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Novel microsatellite markers for the analysis of *Phytophthora infestans* populations

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Co-dominant microsatellite molecular markers for *Phytophthora infestans* were developed and their potential for monitoring the genetic variation in populations was demonstrated in the UK, across Europe and worldwide. Markers were developed according to two strategies. First, several thousand *P. infestans* expressed sequence tag (EST) and bacterial artificial chromosome (BAC) sequences were screened for the presence of simple sequence repeat (SSR) motifs, and, of these, 100 candidate loci were selected for further investigation. Primer pairs developed to these loci were tested against a panel of 10 *P. infestans* isolates and approximately 10% were shown to be polymorphic and therefore appropriate for further testing. Secondly, the construction and screening of a partial genomic library resulted in the development of one additional polymorphic marker. The resulting 12 SSR markers were converted to higher-throughput fluorescence-based assays and used in combination with two previously published markers to characterize a wider collection of 90 *P. infestans* isolates from the UK and six other countries. Several isolates from the closely related species *P. mirabilis*, *P. ipomoea* and *P. phaseoli* collected from around the world were also genotyped using these markers. Amongst the 90 isolates of *P. infestans* examined, considerable SSR diversity was observed, with 68 different genotypes and an average of 3·9 (range 2–9) alleles per locus. When other *Phytophthora* species were genotyped, all loci were successfully amplified and the majority were polymorphic, indicating their transferability for the potential study of other closely related taxa.

Keywords: potato-late blight, co-dominant markers, population genetics, simple sequence repeats

Introduction

Phytophthora infestans, the cause of late blight of potato and tomato, is a worldwide problem made worse by recent introductions of exotic strains originating in Mexico and disseminated internationally by trade (Smart & Fry, 2001; Shattock, 2002; Cooke *et al*., 2003). Despite the importance of *P. infestans* and the fact that much survey work on mating type and fungicide resistance has been carried out, studies of its molecular diversity have been limited by the power of the genetic markers and difficulties in comparing results between laboratories. Work has revealed the presence of both clonal and genetically diverse populations (Knapova & Gisi, 2002; Cooke *et al*., 2003), but the mechanisms responsible for this diversity have not been studied in detail. To understand the biology and ecology of *P. infestans* and the mechanisms and tempo of variation in late-blight populations requires fully characterized powerful markers, e.g. microsatellites, also known as simple sequence repeats (SSRs) (Cooke & Lees, 2004).

SSRs are tandemly repeated motifs of one to six bases found in the nuclear genomes of all eukaryotes tested and are often abundant and evenly dispersed (Tautz & Renz, 1984; Lagercrantz *et al*., 1993). Microsatellite sequences are usually characterized by a high degree of length polymorphism, and are ideal single-locus co-dominant markers for genetical studies. Co-dominance offers a greater resolving power and the data can be used to determine population genetic structure, kinship, reproductive mode and the extent of genetic isolation (Queller *et al*., 1993; Ashley & Dow, 1994; Schlötterer & Pemberton, 1994; Jarne & Lagoda, 1996). Microsatellites have not, until relatively recently, been used for analysis of plant pathogens

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Table 1 Isolates of *Phytophthora infestans* used for simple sequence repeat screening with associated phenotypic data for mating type (A1/A2) and metalaxyl phenotype

Code	Isolate	Source	Origin	Mating type	Metalaxyl phenotype	Year isolated
C1	96.17.5.3	SCRI[a]	Ayr	A1	I	1996
C2	95.17.3.2	SCRI	Edinburgh	A1	S	1995
C3	97.38.2.2	SCRI	Dunkeld	A1	R	1997
C4	96.9.5.1	SCRI	Cononsyth	A1	R	1996
C5	96.13.1.3	SCRI	Glasgow	A1	R	1996
C6	US467	–	USA	A2	R	1998
C7	EC1	CIP[b]	Ecuador	A1	S	1998
C8	AG4	Cornell[c]	Argentina	A2	R	1997
C9	95.16.3.1	SCRI	Edinburgh	A1	S	1995
C10	97.28.1.2	SCRI	Meadowbank	A2	I	1997

S, sensitive; I, intermediate; R, resistant.
[a]SCRI isolates are from Scotland.
[b]Isolates obtained from G. A. Forbes, CIP, Lima, Peru.
[c]Isolates obtained from W. E. Fry, Cornell University, NY, USA.

(e.g. Kaye *et al*., 2003; Guérin *et al*., 2004). SSRs have been characterized for the oomycetes *Plasmopara viticola* (Gobbin *et al*., 2003), *Phytophthora cinnamomi* (Dobrowolski *et al*., 2002), *P. ramorum* (Prospero *et al*., 2004) and polymorphic loci have recently been reported by Knapova & Gisi (2002) for the analysis of *P. infestans* populations on potato and tomato in France. A review of a range of markers used for the characterization of *P. infestans* is given by Cooke & Lees (2004).

The aim of the work described here was to design and validate a set of polymorphic SSR markers for genetic analysis of *P. infestans* and to employ combinations of those markers to assess their potential for assessing genetic variation on a European and global scale. Reliable and rapid molecular marker technology will allow fingerprinting of isolates and their tracking in experimental work, aid population monitoring to investigate short- (field to field) and long-range (country to country) spread of the pathogen and will help to quantify the relative importance of sexual vs. asexual reproduction.

Materials and methods

Phytophthora isolates

The panel of *P. infestans* isolates used for marker development and made available to other researchers to allow comparison of results across laboratories is listed in Table 1. The 90 isolates used for population analysis were obtained from a range of sources: 38 Scottish isolates were collected in 1995–97, and formed a subset of a collection of 500 isolates obtained previously from commercial crops and allotment gardens; these were selected from across three clades defined previously on the basis of scores from 15 AFLP markers. These isolates were previously characterized for mating type, metalaxyl sensitivity, virulence and AFLP as described by Cooke *et al*. (2003). In addition, 30 isolates from England and Wales and nine from Ireland were obtained from disease outbreaks in 2000 and 2002. Thirteen isolates from other countries – Sweden (five), Bolivia (four), Argentina (one), Ecuador (one), Vietnam (one), USA (one) – and four isolates of *P. ipomoeae*, five of *P. mirabilis*, and one isolate of *P. phaseoli* were also included. *P. infestans* cultures were obtained by isolation from late-blight lesions on potato leaves onto rye A agar supplemented with pimaricin (10 μg mL^{-1}) and rifamcin (30 μg mL^{-1}) and were then transferred to, and maintained on, unamended rye A medium at 15°C. Isolates were grown in pea broth medium at 20°C for 1 week prior to DNA extraction (Raeder & Broda, 1985).

Microsatellite marker development

Microsatellite markers were developed according to two strategies: using previously published sequences and through the specific construction and screening of a partial genomic library.

First, the sequence data screened for SSR motifs were obtained from three sources: the *P. infestans* expressed sequence tag (EST) sequences held in the *Phytophthora* Genome Consortium (PGC) database (Waugh *et al*., 2000; http://www.ncgr.org/pgc/index.html); randomly selected genomic DNA fragments from a bacterial artificial chromosome (BAC) library constructed at SCRI (Whisson *et al*., 2001); and other EST sequences (Lam, 2001). The computer program Sputnik (http://espressosoftware.com/pages/sputnik.jsp) was used to identify SSR candidate sequences and 100 of these were selected for further investigation.

Secondly, a partial genomic library was constructed and screened using the approach of Rassmann *et al*. (1991). In brief, genomic DNA fragments 300–500 bp long (restricted with *Alu*I, *Hea*III and *Rsa*I restriction enzymes) were inserted into Bluescript II KS$^-$ vector (Stratagene) and used to produce a library of 8·3 K colonies. This library was screened with a mixture of ^{32}P-labelled $(AC)_{13}$ and $(CT)_{13}$ oligonucleotide microsatellite probes. One hundred and thirty hybridizing (positive) clones (1·57%) were

identified and sequenced. Only eight positive clones included at least 10 repeats, and were therefore used for primer design.

SSR primer design and PCR amplification

Primers were designed to the regions flanking the SSRs in each of the 108 sequences selected using the program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_http://www.cgi) with parameters set for a Tm of 58°C and product sizes ranging from 150 to 250 bp. A panel of 10 *P. infestans* isolates from Scotland and worldwide was selected (Table 1) and their DNA was amplified with each of the 100 candidate primer sets. A negative (water only) control was included in each assay. PCR amplification of these isolates, using each of the selected primer pairs, was based on a standard set of conditions [initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 20 s, 58°C for 25 s and 72°C for 60 s and a final extension step of 72°C for 5 min, in a reaction volume of 25 μL using an MWG-Biotech Primus 96 Thermal cycler (MWG-Biotech)]. The master mix contained the following components: 1 × reaction buffer (16 mM $[NH_4]_2SO_4$, 67 mm Tris-HCl pH 8·8, 0·1% Tween-20; Bioline), 200 μM each dNTPs (Bioline), 0·3 μM each primer (MWG-Biotech), 5·0 mM $MgCl_2$, 250 μg mL^{-1} BSA (Boehringer Mannheim), 1 U Biolase Diamond *Taq* polymerase (Bioline) and 10–100 ng of template DNA.

PCR products obtained for the 10-isolate panel using the 100 primer pairs were screened by visualization on precast Spreadex™· EL 600 wide mini S-50 gels, in a submerged gel electrophoresis (SEA 2000) unit (Elchrom Scientific AG) according to the manufacturer's instructions. Gels were stained for 45 min with SYBR Gold (10 μL of 10 000× concentrate diluted in 100 mL 10 mM TAE) and de-stained in water for 30 min before photographing under UV light. Candidate SSRs were selected for further analysis on the basis of differences in product size and absence of secondary PCR products.

Sequencing of polymorphic regions

To confirm that the PCR products matched the predicted amplicon size and that inter-isolate product size differences (bp) were due to polymorphisms in the SSR region rather than insertions/deletions in the flanking sequences, all polymorphic PCR products were sequenced. Direct sequencing of PCR products was initiated using the relevant SSR primers in a dye-terminator cycle-sequencing reaction (FS sequencing kit, Applied Biosystems) and run on an ABI377 automated sequencer (Applied Biosystems).

High-throughput SSR assay and multiplexing

For automated analysis, forward primers of the 14 polymorphic markers, including Pi4B (TET) and PiG11 (HEX) (Knapova & Gisi, 2002), (Table 2) were labelled at the 5′ end with one of the fluorescent dyes FAM (6-carboxy-fluorescein), TET (4,7,2′,7′-tetrachloro-6-carboxyfluorescein) or HEX (4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein). PCR reactions were carried out as described previously (Loci Pi02, Pi04, Pi16, Pi33, Pi56, Pi63, Pi66, Pi70, Pi89) but substituting the fluorescently labelled forward primer for the unlabelled primer. Markers Pi4B, PiG11 and D13 were amplified using the conditions described by Knapova & Gisi (2002), using an annealing temperature of 50°C for marker D13. Combinations of three markers of varying size and with different fluorescent dyes were tested in a multiplex reaction to allow higher sample throughput. Multiplex reactions were carried out using Qiagen Multiplex PCR master mix according to the manufacturer's instructions (Qiagen).

Genotyping of *Phytophthora* isolates

The 90 *P. infestans* and 10 isolates of other *Phytophthora* species were amplified in a Thermo Fast 96-well nonskirted PCR microplate (AB gene) using each of the primer pairs Pi02, Pi04, Pi16, Pi 26, Pi33, Pi56, Pi63, Pi65, Pi66, Pi70, Pi89, Pi4B, PiG11 and D13, and PCR conditions as described previously. A 3 μL sample, comprising 0·3 μL TAMRA 350 size standard (4 nM), 1 μL 25 μM EDTA with blue dextran (50 mg mL^{-1}), 0·75 μL PCR product and 0·95 μL deionized formamide (99·5%), was denatured at 95°C for 5 min, snap-cooled on ice and loaded into the ABI Prism 377 DNA sequencer and run according to manufacturer's instructions (Applied Biosystems). Peak size and quantitation data generated using Genescan Analysis Software were analysed using Genotyper software for the estimation of allele sizes (both Applied Biosystems).

Marker analysis

The 90 *P. infestans* isolates from the UK and Ireland were grouped into three populations according to geographic origin (England & Wales, Scotland and Ireland). Isolates from other parts of the world were grouped together as a fourth population and isolates from other species were grouped as a fifth. Genotypes were assigned by combining the allelic compositions across 12 loci. Results with markers Pi26 and Pi65 were omitted from the analysis. The total possible number of genotypes generated at each locus and the expected frequencies of the most common and rarest genotypes at the locus were estimated according to allele number and frequency. The likelihood of finding identical, independently generated, multilocus genotypes (nonclonal) when using all 12 loci was estimated for each locus. Gene diversity was estimated according to Nei (1973) and was performed in Popgen (http://cc.oulu.fi/~jaspi/popgen/popgen.htm), and genotype diversity was estimated with a standardized Shannon index as described by Goodwin (1997).

Results

Development of SSR markers

Analysis of the DNA sequences from the sources listed above yielded several thousand sequences containing

Table 2 Details of simple sequence repeat loci from *Phytophthora infestans* based on 90 individuals

Marker	SSR primer sequence	Annealing temperature (°C)	Size range (bp)	Expected size (bp)	Repeat	Number of alleles	Transferability to other species
Pi02[a]	F:CAGCCTCCGTGCAAGA[b] R:AAGGTGCGCGAAGACC	58	142–166	154	$(TG)_{11}$	8	1,2,3[g]
Pi04[c]	F:AGCGGCTTACCGATGG R:CAGCGGCTGTTTCGAC	58	162–170	170	$(GT)_6$	4	1,2,3
Pi16[a]	F:CACAGCACGCGGAATC R:ACGCCGAGTGTCCTGA	58	174–178	177	$(GA)_7$	3	1,2,3
Pi26[a,h]	F:GCAGTAGCCGTAGTCCTCAG R:GTTCCAAATCGTCAACCAAC	58	172	172	$(GT)_9$	NT	1,2,3
Pi33[a]	F:TGCCGACGACAAGGAA R:CGGTCTGCTGCTGCTC	58	203–209	203	$(CAG)_5$	3	1,2,3
Pi56[d]	F:ACAACTATCTATCGGCGTGC R:AGTAGGCTTCACGACCAGC	58	174–176	176	$(AT)_{10}$	2	1,2,3
Pi63[d]	F:ATGACGAAGATGAAAGTGAGG R:ATTCATTATTGGCAATGTTGG	58	148–160	157	$(GAG)_8$	4	1,2,3
Pi65[d,h]	F:AGGAAGAGGAGGAAGAAGAGG R:TCTTTCGTAACGGTTCTTTCC	58	145–151	145	$(AAG)_9$	3	1,2,3
Pi66[d]	F:ACCGACAGCTTCTGAAACC R:AAAATAAGAAGAGATTCGTGCC	58	153–155	153	$(GT)_7$	2	1,2,3
Pi70[d]	F:ATGAAAATACGTCAATGCTCG R:CGTTGGATATTTCTATTTCTTCG	58	189–195	195	$(AAG)_8$	3	1,2,3
Pi89[d]	F:GAGAACGCACAATGTAAGGC R:ACATAAATACACGCTGAACGG	58	179–185	181	$(AT)_9$	3	1,2,3
4B[e]	F:AAAATAAAGCCTTTGGTTCA R:GCAAGCGAGGTTTGTAGATT	58	205–217	218	$(TC)_{34}$	3	1,2,3
G11[e]	F:TGCTATTTATCAAGCGTGGG R:TACAATCTGCAGCCGTAAGA	56	142–166	156	$(TC)_{26}$	8	1,2,3
D13[f]	F:TGCCCCCTGCTCACTC R:GCTCGAATTCATTTTACAGA	50	108–142	136	$(CT)_{27}$	9	1,2,3

[a] *Phytophthora* Genome Initiative database (Waugh *et al.*, 2000).
[b] F, forward primer; R, reverse primer.
[c] Bacterial artificial chromosome library (Whisson *et al.*, 2001).
[d] Expressed sequence tag (Lam, 2001).
[e] Knapova & Gisi (2002).
[f] Specific genomic library.
[g] 1, *Phytophthora ipomoea*; 2, *P. mirabilis*; 3, *P. phaseoli.*
[h] Loci with two to four alleles found, only interpretable as multiallelic phenotype.
NT, not tested.

microsatellite repeats. After a preliminary screening on the basis of sequence quality and the position of the SSR repeat being sufficiently distant from the end of the sequence to allow the design of primers, 100 candidate sequences representing different classes and lengths of primarily di- or trinucleotide repeats were selected. In parallel, the construction and screening of a partial genomic library yielded 140 positive clones, but sequencing revealed only eight clones containing microsatellites with more than 10 repeats.

Subsequent PCR amplification of the *P. infestans* panel of 10 isolates with each of the 108 primer pairs described above resulted in the selection of 12 pairs that yielded a single clear PCR product and revealed polymorphisms amongst the isolates. An example of the polymorphisms seen with the Pi56 marker set is given in Fig. 1, a summary of the properties of the 12 loci and the two existing loci is given in Table 2 and, for reference, the alleles recorded for each of the 10 isolates amplified with each of the markers are given in Table 3.

Analysis of the sequences of the polymorphic PCR products of markers Pi02, Pi04, Pi16, Pi33, Pi56, Pi63, Pi65, Pi66, Pi70, Pi89 confirmed the fragment identity and that the polymorphisms were due to SSR repeat number variation in each case. In a limited number of cases, more than two alleles were consistently identified. Primer set Pi26 (Table 2) generated three to four bands and was therefore not considered suitable for use in population genetic analysis and was not analysed further. Similarly, marker Pi65 generated three bands in some cases and was therefore not included in the final analysis. However, information relating to these markers is included, as multiallelic phenotypes can be useful for isolate tracking studies. In addition, it should be noted that markers Pi63 and 4B generated three bands when used to amplify one of the control isolates (Table 3), but these occurrences

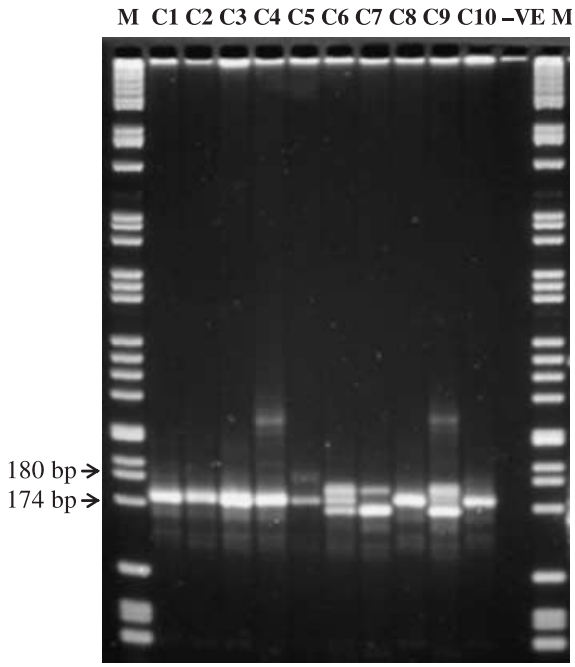

Figure 1 Amplification of DNA from 10 isolates of *Phytophthora infestans* (C1–C10, see Table 1) using marker Pi56. –VE, no DNA control; M, M3 marker (Elchrom Scientific).

were found to be rare in the 90 *P. infestans* isolates tested, and these markers were therefore included in the analysis.

Reproducibility of results was demonstrated on approximately 50 occasions (data not shown) by amplification of DNA from control isolates belonging to the groups C1–C10 (Table 1), using the SSR markers developed, resulting in products of the expected size range. In addition, these control isolates were sent to four European laboratories for testing and the results were comparable.

Genotyping of *Phytophthora* isolates

Ninety isolates of *P. infestans* and 10 isolates of other *Phytophthora* species with a full data set (results from all 12 markers tested) were analysed. When the data were analysed using multilocus genotype tests, 68 genotypes were identified from the 90 *P. infestans* isolates. Novel alleles were found amongst isolates of the other related *Phytophthora* species and none of these isolates had genotypes common with *P. infestans*. As expected, the resolving power of the genotyping increased with marker number: maximum resolution was achieved with 10 loci and the use of more than 11 did not reveal additional genotypes. The relationship between the number of markers used and the number of genotypes identified is given in Fig. 2. The number of possible allelic combinations detected at each locus ranged from three to 45 for *P. infestans* isolates (Table 4), with the total possible number of genotypes being $9{\cdot}18 \times 10^{10}$ when all 12 loci were considered together. The upper and lower probabilities of finding an identical, nonclonal, multilocus genotype in *P. infestans* were $1{\cdot}59 \times 10^{-4}$ and $8{\cdot}0 \times 10^{-35}$, respectively. The most common genotype of *P. infestans* was found six times (once in England in 1999 and five times in Scotland, once in 1995 and four times in 1997). However, three of the Scottish 1997 isolates with this genotype were obtained from the same disease outbreak. Two genotypes were found on five occasions, the first only in England from diverse sites in 1999 (four isolates) and 2002 (one isolate) and the second only in Ireland in isolates taken from diverse sites in 1995 (one), 1996 (two) and 2002 (two).

Gene diversity as calculated by Nei (1973), and averaged across the 12 loci ranged from 0·38 in the Irish isolates (Table 5) to 0·56 in isolates of other species. The average diversity in isolates from England and Wales (0·46) was the same as that found in isolates from across the world.

Discussion

Information regarding the current *P. infestans* population and its evolutionary potential, in terms of increased threats from a rapidly changing population, is useful for informing late blight disease-control strategies. As commented upon by Cooke & Lees (2004), this means that a greater emphasis must be put on studies of *P. infestans* biology. These studies should include improvements in our understanding of the relative contributions and rates of mutation, recombination, natural selection, gene flow, random genetic drift and migration (Burdon & Silk, 1997) to the generation and maintenance of variation in populations (relative importance of asexual vs. sexual reproduction). Suitable genetic markers are therefore needed to facilitate such work.

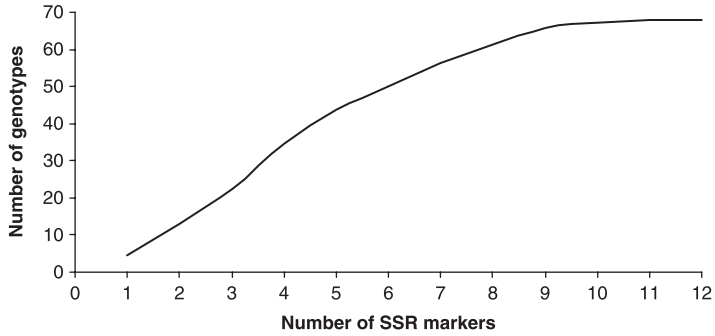

Figure 2 The relationship between number of simple sequence repeat markers used (see Table 2) and ability to distinguish genotypes of *Phytophthora infestans* based on an analysis of 90 isolates.

Table 3 Observed allele sizes (bp) for each control isolate of *Phytophthora infestans* (C1–C10) amplified with each of the 14 SSR markers

	Allele sizes (bp)													
	Marker													
Isolate	Pi02	Pi04	Pi16	Pi26	Pi33	Pi56	Pi63	Pi65	Pi66	Pi70	Pi89	4B	G11	D13
C1	152	166	176	177	203	176	148	148	153	192	179	217	162	136
	162	170	178	179	203	176	151	151	155	195	181	217	162	136
				185				157						
C2	162	166	176	179	203	176	151	145	153	192	179	205	162	118
	164	170	178	181	206	176	157	151	153	192	181	217	162	136
				183										
				187										
C3	162	166	176	179	203	176	151	145	153	192	179	205	162	118
	162	170	178	181	203	176	157	151	155	192	181	217	162	136
				183										
				187										
C4	162	166	176	177	203	176	157	145	155	192	179	205	156	136
	162	170	178	181	203	176	157	145	155	192	181	217	162	136
C5	162	166	176	177	203	176	157	145	155	192	179	205	156	136
	162	170	178	181	203	176	157	145	155	192	181	217	162	136
C6	160	166	174	171	203	176	157	142	155	192	179	213	156	108
	162	170	178	183	203	176	157	145	155	192	181	225	156	112
								151						
C7	162	166	178	177	203	174	157	145	155	192	179	205	158	136
	162	170	178	181	203	176	157	145	155	192	181	213	160	136
				183								215		
				187										
C8	162	170	176	177	203	174	148	145	155	192	179	205	160	136
	162	170	178	179	206	176	157	145	155	192	179	213	160	136
C9	162	166	176	179	203	176	151	145	155	192	179	205	162	118
	162	170	178	183	206	176	157	145	155	192	181	213	162	136
				187										
C10	162	168	178	177	203	176	157	145	155	192	179	205	162	136
	162	168	178	177	203	176	157	145	155	192	179	213	162	136

Previously, genotyping of *P. infestans* has largely been carried out using dominant markers. These are generally characterized by the presence or absence of a specific band on a gel and do not allow discrimination between the homozygous dominant or heterozygous state of a particular locus. Furthermore, it is not clear whether the absence of a band (i.e. a null allele) is always due to the same mutation. However, the development of co-dominant markers allows the discrimination of homozygotes and heterozygotes, since a single allele at a particular locus is amplified in homozygotes, whereas in heterozygotes both alleles are clearly resolved (Duncan *et al*., 1998). Such discriminatory power makes co-dominant markers useful for studying gene flow within populations (Powell *et al*., 1996), as well as linkage analysis (Meksem *et al*., 1995) and mapping studies of specific traits (Raeder *et al*., 1989).

Several co-dominant markers have been used to examine *P. infestans* in the past, but have shown certain limitations. Isozymes are co-dominant (Tooley *et al*., 1985) but there are problems with throughput, band nomination and level of polymorphism (Elansky *et al*., 2001). Single-locus RFLPs are co-dominant and have been used in linkage studies (Carter *et al*., 1999), whilst the moderately repetitive multilocus RFLP probe RG57 (Goodwin *et al*., 1992a) has been useful in investigating genetic diversity of *P. infestans* populations with varying success (Goodwin *et al*., 1992b; Forbes *et al*., 1998; Purvis *et al*., 2001; Cooke *et al*., 2003; Day *et al*., 2004). AFLPs have been scored as co-dominant, but scoring relies on band intensities and is challenging and therefore unreliable. However, they have been used in various studies such as mapping (van der Lee *et al*., 2001a) and diversity (Purvis *et al*., 2001; Cooke *et al*., 2003). These molecular techniques, however, require large quantities of DNA, and often the use of radioactivity (Rosendahl & Taylor, 1997), and can be laborious.

Knapova & Gisi (2002) developed three polymorphic SSR markers with which to analyse *P. infestans* isolates originating from potato and tomato in France and Switzerland. Their work showed that at two loci (including locus Pi4B also used in this study), a total of 10 different alleles were observed in field populations of *P. infestans*. They suggested that sexual recombination and selection rather than long-distance migrations may explain this diversity. These authors found no strong associations between SSR genotype, AFLP pattern, mating type or metalaxyl. As Knapova & Gisi (2002) recorded null alleles with one SSR locus, and some markers show limited allele diversity, it was necessary to expand the existing marker set to allow

Table 4 Number of alleles observed (NA), number of potential genotypes and expected frequencies of most common and rarest genotypes for each simple sequence repeat locus in *Phytophthora infestans*, based on a set of 90 isolates

SSR locus	NA	No. of potential genotypes	Expected frequency of most common genotype	Expected frequency of rarest genotype
Pi02	4	10	0·72	0·0025
Pi04	4	10	0·39	0·0004
Pi16	3	6	0·48	0·0025
Pi33	3	6	0·69	0·0004
G11	9	45	0·25	0·000025
D13	6	21	0·64	0·000025
4B	4	10	0·41	0·2401
Pi56	2	3	0·56	0·0841
Pi66	2	3	0·36	0·0004
Pi89	2	3	0·50	0·0001
Pi63	4	10	0·42	0·0036
Pi70	4	10	0·61	0·0441

a more comprehensive analysis of *P. infestans* populations and isolate collections on a European or wider intercontinental scale. In this study, EST and BAC sequences were exploited for the successful development of an additional 11 SSR markers, the construction and screening of a genomic library yielded an additional marker, and these were used in conjunction with two of the existing markers developed by Knapova & Gisi (2002), bringing the total to 14 markers. The number of markers required for population analysis can vary according to pathogen diversity and the number of alleles per locus. For example, Atallah *et al*. (2004) reported the use of 25 SSR markers for studying the pathogen *Sclerotinia sclerotiorum*, Guérin *et al*. (2004) used 21 markers to examine diversity within *Venturia inaequalis*, whilst Gobbin *et al*. (2003, 2005) reported the use of four or five SSR markers as being sufficient for the analysis of populations of the oomycete *Plasmopara viticola*.

Previous work (Knapova & Gisi, 2002) described the identification of 28 genotypes amongst 176 isolates of *P. infestans* isolated in 1996 and 1997. In this study, 68 genotypes of *P. infestans* were detected from 90 isolates, and confirmed that the panel of SSR markers developed was sufficient for discrimination within UK populations and those on a wider geographic scale. Marker Pi26 consistently amplified more than two alleles and was not considered suitable for population analysis (similarly with marker Pi65). Information regarding these markers was included as it has proved useful in isolate tracking studies (data not shown). In addition, for some isolates, three alleles were amplified at other loci (e.g. Pi63, 4B). These occurrences were rare, and for analysis purposes the genotype was recorded as that occurring most frequently amongst other isolates. This may result in a slight underestimation of diversity in the population, but is unlikely to have a large effect where all markers are used in combination. The presence of one or three alleles at a locus has been previously reported in *P. infestans* (van der Lee *et al*., 2001b) and *P. cinnamomi* (Dobrowolski *et al*., 2002), and van der Lee *et al*. (2004) demonstrated the presence of trisomic linkage groups in 10–16% of progeny from two individual crosses of *P. infestans*. These authors suggest that, as these trisomic progeny were pathogenic on potato, trisomy in *P. infestans* can occur in nature. It is therefore speculated that trisomy could account for the presence of three alleles at some of the loci reported here.

The markers also amplified fragments from closely related *Phytophthora* species, thus demonstrating transferability, although these were not examined further. Fragment length variation in other species may not only relate to sequence changes in the SSR region itself, and will therefore need to be validated by sequencing. However, the fact that the amplified fragment sizes were broadly similar to those of *P. infestans*, and that intraspecific size variation was demonstrated in *P. phaeseoli* and *P. mirabilis*, suggests that the amplified regions also contained polymorphic SSRs. Further work (unpublished data) has also shown

Table 5 Genetic variation of *Phytophthora infestans* isolates estimated with 12 simple sequence repeat loci

	England / Wales (n = 30)		Ireland (n = 9)		Scotland (n = 38)		World isolates (n = 13)	
Locus	NA	Gene diversity	NA	Gene diversity	NA	Gene diversity	NA	Gene diversity
Pi02	2	0·40	2	0·20	3	0·08	3	0·36
Pi04	4	0·61	2	0·50	4	0·60	4	0·54
Pi16	2	0·51	2	0·50	2	0·50	3	0·58
Pi33	3	0·28	2	0·44	2	0·17	2	0·38
Pi56	2	0·40	1	0·00	2	0·33	2	0·40
PI63	4	0·59	3	0·51	4	0·51	3	0·49
Pi66	2	0·49	2	0·35	2	0·44	2	0·50
Pi70	2	0·44	2	0·28	2	0·35	4	0·34
Pi89	2	0·35	2	0·49	2	0·46	2	0·26
G11	7	0·75	3	0·29	5	0·61	7	0·78
D13	2	0·35	2	0·48	2	0·24	5	0·35
4B	3	0·39	2	0·50	3	0·36	4	0·54
Mean	2·9	0·46	2·1	0·38	2·8	0·39	3·4	0·46

NA, number of alleles observed for each marker.

that diversity is increased when isolates from a range of other European collections of *P. infestans* are included in the analysis. This enhanced marker set will therefore form the basis of a pan-European analysis of *P. infestans* under the auspices of the EU concerted action project 'EUCABLIGHT' (www.eucablight.org) in which phenotypic and genotypic information relating to over 12 000 isolates of *P. infestans* has been collated to date. As additional alleles are described by groups across Europe or worldwide, they will be reported to the authors and this information will become publicly available on the project website. In addition, protocols for the use of these markers and their development for use in capillary (ABI3730) systems will shortly become available. For improved throughput and for genotypic testing of large numbers of lesions occurring during an epidemic, DNA from foliar lesions has been stored successfully on FTA cards (Whatman). Recovery and genotyping at a later date without the need for fungal isolation has proved effective (data not shown).

In addition to allowing studies of populations of *P. infestans* on a large scale for the first time, these markers have proved useful in epidemiological studies where isolates with distinct genotypes can be monitored throughout the growth of a crop, and the effects of management practices, such as host resistance and chemical control, on the predominance of isolates throughout an epidemic can be studied.

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