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The Northern Ireland *Phytophthora infestans* population 1998–2002 characterized by genotypic and phenotypic markers

L. R. Cooke[ab]*, D. J. Carlisle[a], C. Donaghy[b], M. Quinn[b], F. M. Perez[c], K. L. Deahl[c]

[a]*Department of Applied Plant Science, Faculty of Science and Agriculture, The Queen's University of Belfast Newforge Lane, Belfast, BT9 5PX;* [b]*Applied Plant Science Division, Department of Agriculture and Rural Development, Newforge Lane, Belfast BT9 5PX, UK; and* [c]*USDA, ARS, PSI, Vegetable Laboratory, Beltsville, MD 20705, USA*

A total of 204 isolates of *Phytophthora infestans* from Northern Ireland, almost all from commercial potato crops, were collected over 5 years (1998–2002). Phenotypic diversity was assessed using mating type and metalaxyl resistance; genotypic diversity was assessed using two allozyme loci (glucose-6-phosphate isomerase, *Gpi,* and peptidase, *Pep*), mitochondrial DNA haplotype and the multilocus RFLP probe RG57. All isolates were A1 mating type and *Gpi 100/100*. The majority were *Pep 100/100*, but four *Pep 83/100* and six *Pep 96/100* isolates were identified. Three mtDNA haplotypes were detected; haplotype IIa was the most common, but each year up to 2001 its frequency declined, with a concomitant increase in Ia isolates. Three isolates had the rare haplotype IIb, but this was only detected in 1998. Metalaxyl resistance and mtDNA haplotype were markedly associated: most haplotype Ia isolates were metalaxyl-resistant, whereas haplotype IIa was more commonly associated with metalaxyl sensitivity. Analysis of a subsample of 91 isolates revealed nine RG57 genotypes, three associated exclusively with haplotype IIa and six exclusively with haplotype Ia. The most common RG57 genotype (51% of isolates) comprised both metalaxyl-resistant and -sensitive haplotype IIa isolates. However, of haplotype Ia isolates, all metalaxyl-resistant phenotypes belonged to one of four RG57 types, one of which was the second most frequent overall (29% of isolates), while all metalaxyl-sensitive isolates belonged to one of two other types. The *P. infestans* population in Northern Ireland appears to consist of a limited number of clones related to, but differentiated from, the populations in mainland Britain and elsewhere in Europe.

Keywords: allozymes, mating type, metalaxyl sensitivity, mitochondrial DNA haplotype, potato-late blight, RG57 analysis

Introduction

In the past 25 years, worldwide changes in populations of the oomycete pathogen *Phytophthora infestans* have been associated with increased problems in controlling late blight on both potatoes and tomatoes. In Northern Ireland, the potential impact of late blight on commercial potato production is reflected in the fact that most seed crops receive eight or nine applications of foliar fungicides and some receive as many as 14 sprays to protect them from foliar and tuber infection by *P. infestans* (L. R. Cooke, unpublished data).

Phytophthora infestans is a diploid, heterothallic organism believed to have originated and evolved in the central highlands of Mexico (Niederhauser, 1991), although a South American origin has also been proposed (Abad & Abad, 1997). Before the 1970s, European populations of this pathogen appear to have consisted exclusively of a single clonal lineage of the A1 mating type, known as US-1, which has the mitochondrial DNA (mtDNA) haplotype Ib, an allozyme genotype [based on glucose-6-phosphate isomerase (*Gpi*) and peptidase (*Pep*) allozyme loci], *Gpi 86/100*, *Pep 92/100* and a characteristic fingerprint based on the multilocus RFLP probe RG57 (Goodwin *et al*., 1994). In recent years, analyses of *P. infestans* isolates from many European countries have generally failed to detect this 'old' clonal lineage of *P. infestans*, and have shown the presence of more diverse, new populations, often containing both A1 and A2 mating type strains.

In a study of the genotypic and phenotypic diversity of *P. infestans* in Northern Ireland in 1995 and 1996 (Carlisle *et al*., 2001), all of the 223 isolates characterized belonged to the 'new population' (as defined by allozyme genotypes and mtDNA haplotypes), but the A2 mating type was not detected, although A2 mating-type strains had previously been found in surveys in Northern Ireland

*E-mail: louise.cooke@dardni.gov.uk

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between 1987 and 1995 (Cooke *et al*., 1995). All isolates from 1995 and 1996 were monomorphic and homozygous at the loci coding for allozymes of glucose-6-phosphate isomerase and peptidase (*Gpi 100/100*, *Pep 100/100*) and 96% were mtDNA haplotype IIa. RAPD-PCR analysis of selected isolates revealed relatively little diversity among the Northern Ireland isolates, which clustered separately from isolates from Great Britain and the European mainland. It was concluded that the Northern Ireland population probably consisted of a series of closely related clonal lineages and that, since the A2 mating type was not detected, sexual recombination was unlikely.

In the Republic of Ireland, the A2 mating type was first isolated in 1988 (O'Sullivan & Dowley, 1991); 35% of 26 isolates from 1988–1989 were A2. Between 1988 and 1994, 249 isolates from the Republic of Ireland were characterized, but A2 isolates were only obtained from cvs Cara, Kerr's Pink and Golden Wonder (O'Sullivan *et al*., 1995) and the majority were obtained from tubers of cv. Cara. Subsequently, Griffin *et al*. (1998) failed to detect the A2 mating type among 114 isolates from 1995 and found that only four (3%) out of 153 isolates from 1996 were A2 mating type; all of these A2 isolates had been obtained from differential blight indicators grown at the Crops Research Centre, Carlow. Griffin *et al*. (2002) further investigated the *P. infestans* population in the Republic of Ireland using mating type, metalaxyl resistance, mtDNA haplotyping and RFLP fingerprinting with the multilocus nuclear DNA probe RG57 (Goodwin *et al*., 1992) to characterize 163 isolates collected between 1995 and 1999, of which 90% were collected in 1996. Their study indicated a low level of diversity in the Irish population of *P. infestans*; only 12 genotypes were detected using RG57, and the four A2 mating-type isolates from 1996 referred to above (the only A2 isolates included) all had the same RG57 fingerprint. They concluded that their results strongly suggested that sexual recombination of *P. infestans* does not occur in Ireland.

In Scotland, characterization of nearly 500 *P. infestans* isolates collected between 1995 and 1997 using mating type, metalaxyl resistance and amplified fragment length polymorphism (AFLP) fingerprinting indicated considerable diversity (Cooke *et al*., 2003). Approximately 20% of isolates were of the A2 mating type, but very few of these were metalaxyl-resistant; it was suggested that the A2 population was possibly restricted from becoming more widely established by its sensitivity to phenylamide fungicides. Among a subsample of 292 isolates examined by AFLP, 56% of isolates had unique fingerprints, but the majority of A2 isolates belonged to a single AFLP group. However, the presence of both mating types within sites, an increasing frequency of isolates with intermediate sensitivity to metalaxyl and the extent of AFLP diversity all suggested that sexual recombination occurred occasionally.

A study by Day *et al*. (2004) of the *P. infestans* population in Great Britain over a similar period (1995–98), using mating type, metalaxyl resistance, mtDNA haplotype and RG57 fingerprinting, revealed that A2 mating-type isolates were rare (3% of 2691 isolates) and all were metalaxyl-sensitive. In a subset of 1459 isolates, 30 fingerprints were detected by RG57, of which four were frequent (77% of isolates), widespread and found in all years. While circumstantial evidence suggested that sexual recombination occurred at some sites, the authors concluded that it was not possible to determine the extent of sexual recombination or its contribution to the evolving gene pool.

The purpose of this study was to extend the previous characterization of the Northern Ireland *P. infestans* population to isolates collected between 1998 and 2002. Isolates were derived largely from samples collected from commercial crops for annual surveys of the frequency of metalaxyl resistance and only one isolate was derived from each site, so it was not possible to examine diversity within sites. To provide more detailed data on diversity and to allow comparison with studies elsewhere, a subset of isolates was fingerprinted with probe RG57 in addition to characterizing isolates by mating type, metalaxyl resistance, allozyme genotype (*Gpi, Pep*) and mtDNA haplotype.

Materials and methods

Collection of isolates

Blighted potato material was collected in the major production areas of Northern Ireland during the growing seasons of 1998–2002 by members of the Department of Agriculture and Rural Development's Potato Inspection Service. Each of the 12 area inspectors was requested to collect a sample of infected foliage (and infected tubers if present) from each of four crops of representative cultivars during crop inspection (mainly in July). Each sample consisted of infected leaves and stems (and infected tubers if present) from a single crop. Information on site, fungicide usage, potato cultivar and blight incidence was collected for each sample. Samples were incubated under high humidity for 24–48 h to encourage sporulation, then isolates were obtained by bulking together the sporangia from all infected foliage from a single crop and initially maintained on detached glasshouse-grown potato leaflets of susceptible main crop cultivars free from R-genes (Cooke, 1986). In addition, when possible, isolates were obtained from infected potato dumps, volunteers, garden crops, breeders' clones and from woody nightshade (*Solanum dulcamara*). Four single-lesion isolates were also obtained from two naturally infected field trial sites in 2002.

After phenylamide-resistance testing (see below), isolates were transferred to rye A agar (Caten & Jinks, 1968) amended with the antibiotics rifampicin (25 mg L^{-1}) and natamycin (25 mg L^{-1}) (Carlisle *et al*., 2001) by placing excised leaf sections directly onto the agar or by picking sporangia off the sporulating leaflets with agar cubes (~2 × 2 × 2 mm). Following incubation at 15°C for several days, culture fragments were transferred to unamended rye agar, antibiotic rye agar or pea agar (Hollomon, 1965) amended with vancomycin (50 mg L^{-1}) and incubated in the dark at 15–18°C for routine maintenance.

A random sample of 20 isolates was used to generate single-zoospore isolates by an adaptation of the method of Caten & Jinks (1968). Sterile distilled water was added to 15-day-old cultures grown on rye agar, which were then incubated at 4°C for 3 h followed by 30 min at room temperature. Aliquots (0·5 mL) of the suspensions were spread evenly over the surface of water agar or thinly poured carrot agar (Erselius & Shaw, 1982) and incubated at 20°C for 12 h to encourage germination of the zoospores. After this period the plates were examined microscopically and single germinating zoospores were cut from the agar and transferred to pea agar plates. These were incubated at 20°C and examined daily for 3 weeks. The zoospores which formed colonies were maintained on rye and pea agar plates as above. In each case, at least three progeny isolates were obtained from the parental isolate and characterized as described below to check for any heterogeneity within the parental isolates.

Mating-type determination

Isolates were grown on unamended rye or carrot agar (Erselius & Shaw, 1982) with known reference isolates of the A1 or A2 mating types (Cooke *et al*., 1995) using two plates for each pairing and placing mycelial plugs (~4 mm diameter) of the two isolates (unknown and tester) approximately 20 mm apart. The dual cultures were incubated at 15°C in darkness for 5–10 days, then examined microscopically for the presence of oospores where the two colonies interacted.

Metalaxyl sensitivity assessment

The floating potato leaf disc method described by Cooke (1986) was used to determine metalaxyl sensitivity. Isolates were tested on 2 and 100 mg metalaxyl L^{-1}; those unable to sporulate on leaf discs at either concentration (but sporulating on untreated discs) were designated sensitive; isolates sporulating on discs at both metalaxyl concentrations were designated resistant, and those sporulating on discs at 2 but not at 100 mg metalaxyl L^{-1} were designated intermediate.

Allozyme assays

Genotypes at two polymorphic allozyme loci, *Gpi-1* (glucose-6-phosphate isomerase, GPI, E.C. 5·3·1·9) and *Pep-1* (peptidase, PEP, E.C. 3·4·3·1), were determined using cellulose acetate electrophoresis (CAE) using the protocols of Goodwin *et al*. (1995) as modified by Carlisle *et al*. (2001). The genotypes of unknown isolates were determined by comparison with reference isolates, including 50·96 (*Gpi 90/100*, *Pep 83/100*), PINN-A (*Gpi 100/100*, *Pep 92/100*), MT95-RED (US-8, *Gpi 100/111/122*, *Pep 100/100*), TW95-024 (US-1, *Gpi 86/100*, *Pep 92/100*) and EC1021 (*Gpi 90/100*, *Pep 96/100*). Isolate 50·96, used as the *Pep 83/100* standard, was originally characterized as such by starch gel electrophoresis by Lebreton & Andrivon (1998), but was also scored as *83/100* in the present study. Isolate EC1021, representative of the EC-1 clonal lineage from Ecuador (Forbes *et al*., 1997), was used as the *Pep 96/100* standard.

Identification of mitochondrial DNA haplotypes

Mitochondrial DNA (mtDNA) haplotypes of isolates were determined by PCR-RFLP using the method of Griffith & Shaw (1998) as modified by Carlisle *et al*. (2001). The DNA haplotype of isolates was assigned by determining the molecular weight of the individual restriction fragments of each PCR-RFLP profile (by comparison with molecular size markers) and referring to the published literature (Griffith & Shaw, 1998).

RG57 fingerprinting

DNA fingerprinting using the moderately repetitive probe RG57 was carried out on a subset of 91 isolates using the method described by Goodwin *et al*. (1992), but with the following modifications. The RG57 insert was PCR-amplified with the oligonucleotide primers M13 (forward and reverse). The product was purified with the Wizard DNA Purification kit (Promega) and labelled with the Random Primer Fluorescein Labeling kit (Antifluorescein-AP; NEN). *Phytophthora infestans* DNA was extracted using GeneElute Plant Genomic DNA Extraction kit (Sigma). The DNA was transferred to Hybond N + Nylon membrane (Amersham Pharmacia Biotech). The manufacturers' instructions for the above kits were followed for membrane transfer, Southern hybridization and nucleic acid detection.

Data analysis

Chi-squared analyses were used to test for the significance of associations between fungicide usage and metalaxyl sensitivity, between geographical area and metalaxyl sensitivity, between geographical area and mtDNA haplotype and between mtDNA haplotype and metalaxyl sensitivity. They were also used to test for associations between each of the polymorphic RG57 bands and metalaxyl sensitivity and mtDNA haplotype.

The data were combined and converted to discrete character state format, and phylogenetic trees were generated using the maximum parsimony (MP) option within PHYLIP (Felsenstein, 2004). Consensus trees were constructed from the 100 best trees generated after 500 replications. Dendrograms were drawn using TREEVIEW version 4. Bootstrap values greater than 70% (Hillis & Bull, 1993) were indicated at branch points.

Results

Source of isolates

A total of 204 isolates from five of the six counties of Northern Ireland (potatoes are not grown commercially in Co. Fermanagh) was obtained and characterized over

Table 1 Potato cultivars from which Northern Ireland isolates of *Phytophthora infestans* were obtained

Cultivar[a]	Region[b] where cultivar grown	Number of isolates
Arran Banner	NW, S	6
Avalanche	S	3
British Queen	NE, NW, S	15
Désirée	NW, S	24
Dunbar Standard	NE, NW	7
Dundrod	NE	5
Home Guard	NE, S	6
Kerr's Pink	NE, NW, S	53
Maris Piper	NE, NW, S	9
Navan	NE, S	4
Nicola	NE, NW, S	13
Pentland Squire	NE	3
Record	NE, NW, S	4
Sharpe's Express	NE	4
Up-to-date	NE, NW, S	7

[a]Cultivars from which three or more isolates were obtained. Other commercially grown cultivars sampled (number of isolates in brackets): Arran Victory (2), Charlotte (1), Dunbar Rover (1), Dunluce (1), Fambo (1), King Edward (2), Lady Rosetta (1), Morene (1), Pentland Dell (2), Pentland Ivory (1), Roscor (1), Santé (1), Saxon (1), Sunbeam (2), unknown (6). In addition, one isolate was obtained from each of cvs Milagro, Orla, Remarka and Santé in naturally infected field trials.
[b]NE, Co. Antrim; NW, Counties Londonderry and Tyrone; S, Counties Armagh and Down.

the 5-year period, with the number per year ranging from 26 to 58 isolates. Of these, 174 isolates were from foliage and five from tubers of commercial potato crops of 31 different cultivars, the most frequent being Kerr's Pink and Désirée (Table 1). In addition, five isolates were from potato dumps, one from a volunteer, four from breeder's clones, four from naturally infected field trials, four from garden potato crops, one from an outdoor-grown tomato fruit (cv. Gardener's Delight) and six from woody nightshade (*S. dulcamara*).

Single-zoospore isolates

At least three single-zoospore isolates were derived and characterized from each of 22 randomly selected parental isolates. All proved to be identical to their parental isolates in tests used subsequently to analyse the field isolates. This indicated homogeneity within the original bulk sporangial isolates.

Mating type and metalaxyl resistance

All of the *P. infestans* isolates proved to be of the A1 mating type (Table 2). Over all years, 50% of isolates tested were sensitive to metalaxyl, 47% contained resistant strains and 3% were classified as intermediate (Table 3); within years the percentage of resistant isolates ranged from 32% in 1998 to 68% (71% including isolates with intermediate sensitivity) in 2001. Chi-squared analysis of frequencies indicated that the proportion of resistant isolates differed between years ($P < 0{\cdot}01$). Over all years, from crops known to have been sprayed with fungicide formulations containing a phenylamide, 67% of isolates were resistant, whereas from crops which had not been phenylamide-treated, only 37% of isolates were resistant (Table 4). Chi-squared analysis indicated a very highly significant ($P < 0{\cdot}001$) association of metalaxyl resistance with sites where a phenylamide fungicide had been applied. Nonetheless, the majority of resistant isolates were obtained from crops which had not received a phenylamide treatment.

Allozyme genotype and mtDNA haplotype

All isolates tested were homozygous (*100/100*) at the *Gpi* locus (Table 2). The vast majority of isolates were also homozygous at the *Pep* locus (*100/100*), but four isolates

Table 3 Number and percentage of Northern Ireland isolates of *Phytophthora infestans* sensitive (S), intermediate (I) and resistant (R) to metalaxyl, 1998–2002

Year	Number of isolates S	I	R	Isolates R + I (%)
1998	32	0	15	32
1999	22	4	9	37
2000	12	0	14	54
2001	11	1	26	71
2002	25	1	32	57
Total	102	6	96	50

Table 2 Characteristics of Northern Ireland isolates of *Phytophthora infestans*, 1998–2002

Mating type	mtDNA haplotype	Allozyme genotype *Gpi*	*Pep*	Number of isolates	Metalaxyl sensitivity[a] S	I	R	Region[b] NE	NW	S	Years found
A1	Ia	*100/100*	*100/100*	55	10	0	45	20	8	27	1998–2002
A1	Ia	*100/100*	*96/100*	6	0	0	6	4	2	0	1999–2001
A1	Ia	*100/100*	*83/100*	4	0	0	4	0	0	4	1998, 2002
A1	IIa	*100/100*	*100/100*	136	90	6	40	44	39	53	1998–2002
A1	IIb	*100/100*	*100/100*	3	2	0	1	0	2	1	1998
Total				204	102	6	96	68	51	85	

[a]S, sensitive; I, intermediate; R, resistant.
[b]NE, Co. Antrim; NW, Counties Londonderry and Tyrone; S, Counties Armagh and Down.

Table 4 Number and percentage of Northern Ireland isolates of *Phytophthora infestans* sensitive (S), intermediate (I) and resistant (R) to metalaxyl derived from crops with or without phenylamide fungicide treatments

	Number (and percentage) of isolates		
Crop management	S	I	R
Treated with phenylamide	19 (33)	0 (0)	39 (67)
Not phenylamide-treated[a]	76 (59)	5 (4)	47 (37)

[a]Excludes 18 crops where treatment was not known.

had the *Pep 83/100* genotype and six had the *Pep 96/100* genotype. The four *Pep 83/100* isolates were all recovered from infected plants at the Agriculture and Food Science Centre, Newforge, one from a plant of a *Solanum* sp. hybrid (*S. sanctae-rosae* × *S. andigena*) in 1998 and the other three from field-trial plots of cvs Milagro, Remarka & Santé in 2002. The six *Pep 96/100* isolates were obtained from widely disparate commercial crops in 1999, 2000 and 2001.

Three mtDNA haplotypes were detected among the isolates analysed (Table 2). Three isolates of haplotype IIb were identified in 1998, but this haplotype was not found in subsequent years. The majority (67%) of isolates were haplotype IIa, the remainder being Ia. Haplotype IIa predominated in each year except 2001, but whereas in 1998 and 1999 over 80% of isolates were IIa, this fell to 62% in 2000 and 45% in 2001, with a recovery to 61% in 2002. Chi-squared analysis of haplotype frequencies indicated that the proportions of isolates with haplotypes Ia and IIa differed between years ($P < 0{\cdot}001$).

RG57 analysis

None of the three haplotype IIb isolates was viable when DNA fingerprinting by the multilocus nuclear DNA probe RG57 (Goodwin *et al*., 1992) was carried out on a subset of 91 isolates (representative of all years), so it was only possible to include haplotype Ia and IIa isolates. Worldwide RG57 analysis of *P. infestans* isolates has shown that 25 restriction fragments/bands can be detected. In the Northern Ireland isolates characterized, six of these bands were not detected (bands 4, 11, 12, 15, 18 and 23), while eight were present in all isolates (bands 1, 5, 13, 14, 20, 21, 24 and 25). The remaining 11 bands (bands 2, 3, 6, 7, 8, 9, 10, 16, 17, 19 and 22) were polymorphic.

In the absence of an internationally agreed system for naming RG57 fingerprints, each type was designated by a code, using NI for Northern Ireland followed by a number. Types differing from the most common types by only one band were designated subtypes, indicated by lower-case suffixes. Thus the most common type was designated NI-1 and its subtype NI-1a.

A total of nine different RG57 fingerprints were detected, of which one (NI-1) occurred in just over 50% of isolates and the second most common (NI-2) in 29% of isolates (Table 5). Of the other seven fingerprints, one was found in 8% and another in 4% of isolates, while the remainder were only represented by one or two isolates.

Associations between markers

All RG57 types were associated with a single mtDNA haplotype (Table 5). Thus all isolates with RG57 fingerprints NI-1, NI-1a and NI-2a had the mtDNA haplotype IIa, while all NI-2, NI-2b, NI-3, NI-4, NI-5 and NI-5a isolates possessed mtDNA haplotype Ia.

Four isolates with the *Pep 96/100* genotype were fingerprinted and these all proved to be RG57 NI-4, mtDNA Ia. Three isolates with the *Pep 83/100* genotype were fingerprinted and these had two distinct and unique fingerprints (NI-5 and NI-5a) and were mtDNA Ia.

MP dendrograms were constructed using the RG57 data alone and using the RG57 data combined with mtDNA haplotype and *Pep* genotype and also with these characters plus metalaxyl resistance. The MP dendrogram constructed with the RG57 data alone had a very similar structure to ones constructed using the other polymorphic characters. Adding metalaxyl resistance did not add significant substructuring and, since as a selectable character the value

Table 5 Genotypes, phenotypes and sources of Northern Ireland isolates of *Phytophthora infestans*, 1998–2002

RG57 type	RG57 fingerprint	mtDNA haplotype	Allozyme genotype		Metalaxyl sensitivity[a]	Number of isolates	Year found	Region[b]
			Gpi	*Pep*				
NI-1	100 010 001 100 110 100 011 001 1	IIa	*100/100*	*100/100*	S, I, R	46	1998–2002	NE, NW, S
NI-1a	100 010 000 100 110 100 011 001 1	IIa	*100/100*	*100/100*	S, R	2	1999, 2000	NE
NI-2	110 010 000 100 110 010 011 101 1	Ia	*100/100*	*100/100*	R	26	1999–2002	NE, NW, S
NI-2a	110 010 000 100 110 110 011 101 1	IIa	*100/100*	*100/100*	S, I	2	1999, 2002	S
NI-2b	110 010 000 100 110 000 011 101 1	Ia	*100/100*	*100/100*	S	1	1998	NE
NI-3	111 010 100 100 110 100 011 101 1	Ia	*100/100*	*100/100*	S	7	1998, 1999, 2002	NE, S
NI-4	111 010 100 000 110 010 011 101 1	Ia	*100/100*	*96/100*	R	4	1999, 2001	NE, NW
NI-5	101 011 110 100 110 100 111 101 1	Ia	*100/100*	*83/100*	R	2	2002	S
NI-5a	101 010 110 100 110 100 111 101 1	Ia	*100/100*	*83/100*	R	1	2002	S

[a]S, sensitive; I, intermediate; R, resistant.

[b]NE, Co. Antrim; NW, Counties Londonderry and Tyrone; S, Counties Armagh and Down.

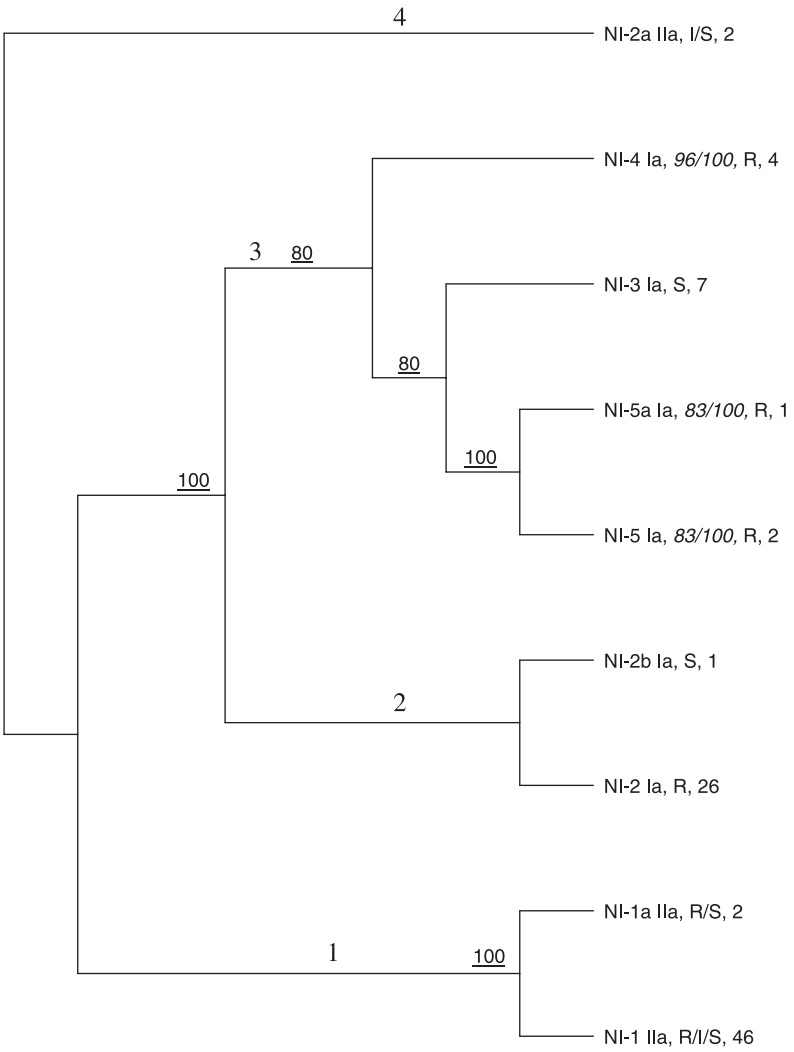

Figure 1 Maximum parsimony dendrogram of mtDNA haplotype, *Pep* genotype and RG57 data for nine *Phytophthora infestans* genotypes from Northern Ireland. Each genotype is designated by its RG57 type (NI-1 to NI-5a), haplotype and metalaxyl resistance, followed by the number of isolates with this genotype. *Pep* allozyme genotype is *100/100*, except where stated in italics after the haplotype. Clusters are arbitrarily numbered (1,2,3 & 4). Bootstrap values (underlined) are shown where these are 70% or greater.

of its inclusion is debatable, only the dendrogram based on RG57 data, mtDNA haplotype and *Pep* genotype is presented (Fig. 1). This dendrogram showed significant grouping of the isolates into four main clusters with significant subclustering. Cluster 1 comprised RG57 types NI-1 and NI-1a, i.e. it included all mtDNA IIa isolates except for those with the NI-2a fingerprint. Cluster 2 comprised RG57 types NI-2 and NI-2b. Cluster 3 comprised RG57 types NI-3, NI-4, NI-5 and NI-5a and was separated into three significant subclusters, one of NI-4, one of NI-3 and one of NI-5 and NI-5a. The final cluster contained only NI-2a. Thus, the mtDNA IIa genotypes were clearly separated from the mtDNA Ia types, which were further subdivided into several genotypes that did not appear to be closely related.

Metalaxyl resistance tended to be associated with mtDNA haplotype Ia (Fig. 2), 85% of Ia isolates being metalaxyl-resistant (Table 2). Chi-squared analysis showed that there was a very highly significant association ($P < 0{\cdot}001$) between metalaxyl resistance and haplotype (the three haplotype IIb isolates were excluded from the analysis). Among the Ia haplotype isolates, metalaxyl resistance was strongly associated with specific genotypes; thus all isolates with the RG57 fingerprint NI-2 were metalaxyl-resistant and all the *Pep 96/100* and *Pep 83/100* isolates were haplotype Ia and metalaxyl-resistant (Table 5). Metalaxyl-sensitive isolates with haplotype Ia were relatively infrequent (15% of Ia isolates); when eight of these were fingerprinted, seven were the only isolates with the RG57 fingerprint NI-3 and the eighth had a unique fingerprint (NI-2b).

In contrast, the majority (66%) of haplotype IIa isolates were metalaxyl-sensitive, but there was no association between metalaxyl sensitivity and specific genotypes (Table 2). Each of the three RG57 mtDNA IIa genotypes included both metalaxyl-resistant and -sensitive isolates, 65% of NI-1 isolates being sensitive and 35% resistant.

Of the 11 polymorphic RG57 bands, three were very highly significantly ($P < 0{\cdot}001$) associated with resistance and two with sensitivity to metalaxyl, while four were very highly significantly ($P < 0{\cdot}001$) associated with mtDNA haplotype Ia and two with haplotype IIa (data not presented).

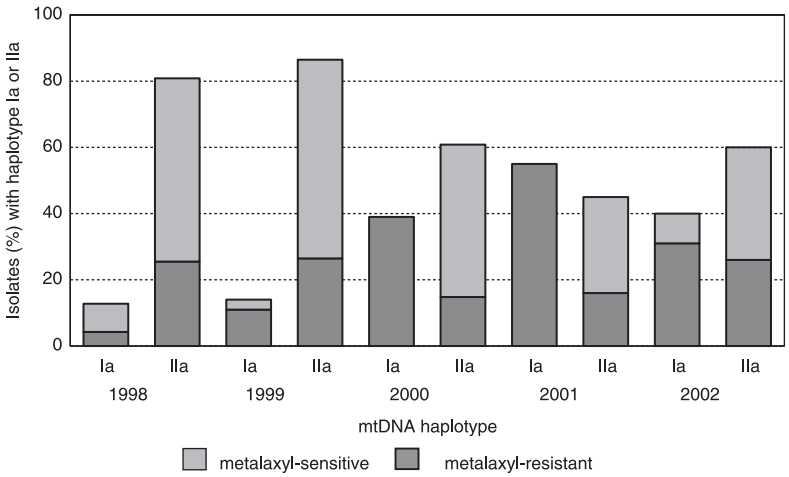

Figure 2 Association between mtDNA haplotype and metalaxyl resistance in Northern Ireland isolates of *Phytophthora infestans*. Bar height indicates the percentage of each haplotype within each year and the shading shows the relative proportions of the haplotypes that were metalaxyl-sensitive and -resistant.

Variation in markers over time and geographical area

The proportion of mtDNA haplotype Ia isolates increased over the years 1998–2001, but declined in 2002 (Fig. 2). Similarly the proportion of metalaxyl-resistant isolates followed this same pattern.

The number of RG57 genotypes detected within years varied from three in 2000 to seven in 1999 and 2002; with such a small number of genotypes it was not considered appropriate to carry out a statistical comparison of diversity over time.

For geographical comparisons, three regions were defined within Northern Ireland, the northeast (Co. Antrim), the northwest (Counties Londonderry and Tyrone) and the south (Counties Down and Armagh). All three regions had very similar proportions of metalaxyl-resistant isolates over the 5-year period (52, 51 and 48% in the northeast, northwest and south, respectively). There was no significant association between metalaxyl sensitivity and region, nor between haplotype and region ($P > 0{\cdot}05$). The two most common RG57 types (NI-1 and NI-2) occurred in all regions and the two other types which comprised more than two isolates also occurred in widely dispersed locations.

Discussion

Except for a few single-lesion or single-tuber isolates, the isolates of *P. infestans* used in the present study were derived from sporangia from a sample of several foliar lesions collected at random within each crop. In a previous study (Carlisle *et al*., 2001), more detailed sampling within crops revealed that isolates with more than one haplotype were detected in three of 29 sites and these were ones where at least two cultivars were growing in close proximity. In the present study, where more than one cultivar was grown in the same field, these were treated as separate samples to reduce the likelihood that multiple infections were sampled. Single-zoospore isolates established from 22 randomly selected isolates indicated that the parental isolates were homogenous for the markers used. In addition, detailed sampling of a naturally infected trial at Greenmount Agricultural College was carried out in 2002 and 80 single-lesion isolates were established from plots of five different potato cultivars. These proved to be identical in terms of mating type, *Gpi* and *Pep* allozyme genotype, mtDNA haplotype and metalaxyl sensitivity, and a subset of 40 were shown to be identical using six microsatellite markers (G. K. Young, personal communication). On this basis, while the possibility of multiple genotypes within a single isolate cannot be excluded, it was considered unlikely. However, caution should be exercised in using multiple-lesion isolates in other studies, since it is only appropriate for a highly clonal population.

No A2 mating-type isolates were found in Northern Ireland in the present study, confirming the results of Carlisle *et al*. (2001) and agreeing with the conclusion of Griffin *et al*. (2002) that the A2 mating type now occurs very rarely, if at all, on the island of Ireland.

Comparison of data from the present study with earlier data (Cooke *et al*., 2000; Carlisle *et al*., 2001) indicated that the proportion of metalaxyl-resistant strains increased in Northern Ireland over the period 1995–2001. For the 2002 season, the anti-resistance strategy endorsed by the Department of Agriculture and Rural Development in Northern Ireland was modified and growers were advised to use no more than two, rather than three, sprays of products containing phenylamide fungicides during the early part of the season only (Cooke, 2003). The proportion of isolates containing metalaxyl-resistant strains was lower in 2002 than in 2001, and this decline continued in 2003 and 2004 (L. R. Cooke, unpublished data).

Only a small number of isolates from Northern Ireland were classified as having intermediate sensitivity to metalaxyl; this was in contrast to the situation in Scotland, where the proportion of intermediate isolates increased from 2% in 1995 to 29% in 1997, and Cooke *et al*. (2003) suggested that they arose from recombination between resistant and sensitive strains. The small number of intermediate strains in the Northern Ireland population may

reflect the lack of recombinant types. In Scotland, none of the intermediate isolates was obtained from a crop known to have been treated with phenylamide fungicides (Cooke *et al*., 2003) and this was also the case in the present study.

In a further comparison between the earlier study (Carlisle *et al*., 2001) and the present one, it was observed that, while in both cases all isolates were monomorphic and homozygous at the *Gpi* locus, in the earlier study all isolates were *Pep 100/100*, whereas three *Pep* alleles (*83*, *96* and *100*) were identified in the present study. This is the first time that isolates with *Pep 83/100* or *Pep 96/100* have been found in Northern Ireland, although Tooley *et al*. (1993) found that a small number of Republic of Ireland isolates from 1989 were *Pep 83/100*. *Pep 83/100* has been reported elsewhere in Europe, in isolates from Great Britain (Carlisle *et al*., 2001), East Germany (Daggett *et al*., 1993), Poland (Sujkowski *et al*., 1994) and France, where it was the most common *Pep* genotype among potato isolates (Lebreton & Andrivon, 1998). Isolates with the allozyme genotype *Pep 96/100* have been reported from Poland (Sujkowski *et al*., 1994) and Hungary (Bakonyi *et al*., 2002b), but are rare in western Europe; the only previous report apparently being of a single isolate collected from Flanders in 1998 (Bakonyi *et al*., 2002a).

The three mtDNA haplotypes, Ia, IIa and IIb, found among the Northern Ireland *P. infestans* isolates, are all characteristic of the new population (Griffith & Shaw, 1998). Isolates of the IIb haplotype (Griffith & Shaw, 1998), which is rare in Europe, occurred in Northern Ireland isolates from 1996 (Carlisle *et al*., 2001), but were found only in 1998 in the present study. The predominance of mtDNA haplotype IIa, which occurred in 96% of isolates in the previous study (Carlisle *et al*., 2001), was much less marked in the years 1998–2002, and in 2001 there were more isolates with the Ia than the IIa haplotype, suggesting that changes in the population were occurring.

Comparison of results of this study with other European studies indicated apparent similarities in the genotypes detected, particularly in Great Britain and the Republic of Ireland. Thus Griffin *et al*. (2002) found their type IE-1 to be the most common among Irish isolates (65%) from 1995 to 1999; this is identical to the most common Northern Ireland RG57 type, NI-1 (51% of isolates), in terms of RG57 fingerprint, mating type and mtDNA haplotype (IIa). The same RG57 fingerprint, named as RF06 and associated only with haplotype IIa, was found by Purvis *et al*. (2001) to be one of the most common among isolates from England and Wales. Cooke *et al*. (2003) reported this fingerprint (their type b) in mtDNA IIa isolates from Scotland. Day *et al*. (2004), who fingerprinted 1459 isolates collected from England, Scotland and Wales in 1995–98, found that their fingerprint RF006, which is identical to NI-1, comprised 9% of the isolates; most of these had the mtDNA haplotype IIa, but a small number had mtDNA Ia. The same fingerprint was also reported as the most common in Norway (N-1) and one of the most common (F-2) in Finland (Brurberg *et al*., 1999). The most frequent RG57 fingerprint found among isolates from Poland collected in 1989–91 by Sujkowski *et al*. (1994) differs from NI-1 only in having band 4 present and, since band 4 is not reproducibly detected by RG57 (Koh *et al*., 1994), these fingerprints are probably the same. While convergent evolution can result in isolates having the same RG57 fingerprint on differing genetic backgrounds (Purvis *et al*., 2001), the widespread occurrence of this fingerprint in northern European *P. infestans* populations and its strong association with a single mtDNA haplotype and mating type suggest that it may represent a widely distributed clonal lineage. The two NI-1a isolates had an identical fingerprint to the IE-1b of Griffin *et al*. (2002), which represented 7% of isolates from the Republic of Ireland in their study. This fingerprint was reported by Day *et al*. (2004) as RF008, but occurred in under 1% of their isolates.

The second major RG57 type, NI-2 (29% of isolates), was initially thought to represent a new RG57 genotype. However, comparison of its fingerprint with others published from European populations revealed that it differed only in bands 16 and 17 from the IE-2 of Griffin *et al*. (2002), which is identical to the RF002 of Day *et al*. (2004). IE-2 and RF002 were both scored as having band 16 and lacking band 17, whereas NI-2 lacked band 16 and has band 17. To clarify the situation, seven isolates identified as RF002 and reported in Day *et al*. (2004) were kindly supplied by Dr D. E. L. Cooke (Scottish Crop Research Institute) and fingerprinted alongside three NI-2 Northern Ireland isolates, one NI-2a isolate (scored as having both bands 16 and 17) and one NI-1 isolate. This showed NI-2 to be identical to RF002 and indicated that the band scored as 16 by Griffin *et al*. (2002) and Day *et al*. (2004) is the one scored as 17 in the present study. The absence of any isolates possessing both bands 16 and 17 from previous studies of isolates from the UK and Ireland may have led to this confusion, which emphasizes the desirability of international collaboration and exchange of standard isolates.

Griffin *et al*. (2002) found IE-2 to be their second most frequent type (11% of isolates) and, as with NI-2, all isolates of this type were metalaxyl-resistant and had the mtDNA haplotype Ia. Day *et al*. (2004) found their RF002 to be the second most common type (18% of isolates) in Great Britain in 1995–98; almost all their RF002 isolates were mtDNA Ia, but a small percentage (0·8%) were IIa. Thus NI-2, like NI-1, appears to represent a European clonal lineage. Two single-band variants of NI-2 with fingerprints which have not been reported elsewhere were found in the present study (NI-2a and NI-2b). Both differed from NI-2 in other characters: the two NI-2a isolates were haplotype IIa and one was metalaxyl-sensitive, while the single NI-2b isolate was haplotype Ia but metalaxyl-sensitive. It is thus unclear if these genotypes are closely related to NI-2.

The third most frequent RG57 type, NI-3, consisted exclusively of metalaxyl-sensitive mtDNA Ia isolates. Griffin *et al*. (2002) identified this fingerprint (designated IE-7) in one of 14 isolates from Northern Ireland supplied for inclusion in their study by the present authors; it

was obtained from woody nightshade (*S. dulcamara*) in Northern Ireland in 1999. Interestingly, of the seven NI-3 isolates, four were obtained from potato crops in disparate locations in 1998 and 2002, but three were from natural infections of the same woody nightshade plant, one obtained in 1999 and two in 2002. No sign of infection of this plant by *P. infestans* was observed in 2000 or 2001. The finding of this genotype on *S. dulcamara* in two separate years 3 years apart suggests that it may be particularly able to infect woody nightshade and that a degree of host adaptation may be involved. Day *et al.* (2004) reported this fingerprint as RF042, associated with mtDNA Ia and occurring in 0·5% of isolates.

Of the remaining RG57 fingerprints, NI-4, NI-5 and NI-5a, which were associated with the unusual *Pep* genotypes, NI-4 and NI-5a do not appear to have been reported previously. NI-5 may be the same fingerprint as NL-39 reported by Zwankhuizen *et al.* (2000), which was associated with a single isolate obtained from the Netherlands in 1994, but differences in band scoring complicate the comparison. Surprisingly, the most common fingerprint (RF039, 47% of isolates) found in England, Scotland and Wales by Day *et al.* (2004) was not found in Northern Ireland in the present study and occurred only in a single isolate (IE-6) in that of Griffin *et al.* (2002). However, NI-5 differs from RF039 only in one RG57 band and, although allozyme genotype was not determined by Day *et al.* (2004), isolates with this fingerprint obtained from the Channel Island of Jersey all had the *Pep* genotype *83/100* (K. L. Deahl & L. R. Cooke, unpublished data), as did NI-5, so they probably represent related genotypes.

In contrast to the similarities with other European genotypes, comparison of Northern Ireland genotypes with US clonal lineages revealed differences in terms of RG57 fingerprints and dilocus allozyme genotypes. Only the A1 *Gpi 100/100* clonal lineage US-6 appears superficially similar to Northern Ireland genotypes, but its *Pep* allozyme genotype is *92/100*, which was not found in any Northern Ireland isolate, and its RG57 fingerprint is quite different. The most common clonal lineage in the US, US-8, is a metalaxyl-resistant A2 type with the *Gpi* genotype *100/111/122*, which has never been reported from Europe. This supports the view that, while both the new European and new US *P. infestans* populations originated from Mexico, they represent separate migrations (Fry *et al.*, 1993).

An association of certain RG57 bands with both metalaxyl resistance and mtDNA haplotype was noted within Northern Ireland *P. infestans* isolates. This is unlikely to indicate that these bands are markers for resistance or haplotype, since comparison with Day *et al.* (2004) showed that these specific associations are not found within RG57 fingerprints of isolates from other regions. The associations with RG57 bands found among Northern Ireland isolates are considered to be indicative of the clonal structure of the population rather than resulting from any real genetic linkage.

Associations between particular genotypes/haplotypes and metalaxyl resistance or sensitivity have, however, occurred elsewhere. Thus, just as all isolates of the Northern Ireland mtDNA Ia genotype NI-2 were metalaxyl-resistant, all isolates with this genotype from the Republic of Ireland (IE-2) and the vast majority from Great Britain with this genotype (RF002) were also metalaxyl-resistant (Griffin *et al.*, 2002; Day *et al.*, 2004). On the other hand, the genotype NI-1/IE-1/RF006 comprised both metalaxyl-resistant and -sensitive isolates in Northern Ireland and the Republic of Ireland, although in Great Britain isolates with this genotype were mainly metalaxyl-sensitive (Griffin *et al.*, 2002; Day *et al.*, 2004). Associations between particular genotypes and metalaxyl resistance may have developed as a result of the clonal population structure. In an asexually reproducing clonal population, the whole genome behaves as though it were effectively linked and selection for one or more characters conferring increased fitness will result in 'hitch-hiking' by others. Thus a character such as metalaxyl resistance may be rapidly disseminated if it is associated in an isolate with increased aggressiveness (Deahl *et al.*, 2002). However, this does not fully explain why metalaxyl-sensitive forms of the genotype NI-2/IE-2/RF002 are so rare, whereas the genotype NI-1/IE-1/RF006 frequently occurs in both metalaxyl-sensitive and -resistant forms. Possibly it may have been occasioned by the difference between migration into the population of an already metalaxyl-resistant genotype compared with *in situ* selection for metalaxyl resistance on a metalaxyl-sensitive genotype.

The very marked clustering which occurred in the MP dendrogram (Fig. 1) lends further support to the view that in Ireland the *P. infestans* population is strongly clonal and exclusively asexual. The structure suggests that several distinct genotypes were introduced by migration and that there is little exchange of genetic information between them, although occasional variants occur, presumably originating by mutation.

The *P. infestans* population in Ireland thus appears to represent a subpopulation of the new European population, containing common European clonal lineages, but clearly differentiated from the populations of Great Britain and mainland Europe in terms of frequencies of mating types (A2 not detected in recent years) and genotypes. Whereas mtDNA Ia is the most frequent haplotype elsewhere in Europe, mtDNA IIa genotypes predominate throughout Ireland, representing 67% of Northern Ireland isolates from 1998 to 2002, 96% of Northern Ireland isolates from 1995 to 1996 (Carlisle *et al.*, 2001) and 78% of isolates characterized by Griffin *et al.* (2002) from the Republic of Ireland. The reasons for this dominance by mtDNA IIa genotypes and for the failure of A2 mating types to persist in the Irish population are not known. Day *et al.* (2004) suggested that sexual recombination probably contributed to the diversity of the *P. infestans* gene pool in Great Britain, but both the present study and that of Griffin *et al.* (2002) provide no evidence for this in Ireland. More detailed sampling and use of additional markers (Cooke & Lees, 2004) would be required to exclude the possibility of very infrequent sexual recombination within the Irish *P. infestans* population.

The differentiation of the Irish *P. infestans* population from other European populations may be a result of clonal selection mediated by restricted introduction of new *P. infestans* genotypes because of controls on importation of seed tubers (Northern Ireland is one of the high-grade seed areas for potato production within the EU and only the higher grades of seed may be planted for either seed or ware) and the distance of Ireland from mainland Europe. However, the drivers of the selection process, be they founder effects or some aspect of pathogenic fitness, remain to be elucidated.

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