Plant Pathology (2002) **51**, 710–719

Potential of sexual reproduction among host-adapted populations of *Phytophthora infestans sensu lato* in Ecuador

R. F. Oliva[a], L. J. Erselius[b], N. E. Adler[a] and G. A. Forbes[a]*†

[a]*International Potato Center (CIP), PO Box 17-21-1977, Quito, Ecuador; and* [b]*Rue Mandel, 34130 Mauguio, France*

To determine the potential of sexual reproduction among host-adapted populations of *Phytophthora infestans sensu lato* in Ecuador, 13 A1 isolates belonging to clonal lineages US-1, EC-1 and EC-3, and 11 A2 isolates belonging to the clonal lineage EC-2, were paired on agar plates to induce crossing. In the first experiment, six A1 isolates (three US-1, two EC-1 and one EC-3) were each crossed with three A2 isolates (total = 18 crosses). Matings involving isolates of the EC-1 lineage produced more oospores of healthy appearance than did matings with isolates of US-1 or EC-3. In the second experiment, the oospores of 35 crosses (21 EC-1 × EC-2; 10 US-1 × EC-2; four EC-3 × EC-2) were dispersed on water agar to assess oospore germination. Overall, germination percentages were low. Only one cross produced enough progeny for evaluation. Twenty-three single-oospore offspring were isolated and evaluated for mating type; electrophoretic patterns of glucose-6-phosphate isomerase (*Gpi*) and peptidase (*Pep*) alloenzyme loci; mitochondrial DNA haplotype; and genomic DNA fingerprint. Multilocus genotype data indicated that all 23 isolates resulted from meiotic recombination. Four progeny with homothallic phenotype appeared to be unstable heterokaryons. Markers at several loci segregated according to simple Mendelian expectations for a diploid organism, but the ratios of three RFLP loci and the *Pep* locus were not consistent with Mendelian expectations. All progeny were nonpathogenic on hosts of the parental genotypes. Reduced mating success and reduced pathogenic fitness of progeny appear to be postmating mechanisms of reproductive isolation in populations of *P. infestans sensu lato* in Ecuador.

Keywords: heterokaryons, host adaptation, mating types, multilocus genotypes, oospore germination, sexual progeny

Introduction

The oomycete *Phytophthora infestans*, the causal agent of potato late blight, is a heterothallic organism that produces oospores when compatible strains of the A1 and A2 mating types come into contact (Gallegly & Galindo, 1958). These oospores may serve as sources of inoculum, with the added risk that they may increase genetic variation within populations. Prior to the 1980s, the A2 mating type had been found only in the Toluca Valley of Central Mexico, where it coexisted in approximately equal frequency with the A1 mating type (Tooley *et al*., 1985; Goodwin *et al*., 1992b). Researchers believe that sexual recombination is common in the Toluca Valley of Mexico because the pathogen population is extremely diverse (Tooley *et al*., 1985; Fry & Spielman, 1991). Over the past two decades the A2 compatibility type of *P. infestans* has been identified in many other countries where the A1 was already present (Fry *et al*., 1993), enabling the pathogen to complete its sexual cycle in regions outside Mexico. At the present time there is circumstantial evidence for sexual reproduction in Europe and North America (Sujkowski *et al*., 1994; Goodwin *et al*., 1995; Miller *et al*., 1997; Andersson *et al*., 1998; Gavino *et al*., 2000; Turkensteen *et al*., 2000).

Nonetheless, the coexistence of the A1 and A2 mating types apparently does not always lead to sexual recombination. Both mating types occur in Japan and Brazil, but to date only clonal populations have been found in these countries (Brasier, 1992). Furthermore, Mosa *et al*. (1993) were unable to germinate oospores from crosses involving A1 and A2 isolates from Japan. In the border zone between northern Bolivia (dominated by A2 compatibility type) and southern Peru (dominated by A1 compatibility type), no evidence of sexual recombination has yet been found (Perez *et al*., 2001).

A similar situation appears to exist in Ecuador, where populations of *P. infestans sensu lato* have been isolated from both cultivated and wild *Solanum* species. To date, four clonal lineages have been described: US-1, EC-1, EC-3 (all A1 mating type), and EC-2 (A2 mating type). The clonal lineage US-1 was distributed worldwide (Goodwin *et al*., 1994) but is becoming increasingly rare. In Ecuador

*To whom correspondence should be addressed.

†E-mail: g.forbes@cgiar.org

Accepted 31 July 2002

© 2002 BSPP

it attacks several hosts, and is the lineage most frequently isolated from tomato. EC-1, which attacks several hosts including cultivated potato in Ecuador, is similar to genotypes found in Europe (Forbes *et al*., 1998). EC-2 attacks wild *Solanum* species that are commonly found in hedgerows surrounding tomato and potato fields. EC-3 has only been found attacking *S. betaceum* (tree tomato), a cultivated perennial crop. While they fit the morphological species description of *P. infestans*, both EC-2 and EC-3 are distinct from any genotype of *P. infestans* yet discovered (Forbes *et al*., 1998; Erselius *et al*., 1999; Ordoñez *et al*., 2000), and have been described only in Ecuador. Doubt remains, therefore, over the species assignation of these lineages (Forbes *et al*., 1998).

Each of the four clonal lineages described in Ecuador attacks one or more hosts in the genus *Solanum*, but no two lineages appear to be primary pathogens of the same host (Erselius *et al*., 1999). For example, US-1 and EC-1 can each infect both tomato and potato, but each lineage is more aggressive on its primary host (Oyarzun *et al*., 1998). This type of host adaptation is quantitative and can be measured with difficulty in a single disease cycle by cross-inoculating detached leaflets (Vega-Sanchez *et al*., 2000). In the field, however, this host adaptation apparently leads to important epidemiological differences, and one rarely finds a pathogen genotype on anything but one of its primary hosts (Oyarzun *et al*., 1998; Vega-Sanchez *et al*., 2000).

Studies carried out to date on *P. infestans sensu lato* in Ecuador have not produced evidence for sexual recombination (Ordoñez *et al*., 2000). As all isolates have been collected from the same eco-region, it seems likely that the lineages do come into contact and that sexual recombination is limited not by geographic isolation, but rather by one or more mechanisms of reproductive isolation. The most evident mechanism appears to be adaptation to particular hosts. Information gathered thus far indicates that host adaptation is particularly strong in EC-2, the only A2 lineage yet found in Ecuador, and EC-3, found only on tree tomato. Inoculation of A2 isolates of EC-2 on many of the hosts of A1 lineages (US-1, EC-1 and EC-3) revealed that the isolates are weakly pathogenic or nonpathogenic on all these hosts (unpublished results). However, the discovery of new species of *Phytophthora* arising from interspecific hybridization between species that have no common host (Sansome *et al*., 1991; Brasier *et al*., 1999; Bonant *et al*., 2000) suggests that this reproductive barrier can be broken. Furthermore, it is possible that there are common hosts that both EC-2 and one of the A1 lineages can infect with a sufficient level of aggressiveness to permit mating. One such host, *S. muricatum*, has yielded EC-2 (A2) and US-1 (A1) type isolates (Adler *et al*., 2002).

While there is some, albeit inconclusive, evidence for host adaptation as pre-mating barrier to sexual reproduction in *P. infestans sensu lato* in Ecuador, no systematic studies have yet been done to assess potential postmating mechanisms of reproductive isolation. The present study was designed to address this information gap. The objective was to test the potential of sexual reproduction among host-adapted late blight populations in Ecuador by studying the *in vitro* production and germination of oospores and the pathogenicity of progeny on detached leaflets.

Materials and methods

Source of isolates

For the two *in vitro* crossing studies described below, a total of 24 isolates of *P. infestans sensu lato*, 13 of A1 and 11 of A2 mating type, were used. These were isolated in the highlands of Ecuador between 1993 and 1999, and were previously characterized for mating type, glucose-6-phosphate isomerase (*Gpi*) and peptidase (*Pep*) alloenzyme loci, and RFLP fingerprint (probe RG57). Isolates were selected to represent all known Ecuadorian lineages (US-1, EC-1, EC-2 and EC-3), and came from different hosts including potato, tomato and several other wild and cultivated *Solanum* species (Table 1). All isolates were part of the *P. infestans sensu lato* collection of the Instituto Nacional Autonomo de Investigaciones Agropecuarias (INIAP), and have been maintained by serial culture on rye A agar (Caten & Jinks, 1968) at 18°C in the dark since isolation.

Oospore production and oospore viability

To assess oospore production and viability, a subset of the 24 isolates described above was used in various crosses. Six A1 isolates (three US-1, two EC-1 and one EC-3) were each crossed with three A2 isolates (EC-2), giving a total of 18 crosses. Mycelial plugs (5 mm diameter) of paired isolates were located 3·5 cm apart in a 9 cm Petri dish (V-8 juice agar) and then incubated at 20°C in darkness (Shattock *et al*., 1986). Each cross was repeated in four Petri dishes. After mycelia from each plug interacted, the point of contact was marked and the dishes were incubated for two more weeks. Two randomly selected agar plugs (0·2 cm^2) were extracted from the marked area and the number of oospores contained within them was estimated with a microscope (×200). Percentages of well formed oospores were determined and oospore viability estimated based on visual oospore appearance, as described by Ordoñez *et al*. (2000). Abnormally thick-walled oospores or empty oospores without an ooplast were considered as nonviable oospores (Erwin & Ribeiro, 1996).

Germination of oospores and establishment of F_1 progeny

To maximize germination of oospores, the best crosses from the experiment described were repeated and added to a number of others, making 35 crosses in total. Twenty EC-1 × EC-2, 10 US-1 × EC-2 and four EC-3 × EC-2 matings were made in V-8 juice broth (without agar as used previously). After 21 days of incubation, oospores and hyphae of each cross were mechanically separated by blending the mycelia in 50 mL sterile deionized water

Table 1 Characterization of isolates of *Phytophthora infestans sensu lato* from Ecuador used as parents in crosses

Solanum Host	Isolate number	Year of collection	Location	Mating type	*Gpi*[a]	*Pep*[a]	Clonal lineage
[b]*Lycopersicon esculentum*	1885	1995	Alchipichi	A1	86/100	92/100	US-1
[b]*S. muricatum*	1876	1995	Puellaro	A1	86/100	92/100	US-1
S. muricatum	1971	1995	Puellaro	A1	86/100	92/100	US-1
S. caripense	2932	1996	Calacali	A1	86/100	92/100	US-1
S. caripense	2942	1996	ND[c]	A1	86/100	92/100	US-1
S. caripense	2944	1996	ND	A1	86/100	92/100	US-1
[b]*S. tuberosum*	1021	1993	Balcashi	A1	90/100	96/100	EC-1
S. tuberosum	2957	1996	C. Colon	A1	90/100	96/100	EC-1
S. tuberosum	2968	1996	C. Colon	A1	90/100	96/100	EC-1
S. colombianum	3079	1997	Papallacta	A1	90/100	96/100	EC-1
S. colombianum	3099	1997	Papallacta	A1	90/100	96/100	EC-1
[b]*S. betaceum*	3105	1997	S. J. Minas	A1	86/100	76/100	EC-3
S. betaceum	3107	1997	S. J. Minas	A1	86/100	76/100	EC-3
S. brevifolium	3072	1997	Nono	A2	100/100	76/100	EC-2
S. brevifolium	3229	1999	Santa Rosa	A2	100/100	76/100	EC-2
S. brevifolium	3260	1999	Chillogallo	A2	100/100	76/100	EC-2
S. brevifolium	3261	1999	Chillogallo	A2	100/100	76/100	EC-2
S. brevifolium	3262	1999	Chillogallo	A2	100/100	76/100	EC-2
S. tetrapetalum	2930	1995	Calacali	A2	100/100	76/100	EC-2
[b]*S. phureja*	1851	1995	Cutuglagua	A2	100/100	76/100	EC-2
Solanum spp.	3234	1999	S. J. Minas	A2	100/100	76/100	EC-2
Solanum spp.	3235	1999	Alchipichi	A2	100/100	76/100	EC-2
Solanum spp.	3236	1999	Alchipichi	A2	100/100	76/100	EC-2
Solanum spp.	232[d]	ND	ND	A2	ND	ND	ND

[a]Glucose-6-phosphate isomerase and peptidase loci, respectively.
[b]Cultivated hosts.
[c]ND, not determined.
[d]Tester isolated and used in extra crosses and mating-type determinations.

(SDW) for 1 min. One mL 1% lysing enzyme (Sigma, L-2665, St Louis, MO, USA) was added to 9 mL oospore suspension to eliminate mycelia and remaining sporangia (Medina & Platt, 1999). Samples were incubated at 20°C for 48 h, mixed with an equal volume of freshly prepared 0·5% $KMnO_4$ solution (Chang & Ko, 1991), and afterwards agitated for 15 min. Free oospores were filtered successively through 180 and 20 μm pore steel filters and washed with several changes of SDW to remove both $KMnO_4$ and lysing enzyme. Samples were then resuspended in 5 mL SDW. Approximately 4000 oospores were spread on 0·6% w/v distilled water agar and incubated with 16 h blue light per day, as described by Shattock *et al.* (1986). Blue light was produced using a standard incandescent bulb covered with two sheets of blue-cellophane paper. The germination rate of each cross was assessed after 1 month of incubation. Hyphal tubes emerging from oospore surfaces, with or without terminal sporangia, were used as an indication of germination. After incubation for 4–30, days germinating oospores were transferred to either rye A or rye B agar (Caten & Jinks, 1968).

Genetic analysis of the progeny

Single-oospore cultures were characterized for *Gpi* on starch gels using the method of Spielman (1991) and *Pep* on polyacrylamide gels, as described by Davis (1964). Mitochondrial DNA (mtDNA) haplotypes were detected using the method of Griffith & Shaw (1998), and DNA fingerprinting was performed with probe RG57, as described by Goodwin *et al.* (1992a). For several markers, specific modifications to published methods that are described by Ordoñez *et al.* (2000) were employed. Mating type was determined by pairing each single-oospore culture on 10% unclarified V-8 juice agar with tester isolates of known A1 and A2 mating types. Multilocus genotypes involving all marker data were compared to detect recombination within the progeny. Segregation ratios at *Gpi*, *Pep* and DNA fingerprint loci were compared with expected frequencies for a diploid organism.

Genetic analysis of 'homothallic' progeny

In four cases single F_1 progeny produced oospores with both A1 and A2 tester strains and when in pure culture. These putative homothallic isolates were examined to determine if they were mixtures of strains of different mating types, mating type heterokaryons or homokaryons with a homothallic genotype. Following the procedures of Pipe *et al.* (2000) for single zoospores isolation, and Fyfe & Shaw (1992) for hyphal tip subculture, 10 single zoospore colonies and five hyphal tip colonies were

established from each of the putative homothallic cultures. Additionally, these homothallic isolates were examined individually for oospore formation in rye A agar medium after 1 year of successive (three to five) propagations. All single-zoospore and hyphal-tip subcultures were characterized for mating type as described above. A sample of 16 single-zoospore subcultures was characterized for *Pep* genotype and DNA fingerprint (RG57).

Pathogenicity of progeny

All single-oospore cultures recovered from the mating *in vitro* were tested for pathogenicity on detached leaflets of the host of each parental isolate. Parental isolates were used as controls. Inoculum was produced by growing isolates for 10 days on rye B agar, amended with 0·05% β-sitosterol. Sporangia were washed and collected on a 20 μm pore steel filter and left in tap water at 4°C for 1 h to induce zoospore release (Ordoñez et al., 2000). The resulting zoospore suspension was calibrated to $2{\cdot}5 \times 10^4$ zoospores per mL using a haemocytometer. One 10 μL drop of the suspension was placed on the abaxial surface of each leaflet. Leaflets were taken from plants ≈45 days old in the glasshouse (*Solanum colombianum*) and from vigorously growing plants in the wild (*S. brevifolium*) around the experimental station of the International Potato Center in Quito, Ecuador. *Solanum brevifolium* is a perennial, and the age of plants was not known. Eight leaflets of each host were inoculated and placed in the lids of four inverted Petri dishes containing water agar in their bases (two leaflets per Petri dish) and incubated at 16°C with 16 h light per day (fluorescent light type 33 daylight). After 10 days of incubation, disease symptoms were recorded and lesion diameters measured as described previously (Oyarzun *et al*., 1998). The assay was repeated.

Statistical analysis

The hypothesis that all A1 lineages were equal as parents for oospore production and oospore viability (appearance) was tested using ANOVA. The general model used was modified from Oyarzun *et al*. (1998):

$$V = u + a + b + a \times b + c(a) + e$$

in which V = variable tested (number of oospores or percentage of well formed oospores); u = overall mean; a = lineage of the A1 isolate; b = A2 isolate; c (a) = A1 isolate nested in lineage; e = residual error. To test the hypothesis that A1 lineages (EC-1, US-1 and EC-3) were equal, $c(a)$ was used as the denominator in the F-test. The hypothesis that oospore production and oospore viability are not related was tested using correlation analysis.

The log-likelihood ratio test (G-test) was used for *Gpi* and *Pep* loci to compare expected and observed allele segregation ratios in the progeny. Assuming dominant inheritance of RG57 loci, the χ^2 test was used to analyse expected and observed frequencies of polymorphic loci for DNA fingerprint bands. Parental isolates were assumed to be diploid.

Results

Oospore production and oospore viability

Sexual structures were observed in all 18 matings involving nine Ecuadorian isolates of *P. infestans sensu lato*. Oospore production varied from 61 to 910 oospores per agar plug (0·2 cm^2) in the crosses 3107 (EC-3) × 1851 (EC-2) and 1021 (EC-1) × 1851 (EC-2), respectively, and the percentage of well formed oospores was 3·1 and 31·5%, respectively (Table 2). Significant differences in number of oospores (P = 0·029) and percentage of well formed oospores (P = 0·041) were observed among A1 lineages. A1 isolates from the EC-1 lineage produced more oospores in crosses with the A2 isolates than did isolates from US-1 or EC-3. The percentage of well formed oospores was correlated with the number of oospores produced (r = 0·80).

Germination success of oospores

Germination of oospores was detected in only six out of 35 crosses (3107 × 3262, 1876 × 3261, 3105 × 3261, 2944 × 3262, 3076 × 3260 and 3099 × 3260), and in five of these germination was less than 0·02%. Only cross 3099 × 3260 generated enough progeny to be analysed. The A1 isolate, 3099, belongs to the EC-1 clonal lineage and was isolated from *S. colombianum*. The A2 isolate, 3260, belongs to the EC-2 clonal lineage and was isolated from *S. brevifolium* (Table 1). Twenty-three single-oospore cultures (P1–P23) were isolated for characterization with molecular markers and for pathogenicity on parental hosts.

Genetic characterization of single-oospore progeny

Based on the analysis of multilocus genotypes involving all the molecular markers, all 23 progeny appeared to be

Table 2 Number of oospores produced and percentage of well formed oospores in crosses involving isolates of *Phytophthora infestans sensu lato* from Ecuador

	A1 isolates					
A2 isolates	021 (EC-1)[a]	2957 (EC-1)	3107 (EC-3)	1885 (US-1)	1876 (US-1)	2932 (US-1)
1851						
No.[b]	910	545	61	195	405	235
%[c]	31·5	21·9	3·1	9·3	13·4	10·3
2930						
No.	583	550	150	155	495	177
%	21·6	23	10·8	4·7	19·5	7·8
3072						
No.	698	884	370	488	429	279
%	29·2	36·3	16·9	9·6	28·6	14·9

[a]Lineage of isolate.
[b]Mean number of oospores produced in 0·2 cm^2 agar plug after 2 weeks of interaction.
[c]Mean percentage of well formed oospores in each cross assessed at the same time as number of oospores.

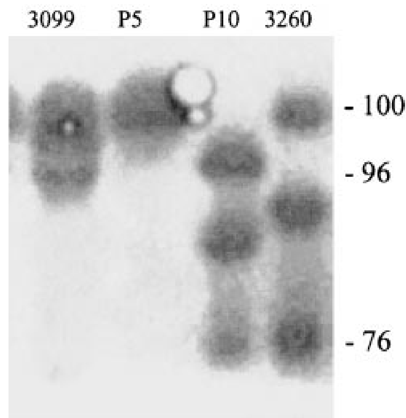

Figure 1 *Peptidase* (*Pep*) pattern from polyacrylamide gel electrophoresis in parental isolates and two recombinant progeny of *Phytophthora infestans sensu lato* from Ecuador. The relative mobility of bands is shown on the right. Parental isolates 3099 and 3260 were 96/100 and 76/100, respectively, while progeny P5 and P10 were characterized as recombinants 100/100 and 76/96, respectively.

hybrids. Polymorphic markers segregated in progeny, but segregation ratios of some markers were not always consistent with simple Mendelian expectations. No single multilocus genotype was identical to either parent. Eleven indisputable meiotic recombinants were identified from their isoenzyme genotype (one *Pep* 76/96 and 10 *Pep* 76/100, *Gpi* 90/100). The other descendants had allelic combinations that could have arisen through selfing (parental types or *Pep* 100/100) but their multilocus genotype, including information from RFLP and other markers, confirmed their recombinant status (Table 3).

Seventeen single-oospore cultures were A1 mating type, two were A2 mating type, and four, characterized as putative homothallic isolates, produced abundant oospores in pure culture and with either A1 or A2 tester isolates (Table 3). These four isolates were studied in more detail.

An observed 1 : 1 ratio of *Gpi* genotypes 90/100 and 100/100 among progeny conformed to the expected ratio of a cross between homozygous and heterozygous parents (Table 4), but as there was no distinctive allele in the A2 parent (100/100), this result did not rule out selfing. The results for the *Pep* locus were more informative, as the genotype 76/96 (one descendant, Fig. 1), and the combination of the 76 *Pep* allele from the A2 parent with the 90 *Gpi* allele from the A1 parent (10 descendants), can only be the result of hybridization. However, the ratio of *Pep* genotypes (76/100, 100/100, 96/100 and 76/96) differed significantly from the 1 : 1 : 1 : 1 ratio expected for two heterozygotic parents (Table 4). In this case, the 76/100 genotype was much more frequent than expected, occurring in 17 of 23 progeny.

Parental isolate 3099 has a mitochondrial haplotype IIa, and parental isolate 3260 has a new haplotype Ic, reported previously by Ordoñez *et al.* (2000). Both

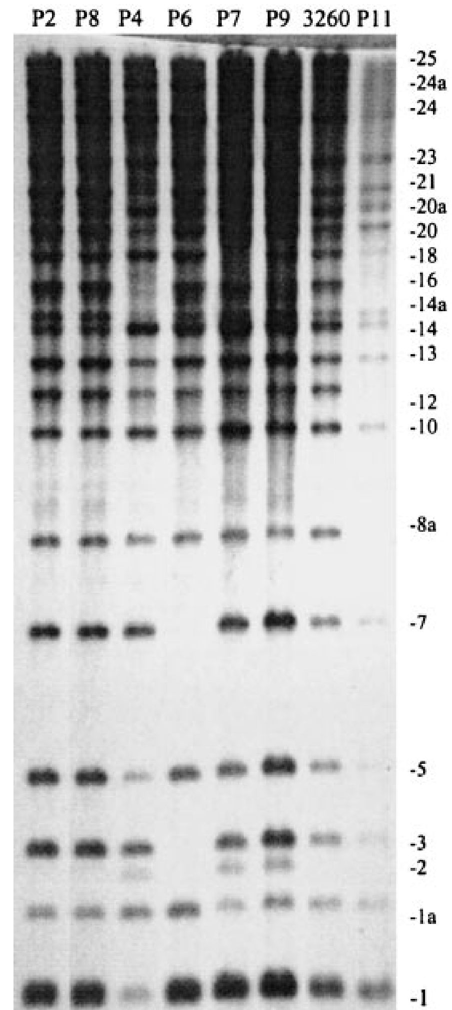

Figure 2 DNA fingerprint pattern obtained with probe RG57 in parental isolate 3260 of *Phytophthora infestans sensu lato* from Ecuador and some single-oospore progeny. P2, P8, P4, P6, P7, P9 and P11 have genotypes different from those previously reported by Ordoñez *et al.* (2000) for Ecuadorian populations. The band numbering system on the right follows Goodwin *et al.* (1992a).

haplotypes were present in the progeny, five and 18 times, respectively (Table 3). All single-oospore cultures were also characterized for DNA fingerprint with probe RG57. Twenty different genotypes were identified among the 23 F_1 progeny (partial results in Fig. 2). Only one culture (P10) had an identical RFLP profile to a parental isolate (3260), however, this single-oospore culture had the recombinant *Pep* genotype (76/96). Following the band nomenclature of Goodwin *et al.* (1992a), the progeny were polymorphic for bands 1a, 2, 3, 7, 8a, 10, 12, 16, 18, 20a and 24a (Table 3). Four bands, 3, 7, 10 and 16, which were present in both parents, were absent in one or more of the progeny, suggesting that the parents were heterozygous at these loci. The segregation ratios of all loci except 10, 12 and 16 were consistent with simple Mendelian expectations (Table 5).

Table 3 Mating type and marker characterization of F_1 progeny and parental isolates of *Phytophthora infestans sensu lato* in Ecuador

Isolate	Mating type	*Gpi* [a]	*Pep* [a]	mt-DNA [b]	(RG57) fingerprint loci [c]																				
					1	1a	2	3	5	7	8a	10	12	13	14	14a	16	18	20	20a	21	23	24	24a	25
3099	A1	90–100	96–100	IIa	1	0	1	1	1	1	0	1	0	1	1	1	1	0	1	0	1	0	1	1	1
3260	A2	100–100	76–100	Ic [d]	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
P1	A1	90–100	96–100	Ic	1	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1
P2	H [e]	100–100	76–100	Ic	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
P3	H	100–100	76–100	Ic	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
P4	A1	90–100	76–100	IIa	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
P5	A1	90–100	100–100	Ic	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	0	1
P6	A2	100–100	76–100	Ic	1	1	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
P7	A1	90–100	76–100	Ic	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1
P8	A1	90–100	76–100	Ic	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
P9	A1	100–100	76–100	Ic	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
P10	A1	100–100	76–96	Ic	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
P11	A1	90–100	76–100	Ic	1	1	0	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	1	0	1
P12	A1	90–100	96–100	Ic	1	0	0	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1
P13	A1	90–100	76–100	Ic	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
P14	A2	90–100	76–100	Ic	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1
P15	A1	90–100	76–100	Ic	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1
P16	A1	90–100	100–100	IIa	1	0	1	0	1	0	0	1	0	1	1	1	1	0	1	0	1	1	1	0	1
P17	A1	90–100	96–100	Ic	1	1	0	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1
P18	A1	90–100	76–100	Ic	1	1	0	0	1	0	1	0	1	1	1	1	1	0	1	0	1	1	1	0	1
P19	A1	90–100	76–100	IIa	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
P20	H	100–100	76–100	Ic	1	1	0	1	1	1	0	1	1	1	1	1	1	0	1	0	1	1	1	0	1
P21	A1	100–100	76–100	IIa	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1
P22	A1	90–100	76–100	Ic	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	0	1
P23	H	100–100	76–100	IIa	1	0	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1

[a] Glucose-6-phosphate isomerase and peptidase loci, respectively.
[b] Mitochondrial DNA haplotype.
[c] Scored as presence (1) and absence (0) of band.
[d] Ic is described in Ordoñez *et al*. (2000).
[e] H, homothallic phenotype.

Table 4 Recombination and segregation at *glucose-6-phosphate isomerase* (*Gpi*) and *peptidase* (*Pep*) alloenzyme loci in the progeny of cross 3099 × 3260

Parents	*Gpi*	*Pep*	Number of progeny	Expected ratio
3099 (A1)	90–100	96–100		
3260 (A2)	100–100	76–100		
Progeny				
	90–100		15	1
	100–100		8	1
				($P = 0{\cdot}14$)
		100–100	2	1
		76–96	1	1
		96–100	3	1
		76–100	17	1
				($P < 0{\cdot}005$)
	90–100	100–100	2	1
	90–100	96–100	3	1
	90–100	76–100	10	1
	100–100	76–96	1	1
	100–100	76–100	7	1
				($P < 0{\cdot}005$)

Table 5 RG57 fingerprint bands of polymorphic loci in the progeny of cross 3099 × 3260

Polymorphic band	Ratio of band in single-oospore progeny		
	[a]Observed	[b]Expected	[c]χ^2
1a	16 : 7	1 : 1	3·6
2	10 : 13	1 : 1	0·4
3	20 : 3	3 : 1	2·1
7	20 : 3	3 : 1	2·1
8a	14 : 9	1 : 1	1·1
10	22 : 1	3 : 1	5·8*
12	18 : 5	1 : 1	7·4**
16	21 : 2	3 : 1	3·8*
18	14 : 9	1 : 1	1·1
20a	12 : 11	1 : 1	0·1
24a	11 : 12	1 : 1	0·1

[a]Ratio of isolates with: without band, total number = 23.
[b]Expected ratios were assumed to be result of dominant inheritance.
[c]The χ^2 value for an expected ratio of 3 : 1 or 1 : 1 with 1 df is 3·84.
*, **, Ratio significantly different from expected at $P < 0{\cdot}05$, $P < 0{\cdot}01$, respectively.

Genetic analysis of 'homothallic' progeny

P2, P3, P20 and P23 were initially characterized as putative homothallic isolates. Isolates P3 and P20 produced A1 and A2 single-zoospore subcultures and A1 hyphal-tip subcultures. Isolates P2 and P23 produced only A2 single-zoospore and hyphal-tip subcultures. No isolate produced subcultures with a homothallic phenotype. After 1 year, only P3 and P20 continued producing oospores in rye A agar medium (Table 6). Sixteen single-zoospore subcultures, selected from P2 and P3, had the same DNA fingerprint (RG57) and *Pep* genotype as P2 and P3, respectively (results not shown).

Pathogenicity of progeny

All progeny of *P. infestans sensu lato* were nonpathogenic or weakly pathogenic on *S. colombianum* and *S. brevifolium*, the hosts of the parental genotypes. Some progeny caused tiny lesions with sparse sporulation, but most caused no visible symptoms of disease. Parental isolates caused normal lesions on their respective host, but neither was pathogenic on the alternative host (results not shown).

Discussion

The results of this study provide evidence for a genetic barrier to sexual reproduction among host-adapted populations of *P. infestans sensu lato* in Ecuador. The A2 isolates of the EC-2 clonal lineage obtained from wild *Solanum* species had limited mating success with the A1 isolates of US-1, EC-1 and EC-3 clonal lineages. Only one of 35 crosses produced sufficient germinating oospores for evaluation. Aborted oospores, nonviable oospores and unbroken dormancy in oospores could provide reasons for the strongly reduced levels of oospore germination observed. At a genetic level, it appears that recessive lethal factors (Erwin & Ribeiro, 1996) may have been fixed in the Ecuadorian population of *P. infestans sensu lato* as a consequence of long-time asexual propagation.

None of the progeny investigated caused lesions on either host of parental isolates. Other researchers have reported nonpathogenic progeny from crosses between different genotypes of *P. infestans* (Al-Kherb *et al*., 1995); *P. cinnamomi* (Linde *et al*., 2001), and from interspecific cross between *P. infestans* and *P. mirabilis* (Goodwin & Fry, 1994). Spielman *et al*. (1990) mentioned that in some

Table 6 Mating type of subcultures of four putative homothallic single-oospore isolates of *Phytophthora infestans*

Isolates	Single-zoospore subcultures (*n* = 10)			Hyphal tip subcultures (*n* = 5)			Oospore production after 1 year
	A1	A2	H[a]	A1	A2	H	
P2	–	10	–	–	5	–	–
P3	8	2	–	5	–	–	+
P20	7	3	–	5	–	–	+
P23	–	10	–	–	5	–	–

[a]H, homothallic phenotype.

matings, avirulence alleles have been found to act as recessives. However, it is unlikely that the lack of pathogenicity in the progeny studied here is due to any phenomenon related to simple R-genes. The sample we studied was small, but still large enough to detect segregation of qualitative genes affecting virulence.

Reproductive isolating mechanisms are known to prevent gene flow within *Phytophthora* (Goodwin & Fry (1994); Goodwin *et al*., 1999; Flier, 2001). Similar mechanisms may be acting in populations of *P. infestans sensu lato* in Ecuador. Apparently, host adaptation provides a strong premating mechanism of reproductive isolation, while low viability and reduced pathogenic fitness of progeny provide strong postmating mechanisms of isolation. All these forces together might be sufficient to restrict sexual recombination and gene flow among clonal lineages in Ecuador. However, the number of crosses we attempted in this study was very small compared with the number that potentially could occur in nature. None of the populations studied here are separated geographically. Furthermore, the discovery of a new interspecific *Phytophthora* hybrid attacking *Alnus* spp., a different host from those of parental species (Brasier *et al*., 1999; Brasier & Kirk, 2001), suggests that host adaptation can be overcome when the interspecific hybrid has the ability to colonize a new host. Andean highland ecosystems have a large number of potential new hosts, many within the genus *Solanum*. Therefore the possibility that any of them may become a novel host for a meiotic recombinant of *P. infestans sensu lato* cannot be excluded.

As also shown in other studies (e.g. Pittis & Shattock, 1994; Flier, 2001), large differences were found in the ability of isolates to form compatible matings. Despite the small sample, it is interesting that the EC-1 lineage is more capable than the EC-3 lineage of interacting with the EC-2 lineage, even though EC-2 and EC-3 appear more closely related by molecular markers (Erselius *et al*., 1999). This is consistent with the hypothesis that the risk of recombination may be greater between genotypes that are phylogenetically distant than between local and introduced genotypes (Brasier *et al*., 1999).

Crosses resulting in large numbers of oospores also produced a higher percentage of well formed oospores, but neither oospore production nor their appearance was related to germination and viability of germlings.

Difference in ploidy between parental isolates may be one reason for the low percentages of well formed oospores and germination. Variation in ploidy level has been demonstrated for different populations of *P. infestans* (Whittaker *et al*., 1991a), although is not itself a barrier to sexual recombination (Whittaker *et al*., 1991b). Among the lineages used in the present study, US-1 had significantly larger sporangia and zoospores than the others (data not shown). This may indicate a ploidy level greater than for the other lineages. EC-2 had relatively small sporangia. Ploidy differences between mating isolates would lead to irregularities in segregation and hence to abortion. Among the descendants obtained from the EC-1 × EC-2 cross, most of the markers segregated according to simple Mendelian expectations for a diploid organism. If there was a difference in ploidy between the parents, it appears that this did not influence segregation among the descendants; any imbalanced genotypes had probably aborted earlier.

Some markers did not segregate as expected, such as *Pep*, for which a much higher percentage of 76/100 genotypes was found among progeny than was expected; but unexpected ratios of alloenzyme markers in previous crosses of *P. infestans* have been associated with deleterious recessive alleles (Spielman *et al*., 1990).

Similarly, single-oospore cultures with a homothallic phenotype are not uncommon (e.g. Shattock *et al*., 1986; Spielman *et al*., 1990; Pipe *et al*., 2000). Homothallism can be produced by a mosaic of A1 and A2 hyphae, heterokaryosis or a single genotype within a homokaryon. No putative homothallic F_1 isolate (P2, P3, P20 or P23) obtained in our study carried all markers possessed by both parental isolates. It appears unlikely, therefore, that they were mixtures of hyphae of different mating types. If they were homokaryons, however, this single homothallic genotype should have been transferred to the uninucleate zoospore, but no such phenotype in either single-zoospore or hyphal-tip subcultures was observed. This suggests that P2, P3, P20 and P23 were heterokaryons with different levels of stability during vegetative growth, resulting in P2 and P23 producing single A2 colonies during asexual propagation (Fyfe & Shaw, 1992; Pipe *et al*., 2000). Mating-type heterokaryons could result from oospores with two zygotic nuclei or with a trisomic nucleus carrying both determinants (Shaw, 1991). However, the possibility that any parent possessed extra copies of the mating type locus cannot be excluded.

Acknowledgements

We wish to thank Dr Wilbert Flier for helpful comments during the revision of this article.

References

Adler NE, Chacón G, Flier WG, Forbes GA, 2002. The Andean fruit crop, pear melon (*Solanum muricatum*), is a common host for A1 and A2 strains *of Phytophthora infestans* in Ecuador. *Plant Pathology* **51**, 802.

Al-Kherb SM, Fininsa C, Shattock RC, Shaw DS, 1995. The inheritance of virulence of *Phytophthora infestans* to potato. *Plant Pathology* **44**, 552–62.

Andersson B, Sandström M, Strömberg A, 1998. Indications of soil borne inoculum of *Phytophthora infestans*. *Potato Research* **41**, 305–10.

Bonant PJM, Hagenaar-de Weerdt M, Man in't Veld WA, Baayen RP, 2000. Molecular characterisation of natural hybrids of *Phytophthora nicotianae* and *P. cactorum*. *Phytopathology* **90**, 867–74.

Brasier CM, 1992. Evolutionary biology of *Phytophthora*: genetic system, sexuality and the generation of variation. *Annual Review of Phytopathology* **30**, 153–71.

Brasier CM, Kirk SA, 2001. Comparative aggressiveness of standard and variant hybrid alder phytophthoras, *Phytophthora cambivora* and other *Phytophthora* species on bark of *Alnus*, *Quercus* and other woody hosts. *Plant Pathology* **50**, 218–29.

Brasier CM, Cooke DEL, Duncan JM, 1999. Origins of a new *Phytophthora* pathogen through interspecific hybridisation. *Proceedings of the National Academy of Sciences, USA* **96**, 5878–83.

Caten CE, Jinks JL, 1968. Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variation. *Canadian Journal of Botany* **46**, 329–48.

Chang TT, Ko WH, 1991. Factors affecting germination of oospores of *Phytophthora infestans*. *Journal of Phytopathology* **133**, 29–35.

Davis BJ, 1964. Disc electrophoresis II. Method and application to human serum protein. *Annals of the New York Academy of Science* **121**, 404–27.

Erselius LJ, Hohl HR, Ordoñez ME, Oyarzun PJ, Jarrin F, Velasco A, Ramon MP, Forbes GA, 1999. Genetic diversity among isolates of *Phytophthora infestans* from various hosts in Ecuador. In: Arthur C, Ferguson P, Smith B, eds. *Impact on a Changing World*. Lima, Peru: International Potato Center, 29–48.

Erwin DC, Ribeiro OK, 1996. *Phytophthora Disease Worldwide*. St Paul, MN, USA: American Phytopathological Society Press.

Flier WG, 2001. *Variation in* Phytophthora infestans: *Sources and Implications*. Wageningen, the Netherlands: Wageningen Agricultural University, PhD Thesis.

Forbes GA, Goodwin SB, Drenth A, Oyarzún P, Ordoñez ME, Fry WE, 1998. A global marker database for *Phytophthora infestans*. *Plant Disease* **82**, 811–8.

Fry WE, Spielman LJ, 1991. Population biology. In: Ingram DH, Williams PH, eds. Phytophthora infestans: *The Cause of Late Blight of Potato*. Advances in Plant Pathology, Vol. 7. London, UK: Academic Press, 171–92.

Fry WE, Goodwin SB, Dyer AT, Matuszak JM, Drenth A, Tooley PW, Sujkowski LS, Koh YJ, Cohen BA, Spielman LJ, Deahl KL, Inglis DA, Sandlan KP, 1993. Historical and recent migrations of *Phytophthora infestans*: chronology, pathways and implications. *Plant Disease* **77**, 653–61.

Fyfe AM, Shaw DS, 1992. An analysis of self-fertility in field isolates of *Phytophthora infestans*. *Mycological Research* **96**, 390–4.

Gallegly ME, Galindo J, 1958. Mating type and oospores of *Phytophthora infestans* in nature in Mexico. *Phytopathology* **48**, 274–7.

Gavino PD, Smart CD, Sandrock W, Miller JS, Hamm PB, Lee TY, Davis RM, Fry WE, 2000. Implications of sexual recombination for *Phytophthora infestans* in the United States: generation of an aggressive lineage. *Plant Disease* **84**, 731–5.

Goodwin SB, Fry WE, 1994. Genetic analyses of interspecific hybrids between *Phytophthora infestans* and *Phytophthora mirabilis*. *Experimental Mycology* **18**, 20–32.

Goodwin SB, Drenth A, Fry WE, 1992a. Cloning and genetic analyses of two highly polymorphic, moderately repetitive nuclear DNAs from *Phytophthora infestans*. *Current Genetics* **22**, 107–15.

Goodwin SB, Spielman LJ, Matuszak JM, Bergeron SN, Fry WE, 1992b. Clonal diversity and genetic differentiation of *Phytophthora infestans* populations in northern and central Mexico. *Phytopathology* **82**, 955–61.

Goodwin SB, Cohen BA, Fry WE, 1994. Panglobal distribution of a single clonal lineage of the Irish potato famine fungus. *Proceedings of the National Academy of Sciences, USA* **91**, 11591–5.

Goodwin SB, Sujkowski LS, Dyer AT, Fry BA, Fry WE, 1995. Direct detection of gene flow and probable sexual reproduction of *Phytophthora infestans* in northern North America. *Phytopathology* **85**, 473–9.

Goodwin SB, Legard DE, Smart CD, Levy M, Fry WE, 1999. Gene flow analysis of molecular markers confirms that *Phytophthora mirabilis* and *P. infestans* are separate species. *Mycologia* **91**, 796–810.

Griffith GW, Shaw DS, 1998. Polymorphisms in *Phytophthora infestans*: four mitochondrial haplotypes are detected after PCR amplification of DNA from pure cultures or from host lesions. *Applied and Environmental Microbiology* **64**, 4007–14.

Linde C, Soo SH, Drenth A, 2001. Sexual recombination in *Phytophthora cinnamomi in vitro* and aggressiveness of single-oospore progeny to *Eucalyptus*. *Plant Pathology* **50**, 97–102.

Medina MV, Platt HW, 1999. Viability of oospores of *Phytophthora infestans* under field conditions in northeastern North America. *Canadian Journal of Plant Pathology* **21**, 137–43.

Miller JS, Hamm PB, Johnson DA, 1997. Characterisation of the *Phytophthora infestans* population in the Columbia Basin of Oregon and Washington from 1992 to 1995. *Phytopathology* **87**, 656–60.

Mosa AA, Kobayashi K, Ogoshi A, 1993. Isozyme polymorphism and segregation in isolates of *Phytophthora infestans* from Japan. *Plant Pathology* **42**, 26–34.

Ordoñez ME, Hohl HR, Velasco A, Ramon MP, Oyarzún PJ, Smart CD, Fry WE, Forbes GA, Erselius LJ, 2000. A novel population of *Phytophthora*, similar to *P. infestans*, attacks wild *Solanum* species in Ecuador. *Phytopathology* **90**, 197–202.

Oyarzún PJ, Pozo A, Ordoñez ME, Doucett K, Forbes GA, 1998. Host specificity of *Phytophthora infestans* on tomato and potato in Ecuador. *Phytopathology* **88**, 265–71.

Pérez WG, Gamboa JS, Fálcon VY, Coca M, Raymundo RM, Nelson RJ, 2001. Genetic structure of Peruvian populations of *Phytophthora infestans*. *Phytopathology* **91**, 956–65.

Pipe ND, Azcoitia V, Shaw DS, 2000. Self-fertility in *Phytophthora infestans*: heterokaryons segregate several phenotypes. *Mycological Research* **104**, 676–80.

Pittis JE, Shattock RC, 1994. Viability, germination and infection potential of oospores of *Phytophthora infestans*. *Plant Pathology* **43**, 387–96.

Sansome E, Brasier M, Hamm PB, 1991. *Phytophthora meadii* may be a species hybrid. *Mycological Research* **95**, 19–30.

Shattock RC, Tooley PW, Fry WE, 1986. Genetics of *Phytophthora infestans*: determination of recombination, segregation and selfing by isozyme analysis. *Phytopathology* **76**, 410–3.

Shaw DS, 1991. Genetics. In: Ingram DH, Williams PH, eds. Phytophthora infestans: *The Cause of Late Blight of Potato*. Advances in Plant Pathology, Vol. 7. London, UK: Academic Press, 131–67.

Spielman LJ, 1991. Isoenzymes and population genetics of *Phytophthora infestans*. In: Lucas JA, Shattock RC, Shaw DS, Cooke LR, eds. Phytophthora. Cambridge, UK: Cambridge University Press, 231–41.

Spielman LJ, Sweigard JA, Shattock RC, Fry WE, 1990. The genetics of *Phytophthora infestans*: segregation of allozyme markers in F_2 and backcross progeny and the inheritance of virulence against potato resistance genes R2 and R4 in F_1 progeny. *Experimental Mycology* **14**, 57–69.

Sujkowski LS, Goodwin SB, Dyer AT, Fry WE, 1994. Increased genotypic diversity via migration and possible occurrence of sexual reproduction of *Phytophthora infestans* in Poland. *Phytopathology* **84**, 201–7.

Tooley PW, Fry WE, Villareal Gonzalez MJ, 1985. Isozyme characterization of sexual and asexual *Phytophthora infestans* populations. *Journal of Heredity* **76**, 431–5.

Turkensteen LJ, Flier WG, Wanningen R, Mulder A, 2000. Production, survival and infectivity of oospores of *Phytophthora infestans*. *Plant Pathology* **49**, 688–96.

Vega-Sánchez ME, Erselius LJ, Rodriguez AM, Bastidas O, Hohl HR, Ojiambo PS, Mukalazi J, Vermeulen T, Fry WE, Forbes GA, 2000. Host adaptation to potato and tomato within US-1 clonal lineage of *Phytophthora infestans* in Uganda and Kenya. *Plant Pathology* **49**, 531–9.

Whittaker SL, Shattock RC, Shaw DS, 1991a. Variation in DNA content of nuclei of *Phytophthora infestans* as measured by a microfluorimetric method using the fluorochrome DAPI. *Mycological Research* **95**, 602–10.

Whittaker SL, Shattock RC, Shaw DS, 1991b. Inheritance of DNA contents in sexual progenies of *Phytophthora infestans*. *Mycological Research* **95**, 1094–100.

Copyright © 2002 EBSCO Publishing