GENETIC ANALYSIS OF IRISH POPULATIONS OF *PHYTOPHTHORA INFESTANS*

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SUMMARY

Phytophthora infestans (Mont.) de Bary, the causal agent of potato late blight is best known for its role in the great Irish famine of 1845-1849 which resulted in the deaths of over 1 million people. Since then, the disease has become established in all potato growing countries and is the most important pathogen of potatoes worldwide. The appearance of resistance to the phenylamide fungicides in the late 1970's indicated that populations of *P. infestans* were changing. An anti-resistance strategy was developed for growers in an effort to reduce the spread of resistant strains. Subsequently the A_2 mating type of the fungus was discovered in 1989 promoting fears that a super strain of the fungus could evolve through sexual reproduction. Populations of the fungus have been monitored from 1981 to 1998 for levels of phenylamide resistance and since 1988 for the A_2 mating type. Physiological race surveys were conducted in 1983 and 1996. Prior to the 1980s no reliable methods were available for adequate identification of genotypes. Development of molecular markers specific to *P. infestans* has made this possible and a survey was conducted on isolates from the 1996 population.

Results confirm that the anti-resistance strategy for phenylamide based fungicides has been effective in preventing the build up of metalaxyl resistant populations of *P. infestans*. During the 1990's the distribution of phenylamide resistance has remained stable at about 50% of crops tested compared to a high of over 80 % in 1981. The level of A_2 in the population has also fallen from a high of 35% of isolates tested in 1989 to a static level of 3-4 % in the 1990's. Physiological race composition has become much more complex since 1983 and 16 different physiological races were found in Ireland in 1996. The population was dominated by race 3.4.7.10.11 which accounted for over 54% of isolates tested. This change has taken place without a corresponding change to varieties with a complex R-gene base.

Twelve different genotypes of the fungus were uncovered using the multilocus probe RG57. Races of the fungus were independent of genotype. One particular genotype IE-2 was predominantly associated with phenylamide resistance. The low population diversity discovered suggests that sexual reproduction between A_1 and A_2 types has not been a major factor in disease epidemiology to date. Super strains similar to those identified in the USA could not be confirmed.

The overall level of variation in the Irish isolates of *Phytophthora infestans* would confirm that the population has become progressively more diverse during the last forty years. However, the population is much less complex than that found in the highland tropics of central Mexico.

INTRODUCTION

Potato late blight, caused by the oomycete fungus *Phytophthora infestans* (Mont.) de Bary, is the notorious fungus responsible for the Irish potato famine of 1845-'49 and it is still the most important disease affecting the potato worldwide. The fungus exists as distinct populations which equal the sum of the strains present in a given geographic area. Each strain can be further divided depending on physiological race, fungicide resistance, mating type and genetic markers. Physiological races are identified by the presence of one or more of the 11 known virulence genes. *P infestans* is a heterothallic fungus and exists as two different mating types. Each strain can therefore be subdivided into the two mating types A_1 and A_2. Strains can also be divided into different genotypes by genetic fingerprinting which is based on the presence or absence of different genetic markers.

Late blight is indigenous to central Mexico (Goodwin *et al.,* 1994) and it is believed that a single strain of the fungus (US-1) migrated from Mexico to Europe via North America in the early 1840's. The cool, humid climate of north eastern Europe was particularly suitable for the development and spread of the disease and it has remained endemic in Ireland for over 150 years. Studies have shown the original strain of the fungus introduced from the new world (US-1) dominated the European population until the late 1970's (Fry & Goodwin, 1995).

Research on physiological races confirmed that there were only two physiological races present in Ireland by the middle of the twentieth century (Doling, 1957). This would suggest that there was little genetic variation in the Irish population at that time. This situation appeared to be changing in the 1980's with the first record of phenylamide resistance in commercial potato crops in Ireland (Dowley & O'Sullivan, 1981) and the confirmation that physiological races had become very much more complex (O'Sullivan & Dowley, 1983). Some years later the presence of the second mating type (A_2) was confirmed in Ireland (O'Sullivan & Dowley, 1991). For many years it was thought that the potato late blight fungus, reproduced sexually only in Mexico, where two mating types A_1 and A_2 occur in a ratio of approximately 1:1 (Gallegley & Galindo, 1958). The apparent absence of sexual reproduction in temperate and cold regions was attributed to the absence of the A_2 mating type from these regions (Smooth *et al*., 1958). Since then, A_2 strains have been reported in a growing number of European countries; Switzerland (Hohl & Iselin, 1984), Scotland (Malcolmson, 1985), England & Wales (Tantius *et al*., 1986), the Netherlands (Frinking *et al*., 1987) and in Ireland (O'Sullivan & Dowley, 1991). Surveys conducted worldwide using molecular markers have revealed increased population diversity in both A_1 and A_2 mating types (Forbes *et al*., 1998). This indicated a possible global migration of new strains of the fungus. The presence of the two mating types raised the possibility of fitter and more

aggressive strains arising by sexual reproduction. Sexual reproduction could also lead to soil borne inoculum in the form of oospores giving rise to earlier epidemic development.

The objective of this project was to establish the level of genetic diversity present in the Irish population of *P. infestans* using physiological and molecular markers and to quantify the possibility of sexual reproduction leading to increased problems in disease control.

METHODS

Phenylamide resistance

Crops in the main potato growing areas of the country were sampled at random. Four samples of 100 infected leaves were taken from each hectare sampled. A suspension of ca 50,000 sporangia ml^{-1} was prepared from each sample by brushing the sporangia from each leaflet into distilled water. The suspension was then incubated for 3 h at 10^0C to promote zoospore formation.

Prior to inoculation, 15-mm discs were cut from fully expanded leaflets of the cv. Kerr's Pink using a cork borer. Five leaf discs were then floated adaxial surface downwards on 10 ml of 0, 5 and 100 µg ml^{-1} metalaxyl in 5-cm petri dishes. One 20 µl drop of inoculum from each sample was placed on the abaxial surface of each leaf disc and the petri dishes were covered and incubated for 7 days at 18^0C, a 12 h daylength and a light intensity of 150 lux. A sample was considered to contain phenylamide resistance when leaf discs developed complete necrosis accompanied by sporulation at all levels of metalaxyl. Sensitive samples showed slight necrosis and no sporulation on the two levels of metalaxyl.

Mating type

Isolates of *P. infestans* were obtained from infected potato foliage and tubers and isolated onto rye A (Caten & Jinks, 1968) or pea (Malcolmson, 1979) agars containing rifampicin (50 µg ml^{-1}). Isolates were maintained in culture on rye A or pea agars. Mating types were determined by pairing a pure culture of each isolate with reference A_1 and A_2 isolates on V8 agar (Ribeiro, 1978) or rye A agar. Paired cultures were incubated at 15-20^0C for 10 days and checked microscopically for the presence of oospores at the contact zones between the unknown and reference isolates. Mating type was determined for 704 isolates of *P. infestans* collected from 261 potato crops in 16 counties between 1989 and 1998. Metalaxyl resistance was also determined for these isolates.

Physiological race determination

Races of *P. infestans* were determined by their reaction on leaf discs of indicator plants possessing R genes 1 to 11. Suspensions in sterile distilled water containing approximately 1 x 10^6 sporangia ml^{-1} were prepared from 3-week old cultures. These were incubated at 10°C for 3 h to induce zoospore formation. Leaf discs, floating on distilled water (5 per petri dish, 1 petri dish of each indicator for each isolate) were inoculated on the abaxial surfaces with 20 µl drops of each zoospore suspensions. The leaf discs were then incubated in a lighted incubator simulating daylight at 18-20°C and examined after one week. Races were determined by the presence or absence of hypersensitive reactions without sporulation on the leaf discs of differential indicator plants.

Genomic DNA analysis

Isolates of *P. infestans* for DNA extraction were grown for 15 days in pea broth (200 g frozen peas were autoclaved in 1l distilled water at 121°C for 15 min., the supernatant was collected, made up to 1l with distilled water and re-autoclaved) amended with 12.5 µg ml^{-1} rifampicin, 12.5 µg ml^{-1} ampicillin and 25 µg ml^{-1} Pimaricin. DNA extractions and RG57 Southern Blotting were carried out using the protocols of Pipe *et al.*, (In Press). Bands were scored by presence or absence but not intensity. Data analysis and trees were produced using NTSYSpc (Exeter software).

Mitochondrial DNA haplotype

Four different forms of mitochondrial (Mt) DNA have been found in strains of *P. infestans*. Type II differs from type I by possessing an extra 1kb insert of DNA. Type 1 may be further differentiated into Ia and Ib, the latter possessing an extra *Msp*I restriction site. Type II was further differentiated into type IIa and IIb with IIb possessing an extra *Cfo*1 site (Carter *et al.*, 1990). A method to determine Mt DNA type was developed by Griffith & Shaw (1998). This method which relies on PCR amplification of sections of mitochondrial DNA and subsequent restriction enzyme digestion was utilised to analyse isolates.

RESULTS

Phenylamide resistance

The number of crops sampled per annum depended on disease incidence and varied between 18 and 95 (mean 50). The % of crops with resistance present was

highest in 1981 but fell rapidly in the absence of phenylamide use between 1981 and 1984 (Dowley & O`Sullivan, 1985). Phenylamides were reintroduced to the Irish market in 1985 following an early and severe outbreak of late blight. Despite the existence of an anti-resistance strategy, advocating non-curative use of phenylamide base products, they was a high level of curative use between 1985 and 1989 (Dowley & O`Sullivan, 1991). This resulted in a rapid increase in the number of crops with phenylamide resistance present (Fig.1). From 1990 onwards there was close adherence to the anti-resistance strategy of using the phenylamide/mancozeb mixtures for the first three sprays of the programme and avoiding curative use and use on early crops. As a result the distribution of phenylamide resistance again declined.

In the 1990's phenylamide resistance was confined to about 50% of crops tested. This figure increased slightly when late blight appeared early in the season and coincided with the application of one of the three phenylamide/mancozeb sprays. A drop in the distribution of phenylamide resistance was recorded when late blight appeared late in the season. Field trials confirmed that where phenylamide/mancozeb mixtures were used according to the anti-resistance strategy there was excellent control of both foliage and tuber blight, even in the presence of phenylamide resistant strains (Dowley, 1994). Within crops where resistance was confirmed, the level of resistance rarely exceeded 50% of the *Phytophthora* population. This could explain why phenylamide based fungicides continued to give good disease control even when metalaxyl resistant strains were known to be present.

Examination of seasonal variation in the occurrence of metalaxyl resistance also confirmed that resistance was more widespread towards the end of the season when the application of phenylamide based products had ceased. This factor would also contribute to greater efficacy of the phenylamide based products when applied early in the season

Mating types

The A_2 mating type was first confirmed in the Republic of Ireland in 1988/'89. The incidence of A_1, A_2 and self-fertile mating types among the 26 isolates first tested is given in Table 1. The majority of leaf isolates were of the A_1 mating type while the A_2 type was dominant among tuber isolates. Only one of the isolates was self-fertile. This culture had an adpressed growth pattern on pea agar, formed very few sporangia and produced oospores in abundance in single culture (O'Sullivan & Dowley, 1991). The overall frequency of A_2 strains was 35%. This is higher than the 10% frequency of these strains reported from England & Wales (Shattock *et al*., 1990) and the Netherlands (Therrien *et al*., 1989). However, when isolates

from the cv. Cara, all of which came from one area, are excluded, the frequency of A_2 strains is similar to that recorded in these other countries.

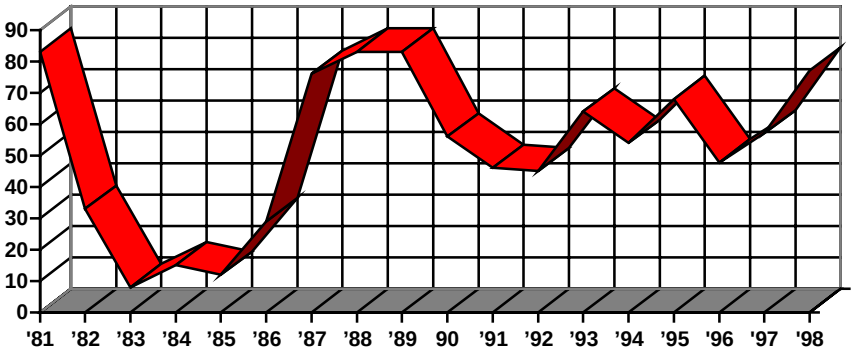

Fig. 1: % Crops with phenylamide resistance present 1981-1988

Table 1: Mating type of *Phytophthora infestans* in Ireland 1988 and 1989

Cultivar	Tissue	A_1	A_2	Self-fertile
Cara	Tuber	2	6	0
Cara	Leaf	0	1	0
Kerr's Pink	Leaf	9	1	1
Record	Leaf	1	0	0
Golden Wonder	Leaf	2	1	0
King Edward	Leaf	1	0	0
Arran Victory	Leaf	1	0	0
Total		**16**	**9**	**1**

During the last ten years *P. infestans* isolates were obtained from 13 potato cultivars, mainly Kerr's Pink, Record, Cara and Rooster together with some unnamed breeding clones and R-gene differentials. The A_2 mating type was found in isolates from only three commercial cultivars, Kerr's Pink (3.6%), Golden Wonder (1.4%) and Cara (39.3%). None of the A_2 isolates were resistant to phenylamides. The incidence of

the A_2 mating type of *P. infestans* has decreased in the Republic of Ireland from 35% of isolates tested in 1989 to 4% of those tested in 1997 (Fig. 2). It must be noted that one-third of isolates tested in 1988-89 were from cv. Cara and 75% of these were of the A_2 mating type. If isolates from cv. Cara are excluded then the A_2 occurred with a frequency of 11%. The presence of both mating types permits genetic recombination with the possible emergence of new, more aggressive or fungicide-resistant strains of *P. infestans*. Oospore production and survival in the soil could also provide a new source of inoculum for the initiation of epidemics. However, the low incidence of the A_2 mating type suggests that genetic recombination in *P. infestans* under natural conditions in Ireland must be a very rare event, if it occurs at all (O'Sullivan *et al.*, 1995). Isolates collected in the Republic of Ireland in 1988 and 1989 were also of the new population type (Tooley *et al*., 1993) suggesting that the A_2 mating type, which appears to have arrived with the new population, may have been present in Ireland for some time.

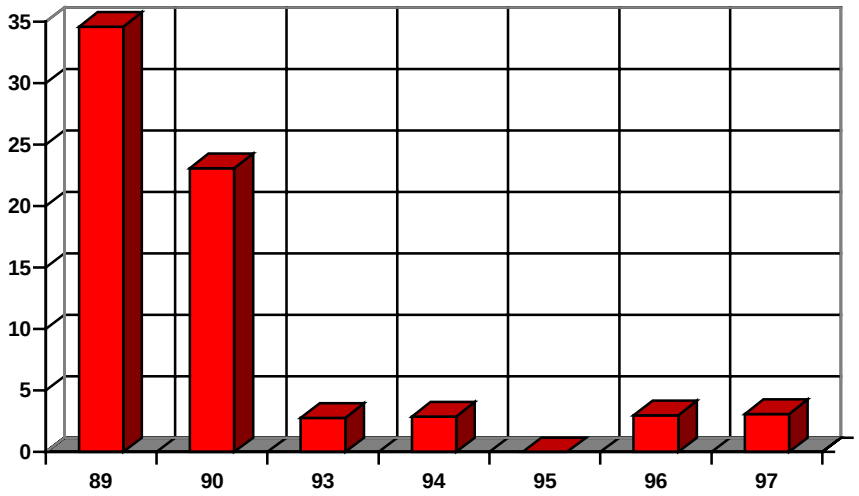

Fig. 2: % frequency of the A_2 mating type 1989-1997

Physiological races

All isolates analysed were from the 1996 population. Four of the isolates analysed were of the A_2 mating type and these were all from the same sample site in Carlow. Thirty-two of the 143 isolates were metalaxyl resistant. All A_2 isolates were metalaxyl sensitive. Physiological race type was independent of other traits. Analysis of race diversity indicates that the population is much more diverse than when previously sampled in 1983. Diversity indices calculated using the Shannon and Gleason index recorded values of H_S= 0.86 and H_G = 0.91 in 1983 (O'Sullivan & Dowley, 1983; Andrivon, 1995). This had increased to H_S = 1.67 and H_G = 3.02 by 1996 indicating an increase in population diversity. However when compared to indices calculated for Mexican populations where H_S = 3.45 and H_G =10.82 it shows that Mexican populations are still far more diverse. A_2 isolates when compared to A_1 isolates were found to be more complex and more likely to contain rare virulences such as 1, 2, 5 and 6. The mean number of virulences per isolate was 5 while the mean number of virulence factors per race was 5.4. The rarest virulences were also found only in the most complex races. This has been found in most race surveys conducted worldwide and suggests the influence of mutation events on race structure (Andrivon, 1995). Race 3.4.7.10.11 was found to be the most common race in the population accounting for 54% of isolates tested (Table 2) with distribution over both phenylamide resistant and sensitive strains. While the presence of 10 virulence genes were confirmed, virulence 1 and 2 were found only at very low levels (Fig. 3) and predominantly in phenylamide resistant and A_2 isolates. This was considered unusual as few commercial potato cultivars grown in Ireland possess R-genes and those that do contain either R_1, R_2, R_3, or a combination of the three. Virulence 9 was not found while virulence 8 was only found in one isolate. Most virulences found in Irish isolates appear unnecessary for successful survival on the potato cultivars commonly grown in Ireland.

Table 2: Frequency (f) of races of *Phytophthora infestans* in 1996 as percentage of total.

Race	f	Race	f
4.10	0.7	4.6.7.10.11	0.7
3.4.7	2.7	1.3.4.7.10.11	1.4
4.10.11	14.2	3.4.5.7.10.11	10.8
4.5.8.11	0.7	3.4.6.7.10.11	0.7
3.4.10.11	3.4	3.4.5.6.7.10.11	3.4
4.7.10.11	4.1	1.2.3.4.6.7.10.11	1.4
3.4.7.10.11	54.1	1.3.4.5.6.7.10.11	0.7
4.5.7.10.11	0.7	1.2.3.4.5.6.7.10.11	0.7

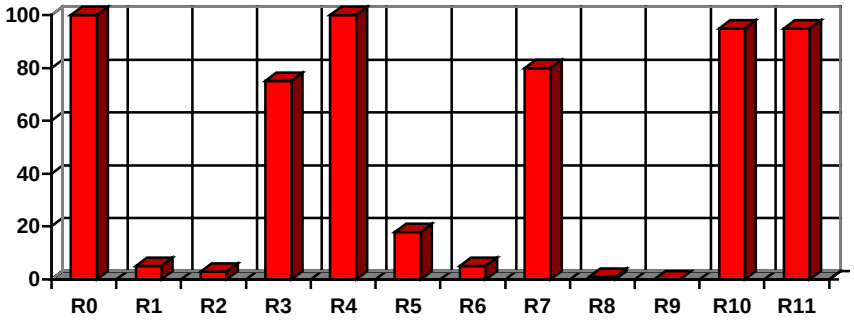

Fig. 3: Frequency of virulence to R-genes in the 1996 population of *P. infestans*

DNA analysis

Most of the 115 isolates analysed were from the 1996 population. A small number from the 94, 95 and 99 population was also included. Two different mitochondrial haplotypes of four possible types were identified (Fig. 4). Haplotype IIa was predominantly associated with metalaxyl sensitivity. The A_2 isolates and most metalaxyl resistant strains were of the Ia type (Table 3). Twelve different genotypes to date have been uncovered using the RG57 probe. The probe identifies 25-30 bands (Fig 5). Two bands not normally scored in other studies were included in this study due to their presence or absence in some of the isolates tested. These were band 14a and 25a. Isolates are differentiated on the basis of a 25-30 unit long binary code, each unit denoting the presence or absence of a band. Analysis using Jaccards coefficient and UPGMA clustering shows that Types IE-1a, IE-1b and IE-1c may be derived from type IE-1 due to the similarity of characteristics, they only differ in fingerprint by one band (Fig. 6). Similarly this is true for type 2 and 2a. IE-5 differs from IE-1 by two bands. Type 3 and 4 appear also to be genetically similar. However while all the other types, which cluster together share the same phenotype for phenylamide resistance, Mt DNA type and mating type, IE-3 and IE-4 differ for mating type. They may be independently arising fingerprint types or possibly the same strain which switched mating type by somatic recombination. Due to the clonality of phenylamide resistance strains the data seems to suggest that resistance is largely contained within one genotype and related strain. Only two of the fifteen IE-2 types were metalaxyl sensitive, and

only two of the seventy two IE-1 isolates were metalaxyl resistant and both of these were from the north of Ireland. All A_2 isolates typed from Ireland to date have been of type IE-3. Type IE-1, IE-2 and IE-3 were found to be major components of the UK population in 1996, 1997 and 1998 (D.S. Shaw & J.P. Day, pers. Comm.). Types IE-5a, IE-6 and IE-7 were found in 1999 only. Types IE-5a and IE-6 were isolated from a field trial in Co. Carlow. An isolate from woody nightshade in 1998 (isolate courtesy Dr. Louise Cooke) was also type IE-5a. Type IE-7 was a potato isolate also provided by Dr. Louise Cooke, Queens University of Belfast. Type IE-6 was found to dominate the *P. infestans* population in the south of England during the years mentioned above. As type IE-6 was previously detected in the UK it suggests that it did not arise here by sexual recombination among existing strains. The detection of these strains in Ireland may indicate that the population is more diverse now than in 1996 and that strains may be migrating from the UK to Ireland.

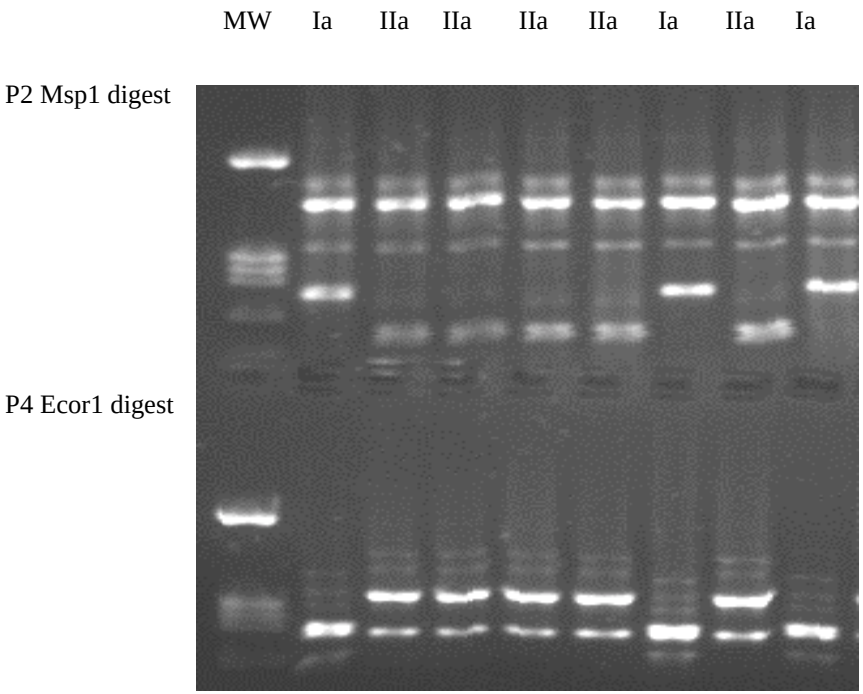

Fig. 4: Mitochondrial DNA polymorphisms revealed by PCR RFLP of eight Irish isolates of *Phytophthora infestans* representing the two haplotypes found

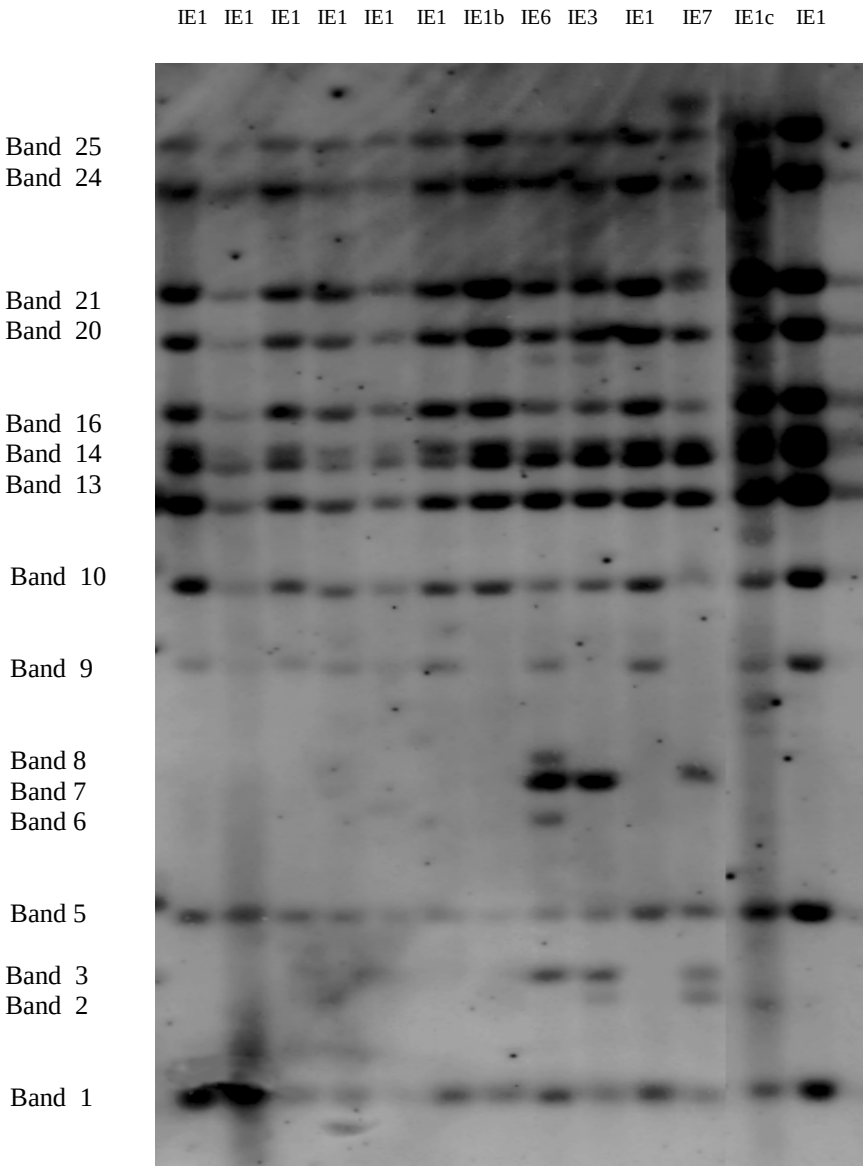

Fig. 5: RG57 Southern hybridisation of EcoR1 digested *P. infestans* genomic DNA

Table 3: Summary of RG57 fingerprint patterns and associated traits of Irish *Phytophthora infestans* isolates (The old population represented by US-1 and a strain from the new North American population US-6 are shown for comparison).

RG57 Fingerprint	Type	Mt Haplotype	Metalaxyl Sensitivity	% f	Mating Type
1000 10001 100110 1000 110011	IE-1	IIa	S/R	65.2	A_1
1100 10001 100110 1000 110011	IE -1a	IIa	S	2.6	A_1
1000 10000 100110 1000 110011	IE-1b	IIa	S	7	A_1
1000 10001 101110 1000 110011	IE-1c	IIa	S	2.6	A_1
1100 10000 100110 1000 111011	IE -2	Ia	R/S	10.4	A_1
1100 00000 100110 1000 111011	IE- 2a	Ia	R	1.7	A_1
1110 10100 100110 1001 111011	IE-3	Ia	S	3..5	A_2
1110 11101 100110 1001 111011	IE-4	Ia	S	1.74	A_1
1100 10001 101110 1001 110011	IE -5	IIa	R	1.74	A_1
1100 10001 101110 1011 110011	IE-5a	IIa	S	1.74	A_1
1010 11111 100110 1001 111011	IE-6	Ia	N/A	.87	A_1
1110 10100 100110 1000 111011	IE-7	Ia	S	.87	A_1
1010 11100 100110 0010 110011	US-6	IIb	S	N/A	A_1
1010 10101 100110 1000 110011	US-1	Ib	R/S	N/A	A_1

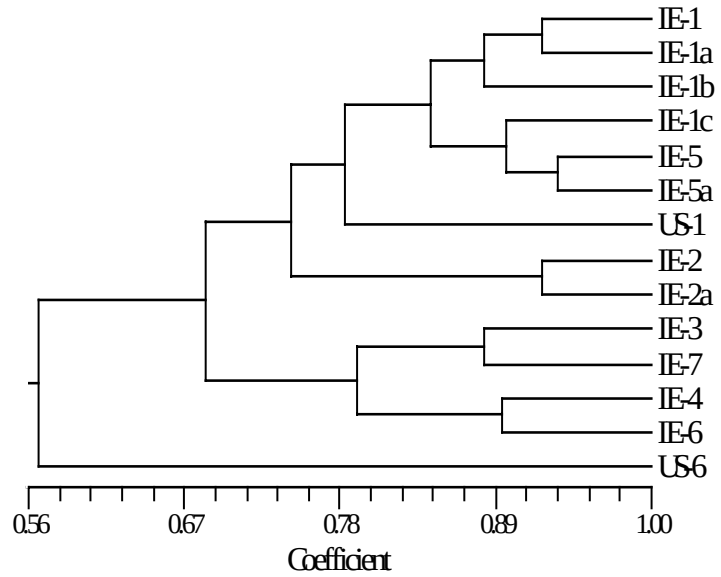

Fig. 6: Jaccard UPGMA tree of *Phytophthora infestans* RG57 data.

DISCUSSION

Within each individual year the distribution of metalaxyl resistance consistently increased as the season advanced. This would suggest that phenylamide resistant strains were less adapted to over-wintering compared with the sensitive strains. Curative use of the phenylamide fungicides also had a significant effect on increasing the distribution of metalaxyl resistance. This miss-use of the fungicide was common between 1985 and 1990 and resulted in very widespread distribution of resistance (Fig 1). However, where there was adherence to the anti-resistance strategy developed at Oak Park, the distribution of resistance was found to reduce. This strategy permits the continued use of the phenylamide-based fungicides while also achieving excellent foliage and tuber blight control.

Physiological race specialisation has become much more complex in the latter half of the twentieth century. In the mid-fifties there were 2 physiological races present in Ireland while in 1996 this figure had increased to 16 (Table 2). This increase in virulence has taken place without the introduction of varieties with complex R-genes and confirms that there has been a major change in the genetic variation of the fungus over the last 40 years. Metalaxyl resistance was distributed across a wide range of physiological races and this would suggest that resistance did not arise from a single mutation but was probably present in the population prior to the introduction of the phenylamide based fungicides.

Genetic variation may be further increased by the appearance of the A_2 mating type which permits sexual hybridisation and genetic recombination. This could see the development of a fitter and more aggressive strain of *P. infestans* that might be more difficult to control with current fungicides. The existence of oospores which can remain viable in the soil for years could also change the epidemiology of the disease and make it necessary to develop new control programmes. However, the low incidence of the A_2 mating type (Fig. 2) would suggest that it is unlikely to lead to widespread genetic variation in the immediate future. The future frequency and distribution of the A_2 mating type will need to be monitored so that any change in this position can be anticipated.

It appears that differences in physiological race are not clearly reflected by genotype. However isolates of the Mt DNA type Ia tend to possess rarer virulences and more complex race phenotypes. Several different race types have been found in each of the fingerprint groups shown in Table 3. The population is clearly more diverse than the early 80's population but not yet as variable as the Mexican population. Isolates corresponding to the US-1 strain were not found and this concurs with the findings of Tooly *et al.* (1983). This suggests that the old population has been replaced by new fitter strains. However, the RG57 probe

showed that the so called ‘super strains’ such as US-6 and US-8 were absent from the 1996 population. US-1 strains from the early 1980's Irish population have been found to be phenylamide resistant which suggests that resistance arose here and was present before the new migrations took over. Low levels of A_2 in the population suggest that it does not play a significant role in the epidemiology of the disease. This is also supported by the low number of different genotypes in the population indicating absence of sexual reproduction at present. There is a high degree of similarity between Irish and British populations. As a result the major source of genetic variation in the Irish population may be due to migration of new strains form the UK and mainland Europe and not from the direct import of seed potatoes from Mexico or South America.

CONCLUSIONS

- A new population has completely replaced the old Irish population
- Genetic diversity of the Irish population of *Phythophthora infestans* has progressively increased but is still less diverse than the Mexican population.
- Differences between closely related strains may have arisen from somatic recombination
- A major source of genetic variation is migration of new strains from Europe and the UK
- There has been a steady increase in the complexity of physiological races
- Rarest virulences found only in most complex races
- Incidence of A_2 has decreased steadily since first confirmed in 1989
- A_2 mating type exhibits more complex races
- Phenylamide resistance increased as the season advanced and following curative application of phenylamides
- Phenylamide resistance decreased and good disease control was achieved where the anti-resistance strategy was followed
- Phenylamide resistant strains may be restricted to genotype
- Use of phenylamide sprays may exert selection pressure on population keeping A_2 levels low and resistant types such as IE-2 high
- The risk of oospores over-wintering and initiating an epidemic is low
- The possibility of genetic recombination and the emergence of a "super strain" is a reality, but unlikely to happen quickly.

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