day & Shattock, 1997; Turkensteen *et al*., 2000). An increase in genetic variation at the molecular level and a more complex structure were observed in the new *P. infestans* populations (Drenth *et al*., 1993b; Knapova & Gisi, 2002). Until 1980, only the A1 mating type was detected in European populations. Since then the A2 mating type has been found, although initially only in a few isolates, in almost all European countries, and later in many other parts of the world (Gisi & Cohen, 1996). The proportion of A2 mating type isolates collected from commercial potato fields has remained low in countries such as the UK, France, Germany and Switzerland (Gisi & Cohen, 1996; Lebreton *et al*., 1996; Duvauchelle *et al*., 1997;

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Day *et al*., 2001; Knapova & Gisi, 2002), whereas in the Netherlands and Scandinavia it has reached 50% and above in current populations (Drenth *et al*., 1993a; Hermansen *et al*., 2000; Turkensteen *et al*., 2000).

The presence of both mating types in European populations of *P. infestans* enables the pathogen to reproduce sexually and, as a consequence, to produce oospores (Drenth *et al*., 1995; Andersson *et al*., 1998; Strömberg *et al*., 1999; Hermansen *et al*., 2000; Turkensteen *et al*., 2000). Cross-infection of potato and tomato by *P. infestans* is of practical significance in areas where both hosts are cultivated in close vicinity. Strong indications are available for the existence of an adaptation of *P. infestans* to one or other host. Legard *et al*. (1995) compared isolates from North America, Mexico and the Netherlands, and differentiated between tomato-aggressive and nonaggressive isolates. All isolates were aggressive on potato, but few on tomato. Therefore tomato-aggressiveness was claimed to be a trait that might have evolved only recently in *P. infestans*. Among Ecuadorian isolates, all produced larger lesions on the host from which they were isolated (Oyarzun *et al*., 1998). Isolates collected in Taiwan from tomato expressed a distinct host specificity (Ann *et al*., 1998). In French and Swiss populations, isolates were most aggressive on the host of their origin, and significantly lower for isolates from potato on tomato (Knapova & Gisi, 2002). Similar results were observed recently for isolates from Israel (Cohen, 2002). In some regions of the Netherlands, Fry *et al*. (1993) detected tomato- and potato-specific genotypes, but in other regions the genotype structure was the same on both hosts. Using molecular markers (simple sequence repeats, SSR), Knapova & Gisi (2002) were able to differentiate 22 genotypes in Swiss and French field populations, of which seven were specific for tomato, 11 for potato, and four were isolated from both hosts.

Sexual crosses have been made *in vitro* for *P. infestans* in order to investigate inheritance of several phenotypic characteristics such as resistance to metalaxyl (Shattock, 1988; Fabritius *et al*., 1997; Lee *et al*., 1999); allozyme markers (Spielman *et al*., 1990); and mating type (Judelson *et al*., 1995), and for genetic mapping of avirulence genes (Al-Kherb *et al*., 1995; Van der Lee *et al*., 2001). As with all oomycetes, *P. infestans* is a diploid organism but can also be polyploid, and it has an extremely large genome. When A1 and A2 mating type isolates are paired, each may produce antheridia, oogonia or both, which later fuse to form hybrid oospores (Judelson, 1997). Antheridia and oogonia from the same parent may also fuse, resulting in selfing. The sexual preference of an isolate depends on the relative preference of the mating partner, but also on the position of gametangia within the mating culture (Judelson, 1997). These characteristics make segregation analyses in *P. infestans* rather complex.

This paper describes oospore production and germination of selected crosses to determine whether isolates from potato and tomato have potential for genetic recombination. This may help explain the increase in genetic diversity of recent *P. infestans* field populations on potato and tomato in Europe. In addition, the F_1 and F_2 progeny of a selected cross were characterized phenotypically and genotypically to study segregation of several important characteristics. Phenotyping included mating type, sensitivity to the phenylamide fungicide metalaxyl-M, fertility, aggressiveness on potato and tomato, and virulence on potato R-gene differentials. Genotypically, the progeny were characterized for mitochondrial DNA haplotype using RFLP–PCR (restriction fragment length polymorphism–polymerase chain reaction), AFLP (amplified fragment length polymorphism) and SSR (microsatellite) markers.

Materials and methods

Origin of *P. infestans* isolates

Five and nine *P. infestans* isolates from potato and tomato, respectively, were selected from a large number collected in France, Switzerland and Germany in 1997. Isolation was carried out as described by Knapova & Gisi (2002). Pure cultures were incubated on V8 agar (Ribeiro, 1978) in the dark for 12 days at 18°C. Agar plugs from these cultures were stored under liquid nitrogen prior to use. One isolate from Switzerland (1996) and one from Japan (1990) were included in the study as reference isolates (potato origin). All 16 isolates were characterized phenotypically and genotypically (Table 1) and were used for sexual crosses. For segregation studies, a fertile cross (C14) was selected, consisting of parents P7 and T4 with differences in sensitivity to metalaxyl-M (resistant and sensitive, respectively); mitochondrial haplotype (IIa and Ia); SSR genotype (A-01 and D-03); and host origin (potato and tomato). A total of 43 putative F_1 progeny isolates were generated and maintained on V8 agar or stored under liquid nitrogen for later use. With F_1 progeny isolates several sibcrosses were made, of which cross 8 was most fertile and produced 51 putative F_2 progeny isolates. The two parental isolates 2.45 (A1) and 2.50 (A2) were both intermediate in sensitivity to metalaxyl-M and without host preference.

Phenotypic characterization of isolates

All field isolates and the F_1 and F_2 progeny were characterized for mating type (A1, A2) as described by Knapova & Gisi (2002). Sensitivity to metalaxyl-M was evaluated by placing mycelial plugs on V8 agar amended with five different concentrations (100, 10, 1, 0·1, 0·01 mg a.i. L^{-1}) of metalaxyl-M (formulated as EC 480) and on fungicide-free V8 medium. Radial growth was measured after 12 days of incubation at 18°C in the dark. Dose–response curves and EC_{50} values were calculated on the basis of growth inhibition in three independent experiments with three replicates per treatment. The isolates were assigned to three groups: sensitive (s, $EC_{50} \leq 0{\cdot}02$ mg L^{-1}); intermediate (i, 0·02 mg $L^{-1} < EC_{50} \leq 10$ mg L^{-1}); and resistant (r, $EC_{50} > 10$ mg L^{-1}).

For the assessment of virulence, detached leaflets of an R-gene differential set of potato clones, R1 to R11, each

Table 1 Phenotypic and genotypic characterization of *Phytophthora infestans* isolates collected from potato (P) and tomato (T) fields in Switzerland (CH), France (F) and Germany (D) in 1997

Country	Isolate	Race structure[a]	Number of virulence factors	Sensitivity[b]	Mating type	SSR genotype[c]	mt haplotype[d]
CH	P1[e]	3.4.10.11	4	s	A1	D-02	Ib
F	P2	1.3.4.7.10.11	6	s	A1	nd[f]	nd[f]
F	P3	1.3.4.7.8.10.11	7	s	A1	nd	nd
CH	P4	1.2.3.4.7.8.10.11	8	r	A1	nd	nd
CH	P5	1.2.3.4.6.7.8.10.11	9	r	A1	nd	nd
CH	P6	1.3.4.7.10.11	6	s	A2	D-02	Ia
J	P7[g]	1.2.3.4.7.8.10.11	8	r	A2	A-01	IIa
CH	T1	4	1	s	A1	B-01	Ia
CH	T2	1.4	2	s	A1	D-01	Ia
CH	T3	1.3.4	3	s	A1	B-03	Ia
D	T4	1.2.3.4.6.7.10.11	8	s	A1	D-03	Ia
CH	T5	1.3.4.7.10.11	6	s	A1	A-03	Ia
CH	T7	4	1	s	A2	D-03	Ia
F	T8	3.7	2	s	A2	B-03	Ib
CH	T9	1.3.4.7	4	s	A2	A-03	Ia
F	T10	1.3.4.7	4	s	A2	A-03	Ia

[a]Determined with potato, R-gene differentials (see Materials and methods).
[b]s, sensitive; r, resistant to the phenylamide fungicide metalaxyl-M.
[c]SSR, simple sequence repeats, nomenclature described in Table 2.
[d]Mitochondrial haplotype, according to Griffith & Shaw (1998).
[e]Isolate received in 1986.
[f]nd, not determined.
[g]Isolate received from Japan in 1990.

carrying a single resistance gene, were used as described by Knapova & Gisi (2002). Aggressiveness of field isolates and progeny was tested on 2·5 cm diameter leaf discs of potato cv. Bintje and tomato cv. Baby. Leaves number 4 and 5 (from the top) of 5-week-old plants were used to cut leaf discs. These were placed lower surface up on water agar in Petri dishes. For inoculation, a 50 μL droplet of a sporangial suspension (20 000 sporangia mL^{-1}) was pipetted onto the centre of each leaf disc. The dishes were incubated for 6 days at 16°C and a photoperiod of 16 h per day. For every isolate and both hosts, three Petri dishes containing five leaf discs were inoculated. The infected leaf area was assessed visually and was defined as necrotic plus green area covered with sporangia. The isolates were tested in three independent experiments, and the data were pooled. Statistical evaluations for significant differences between isolates and hosts were performed with the Mann–Whitney Rank sum test at $P < 0{\cdot}001$.

Genotypic characterization of isolates

Field isolates and F_1 and F_2 progeny were characterized for mitochondrial haplotype by RFLP–PCR, and nuclear markers based on AFLP and SSR (microsatellites) (Knapova *et al*., 2001; Knapova & Gisi, 2002). The AFLP method was performed with minor modifications. The volume for the restriction/ligation reaction was 22 μL containing 1 × T4 ligation buffer with 10 mM ATP, 100 ng genomic DNA, 2 U *Mse*I, 10 U *Eco*RI and 130 U T4 DNA-ligase (all enzymes and buffers from New England BioLabs, Beverly, MA, USA), 0·2 μM *Eco*RI-adapters, 2 μM *Mse*I-adapters, 50 mM NaCl, and 1·1 μg BSA. The mixture was incubated overnight at 37°C. For AFLP genotyping, a total of 25 and 18 AFLP markers were used for the F_1 and F_2 progeny, respectively. For microsatellite (SSR) analyses, the primer pairs 4B (forward: 5′-AAA ATA AAG CCT TTG GTT CA-3 labelled with HEX; reverse: 5′-GCA AGC GAG GTT TGT AGA TT-3′) and 4G (forward: 5′-CGC TGT GTG GAT GAC AAG TA-3′ labelled with NED; reverse: 5′-TCG ACC TGA CAT ACG AGC TA-3′) were used for amplifying the polymorphic TC microsatellites 4B and 4G. A total of 10 different alleles at two loci were chosen for genotype classification (Table 2), of which alleles 206, 214, 218 and 226 were at locus 4B, and alleles 157, 159, 161, 163, 165 and 177 were at locus 4G.

Sexual crosses

Crosses between selected A1 and A2 field isolates were made on V8 agar in the same way as for mating type determination. Crosses were made with isolates that were subcultured once after cryoconservation. In total, 19 crosses were made, eight of which (C1–C8) involved isolates collected from potato (P1–P7); five (C15–19) isolates from tomato (T1, T2, T4, T5, T7–T10); and six (C9–C14) isolates from the two different hosts (P2, P6,

Table 2 Single sequence repeat (SSR) genotype classification for *Phytophthora infestans* isolates based on allele frequency at microsatellite loci Pi4B and Pi4G

	Pi4B[a]				Pi4G					
Genotype	206	214	218	226	157	159	161	163	165	177
A-01			X							
A-03			X				X			
B-01		X								
B-03		X					X			
D-01		X	X							
D-02		X	X					X		
D-03		X	X				X			

[a]More information on the SSR classification is given by Knapova & Gisi (2002).

Table 3 Oospore production on V8 agar (after 12 days) and oospore germination on water agar (after 14 days) for crosses (C1–C20) of *Phytophthora infestans* isolates collected from potato (P) or tomato (T)

Cross	Mating type (A1, A2) and sensitivity (s,r)[a]	Number of oospores (mm^{-2})[b]	Germination of oospores (% of total)[b]
P × P			
C1 P1 × P6	A1s × A2s	4 ± 2	0
C2 P2 × P6	A1s × A2s	30 ± 6	10 ± 3
C3 P3 × P6	A1s × A2s	19 ± 3	6 ± 0
C4 P1 × P7	A1s × A2r	4 ± 3	0
C5 P2 × P7	A1s × A2r	6 ± 4	0
C6 P4 × P6	A1r × A2s	6 ± 2	0
C7 P5 × P6	A1r × A2s	0	–[c]
C8 P5 × P7	A1r × A2r	0	–
		m = 8·6	*m* = 2·7
P × T			
C9 P2 × T9	A1s × A2s	5 ± 0	4 ± 1
C10 P6 × T1	A2s × A1s	18 ± 3	0
C11 P6 × T2	A2s × A1s	14 ± 2	6 ± 1
C12 P6 × T3	A2s × A1s	17 ± 9	0
C13 P6 × T4	A2s × A1s	19 ± 7	24 ± 3
C14 P7 × T4	A2r × A1s	10 ± 4	4 ± 1
		m = 13·8	*m* = 6·3
T × T			
C15 T1 × T7	A1s × A2s	11 ± 5	10 ± 3
C16 T2 × T9	A1s × A2s	3 ± 3	0
C17 T4 × T7	A1s × A2s	8 ± 1	24 ± 4
C18 T4 × T8	A1s × A2s	12 ± 2	11 ± 2
C19 T5 × T10	A1r × A2s	4 ± 2	9 ± 0
		m = 7·6	*m* = 10·8
Reference mating			
C20 P6 × T7	A2s × A2s	0	–

[a]s, sensitive; r, resistant to the phenylamide fungicide metalaxy-M.
[b]Means of five replicates with standard deviation. [c]Not determined.

P7, T1–T4, T9) (Table 3). An A2 × A2 combination (C20) was used as negative control (identical mating types). Oospores were counted microscopically in five replicates after incubation in the dark at 18°C for 12 days.

To assess oospore germination, a 5 × 30 mm piece of agar was cut from each plate, added to 4 mL distilled water and vortexed. The samples were then filtered through a nylon net (mesh size 45 μm) to remove mycelial fragments. Samples containing 1 mL oospore filtrate and 1 mL 0·6% Novozym solution (later 1·7% Glucanex, Novo Nordisk, A/S, Bagsværd, Denmark) were incubated overnight and then centrifuged three times for 3 min each (3000 ***g***). After each centrifugation the supernatant was removed and the pellet resuspended in 1 mL distilled water. Ten droplets (10 μL each) of the final preparation were pipetted onto water agar plates (10 plates for each sample). Germinating oospores were counted microscopically after incubation for 14 days at 18°C. For cross C14, germinating single oospores were transferred with a glass needle from water to V8 agar containing rifampicin (10 mg L^{-1}), ampicillin (125 mg L^{-1}) and pimaricin (10 mg L^{-1}). A total of 43 F_1 progeny were successfully isolated and analysed for mating type and AFLP and SSR genotype. Because of contamination, only 35 isolates were assessed for sensitivity to the phenylamide fungicide metalaxyl-M and only 27 for mtDNA haplotype.

Four different sibcrosses of F_1 progeny isolates were carried out: 2.47 × 2.50; 2.41 × 2.50; 2.53 × 2.54; and 2.45 × 2.50. For cross 2.41 × 2.50, a germination rate of 3.5% was observed and only five F_2 progeny isolates could be produced. Cross 2.47 × 2.50 (germination rate 3.0%) yielded only two, and cross 2.53 × 2.54 (germination rate 2.8%) no progeny isolates at all. However, in cross 2.45 × 2.50 (cross 8), a germination rate of 3.7% was observed and 54 F_2 progeny were successfully isolated, of which three were not viable.

Results

Oospore production and germination in F_1 progeny

All isolates grew well, and formation of oospores on V8 agar occurred after 12 days of incubation for most crosses. The number of oospores and percentage germination *in vitro* varied greatly among the 19 *P. infestans* crosses tested (Table 3). The highest number of oospores was produced by cross C2 (P × P), followed by C3 (P × P), then C13, C10 and C12 (all P × T). In two crosses (C7 and C8, both P × P crosses) no oospores were formed, even after an additional 7 days incubation. On average, the number of oospores was highest in P × T crosses (average 13·8 oospores mm^{-2}).

Oospore germination was generally low, and none geminated in seven crosses (C1, 4, 5, 6, 10, 12 and 16). However, in two crosses, C17 (T × T) and C13 (P × T), 24% of oospores germinated after incubation for 14 days on water agar (Table 3). In general, oospore germination was lowest in P × P crosses and highest in T × T crosses. Crosses that produced most oospores generally had higher percentage germination, with the exception of crosses 10 and 12, which had a high number of oospores but no germination, and cross 17, which produced a low number of oospores but had high percentage germination

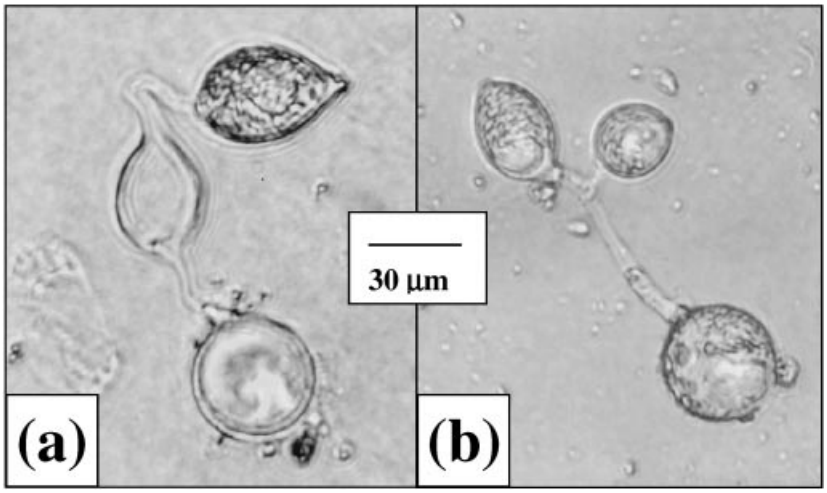

Figure 1 Oospores of *Phytophthora infestans* germinating on water agar by producing a germ tube with sporangia (a) in series and (b) by branching.

(Table 3). Most oospores germinated by formation of one or more sporangia (Fig. 1a,b, respectively) that either formed another sporangium (Fig. 1a) or allowed mycelial growth by formation of a germ tube. Some oospores formed germ tubes and long hyphae without producing sporangia. Taking oospore production and germination together as a measure of fertility of sexual crosses, only 10 out of the 19 crosses were fertile. There were only two fertile crosses, C14 and C19, with parents carrying different sensitivities (s and r, respectively) to metalaxyl-M, and C14 was selected for segregation analyses because the parents were different in mitochondrial haplotype (IIa for P7 and Ia for T4) and originated from different hosts.

Segregation of phenotypic traits in F_1 and F_2 progeny

A total of 43 single-oospore F_1 progeny isolates was successfully produced from cross C14, of which 25 belonged to mating type A1 and 18 to A2. The F_1 parents were similar in race structure and both were highly complex races with virulence on eight potato differentials. Parent P7 was virulent on differential R8 and parent T4 on differential R6; otherwise virulence spectra were identical (Table 1). Of 12 progeny tested, none was virulent on differential R6, and only four were virulent on differential R8. In the F_2 progeny of cross 8, mating type distribution was 24 A1 and 21 A2. Six isolates produced oospores without the presence of the opposite mating type (self-fertile or homothallic isolates).

The sensitivity of the F_1 parents to the phenylamide fungicide metalaxyl-M differed strongly, with EC_{50} values of 0·0013 mg L^{-1} for the sensitive T4 and 37·6 mg L^{-1} for the resistant P7 parents (Fig. 2). For technical reasons, only 35 of the 43 F_1 progeny isolates were successfully tested for sensitivity to metalaxyl-M. Almost all (31 of 35) isolates gave an intermediate response to metalaxyl-M (EC_{50} ranging from 0·037 to 2·08 mg L^{-1}), which was significantly different at $P = 0·005$ from both parents (Fig. 2). Offspring numbers 7 and 44 were resistant to metalaxyl-M, A2 mating type, and had SSR genotype A-01, identical to the resistant parent. Offspring numbers 3 and 5 were sensitive, A1 mating type, and had SSR genotype D-03, identical to the sensitive parent. Therefore these four offspring probably emerged through selfing, whereas all others can be considered as hybrid F_1 progeny. The very close (but not identical) AFLP banding pattern of isolates 3, 5, 7 and 44 compared to their respective parents (Fig. 4) is further support for the selfing hypothesis.

The two parental F_1 isolates of the sibmating cross 8 were intermediate in sensitivity, with EC_{50} values of 2·32 and 2·54 mg L^{-1}, respectively. The EC_{50} values of four of the 51 F_2 isolates analysed were significantly higher (8·61–40·17 mg L^{-1}) than those of the parents, and were defined as resistant. Another four F_2 isolates yielded significantly lower EC_{50} values (0·04–0·12 mg L^{-1}), and were considered sensitive (Mann–Whitney rank sum test, $P = 0·001$). All other 43 F_2 isolates did not differ from the parents and were intermediate in sensitivity to metalaxyl-M. According to SSR and AFLP analyses, as well as determination of mating type and sensitivity to metalaxyl-M, four F_2 isolates emerged through selfing, two each of the parental isolates 2.45 and 2.50.

For the F_1 progeny isolates, the infected leaf area (ILA) ranged from 8 to 70% on tomato and from 2 to 85% on potato. In general, the most aggressive isolates on potato were also most aggressive on tomato, and weak isolates were weak on both hosts. The aggressiveness of most F_1 progeny isolates was higher than that of the potato parent, P7 (30% ILA), but lower than that of the tomato parent, T4 (71% ILA). Of F_1 progeny isolates, 23% attacked potato significantly better than tomato, 15% showed a higher aggressiveness on tomato, and 62% did not express any host preference (Table 4). In general, aggressiveness of F_2 progeny isolates was lower compared to F_1; the infected leaf area on tomato ranged from 1 to 65%, and on potato from 0 to 69%. A significant proportion of isolates (24%) infected both hosts very poorly (ILA < 5%), and were considered as nonpathogenic (Table 4). The proportion of highly aggressive isolates (ILA > 50%) decreased from 46% in the F_1 to 7% in the

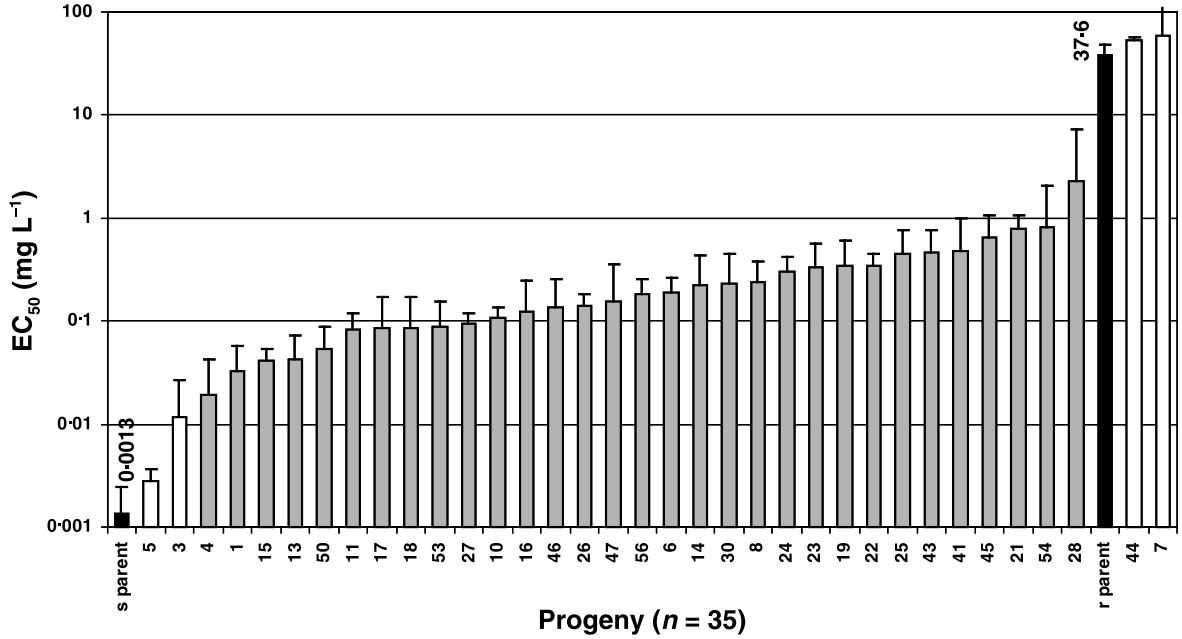

Figure 2 Sensitivity of *Phytophthora infestans* to metalaxyl-M (EC_{50}, mg L^{-1}) of the sensitive (s) parent T4, the resistant (r) parent P7, and the F_1 progeny (n = 35). Offspring 3 and 5 are selfs of the sensitive parent (T4); 7 and 44 are selfs of the resistant parent (P7). Values are means of three tests, bars are standard deviations.

Table 4 Proportion of isolates in the F_1 and F_2 progeny of *Phytophthora infestans* expressing different levels of aggressiveness (% infected leaf area, ILA) on tomato and potato leaf discs (host preference)

		No preference		Tomato		Potato	
Progeny[a]	Non-pathogenic (<5%)[b]	5–50%[b]	>50%	5–50%[b]	>50%	5–50%[b]	>50%
F_1 (n = 13)	0	39	23	15	0	0	23
F_2 (n = 41)	24	32	5	37	0	0	2

[a]Selfs and self-fertiles are not included.
[b]Percentage infected leaf area.

F_2 progeny. There was a shift in host preference (potato : tomato : no preference) from 23 : 15 : 62% of isolates in F_1 progeny to 2 : 37 : 37% in F_2 (Table 4), or 3 : 48: 49% if nonpathogenic isolates are excluded.

Segregation of genotypic markers in F_1 and F_2 progeny

Almost all (25 out of 27) F_1 progeny were of the same mitochondrial haplotype, Ia, as the T4 parent. Only two isolates were haplotype IIa as the P7 parent (Fig. 3). Inheritance of mtDNA fragments is uniparental and maternal for a unisogamous organism such as *P. infestans*. In cross C14, the T4 parent (A1 mating type) apparently served as 'female' and the P7 parent (A2 mating type) as 'male' for almost all progeny. The two progeny which were haplotype IIa, numbers 18 and 27 (arrows in Fig. 3) were both A2 mating type, SSR genotype A-03, and almost identical in AFLP banding pattern (Fig. 4). They emerged either through paternal inheritance with the P7 parent serving as male (leakage from antheridia), or through maternal inheritance with a change in sexual behaviour of P7 serving as female.

Parent P7 had SSR genotype A-01 with one allele (218) at locus 4B and a 'null allele' (no allele) at locus 4G, whereas parent T4 had SSR genotype D-03, which is heterozygous at locus 4B (alleles 214 and 218) and has one allele (161) at locus 4G (Tables 1, 2 and 5). The F_1 progeny (39 isolates, four selfs not included) was composed of six different SSR genotypes: the parental genotypes A-01 and D-03 (six and seven isolates, respectively); genotypes A-03 and D-01 (nine and 15 isolates, respectively); and two unexpected genotypes, B-01 and B-03 (one isolate each) (Table 5). For locus 4B, the segregation pattern of alleles was 15 : 22 : 2, and for locus 4G it was 22 : 17, which represents a largely 1 : 1 Mendelian segregation ratio. The parents of the F_2 progeny, 2.50 and 2.45, belonged to the same SSR genotype (A-01 and D-03, respectively) as the P7 and T4 parents of the F_1 progeny (Table 5). In the F_2 progeny the two parental genotypes, A-01 and D-03, were detected (21 and 12 isolates, respectively), and in addition two new genotypes, A-03 and D-01, emerged (three and five isolates, respectively) (Table 5). The segregation pattern was 26 : 15 for locus 4G and 24 : 17 for locus 4B, which represents a

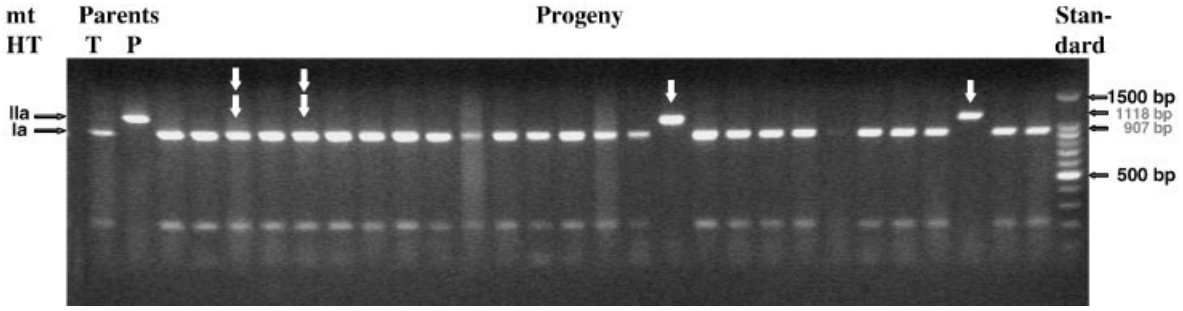

Figure 3 Uniparental inheritance of mitochondrial DNA haplotype (mt HT) in the F_1 progeny (n = 27) of cross C14 (Table 3) with parents T4 (mt HT Ia) × P7 (mt HT IIa) of *Phytophthora infestans.* The first two lanes are parents (T4, P7) and the last lane is a DNA standard ladder. The 27 lanes in between are progeny 1–29 from left to right (isolates 7 (self of P7 parent) and 12 are not included). Lanes 5 and 7 (isolates 3 and 5, double arrows) are selfs of the T4 parent (mt HT Ia). Two isolates in lanes 18 and 27 are mt HT IIa (marked with arrows, both A2 mating type as P7), all others are mt HT Ia.

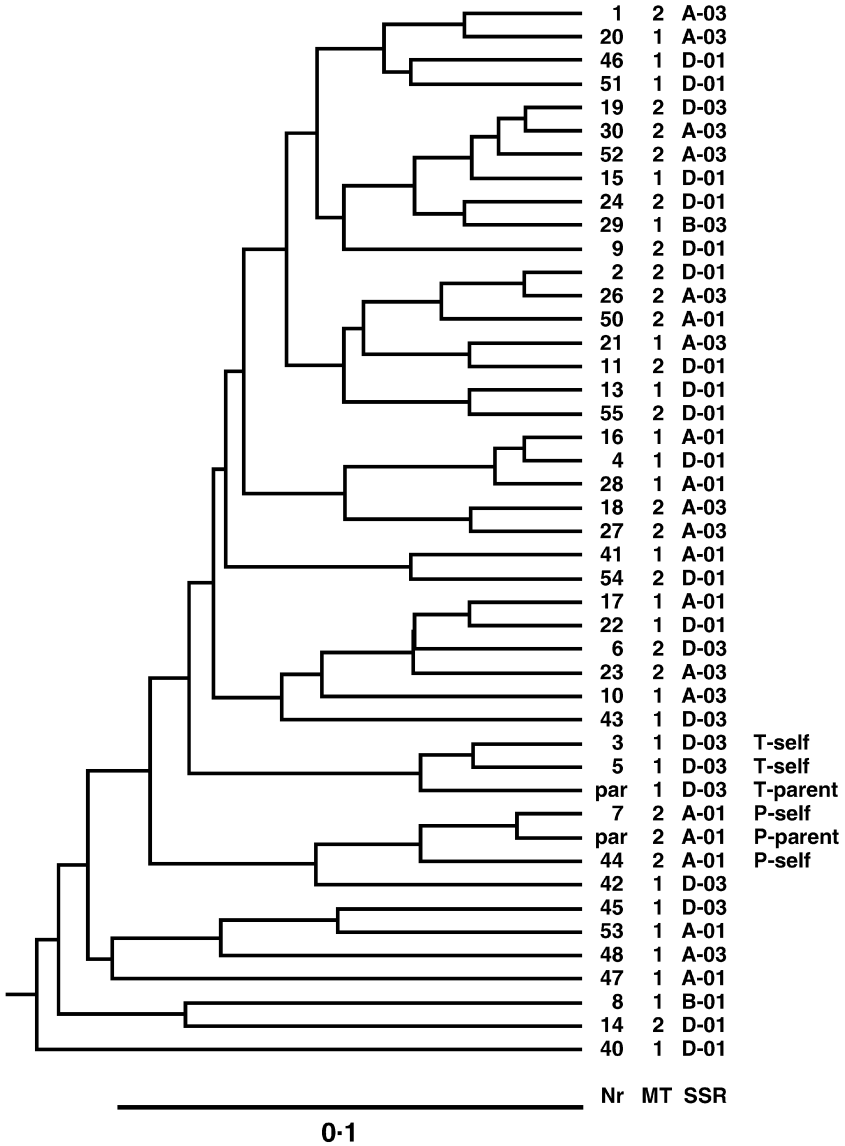

Figure 4 Genetic similarity of AFLP genotypes in the F_1 progeny (n = 43, as number Nr, including four selfs) of *Phytophthora infestans* from mating C14 (Table 3) with the parents T4 and P7. The mating type (MT) of isolates is given as 1 for A1 and 2 for A2; SSR genotypes are defined in Table 2. All isolates are mitochondrial DNA haplotype Ia, except isolates 7 and 44 which are selfs of P7-parent, and 18 and 27 which are mt haplotype IIa.

Table 5 Single sequence repeat (SSR) genotypes, allele combination, number of isolates and segregation pattern in F_1 progeny of cross C14 and in F_2 progeny of sibcross 8 of *Phytophthora infestans*

Locus	Parents		Allele (bp)[a] in progeny						Segregation	
									Exp.	Obs.
	P7	T4							F_1 (n = 39)[c]	
4B	218	214	218	218	214	214	214	214	218/218 : 214/218	(A-01 + A-03) : (D-01 + D-03)
	218	218	218	218	218	218	214	214	= 1 : 1	: (B-01 + B-03)
										= 15 : 22 : 2
4G	0[b]	161	0	161	0	161	0	161	unknown	(A-01 + D-01 + B-01)
		0[b]		0		0		0		: (A-03 + D-03 + B-03)
										= 22 : 17
Genotype	A-01	D-03	A-01	A-03	D-01	D-03	B-01	B-03		
No. isolates[c]			6	9	15	7	1	1		
	2.50	2.45							F_2 (n = 41)[d]	
4B	218	214	218	218	214	214			218/218:214/218	(A-01 + A-03) : (D-01 + D-03)
	218	218	218	218	218	218			= 1 : 1	= 24 : 17
4G	0[b]	161	0	161	0	161			0 : 161/0	(A-01 + D-01) : (A-03 + D-03)
		0[b]		0		0			1 : 1	= 26 : 15
Genotype	A-01	D-03	A-01	A-03	D-01	D-03				
No. isolates[d]			21	3	5	12				

[a]Allele sizes in bp; the locus is homozygous or heterozygous if the two alleles have the same or a different size, respectively.
[b]0 = 'null allele' for locus 4G and the A-01 parent; also confirmed with a second primer pair producing a longer amplicon.
[c]Total number of progeny excluding four selfs.
[d]Total number of progeny excluding four selfs and six self-fertile isolates.

largely 1 : 1 Mendelian segregation ratio (χ^2 = 3·13, P = 0·08).

Based on 25 AFLP markers, the 43 F_1 progeny isolates (including four selfs) were closely related to each other and clustering was not possible (Fig. 4). The four selfs were very close, but not identical to the two parents, P7 and T4 (one band missing from each self compared to parents). There was no obvious association between AFLP and SSR genotypes, nor between AFLP genotype and mating type (Fig. 4). Of the 25 AFLP markers, 16 were homozygous in the parents and segregated in an almost perfect 1 : 0 ratio (11 markers with a ratio 39 : 0, four with 38 : 1 and one with 37 : 2) in F_1 progeny (Table 6). Four out of the seven remaining markers segregated in the expected 1 : 1 ratio, the other three (203, 411, 427) behaved differently. The expected 1 : 2 : 1 segregation pattern was observed for marker 343 (11 : 21 : 7), but not for 309 (14 : 25 : 0) (Table 6). Thus inheritance of AFLP markers in the F_1 progeny was largely Mendelian (for 21 of 25 markers). For the F_2 progeny (n = 41, excluding six self-fertiles and four selfs), 18 polymorphic bands were used as markers to identify recombinants. Ten out of 18 AFLP markers segregated in the expected 1 : 1 ratio and four in a 3 : 1 ratio. A significant distortion was observed for four markers only (results not shown). Two additional isolates were almost identical in AFLP banding pattern to each of parents 2.50 and 2.45. Two displayed the same SSR genotype, mating type and sensitivity to metalaxyl-M as parent 2.45, the other two were identical to parent 2.50. Therefore the four isolates were classified as selfs.

Discussion

The segregation of phenotypic characteristics and genotypic markers in the F_1 progeny was analysed for cross C14, which was selected because the parents, P7 (mating type A2) and T4 (mating type A1), differed in sensitivity (resistant vs. sensitive) to the phenylamide fungicide metalaxyl-M, mitochondrial DNA haplotype (IIa vs. Ia), SSR genotype (A-01 vs. D-03), and host preference (potato vs. tomato). The segregation in the F_2 progeny was analysed for sibcross 8 because the F_1 parents, 2.50 and 2.45, did not differ in sensitivity to metalaxyl-M and host preference, and were the same SSR genotype as the F_0 parents (A-01 and D-03, respectively). This combination of parents was apparently the most productive in sexual recombination and yielded enough isolates in F_1 and F_2 progeny for studying segregation. The other parental pairs were less fertile or suffered a higher mortality among the progeny, possibly due to the known consequences of inbreeding in sibcrosses (Eckert, 1994).

The genetics of mating type in *P. infestans* is complex and the A1 : A2 ratio in progeny can vary significantly, ranging from close to 1 : 1 to an excess of A1 over A2 (Shaw, 1991; Judelson *et al*., 1995). In this study, a ratio of 25 : 18 was observed in the F_1 and 24 : 21 in the F_2 progeny. A low frequency of A2 mating type isolates was repeatedly observed in populations of some European countries such as the UK, France, Germany and Switzerland (Gisi & Cohen, 1996; Knapova & Gisi, 2002), and this may be partly a result of unbalanced sexual recombination. Outcrossing and selfing can both occur within

Table 6 Segregation of 25 AFLP markers in F_1 progeny of cross C14 (T4 × P7) of *Phytophthora infestans*

AFLP markers (bp)	Parents, genetic model[a]		Segregation of F_1 progeny[b]			
	T4	P7	Expected		Observed	
163	*AA*	*AA*	*AA*	1 : 0	*AA*	38 : 1
203	*Aa*	*AA*	*AA,Aa*	1 : 1	*AA,Aa,aa*	18 : 20 : 1[c]
233	*AA*	*AA*	*AA*	1 : 0	*AA*	39 : 0
238	*Aa*	*aa*	*Aa,aa*	1 : 1	*Aa,aa*	15 : 24
256	*AA*	*AA*	*AA*	1 : 0	*AA*	39 : 0
259	*AA*	*AA*	*AA*	1 : 0	*AA,aa*	38 : 1
284	*AA*	*AA*	*AA*	1 : 0	*AA*	39 : 0
289	*AA*	*AA*	*AA*	1 : 0	*AA*	39 : 0
309	*Aa*	*Aa*	*AA,Aa,aa*	1 : 2 : 1	*AA,Aa,aa*	14 : 25 : 0[c]
328	*AA*	*AA*	*AA*	1 : 0	*AA*	39 : 0
338	*AA*	*AA*	*AA*	1 : 0	*AA*	39 : 0
343	*Aa*	*Aa*	*AA,Aa,aa*	1 : 2 : 1	*AA,Aa,aa*	11 : 21 : 7
368	*AA*	*AA*	*AA*	1 : 0	*AA,Aa*	38 : 1
380	*Aa*	*aa*	*Aa,aa*	1 : 1	*Aa,aa*	21 : 18
390	*AA*	*Aa*	*AA,Aa*	1 : 1	*AA,Aa*	19 : 20
394	*AA*	*AA*	*AA*	1 : 0	*AA,Aa*	38 : 1
400	*AA*	*AA*	*AA*	1 : 0	*AA,Aa*	37 : 2
411	*Aa*	*aa*	*Aa,aa*	1 : 1	*Aa,aa*	28 : 11[c]
427	*Aa*	*aa*	*Aa,aa*	1 : 1	*Aa,aa*	13 : 26[c]
436	*aa*	*AA*	*Aa*	1 : 0	*AA,Aa,aa*	39 : 0
448	*AA*	*AA*	*AA*	1 : 0	*AA*	39 : 0
451	*Aa*	*aa*	*Aa,aa*	1 : 1	Aa,aa	15 : 24
457	*AA*	*AA*	*AA*	1 : 0	*AA*	39 : 0
486	*AA*	*AA*	*AA*	1 : 0	*AA*	39 : 0
493	*AA*	*AA*	*AA*	1 : 0	*AA*	39 : 0

[a]*AA* homozygous; *Aa* heterozygous; *aa*: homozygous absent.
[b]Total number of progeny was 39, excluding four selfs.
[c]Significantly different from expected segregation ratio (χ^2 test), all other ratios not significantly different.

mating cultures (Judelson, 1997). In previous studies the frequency of oospore-derived cultures that resulted from selfing ranged from low (Shattock *et al*., 1986) to more than half of all cultures (Whittaker *et al*., 1994). In the crosses here, about 10% of isolates in both F_1 and F_2 progeny were selfs. They were differentiated from outcrosses and cultures arising from residues of parental mycelia by the segregation of SSR and AFLP markers, and sensitivity to metalaxyl-M. The F_1 selfs were either sensitive or resistant, as were the parents, whereas all true sexual recombinants had an intermediate response to metalaxyl-M. Mating type and SSR genotype of the selfs in F_1 and F_2 progeny were identical to their parents, whereas AFLP banding pattern was very similar but not identical to that of the parents. In the evolution of field isolates and genetic diversity of populations, not only outcrossing but also selfing should be considered. The latter process may be a more likely event in populations, assuming that selfing is similar in frequency *in planta* as observed *in vitro*.

Inheritance of resistance to metalaxyl has been described as being controlled by one single, incompletely dominant gene (Shaw & Shattock, 1991). This observation was made because the majority of the F_1 progeny from a sensitive and resistant parent was intermediate in sensitivity, and crosses between two F_1 isolates yielded a 1 : 2 : 1 distribution of sensitive, intermediate and resistant F_2 progeny isolates (Shattock, 1988). In another report, resistance to metalaxyl was associated with one semidominant locus combined with the effect of several minor loci resulting in a continuous segregation for sensitivity in F_1 progeny (Fabritius *et al*., 1997). In a separate study, one parent was heterozygous with one single gene carrying a dominant allele, and the segregation was 1s : 1r in F_1 progeny and 1s : 3r in F_2 progeny (Lee *et al*., 1999). Although the F_1 isolates of cross 14 were intermediate in sensitivity, suggesting that one single, incompletely dominant gene controls metalaxyl resistance, the segregation pattern in the F_2 generation was not 1 : 2 : 1, but approximately 1 : 10 : 1. The distortion from the Mendelian distribution may be explained by the quoted effects of minor loci (Fabritius *et al*., 1997) or the presence of more than one major locus for metalaxyl resistance (Judelson & Roberts, 1999). It is not known how frequent the various resistance alleles are in resistant individuals of different origins, but it is apparent that sensitive offspring will always reappear after recurrent sexual recombination in subsequent generations, and resistant individuals will not disappear independently of the model of inheritance described above. If products containing phenylamide fungicides such as metalaxyl-M are used within

recommendations (e.g. limitation of number of applications), treated populations where recombination is occurring would be expected to remain mixed in sensitivity (Gisi & Cohen, 1996). In addition, migration of individuals will contribute to mixed sensitivities in clonal populations.

SSR and AFLP markers are unambiguous nuclear tools that can be used to identify and characterize the segregation patterns in hybrid progeny. If only one allele is detected for a specific locus in an SSR genotype, it may represent a homozygous state or a combination with a null allele. Based on the segregation pattern of the SSR markers used here, the parent P7 was homozygous at locus 4B and carried a null allele at locus 4G, whereas the other parent (T4) was heterozygous at locus 4B and homozygous at locus 4G. For locus 4B, the segregation of alleles in the F_1 progeny was 15 : 22 : 2 (homozygote × heterozygote), and for locus 4G it was 22 : 17 (null allele × homozygote; selfs not included in these ratios). This apparent 1 : 1 Mendelian segregation suggests that all offspring are 'true' outcrosses and that the null allele (locus 4G) can be regarded as heterozygous. The two unusual isolates which are homozygous (allele 214/214) at locus 4B could have emerged through loss of four base pairs, selfing, or if the parent P carries a null allele at locus 4B. The latter two possibilities are considered unlikely because the two genotypes were intermediate in sensitivity to metalaxyl-M, which precludes selfing, and their frequency (one isolate per genotype) in F_1 progeny was too low to support the hypothesis of a null allele. In addition to the two unexpected SSR genotypes (B-01 and B-03), another two new genotypes, A-03 and D-01 (nine and 15 isolates, respectively), and the two parental genotypes, A-01 and D-03 (six and nine isolates, respectively), emerged from this cross in the F_1 progeny. Because the parents of both F_1 and F_2 were the same SSR genotypes (A-01 and D-03, respectively), segregation of the alleles was expected to be similar for the F_2 progeny. At locus 4B it was a 1 : 1 Mendelian segregation (24 : 17), whereas at locus 4G the null allele was about twice as frequent as allele 161 (26 : 15). The reasons for this distortion are unknown. The SSR genotypes generated in the F_1 and F_2 progeny were also detected in isolates of *P. infestans* collected recently in Switzerland and France (Knapova & Gisi, 2002). In that study the A-03 was the predominant SSR genotype collected from potato. It is perhaps surprising that a cross between an isolate from Germany (parent T4) and from Japan (parent P7, this study) has produced SSR genotypes that are found in recent populations in France and Switzerland (Knapova & Gisi, 2002). Nevertheless, the parents used for the cross had SSR genotypes (D-03 and A-01) which are present, although only in few isolates, in the field population. This observation may be explained by the complex nature of the genome and high heterozygousity of *P. infestans*, and suggests that recombination may be more important than migration in determining the structure of local populations. The structure of current European populations may have emerged partially from local processes, including sexual recombination and selection for higher fitness.

Of the 25 AFLP markers used to characterize segregation in the F_1 progeny, 16 were homozygous and nine heterozygous in the F_0 parents; they segregated in either a 1 : 0 ratio (a few offspring had one or two very fine bands that were not present in the others), a 1 : 1, or a 1 : 2 : 1 ratio. Four markers had unexpected segregation ratios. In the F_2 progeny, there were four (out of 18) AFLP markers with an unexpected segregation, the others segregated in the expected 1 : 1 or 3 : 1 ratios. There are several possibilities described in the literature for abnormalities in segregation, including linked deleterious, recessive alleles, mitotic irregularities, chromosome rearrangements and unsuspected epistatic loci (Spielman *et al*., 1990). Inaccurate technical procedures such as contamination with foreign DNA or incomplete digestion of DNA may also modify results. It is not known what causes the unexpected segregation pattern of some markers in this study. Additional crosses with the same and also with other genotypes should be produced to investigate the inheritance of phenotypic and genotypic traits and the frequency of unexpected segregation in *P. infestans*.

In oomycetes, inheritance of mitochondrial genes is known to be maternal in most cases (Birky, 1995). In this study, 25 of 27 isolates of the F_1 progeny received mitochondrial DNA fragments from one parent and two from the other parent. Biparental inheritance did not occur. It is assumed that mtDNA haplotype in *P. infestans* is inherited maternally as a rule, but that during fertilization paternal inheritance is also possible to a certain degree through leakage of mitochondria from antheridia. Alternatively, a localized change of sexual behaviour of the parents may also explain the observed results. In this case the A2 parent would have produced oogonia in localized areas of the culture. Although sexual preference and compatibility type are determined by unlinked loci, A1 and A2 isolates generally act as predominant females and males, respectively (Judelson, 1997).

In most crosses produced in this study, abundant oospores were formed in agar cultures after 12 days of incubation. Nevertheless, a few crosses did not produce oospores, although the parents were of opposite mating type. Furthermore, germination of oospores was generally low (and nil for some crosses) and depended on the combination of parental isolates. Nonviability in progeny may be caused by balanced lethals due to a deficiency in essential genes (Judelson *et al*., 1995). However, other factors may be as important for fertility, such as age of cultures (time between isolation from host and mating), mycelial growth rate, production of compatibility substances and diffusible sex hormones (Judelson, 1997), and adequate growth conditions *in vitro* similar to those present *in planta*. Even in host tissue, the production of oospores can vary significantly. Abundant oospores form in the pericarp and around the seeds of tomato fruits, whereas in potato tubers production appears to be limited (Levin *et al*., 2001). In this study (Table 3), crosses between isolates originating from potato were generally less fertile (based on *in vitro* production and germination of oospores) than those between isolates from potato and

tomato, or tomato and tomato. It is not known which factors control fertility in *P. infestans* isolates, but origin and high fitness of parents are likely to play a major role.

Although two F_1 isolates without host preference were crossed, only one F_2 isolate was found that was more aggressive on potato than on tomato, whereas half the F_2 progeny preferred tomato. It would be interesting to examine host preference in matings between potato isolates, as it is not known whether segregation for tomato preference would still occur. In contrast, would a cross between two isolates preferring tomato result in progeny exclusively preferring tomato? Unfortunately, the mating type and host preference of the F_1 isolates made it impossible to study these questions. It would also be interesting to verify, by using more crosses and a larger number of isolates in F_1 and F_2 progeny, if the increased proportion of tomato-preferring isolates observed in the F_2 progeny is a general trend for sexual recombination in *P. infestans*, or a result of using particular parental isolates. Host preference is not a differentiating character but shows a continuous gradation in its expression, as is known for traits that are inherited polygenically. Vega-Sánchez *et al.* (2000) described this phenomenon as 'host adaptation' and concluded that it is determined not by the ability to cause disease, but rather by quantitative differences in pathogenic fitness. They further suggest that an adaptation to tomato is associated with a loss of pathogenic fitness on potato. In general, sexual recombination in *P. infestans* may yield highly aggressive progeny isolates that may express a preference for potato or tomato, further enhanced by selection through the host.

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