

DNA POLYMORPHISMS AS GENETIC MARKERS

IN *PHYTOPHTHORA INFESTANS*

A thesis submitted for the degree of
Doctor of Philosophy
of the University of London

by

Deidre Anne Carter

October, 1990

Biology Department,
Imperial College of Science, Technology and Medicine,
London SW7 2BB,
United Kingdom.

ABSTRACT.

P. infestans is a major pathogen of potatoes and tomatoes, and occurs worldwide wherever these crops are grown. Despite considerable efforts in the fields of population biology and genetics, work to date on this organism has been hampered by a lack of easily scored, selectable markers exhibiting sufficient polymorphism between isolates. Variation at the DNA level, detected as restriction fragment length polymorphisms (RFLPs) has been used to aid studies in a number of organisms, including the lower fungi, and the work presented here investigates their application to *P. infestans*. The occurrence of RFLPs was initially analysed in the mitochondrial and nuclear genomes of 24 isolates obtained globally and from around the UK. Polymorphism in mtDNA was sufficient to divide these isolates into four groups. nDNA polymorphisms supported and extended this grouping, and almost all isolates could be distinguished from one another using 5 different nDNA probes. Information on heterozygosity, ploidy levels, heterokaryosis, and the exchange of genetic material in the field was also obtained from the RFLP analysis of these isolates. 19 RFLP markers and 6 avirulence gene loci were then used to establish a preliminary linkage map for *P. infestans*. DNA was extracted from 80 progeny from a cross between two *P. infestans* isolates; Ca65 (A1; avr- 1, 4, 11; Gpi-1: 100-100) and 550 (A2; avr-1, 2, 3, 4, 5, 7, 10, 11; Gpi-1: 86-86) and was analysed for the inheritance of the avirulence genes and RFLP loci. Five small linkage groups were obtained containing a total of 16 loci. 28 progeny were omitted from the genetic analysis because of unexpected hybridisation profiles indicating triploidy or aneuploidy. Coinheritance of extra alleles and the mtDNA genotypes in these progeny indicated that the extra alleles arose in the oogonial parent. Pulsed field gel electrophoresis was initiated, and some apparent separation of DNA could be seen.

ACKNOWLEDGEMENTS

I am indebted to my supervisors, Professor Ken Buck, Dr. David Shaw, Dr. Richard Shattock, and Dr Simon Archer for their help and support during the course of my work and the preparation of this manuscript.

I am also very grateful to Dr. Helen Harley, Dr. Lodewijk Sandkuyl and Russell Snell for help with the linkage analysis, and to Chimida Fininsa for the work on the inheritance of virulence.

Thanks also to all members of labs 1.4, 1.7, 1.9 and the ground floor for help, encouragement and entertainment throughout the past three years, in particular to Paul for help with figures and tables, and to Jo and Mitla for tolerating all my mess and being such good lab mates.

Finally, thank you to all my friends and family for putting up with me and my behaviour during all phases of this PhD.

CONTENTS

	Page
ABSTRACT	1
ACKNOWLEDGEMENTS	2
CONTENTS	3
LIST OF FIGURES	6
LIST OF TABLES	10
CHAPTER 1	
INTRODUCTION	11
1.1 The <i>P. infestans</i> - <i>Solanum</i> pathosystem and the genetics of virulence	14
1.2 The development of markers	17
1.2.1 Conventional markers	17
a. Application to studies in population biology	17
b. Application to genetic studies	18
1.2.2 Molecular markers	19
a. Isozymes	20
b. Restriction fragment length polymorphisms	24
1.3 Aims of the current study	34
CHAPTER 2	
MATERIALS AND METHODS	35
2.1 Abbreviations	35
2.2 Names and addresses of commercial companies	36
2.3 Safety considerations and miscellaneous general methods	37
2.4 <i>P. infestans</i> isolates	38
2.5 Media	38
2.6 Culture conditions	38

2.7	DNA isolation	39
2.7.1	Large scale extraction for mtDNA purification	39
2.7.2	Small scale genomic extraction	40
2.8	Restriction enzyme digestion and gel electrophoresis	41
2.9	Construction of <i>P. infestans</i> recombinant DNA clones	42
2.9.1	Random cloning for population studies	42
2.9.2	Size-selected cloning for genetic mapping	42
2.10	Southern transfers, dot-blots, hybridisation and autoradiography	43
2.11	Linkage analysis	44
2.12	Pulsed field gel electrophoresis	45
2.12.1	Preparation of inserts	45
a.	Using isolated nuclei	45
b	Using protoplasts	46
2.12.2	Electrophoresis conditions	46
2.13	Nomenclature	46
 CHAPTER 3		
	RFLPs IN MITOCHONDRIAL DNA	47
3.1	Choice of isolates	47
3.2	DNA isolation	47
3.3	Restriction endonuclease digestion and RFLP analysis	50
3.4	Discussion of chapter 3	66
 CHAPTER 4		
	RFLPs IN NUCLEAR DNA	70
4.1	DNA isolation and restriction endonuclease digestion	70
4.2	Production and analysis of recombinant clones	70
4.3	RFLP analysis of isolates	72
4.4	Discussion of chapter 4	83

CHAPTER 5	
DNA MARKERS FOR GENETIC MAPPING	86
5.1 Choice of parents	86
5.2 DNA extraction	86
5.3 Screening for RFLP recombinants	91
5.4 Constructing the linkage map	97
5.4.1 Autoradiographs of the RFLPs	97
5.4.2 Linkage analysis of the polymorphic loci	119
5.5 Discussion of chapter 5	122
CHAPTER 6	
PULSED FIELD GEL ELECTROPHORESIS	130
6.1 Preparation of nuclei and protoplasts	130
6.2 Electrophoresis of whole chromosomes	130
6.3 Discussion of chapter 6	139
CHAPTER 7	
OVERALL DISCUSSION AND SUMMARY	143
REFERENCES	151
APPENDICES	169

LIST OF FIGURES

	Page
1.1 Detection and interpretation of RFLPs in nuclear DNA	29
3.1 CsCl-bisbenzimidide density gradient of <i>P. infestans</i> mt and nDNA	49
3.2 Agarose gel electrophoresis of mtDNA from <i>P. infestans</i> digested with <i>HindIII</i>	52
3.3 Agarose gel electrophoresis of mtDNA from <i>P. infestans</i> digested with <i>BclI</i>	53
3.4 Agarose gel electrophoresis of mtDNA from <i>P. infestans</i> digested with <i>BglII</i>	53
3.5 Agarose gel electrophoresis of mtDNA from <i>P. infestans</i> digested with <i>ClaI</i>	55
3.6 Agarose gel electrophoresis of mtDNA from <i>P. infestans</i> digested with <i>EcoRI</i>	55
3.7 Restriction map of <i>P. infestans</i> Type I mtDNA	56
3.8 Agarose gel electrophoresis of mtDNA from <i>P. infestans</i> digested with <i>Avall</i>	58
3.9 Agarose gel electrophoresis of mtDNA from <i>P. infestans</i> digested with <i>MspI</i>	59
3.10 Agarose gel electrophoresis of nDNA from <i>P. infestans</i> digested with <i>MspI</i>	60
3.11 Southern blot of nDNA from <i>P. infestans</i> digested with <i>MspI</i> and hybridised with mtDNA	60
3.12 Agarose gel electrophoresis of nDNA from <i>P. infestans</i> digested with <i>HhaI</i>	62
3.13 Agarose gel electrophoresis of mtDNA from <i>P. infestans</i> digested with <i>HhaI</i>	62
3.14 Agarose gel electrophoresis of Type II mtDNA from <i>P. infestans</i> digested with <i>RsaI</i>	64
4.1 Agarose gel electrophoresis of nDNA from <i>P. infestans</i> digested with <i>HindIII</i>	71
4.2 Dot-blot of <i>P. infestans</i> clones, hybridised with radiolabelled nDNA	71
4.3 Southern blot of <i>BglII</i> -digested nDNA from <i>P. infestans</i> isolates, hybridised with pIN1	73
4.4 a Southern blot of <i>MspI</i> -digested nDNA from <i>P. infestans</i> isolates, hybridised with pIN41	74
b. Diagram showing size and numbering of bands detected in a)	74
4.5 a Southern blot of <i>MspI</i> -digested nDNA from <i>P. infestans</i> isolates, hybridised with pIN2	76
b. Diagrammatic model to explain the formation of bands detected in a)	76
4.6 a Southern blot of <i>BglII</i> -digested nDNA from <i>P. infestans</i> isolates, hybridised with pIN2	79
b. Diagrammatic model to explain the formation of bands detected in a)	79

4.7 a Southern blot of <i>MspI</i> -digested nDNA from <i>P. infestans</i> isolates, hybridised with pIN29	81
b. Diagram showing size and numbering of bands detected in a)	
4.8 a Southern blot of <i>MspI</i> -digested nDNA from <i>P. infestans</i> isolates, hybridised with pIN11	82
5.1 a. Agarose gel electrophoresis of DNA from Ca65, 550, and 80 progeny digested with <i>MspI</i>	89
5.1 b. Southern blot of <i>BglII</i> -digested DNA from Ca65, 550 and 80 progeny hybridised with pSTA99	90
5.2 Agarose gel electrophoresis of 95 pUC19 recombinant clones containing 0.5 kb inserts of <i>P. infestans</i> DNA	92
5.3 Southern blot of recombinant clones hybridised with radiolabelled <i>P. infestans</i> DNA	92
5.4 "Test" blots of DNA from <i>P. infestans</i> isolates Ca65 and 550 digested with <i>MspI</i> , <i>BglII</i> and <i>HindIII</i>	94
5.5 Southern blot of DNA from 9 progeny digested with <i>HindIII</i> and <i>MspI</i> and hybridised with pIN5-655	96
5.6 a Southern blot of <i>MspI</i> -digested DNA from Ca65, 550, and 80 progeny hybridised with pIN2.	98
b Diagram showing numbering of bands detected in a).	98
5.7 a Southern blot of <i>MspI</i> -digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-199	101
b Diagram showing numbering of bands detected in a).	101
5.8 a Southern blot of <i>MspI</i> -digested DNA from Ca65, 550, and 80 progeny hybridised with pIN11.	102
b Diagram showing numbering of bands detected in a)	102.
5.9 a Southern blot of <i>MspI</i> -digested DNA from Ca65, 550, and 80 progeny hybridised with pIN29.	103
b Diagram showing numbering of polymorphic bands detected in a).	103
5.10 a Southern blot of <i>BglII</i> -digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-6.	104
b Diagram showing numbering of polymorphic bands detected in a).	104
5.11 a Southern blot of <i>MspI</i> -digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-13.	105
b Diagram showing numbering of polymorphic bands detected in a).	105
5.12 a Southern blot of <i>MspI</i> -digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-26.	106
b Diagram showing numbering of bands detected in a).	106

5.13 a Southern blot of <i>Bgl</i> II-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-174.	1 0 7
b Diagram showing numbering of bands detected in a).	1 0 7
5.14 a Southern blot of <i>Hind</i> III-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-296.	1 0 8
b Diagram showing numbering of bands detected in a).	1 0 8
5.15 a Southern blot of <i>Msp</i> I-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-313.	1 0 9
b Diagram showing numbering of bands detected in a).	1 0 9
5.16 a Southern blot of <i>Bgl</i> II-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-362.	1 1 0
b Diagram showing numbering of polymorphic bands detected in a).	1 1 0
5.17 a Southern blot of <i>Msp</i> I-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-62.	1 1 2
b Diagram showing numbering of bands detected in a)	1 1 2.
5.18 a Southern blot of <i>Bgl</i> II-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-216.	1 1 3
b Diagram showing numbering of polymorphic bands detected in a).	1 1 3
5.19 a Southern blot of <i>Bgl</i> II-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-304.	1 1 5
b Diagram showing numbering of bands detected in a).	1 1 5
c Original "test blot" showing hybridisation profile on Ca65 and 550 DNA digested with <i>Msp</i> I, <i>Bgl</i> II and <i>Hind</i> III.	1 1 5
5.20 a Southern blot of <i>Msp</i> I-digested DNA from Ca65, 550, and progeny numbers 260 - 285, hybridised with pIN5-317	1 1 6
b) Diagram showing numbering of bands detected in a).	1 1 6
5.21 a Southern blot of <i>Msp</i> I-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-655.	1 1 8
b Diagram showing numbering of bands detected in a).	1 1 8
6.1 Pulsed field gel electrophoresis of <i>Saccharomyces cerevisiae</i> , <i>Schizosaccharomyces pombe</i> and <i>P. infestans</i> . DNA using electrophoresis conditions for separating <i>S. pombe</i> chromosomes	1 3 1
6.2 Pulsed field gel electrophoresis of <i>Saccharomyces cerevisiae</i> , <i>Schizosaccharomyces pombe</i> and <i>P. infestans</i> . DNA using electrophoresis conditions for separating <i>S. pombe</i> chromosomes and using Chromosomal Grade Agarose (Biorad).	1 3 3
6.3 Pulsed field gel electrophoresis of <i>Saccharomyces cerevisiae</i> , <i>Schizosaccharomyces pombe</i> and <i>P. infestans</i> . DNA using electrophoresis conditions for separating <i>S. cerevisiae</i> chromosomes	1 3 4
6.4 a Pulsed field gel electrophoresis of <i>Saccharomyces cerevisiae</i> , <i>Schizosaccharomyces pombe</i> and <i>P. infestans</i> . DNA using	

electrophoresis conditions for separating 2 - 4 Mb DNA fragments	136
b Southern blot of a) hybridised with total radiolabelled <i>P. infestans</i> DNA.	136
c Southern blot of a) hybridised with radiolabelled rDNA from <i>Drosophila melanogaster</i> .	136
d Southern blot of a) hybridised with radiolabelled pSTA99,	136
6.5 Pulsed field gel electrophoresis of <i>Saccharomyces cerevisiae</i> , <i>Schizosaccharomyces pombe</i> and <i>P. infestans</i> .DNA using electrophoresis conditions for separating 10 Mb DNA fragments	138

LIST OF TABLES

	Page
3.1 Properties of the different global isolates of <i>P. infestans</i>	48
3.2 Restriction fragment sizes of mtDNA from <i>P. infestans</i>	51
3.3 mtDNA types in the different isolates of <i>P. infestans</i>	65
4.1 Grouping of isolates according to which fragments hybridise with pIN41	74
4.2 Grouping of isolates according to <i>MspI</i> fragments which hybridise with pIN2	77
4.3 Grouping of isolates according to <i>BglII</i> fragments which hybridise with pIN2	80
4.4 Grouping of isolates according to <i>MspI</i> fragments which hybridise with pIN29	81
4.5 Grouping of isolates according to <i>MspI</i> fragments which hybridise with pIN11	82
5.1 Segregation of avirulence and virulence in <i>P. infestans</i> hybrid F1 progeny of Ca65 x 550 to 9 potato differentials	87
5.2 Linkage analysis of RFLP and avirulence gene loci	120
5.3 Progeny with more than 2 alleles at RFLP loci	125
5.4 Mitochondrial inheritance in progeny with extra alleles	127

CHAPTER 1 : INTRODUCTION.

In the 1840s, a novel phytopathogenic fungus left an indelible mark on world history and on the science of plant pathology when it ravaged the potato crops of Ireland and Europe. The fungus subsequently isolated was initially labelled *Botrytis infestans* by Montagne (1845), and reclassified *Peronospora infestans* by Unger in 1847 and Caspary in 1852. Much later, in 1876, it was renamed *Phytophthora infestans* by de Bary, and became the type species of the new genus *Phytophthora*.

Phytophthora species belong within the Oomycete class of fungi. This order was traditionally distinguished from the higher fungi by oogamy and a predominantly aquatic mode of life. Recent molecular and biochemical evidence indicates that the Oomycetes may be more closely related to the chrysophytes (golden brown algae) than to the higher fungi. The Oomycetes have apparently evolved a mode of life similar to that of the higher fungi, but independently from them (Gunderson *et al.*, 1987).

The Oomycetes are divided into two orders, the Saprolegniales and the Peronosporales. The Saprolegniales are mainly saprophytes, including only *Aphanomyces* as a plant pathogen. Within the Peronosporales, a gradation of morphological specialisation and parasitism occurs. This ranges from the *Pythium* species which are relatively unspecialised soil fungi, to the downy mildews, these being obligate parasites of leaves and stems with a prolonged biotrophic phase and a high degree of host specialisation. The majority of *Phytophthora* species fit between these two extremes, being predominantly root pathogens but with a greater degree of host specialisation than *Pythium*. A few, including *P. infestans*, have evolved to attack leaves, and thus resemble the mildews in their biology, but are not obligate parasites and have only a short biotrophic phase.

Morphologically, the *Phytophthora* genus is distinguished from *Pythium* by the complete differentiation of zoospores within the sporangium prior to their expulsion. *Phytophthora* is distinguished from the true downy mildews by the indeterminate branching of its sporangiophores.

The different species belonging to *Phytophthora* are also distinguished from one another primarily by morphology. The main criterion for this differentiation is the papillate nature of the sporangium. Other characteristics used include the nature of the sporangiophores, antheridia, oogonia, oospores, chlamydospores, hyphal swellings, and the rate of growth at different temperatures (Newhook, Waterhouse & Stamps, 1978).

P. infestans is one of only two *Phytophthora* species producing spores that are detachable and airborne; the other being *P. phaseoli*. This is therefore the main criterion used in its identification. A number of other morphological features separate *P. infestans* and *P. phaseoli*.

Like other Oomycetes, *Phytophthora* species are diploid, although field isolates of *P. infestans* obtained outside Mexico are often of higher ploidy, and values as high as $n=8$ have been observed in some isolates (Therrien *et al.*, 1989, Tooley, Therrien & Ritch, 1989, Whitticker, Shattock & Shaw, 1990). Evidence from cytological staining and comparisons with chicken erythrocyte standards suggests that the nuclear DNA content of *P. infestans* is very high relative to other fungal plant pathogens and to other species of *Phytophthora*. Tooley & Therrien (1987) estimated the mean DNA content in Mexican (diploid) isolates at 0.52 pg DNA per nucleus, which is equivalent to approximately 4.7×10^8 base pairs per diploid genome. This is 10-20 times the DNA content of a number of species within the higher fungi studied to date (the exceptions being *Ustilago maydis*, with 0.19 pg per haploid nucleus, and *Entomophaga aulicae*, with extremely large haploid nuclei containing an estimated 8.2 pg DNA; Murrin *et al.*, 1986). It is also about 1.3 times that

reported for *Saprolegnia* sp. (Tanaka *et al.*, 1982), 7 times that of *Bremia* and *Achlya* (Tooley, unpubl.; Hudspeth, Timberlake & Goldberg, 1977), and 2.5 times that of *P. megasperma* f. sp. *glycinea* (Rutherford & Ward, 1985)

The majority^{of} *Phytophthora* species are phytopathogenic, and over 1000 different plant-*Phytophthora* pathosystems are known, many of which are important economically. Since its initial dramatic introduction, *P. infestans* has successively outwitted attempts to control it via breeding programs and chemical fungicides. Major blight epidemics are still commonplace throughout all of the potato growing regions of the world. Pathologists, breeders and growers now realise that a much better understanding of the host-pathogen interaction is needed, which may eventually be used to develop new control strategies and achieve a level of host resistance that is both durable and highly effective.

1.1 The *P.infestans*-*Solanum* pathosystem and the genetics of virulence

P. infestans is pathogenic on both potatoes and tomatoes, however the pathosystem with the potato host has been more extensively studied and will be the interaction discussed here.

It is generally accepted that the *P.infestans*-*Solanum* pathosystem originated in Central Mexico. Although this is not the native land of the potato plant, it appears to be the country of origin of *P. infestans*. This is supported by a far greater variability among *P. infestans* isolates from Central Mexico than occurs among isolates from the rest of the world (Tooley *et al*, 1985). Also, the A2 mating type apparently has long been present in Central Mexico but until recently was largely absent in other countries.

Sporangia are the primary agents of dispersal of *P. infestans*. These are shed into dry air, and allow dissemination over considerable distances. Records indicate that during the 1840s the disease spread at a rate of around 500 km per year, a speed typical of wind-dispersed fungi (Gregory, 1984). Upon germination, which requires free water, sporangia may produce a germ tube. More typically however, and always at temperatures below 16°C, zoospores are liberated, which swim on the plant surface, encyst, and later form germlings. These penetrate the epidermal cells directly, or more rarely enter through stomata. In a susceptible host, rapid growth of hyphae and the production of haustoria ensue. Haustoria invade individual host cells and are thought to be the agents of uptake of water and nutrients. The subsequent death of infected cells produces extensive lesions in 5 - 7 days following infection. Potato tubers may become infected by zoospores washed down by rain, or by direct contact with infected foliage at harvest. Infection and colonisation of the tuber occurs by essentially the same process as in leaf tissue. The blighted

tubers have a reddish-brown granular rot beneath discoloured skin, and invariably become secondarily infected by soft-rotting bacteria, especially during storage. Crop loss may thus occur by direct damage to the potato tuber, or by damage to the plant through foliar attack. Overwintering of the fungus in infected tubers makes them an important source of infection in subsequent seasons.

Following infection of a resistant host, fungal growth is much more severely restricted. Rapid host cell necrosis, or hypersensitivity, occurs, although this may not necessarily be the cause of fungal death *per se*. Phytoalexin production, the encasement of haustoria, and modifications to cell wall chemistry have also been implicated in resistance (Coffey & Wilson, 1984)

The resistance of *Solanum* to *P. infestans* may be determined generally ("horizontal" resistance) or specifically ("vertical" resistance). General resistance is effective against all isolates of the fungus and appears to involve the interplay of a number of host factors, which could range from physical barriers to chemical inhibitors. As such, it is more durable and more stable than specific resistance, but is difficult to analyse genetically.

The specific resistant reaction in the *Solanum* host, characterised by a marked resistance to some but not other isolates (races) of the pathogen, is determined by a series of independently acting resistance genes. These appear to complement a series of corresponding single genes for avirulence in the pathogen. This conforms to the classic gene-for-gene interaction first described by Flor (1954) in the interaction between flax and its rust pathogen. The *P. infestans* - *Solanum* pathosystem is generally described using the standardised nomenclature of Black *et al.*, (1953). A specificity in the fungus is assigned a number that corresponds to the R-gene whose hypersensitive reaction it is capable of overcoming. Thus race 1,2 attacks potatoes that are R1 or R2 or R1R2 but cannot attack those that carry R3 or

R4. Black's differential series contained 11 such R genes, designated R1 - R11.

As they are independently acting and determined by single loci, avirulence genes make ideal candidates for genetic analysis. The genetics of specific virulence in *P. infestans* has therefore been studied extensively. Initial work examining the inheritance of virulence to genes R1 - R4 produced evidence of recombination and segregation of virulence phenotypes, but was unable to define a diploid pattern of inheritance of single genes (Romero & Erwin, 1969; Laviola and Gallegly, 1983). Improved methods of performing crosses and establishing progeny has more recently allowed analysis of large F1 progenies and some F2 and backcross progenies. Al-Kherb (1988) investigated virulence against potato genes R1-R4 in five crosses involving a total of 694 progeny. His results indicated single locus control with avirulence dominant in each case. Spielman *et al.*, (1990), however, observed segregation ratios of virulence to avirulence on R2 and R4 which suggested that virulence was dominant against both these host resistance genes. Avirulence appeared dominant against R3, but progeny segregated differently when different parents were used, indicating a second locus may be involved. Segregation for virulence against R1 did not indicate which phenotype was dominant.

The contradictions between the results of Al-Kherb and Spielman *et al.*, may indicate that more than one locus is involved in the control of virulence against R2 and R4. The two groups used different parental isolates, which may have been segregating for different loci conditioning the same disease interaction.

1.2 The development of markers.

1.2.1. Conventional markers

a. Application to studies in population biology.

Interest in the population biology of *P. infestans* has been heightened in recent years by the discovery of the A2 mating type throughout Europe (Hohl & Iselin, 1984, Shaw *et al.*, 1985) and more recently in Japan (Mosa *et al.*, 1989) and the USA (Deahl *et al.*, 1990). Previously it was believed that the A2 was confined to Mexico, where it occurs in equal numbers with the A1. Only A1s appeared to exist elsewhere, although whether the A2 was present at a low level but simply not detected is not known. It would be interesting to know how and when the A2 arrived, and also whether there has been a concomitant increase in the level of sexual recombination occurring in the field. This is of obvious importance to the pathologist, as sexual recombination could cause an increase in the variation and adaptive potential of the fungus, and the thick-walled sexual oospore may allow greater persistence in the soil.

The morphological simplicity of *P. infestans* has made analysis of variation and relatedness within populations difficult. Virulence phenotypes (Tooley, Sweigard and Fry, 1986) and ploidy levels (Tooley & Therrien, 1987; Whitticker, Shattock & Shaw, 1990) have been used to investigate naturally occurring populations, but such analyses can be very time consuming and results are not always clear-cut. Possession of virulence specificities can also be subject to selection pressure so that otherwise totally different isolates may possess similar or identical virulence profiles because of the host resistance phenotype predominant in the field.

It would be desirable in population studies to have a means of "typing" isolates, using characteristics that are stable, easy to detect and not subject to selection pressure. Movement of populations in the field setting, their relatedness with one another, and the extent of recombination between members of different populations, could then be easily and reliably assessed.

b. Application to genetic studies.

Ironically, geneticists have benefitted greatly by the appearance of the A2 outside Mexico. This allowed quarantine restrictions, prohibiting the import of the Mexican A2 to most countries, to be lifted. Subsequent improvements in methods for performing crosses and regenerating oospores has allowed work to progress from defining mechanisms of generating variation in asexual populations, to the establishment of progenies and analysis of inheritance following sexual recombination.

Geneticists have the advantage that they can perform genetic crosses and analyse inheritance using phenotypic variants that would be far too rare for the population biologist to use in field studies. A number of morphological and growth characteristics that occur naturally but at a low frequency have been studied genetically (reviewed by Shaw, 1990). Mutations can also be induced artificially using agents like nitrosoguanidine. Induced drug resistance mutations, which tend to be inherited dominantly, have been used as markers for examining the occurrence of heterokaryosis and parasexuality in asexual crosses (Poedinok & Dyakov, 1981, Shattock & Shaw, 1976), and hybridity following sexual crosses (Ko, 1988). Virulence phenotypes have likewise been used as markers to study vegetative hybridity (Malcolmson, 1970).

A major complication in genetic studies of *P. infestans* is the diploid nature of the organism. This makes the inheritance of recessive markers, for

example auxotrophic markers, difficult to assess, limiting their use in genetic mapping work. Also, *P. infestans* is naturally outbreeding, and attempts to backcross or mate siblings often generate unexpected results (Shattock, 1988, Spielman *et al.*, 1990), probably due to the presence of deleterious recessive alleles (Spielman *et al.*, 1990)

1.2.2. Molecular markers.

The techniques of molecular biology allow the detection of very minor changes in the genetic make-up of an organism. Such changes do not generally affect morphology or biochemical metabolism, but may result in alterations to the macromolecules of the organism.

The macromolecules assayed are generally either proteins or nucleic acids. Enzyme variants, known as isozymes, have been detected in a number of phytopathogenic fungi including *Phytophthora*, and have been used in studies on population dynamics and the nature of inheritance. Changes in DNA sequence organisation are generally detected as restriction fragment length polymorphisms (RFLPs). Although slower to gain use, the potential benefits of RFLPs are now beginning to be realised, and they are being increasingly employed in work on phytopathogenic fungi.

Both classes of molecular markers possess considerable advantages over conventional markers for population and genetic studies. They are far more numerous than conventional markers, and as they are not generally associated with any deleterious effects many markers can be considered simultaneously. They can therefore be used to generate genetic maps on which conventional markers can be placed. These maps may then be used to locate and isolate genes of interest. Their numerous and polymorphic nature can also allow isolates to

be "typed". This makes it possible to follow the movement of isolates in population studies, and assess the relatedness of isolates with one another.

Molecular markers are expressed codominantly, allowing the identification of all possible alleles at the locus under study. Homozygotes can therefore be distinguished from heterozygotes, and all possible genotypes resulting from a genetic cross can be identified without the need for sibcrossing or backcrossing. Hybrids can be readily distinguished from parentals or selfs, and aberrant segregation ratios can be screened for. Heterozygously determined markers also allow conclusive proof of diploidy.

Finally, molecular markers are relatively easy to obtain. They are usually not tissue-specific and can be extracted from small amounts of starting material. Molecular techniques are being increasingly refined and new techniques developed to increase further our ability to detect molecular changes.

a. Isozymes

Isozymes are defined as variant forms of an enzyme which share the same catalytic function. Alteration to the size or charge of the enzyme allows the variants to be distinguished by electrophoresis through starch or polyacrylamide gels.

Isozymes can arise from alleles of a single locus (allozymes) or may arise from a multigene family, in which case a more complex electrophoretic pattern may result.

Monomeric or dimeric allozymes are most commonly used in population and genetic studies. A monomeric enzyme produces a single band following electrophoresis if encoded homozygously, and two bands if heterozygous. Dimeric enzymes consist of two joined gene products or subunits. These

combine randomly, so that homozygotes are single - banded but three bands occur in the heterozygote; two representing homodimers and a third band running equidistant between these which is heterodimeric. Isozyme nomenclature assigns the allele producing the most common electrophoretic variant a value of 100. Other alleles are then assigned greater or lesser values based on their relative migration.

The first investigation into uncovering isozymes in *P. infestans* and using them in population analysis was done by Tooley *et al.*, in 1985. 38 different enzyme systems were assayed, of which 17 produced activity, and 24 loci were resolved. These were used to compare asexual populations from North America and Europe with populations from Mexico, where both sexes occur. The majority of loci, however, were either not polymorphic between the two populations, or gave banding phenotypes that were too poorly resolved to allow valid comparisons. Only four loci were therefore used : Gpi-1 (glucosephosphate isomerase) and Pep (peptidase), which were polymorphic in both the sexual and asexual populations, and Me (malic enzyme) and Xdh (xanthine dehydrogenase) which were polymorphic only in the sexual populations. The observation that greater variation at all enzyme loci existed in the sexual population, and that the distribution of alleles was consistent with the Hardy - Weinburg equilibrium, allowed the authors to hypothesise that random mating was indeed occurring between the two mating types present in Mexico. The electrophoretic phenotypes produced by Gpi-1 and Pep, which both encode dimeric enzymes, gave further support to the belief that the vegetative phase of *P. infestans* is diploid.

Since this time, isozymes have proved an invaluable tool in studies of inheritance and the analysis of *P. infestans* populations. Shattock *et al.* (1986a) used polymorphism at Gpi-1 and Pep to investigate the nature of selfing following intraspecific stimulation. All *P. infestans* isolates are

bisexual, producing antheridia and oogonia, and can be induced to self by the presence of a compatible mating type. Separation of the two mating types by a polycarbonate membrane prevents sexual recombination and only selfed oospores result. Shattock *et al.* (1986a) examined segregation at the Pep locus in single oospore cultures produced by induced intraspecific selfing. Two of the progeny were homozygous, whereas the remaining seven were heterozygous (92/100) like the parent. From this it appears that selfs are the products of true automixis following reduction division and fertilization.

Isozymes have also been used to distinguish selfs from hybrids (Shattock *et al.*, 1986b), where their codominant nature makes interpretation straightforward and unambiguous. This is important in genetic analysis, as the proportion of phenotypes in a progeny will depend on the relative frequencies of selfing and true sexual recombination. By choosing parents with different but homozygous isozyme genotypes, progeny can be rapidly screened for hybrids and selfs. The authors found selfing to occur in most sexual crosses, but at a low frequency.

In a similar way, isozymes can also be used to examine progeny in situations where unexpected segregation ratios are believed to have occurred. Spielman *et al.* (1990) followed the inheritance of Gpi-1 and Pep in backcross and sibcross progeny, and found aberrant segregation in a number of these crosses. Obviously such information is vital if one wishes to use progeny generated by back- and sibcrossing in genetic analyses.

Isozymes are being increasingly employed in population studies of *P. infestans*, and are helping to piece together our knowledge of the history of *P. infestans* populations and the changes that are still occurring to these populations.

Tooley *et al.* (1989) used isozymes along with mating type, race structure, and ploidy to examine populations from Europe, the United States, Peru and Mexico. They found the phenotypic distribution of isozyme loci in Peruvian

isolates to be very similar to those seen in Europe and the U.S., suggesting late blight was introduced into Europe and the U.S. from Peru. Their data also supported the hypothesis that Mexico, and not the Andes, was the centre of origin of the *P. infestans* - *Solanum* pathosystem, as Peruvian isolates showed less diversity in terms of isozymes and virulence phenotypes than the Mexican isolates. Also, the A2 mating type was absent from Peruvian populations.

A four year survey of isolates from England and Wales likewise showed very limited variation in isozyme profiles, supporting the concept of restricted gene flow in populations outside Mexico (Shattock *et al.*, 1990).

Spielman (1990), used the data from Tooley *et al.* (1985) and Shattock *et al.* (1990), and analysed the isozymes in a large number of additional *P. infestans* isolates from Europe, North America, South America, and Asia. A comparison of the Gpi-1 and Pep profiles over time indicated that the *P. infestans* populations in some of these countries were undergoing changes. It appeared that the original populations were being invaded, and in some cases almost completely replaced by new populations, revealed by a shift in predominant isozyme profiles expressed by the two populations. This led Spielman to hypothesise that a migration event had occurred in these countries, introducing both new isozyme genotypes and the A2 mating type.

The information gained by the use of isozymes has thus been considerable. Their main limitation in *P. infestans* work is the lack of easily resolved, polymorphic isozyme systems, and, at Gpi-1 and Pep, a rather limited level of polymorphism in the majority of *P. infestans* populations. More numerous alleles are essential for genetic mapping work, and would help define more accurately the relationships between *P. infestans* populations. It is toward these goals that DNA restriction fragment length polymorphisms are now being employed.

b. Restriction fragment length polymorphisms.

Variation at the protein level has its origin in changes to the DNA of the organism. Base substitution, rearrangement, deletion or insertion may occur in the specifying gene, altering its protein product. Molecular techniques now allow these changes to be detected and analysed at the DNA level.

Not all changes to nucleic acids will result in altered proteins. The redundancy of the genetic code allows some DNA modifications to be "silent", so that base substitution does not affect the amino acid sequence. Alterations may also occur in non-transcribed regions, such as introns, regulatory sequences, flanking sequences, pseudogenes, and some types of satellite and repetitive sequences. All such changes would go unnoticed in protein analysis, but are equally detectable in nucleic acid analysis as those producing phenotypic modifications. Furthermore, since unexpressed DNA may be more free from functional constraints, it may accumulate alterations to a greater extent that is possible within functional genes.

The easiest way to detect DNA polymorphism is by comparing the digestion profiles produced when the DNA under study is cut to completion using type II restriction endonucleases. These enzymes recognise certain nucleotide sequences, known as restriction sites, and will cleave the DNA wherever such sites occur. A consistent number of double-stranded DNA fragments will therefore result, each flanked by a severed restriction site. Electrophoresis through an agarose gel separates these restriction fragments according to size, producing a profile that is particular for the nucleic acid sequence and the restriction enzyme being used.

Alteration to a restriction site will prevent recognition and cleavage from occurring, producing a single fragment from the two fragments that originally flanked the altered site. Conversely, new sites may be generated, causing an

increase in the number of restriction fragments. Deletions and insertions will likewise alter restriction fragment sizes. All such changes are collectively denoted restriction fragment length polymorphisms, or RFLPs.

RFLPs were first recognised and used in the genetic analysis of adenovirus mutants. Temperature sensitive mutations were found to be linked to specific restriction fragment length differences. These differences could then be used to locate the mutations on a restriction map of the virus (Grodzicker *et al.*, 1974). RFLPs were later used to show the maternal inheritance of mammalian mitochondrial DNA (Hutchinson *et al.*, 1974), and the Mendelian inheritance of ribosomal DNA in yeast (Petes & Botstein, 1977). In 1980, Botstein *et al* proposed that RFLPs could be used to create a genetic linkage map of human DNA. RFLPs have since found increasing application in studies encompassing a diverse range of organisms.

RFLPs in mitochondrial DNA.

The examination of DNA polymorphisms in mitochondrial genomes has become increasingly popular in population and evolutionary biology studies. Although it is not generally high enough to distinguish every individual, mtDNA variability can be used to differentiate between populations within a species, and to distinguish sub-species, species, and higher taxa.

Mitochondrial genomes from different organisms vary considerably in size, but generally they are small enough to allow polymorphisms to be viewed by restriction enzyme cleavage and gel electrophoresis alone. MtDNA studies therefore do not need to employ cloned, radiolabelled fragments, which are necessary to distinguish polymorphisms in nuclear DNA. Their small size also means mtDNAs can be viewed in their entirety, and polymorphisms can be mapped to distinct sites on the genome.

The majority of species appear to inherit their mitochondrial genomes

uniparentally (Mitchell & Mitchell, 1952; Manella, Pittenger & Lambowitz, 1979), that is, without recombination (although some exceptions do occur (Taylor 1986)). Movement of a particular mitochondrial genome through a population can therefore be traced in subsequent generations of that population. This allows genealogies of individuals to be constructed in a mode analogous to using male surnames in genealogy studies of human populations.

Determining whether mitochondrial genome variants are transmitted between members of a species also allows an assessment of the level of sexual recombination occurring in the field. The restriction of mtDNA types within certain populations can be used to estimate the number of mating populations that exist within that species (Garber & Yoder, 1984).

MtDNA can also be used in genetic studies as a cytoplasmic marker. The coinheritance of phenotypic characteristics with mtDNA variants provides evidence that the characteristics are encoded by cytoplasmically determined genes.

MtDNA can be isolated in a relatively straightforward procedure using a bisbenzimidazole-CsCl density gradient. Bisbenzimidazole is a fluorescent dye which binds preferentially to AT-rich DNA sequences. The fluorescent dye DAPI, which appears to bind DNA in a similar manner, may also be used but it is considerably more expensive than bisbenzimidazole (Garber & Yoder, 1983). As mitochondrial genomes generally contain a higher proportion of AT bases than nuclear DNA, they bind relatively more bisbenzimidazole (or DAPI). This decreases their buoyant density, causing them to band above the nuclear DNA on a CsCl density gradient, from which they can be removed in a reasonably pure form.

MtDNA RFLPs have been employed in population and evolutionary studies of a wide range of organisms (Avice *et al.*, 1979; Sederoff *et al.*, 1981; Latorre, Moya & Ayala, 1986; Cann, Stoneking & Wilson, 1987; Ball *et al.*, 1988).

In the fungi, mtDNA polymorphisms have mostly been used to assess taxonomic relationships. Fungal taxonomy has traditionally relied on morphological features, which can be difficult to interpret and are often misleading. MtDNA RFLPs have been used to assess relationships between members of different genera, (Hoeben & Clark-Walker, 1986) members of different species, (Weber *et al.*, 1986; Anderson, Petsche & Smith, 1987; Croft, 1987; Forster *et al.*, 1987; Smith & Anderson, 1989), and between isolates belonging to a single species (Hulbert & Mitchelmore, 1988; Forster *et al.*, 1989; Varma & Kwon-Chung, 1989; Bates, Buck & Brasier, 1990). Studies in fungal population biology have also used mtDNA polymorphisms to determine relationships between global populations (Garber & Yoder, 1985), and to assess the possible origins of newly arising phenotypes (Bates, Buck & Brasier, 1990).

A number of studies on the mtDNA of Oomycetes have been conducted (Boyd *et al.*, 1983; Hudspeth *et al.*, 1987; Forster *et al.*, 1987; McNabb & Klassen, 1988; Forster *et al.*, 1989). These studies indicate that Oomycete mtDNA groups into at least two distinct organisational patterns : 1) The *Achlya* pattern. This is characterised by the presence of large inverted repeats, allowing the mitochondrial genome to exist as two orientational isomers. Members of the genera *Achlya*, *Leptolegnia*, *Pythium*, and *Sapromyces* have mitochondria with this inverted repeat (Hudspeth *et al.*, 1987; Boyd *et al.*, 1983) 2) The *Phytophthora* pattern, which lacks the inverted repeat and thus is more typical of mitochondrial genomes. Within the Oomycetes, this pattern is confined to species belonging to *Phytophthora* and *Apodachyla* (Hudspeth, 1985). As it is generally conserved, the inverted repeat is believed to be an ancestral character which has been lost from the *Phytophthora* and *Apodachyla* mitochondrial genomes (McNabb & Klassen, 1988).

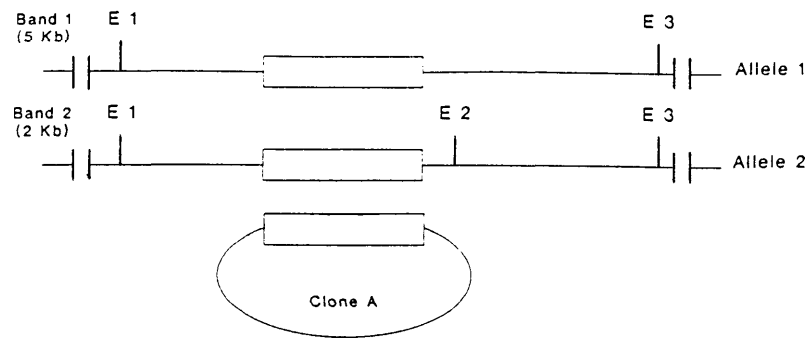
MtDNA has previously been isolated from *P. infestans*, and was characterised as a circular molecule of 36.2kb with a GC content of 22.4% (Klinczac & Prell, 1984). Restriction fragment profiles were compared between five different pathotypes using eleven restriction enzymes, but no RFLPs were detected between these isolates.

RFLPs in nuclear genomes.

The nuclear genomes of higher organisms are too large to detect RFLPs by gel electrophoresis and ethidium bromide staining alone. Complete digestion of nDNA produces a smear of fragments on an agarose gel, and polymorphic sequences are detected by hybridisation with homologous radiolabelled fragments. The procedure for determining nuclear RFLPs is illustrated in Figure 1.

RFLPs in nuclear DNA were initially detected by hybridisation with clones containing functional genes (Jeffreys, 1979; Ullrich *et al.*, 1980). Conservation of many genes between species means the same clone can be used as a heterologous probe for a diverse range of organisms. Cloned cDNA sequences of unknown function have likewise been used. RFLP studies now however most commonly employ random genomic clones. The insert sequences of these clones are "anonymous", in that they do not represent any known gene function. They may contain coding or non-coding parts of a gene, or be unassociated with any gene. As they are less constrained by functional requirements, non-coding and non-gene sequences are likely to be more polymorphic than the coding sequences present in functional genes and in cDNA clones (Li, Luo & Wu, 1985). Genomic clones may also contain sequences that are repeated throughout the genome, which can be useful for "typing" or "fingerprinting" individuals. The extreme example of hypervariable repetitive DNA is the minisatellite DNA used by Jeffreys in the construction of

1) Possible Alleles at Hybridising Locus

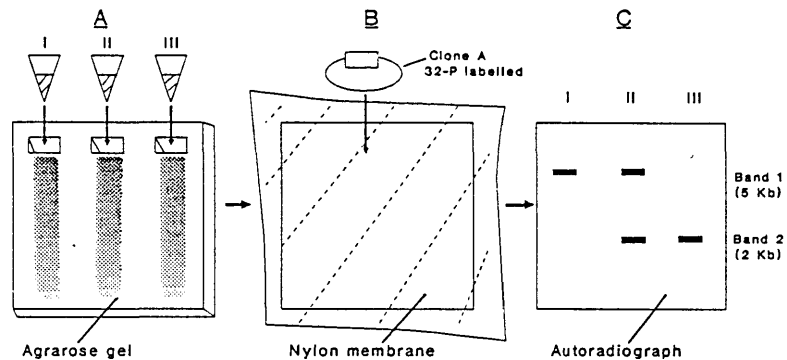


E1, E2 & E3 - sites for restriction enzyme 'E'

E2 is polymorphic

□ - hybridising regions

2) Use of Radiolabelled Clone A to Detect RFLPs



A: DNA from three diploid individuals digested with enzyme 'E' is size-fractionated by gel electrophoresis

B: DNA is transferred by capillary action to a nylon membrane
Blot is hybridised with a radiolabelled clone A

C: Autoradiography reveals restriction fragments hybridising with clone A

3) Interpretation

INDIVIDUAL	PHENOTYPE	GENOTYPE						
		E1	E2	E3	/	E1	E2	E3
I	Band 1	+	-	+	/	+	-	+
II	Band 1 & Band 2	+	-	+	/	+	±	±
III	Band 2	+	±	±	/	+	±	±

• : Presence of site

- : Absence of site

± : Site is outside hybridising region therefore presence or absence cannot be determined.

Figure 1.1 : Detection and interpretation of RFLPs in nuclear DNA.

his "fingerprinting" probes. The variation detected by such probes is so great that they will theoretically distinguish between any two individuals (Jeffreys, 1985). The validity of the resulting fingerprint is such that it is now being used in court in paternity cases and as forensic evidence (albeit with some problems, Lander, 1989). The minisatellite probes are also capable of detecting polymorphic regions in birds, dogs, cats, mice and plants (Burke & Bruford, 1987; Jeffreys *et al.*, 1987; Dallas, 1988; Rogstad *et al.*, 1988). Recently they have been used successfully to distinguish between two subgroups of the filamentous plant pathogen, *Colletotrichum gloeosporioides* (Braithwaite & Manners, 1989)

A number of studies employing mtDNA polymorphisms to analyse fungal populations have used RFLPs in nuclear DNA to back-up and extend their work (Forster, Oudemans & Coffey, 1990; Hulbert and Mitchelmore 1988; Bates, Buck & Brasier, 1990). Sequence variation is often considerably more pronounced among nuclear DNAs than in the corresponding mtDNAs, due probably to the cloning of non-essential nDNA sequences. A comparison of dendograms based on nuclear and mitochondrial RFLPs in *Phytophthora capsici* revealed an almost identical branching pattern (Forster & Coffey, 1990), indicating that the different analyses produce comparable results.

nDNA polymorphisms have also been used in population and epidemiological studies of obligate biotrophs, where low yields of fungal material make mitochondrial analysis impractical. O'Dell *et al.*, (1989) used repetitive nDNA sequences in population studies of *Erysiphe graminis* on different host plants. Repetitive RFLPs are likewise being exploited to investigate isolates of barley rust (J. Smith, pers. comm.).

Genetic studies require polymorphisms that can be assigned to distinct, single loci. Clones containing insert sequences that are present in a single copy per haploid genome are therefore desirable, although repetitive sequences can be

used if the polymorphisms they detect can be established as being allelic (Landry *et al.*, 1987)

Clones containing single-copy sequences can be screened for in a library of random genomic clones by hybridising the library with total radiolabelled nDNA. A very weak hybridising signal indicates the clone contains a DNA sequence that is repeated only once or very few times throughout the genome (Landry & Micheltore, 1985). Generally, clones with small inserts (< 1000 bp) are desirable as they are less likely to contain interspersed repetitive sequences.

Single copy sequences can also be obtained by producing cDNA clones. As their inserts represent expressed genes, hybridisation with these clones should detect only one or a few copies per genome. Libraries of cDNA clones may, however, be of lower complexity than genomic libraries, with certain insert sequences predominant due to an abundance of their mRNAs (Micheltore, 1988). cDNA clones may also produce weak signals when hybridised to genomic DNA due to the presence of introns (Helentjaris, 1987).

Once obtained, single-locus RFLPs can be used to follow inheritance in progeny and assess linkage relationships in the same way as any other genetic marker. As RFLPs are codominant however, all possible genotypes arising from a cross can be identified. This provides considerably more information per locus, and eliminates the need for back- and sib-crossing.

RFLP mapping has become extremely important in human genetics, where linkage with disease phenotypes is used to locate and isolate genes associated with genetic disorders (Cantor, 1984, Riordan *et al.*, 1989). RFLP maps are likewise being constructed for a number of crop plants, with the intention of using them for genetic manipulation and strain improvement (Helentjaris *et al.*, 1985; Evola, Burr & Burr, 1986; Landry *et al.*, 1987).

New techniques are being continually developed which augment mapping

work; most importantly pulsed field gel electrophoresis (PFGE), (Schwartz & Cantor, 1984) and the polymerase chain reaction (PCR) (Saiki *et al.*, 1985; Mullis *et al.*, 1986). PFGE allows the separation of very large DNA fragments in agarose gels by subjecting the DNA to alternating pulses of electric field from diametrically opposed directions. In the lower eucaryotes, whole chromosomes may be separated, and an electrophoretic karyotype of the organism can be generated. Blotting and hybridisation of the pulsed field gel with the RFLP-detecting sequences can then allow the polymorphic loci to be directly placed on the large resolved DNA sequences.

PCR can provide selected amplification of repeated regions in the genome known to be polymorphic, for example the (CA)_n repeats found in the human genome (Weber & May, 1989). It also allows analysis to be conducted on very small amounts of starting material, reducing the time and labour necessary to prepare DNA samples.

The lack of suitable conventional markers makes RFLPs particularly valuable in the genetic mapping of phytopathogenic fungi. Saturated genetic maps can then allow phenotypic characteristics of interest, such as virulence and mating type, to be located and eventually, isolated and analysed.

Hulbert *et al.*, (1988) developed a preliminary linkage map for *Bremia lactucae* by analysing cosegregation of 52 RFLP loci, 8 virulence genes and the mating type locus. Thirteen small linkage groups were established, which included 47 of the markers analysed. One of the RFLP loci was established as being linked to the virulence locus, A6, at an estimated distance of 6.5 cM, although how this correlates with physical distance in *B. lactucae* is not known. Hulbert *et al.*, (1988) utilised both cDNA and random genomic clones, which had advantages and disadvantages in terms of their efficiency in identifying RFLPs. Genomic clones often carried repetitive sequences, although the probability of this was reduced by using clones with small inserts. cDNA clones were less likely to detect repetitive sequences, however

the complexity of the cDNA library was much lower than the genomic library, and some clones carried identical sequences.

Raeder, Thompson and Broda (1989) constructed an almost saturated RFLP map of *Phanerochaete chrysosporium*, with 90% of the markers showing linkage. The small size of the *P. chrysosporium* genome, and the ability to utilise haploid material derived from single basidiospore-grown mycelia, meant only 38 RFLP markers were necessary to achieve this level of linkage. The map has been used to locate a cellulase gene and a mating type locus, and to establish that a family of lignin peroxidase genes occurs arranged in two unlinked clusters.

The well-defined genetics of virulence in *P. infestans* makes it an ideal candidate for the development of a genetic map based on RFLPs, with the eventual goal of locating and isolating the avirulence genes. As crosses can be performed easily and efficiently, and large amounts of mycelia can be produced on artificial media, *P. infestans* could provide a model system for studying the interaction between the avirulence genes of a fungal pathogen and the R genes of its host plant.

1.3 Aims of the current study.

The work outlined in this study was carried out through the following approaches :

1. To investigate the occurrence of RFLPs in the mitochondrial and nuclear genomes of *P. infestans*, using isolates from diverse geographic locations.
2. To use these mt and nDNA RFLPs to analyse the relatedness of the different geographic isolates, to investigate the spread of *P. infestans* globally, and to assess the extent of sexual recombination between isolates occurring in the field.
3. To analyse the nature of the genetic material in *P. infestans*; eg. relative amounts of single copy and repetitive nDNA, levels of heterozygosity in field isolates, ploidy levels, and the occurrence of heterokaryosis.
4. To establish a preliminary linkage map for *P. infestans*. based on RFLP and avirulence gene loci, using progeny from a sexual cross between isolates Ca65 (virulent on r0, R1, R4 and R11; Gpi-1 : 100-100) and 550 (virulent on r0, R1, R2, R3, R4, R5, R7, R8, R10 and R11; Gpi-1 : 86-86).
5. To initiate the separation of whole chromosomes from *P. infestans* by pulsed field gel electrophoresis, and, if successful, to use blots of these gels to physically locate RFLPs on *P. infestans* chromosomes.

CHAPTER 2 : MATERIALS AND METHODS.

2.1 Abbreviations.

bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary DNA
CHEF	contour-clamped homogenous electric field
CTAB	cetyltrimethylammonium bromide
EDTA	ethylenediaminetetraacetic acid disodium salt
ESP	EDTA, sarcosine and Proteinase K
g	gram(s)
h	hour(s)
kb	kilobase pairs
LMT	low melting agarose
MES	2-(<i>N</i> -Morpholino)ethane sulphonic acid
Mb	Megabase pairs
min	minutes
ng	nanograms
PAS	p – aminosalicylic acid
psi	pounds per square inch
PFGE	pulsed field gel electrophoresis
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	seconds
TNS	triisnaphthalene sulphonic acid sodium salt
Tris	tris(hydroxymethylaminomethane)
U	units
µg	micrograms
UV	ultraviolet

2.2 Names and addresses of commercial companies.

Amersham international :	Lincoln Place, Green End, Aylesbury, Bucks. HP20 2TP.
BDH :	Freshwater Rd, Dagenham, Essex RM8 1RZ.
Beckman :	Progress Rd., Sands Industrial Estate, High Wycombe, Bucks HP12 4SL.
BioRad Labs Ltd. :	Greenhill Crescent, Holywell Industrial Estate, Whatford, Hertfordshire WD1 8RD
Cambio :	34 Millington Rd, Cambridge, CB3 9HP.
Cambridge Bioscience :	42 Devonshire Rd, Cambridge, CB1 2BL.
Difco Laboratories :	PO Box 14B, Central Ave., East Mosely, Surrey KT8 0SE.
Gallenkamp :	Belton Rd. West, Loughborough, Leisestershire LE11 0TR
Genetic Research Instrumentation :	Gene House, Dunmow Rd., Felsted Nr., Dunmow, Essex CM6 3LD.
FMC BioProducts :	5 Maple St, Rockland, ME, USA.
Fugi :	α/- Genetic Research Instrumentation.
Ilford Ltd. :	Christopher Martin Rd., Basildon, Essex SS14 3ET.
Kodak Ltd. :	Kodak - pathe, 71102 Chalon-sur-Saone, France
Northumbria Biologicals Ltd. :	South Nelson Industrial Estate, Cramlington, NE23 9HL.
Novo Biolabs Ltd. :	DK 2880, Bagsvaerd, Denmark.

Pharmacia :	Pharmacia House, Midsummer Blvd., Milton Keynes MK9 3HP.
Schleicher & Schuell :	Anderman and Company Ltd., Surrey, KT2 6NH
Sigma :	Fancy Rd., Poole, Dorset, BH7 7NH.
Stratagene :	Cambridge Innovation Park, Milton Rd, Cambridge CB4 4GF
Whatman Labsales Ltd. :	Colred Rd., Parkwood, Maidstone, Kent ME15 9XN.
X-OGRAPH	c/- Genetic Research Instrumentation.

2.3 Safety considerations & miscellaneous general methods.

A laboratory coat was worn at all times and disposable gloves were worn when handling dangerous chemicals, radioactive isotopes, and when carrying out recombinant DNA work. Safety glasses were worn when using phenol and liquid nitrogen, and when working with radioactive isotopes. All radioactive isotopes were used behind a perspex screen, and a Geiger counter was used to check for contamination. All radioactive wastes were disposed of in accordance with Imperial College Safety Regulations.

An Envair Class II microbiological safety cabinet was used for all work involving recombinant bacteria and *P. infestans* isolates, and all waste media and materials were autoclaved prior to disposal.

The work with *P. infestans* was registered with the Ministry of Agriculture (Licence No. PHF 1250/103(99)) , and recombinant DNA work was registered with the Advisory Committee on Genetic Manipulation (ACGM). The required medical examination was taken for work involving recombinant DNA.

Most laboratory reagents were supplied by Sigma or BDH and were of analytical grade.

All plasticware, glassware and the majority of solutions were sterilized by autoclaving at 15 psi, 121°C for 20 min. Solutions which could not be autoclaved were filter sterilized using Millipore 0.2 µm filters.

Analytical grade phenol was purchased from BDH, and was equilibrated with 1 M Tris-Cl, pH 8.0 followed by 0.1 M Tris-Cl, pH8.0 until the pH of the aqueous phase was greater than or equal to 7.5. Equilibrated phenol was stored in aliquots wrapped in foil at -20°C, and prior to use was stored at 4°C.

2.4 *P. infestans* isolates.

Isolates were from the University of North Wales culture collection. Their origins, mating type, ploidy, alleles of glucose phosphate isomerase and peptidase loci and virulence phenotypes are given in Table 3.1.

2.5 Media.

Rye agar (Difco) was prepared as described by Caten & Jinks (1968). Antibiotics were incorporated where necessary at the following concentrations: Rifampicin 25µg/ml, Vancomycin 50 µg/ml, Nystatin 50 µg/ml.

Pea water broth was made by autoclaving 300g of frozen green peas in 1 litre of water (121°C, 15 p.s.i., 20 min). The supernatant was collected by decantation into sterile Petri dishes.

2.6 Culture conditions.

P. infestans isolates, held in liquid nitrogen storage or under mineral oil on rye A agar, were transferred to rye A agar slopes and cultured at 18°C. For DNA

extractions isolates were further grown in static liquid culture on pea water broth in Petri dishes (25 ml of broth per dish) for 10 to 14 days at 18° C. Cultures were harvested by filtration through muslin, washed with distilled water and frozen under liquid nitrogen. Frozen mycelium was stored at -70° C. For large scale DNA extractions, the mycelia was lyophilised. 40 Petri dish cultures yielded about 10g of lyophilised mycelium.

2.7 DNA isolation.

2.7.1 Large scale extractions for mtDNA purification.

Total DNA was isolated by a modification of the procedure of Forster *et al* (1987). Freeze dried fungal material (5 to 10 g) was ground under liquid nitrogen and extracted for 1 h at 65° C in 10 ml/g pre-heated extraction buffer (0.7M NaCl, 1% cetyltrimethylammonium bromide (CTAB), 50 mM Tris-Cl pH 8.0, 250 mM EDTA pH 8.0, 1% 2-mercaptoethanol). The suspension was extracted three times with 1 vol. chloroform : isoamyl alcohol (24:1 by vol.) and the phases were separated by centrifugation at 13 000*g* for 20 min at 20° C. 1 vol. solution B (1% CTAB, 50 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0) was added to the supernatant, mixed by inversion, and incubated at room temperature for at least 30 min to precipitate the DNA. The DNA was pelleted by centrifugation at 2 000 *g* for 6 min and then washed with 15 ml per tube of a 1 : 1 mixture by vol. of extraction buffer and buffer B. Mt DNA was separated from nuclear DNA by centrifugation in CsCl gradients containing bis-benzimide (Garber & Yoder, 1983). The DNA pellet was resuspended in 16 ml of TE buffer (10mM Tris Cl pH 8.0, 1mM EDTA pH 8.0) containing 14.9 g CsCl (final density 1.6 g/ml) by shaking at 42° C for at least 30 min, and then transferred to two quick-seal polyallomer ultracentrifuge tubes. After addition of 1.2 mg bis-benzimide to each tube, the solution was centrifuged at 200 000*g* at 25° C for 16 h in a Beckman Ti80 rotor. The mtDNA and nDNA bands were removed

separately and further purified by a second cycle of centrifugation in TE buffer containing CsCl, followed by dialysis at 4⁰ C against TE buffer.

2.7.2 Small scale genomic DNA extraction.

Mycelium harvested from a single Petri dish (approx. 2 to 3 g) was used in the small-scale extractions. The mycelial pad was rinsed, drained thoroughly, and ground under liquid nitrogen to a fine powder. DNA was extracted by a procedure based on that of Kolar *et al.*, (1988). Extraction buffer (200 mM Tris-Cl pH 8.5, 250 mM NaCl, 50 mM EDTA, 48 mg PAS/ml, 8 mg TNS/ml) was added to the mycelial powder and the suspension was mixed to homogeneity using a sealed glass pasteur pipette. The extraction buffer was prepared fresh each time by adding the PAS and TNS to the sterile Tris-Cl/NaCl/EDTA solution, vortexing well, and leaving on ice for 15 min prior to use to settle the precipitate. The mycelial extract was then mixed with an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1 by vol.) and centrifuged at 10 000*g* for 10 min to separate the phases. The upper phase was transferred to fresh eppendorf tubes, 1 g/ml CsCl and 0.8 mg ethidium bromide were added, and the tubes were left on ice for 10 min. The suspensions usually became cloudy, and were centrifuged for 10 min to remove the precipitate, which collected at the top of the tube. The resulting clear fluid was then transferred to a Beckman Polyallomer 11 x 32 mm quick-seal ultracentrifuge tube. It was often necessary to add an extra 0.5 mg of ethidium bromide, as much of what was originally added became bound to the precipitate and was removed along with it. The solutions were centrifuged at 356 000*g* for 16 h at 20⁰C, in a 100.2 rotor and using a TL-100 ultracentrifuge. DNA bands were removed by syringe with a 23 gauge needle. Ethidium bromide was removed by three extractions with an equal volume of water-saturated butanol, and the DNA was dialysed overnight against 2 litres of TE buffer. Following dialysis, a considerable amount of polysaccharide material was still present in the solution, and the DNA was recovered by precipitation with 0.1 vol. 3 M sodium acetate pH 5.2 and one vol. of isopropanol.

The precipitated DNA formed a clump, and was removed by winding onto a Gilson p200 tip. The DNA was fully dissolved in 0.5 ml TE buffer, and re-precipitated using 0.1 vol. 3 M sodium acetate pH 5.2 and two vol. of absolute ethanol. The DNA was again removed using a Gilson p200 pipette tip, and was rinsed briefly in 70 % ethanol and resuspended in 200 µl sterile distilled water.

2.8 Restriction enzyme digestion and gel electrophoresis.

Restriction enzymes were purchased from a number of different companies (Northumbria Biochemicals Ltd., Cambio, Biorad Ltd, Beckman), and were used according to the manufacturer's recommendations. Generally, 250 ng mtDNA of 5 - 10 µg nDNA was digested for 2 - 5 hours using 2 U/µg of restriction enzyme. Electrophoresis was carried out in horizontal agarose gels in TBE buffer (8.9 mM Tris-borate, 2 mM EDTA, pH 8.3). Electrophoresis conditions, gel size, and % agarose varied according to the nature of the DNA samples being analysed. MtDNA and *MspI*- digested nDNA used in the population analyses were electrophoresed in 25 cm, 0.7% agarose gels at 5 volts/cm for 16 h. Gels of the same size and % agarose were used for *HindIII* and *BglII* nDNA digests in the population studies, but these were electrophoresed at 5 volts/cm for 25-30 h to eliminate fragments of less than 1 kb. In the genetic mapping work, five 8 cm gels were run, with one lane each of the two parents, 16 lanes of the progeny, and two lanes of DNA markers (kb ladder purchased from Bethesda Research Laboratories). Gels of *HindIII* and *MspII* digested DNA, in which the radiolabelled probes were expected to hybridise with small fragments, were made using 1.0% agarose, and were electrophoresed at 6 volts/cm for 1 - 2 h. 0.7% agarose gels were used for *BglII*-digested DNA, where hybridisation with larger fragments was expected, and electrophoresis was at 3.5 volts/cm for 3 - 4 h.

250 ng of mtDNA was loaded per lane. Gels for population studies used 5 µg nDNA

per lane, and those for genetic mapping used 3 µg nDNA per lane. Phage lambda DNA digested with *Hind*III or *Av*all, and kilobase ladder DNA were used as size standards. Gels were stained with ethidium bromide and were viewed using a UV transilluminator (Maniatis *et al.*, 1982).

2.9 Construction of *P. infestans* recombinant DNA clones.

2.9.1 Random cloning for population studies.

nDNA from *P. infestans* isolate 550 was fully digested with *Hind*III and the restriction fragments were ligated into the *Hind*III site of pUC19 using the method of Cebianchi & Wilson (1987). Transformation of *Escherichia coli* strain DH5α with the recombinant plasmids was done following the Standard Transformation Protocol of Hanahan (1985).

2.9.2 Size-selected cloning for genetic mapping.

P. infestans nDNA was fully digested with *Hind*III, but small fragments were selected for cloning in order to reduce the likelihood of cloning repetitive sequences. The digested DNA was size-fractionated on agarose gels, and a small strip of NA45 (Schleicher & Schull) paper was inserted into the lane of DNA at approximately 0.5 kb. Electrophoresis buffer was drained to just below the level of the gel, and electrophoresis was continued for 5 min. The NA45 paper was removed and placed in an eppendorf tube containing 0.5 ml of TES (10 mM Tris-Cl pH8.0, 1 mM EDTA pH 8.0, 0.5 M NaCl), and was incubated at 65°C for 20 min. DNA was precipitated by the addition of 1 ml absolute ethanol, was washed with 1 ml of 70% ethanol, and was resuspended in 20 µl of sterile distilled water. 1 µl was used in the ligation reaction with *Hind*III-digested pUC19 (Cebianchi & Wilson, 1987). *E. coli* strain DH5α was transformed with the recombinant plasmids by electroporation using the BioRad Gene Pulser, and following the instructions of the manufacturer.

2.10 Southern transfers, dot-blot, hybridisation and autoradiography.

Digested DNA in agarose gels was transferred to Hybond N+ membranes using the SSPE method given in the manufacturers recommendations (Amersham International). This involved 1). acid nicking of the DNA by soaking the gel in 0.25 M HCl for 30 min. 2). denaturation with 0.5M NaOH, 1.5M NaCl for 30 min. 3). neutralisation with 1.5M NaCl, 0.5M Tris-HCl pH 7.2, 0.001M EDTA for 2 x 15 min. Gels were rinsed briefly with distilled water between each of the above steps. Capillary blotting of the DNA was done by placing the gel onto a wick of Whatman 3MM filter paper saturated with blotting buffer (20 x SSPE : 3.6M NaCl, 0.2M Sodium phosphate, 0.02M EDTA pH7.7) which overhung into a reservoir of blotting buffer. Hybond N+ membrane was carefully placed onto the gel surface. Three pieces of Whatman 3MM paper, cut to the same size as the Hybond N+, were placed on top of the membrane, followed by a 5 cm stack of absorbant paper towels. A small (approx. 200g) flat weight was balanced on top of the towels, and transfer was performed for 16 - 24 h. After blotting, the membrane was alkali fixed by placing it onto a pad of Whatman 3MM soaked in 0.4M NaOH for 20 min. The membrane was rinsed briefly (less than 1 min) in 5 x SSPE with gentle agitation, then UV fixed using a Stratafix apparatus (Stratagene) on the autofix setting.

Dot - blots were constructed by spotting approximately 1.0 µg of each denatured recombinant plasmid DNA onto Hybond N+ membrane. Initially this was done by hand in 1 µl aliquots. Later, the BioRad Dot-blot manifold was available, and was used according to the manufacturers recommendations.

Radiolabelled probe DNA (*P. infestans* nDNA, cloned *P.infestans* DNA, phage lambda or kilobase ladder marker DNA) was prepared by random priming (Feinberg & Vogelstein, 1983). Probes were prepared from *P.infestans* recombinant clones

by digesting the clones fully with *Hind*III and separating the insert and vector bands by electrophoresis in 1.0% NuSieve GTG agarose (FMC BioProducts) using TAE electrophoresis buffer (60 mM Tris-acetate, 1 mM EDTA pH 8.0). The *P. infestans* insert DNA was excised, water added at 3 ml/g, and the gel was boiled for 7 min. The gel solution was incubated at 37°C for 10 min or longer, and could then be used directly in labelling reactions. Generally, between 50 - 100 ng of DNA was labelled, and incorporation of at least 50% was achieved. Radiolabelled probes were hybridised with the membrane overnight following the Hybond N+ protocol. All hybridisations were done in shallow plastic boxes with tightly fitting lids (Stewart Plastics Ltd) using 20 - 50 ml hybridisation buffer (5 x SSPE, 0.5% [w/v] SDS, 5 x Denhardt's solution : 0.1 % [w/v] bovine serum albumin, 0.1 % [w/v] Ficoll, 0.1 % [w/v] polyvinylpyrrolidone). Blots were pre-hybridised for at least 1 h before radioactive probe was added. Hybridisation was done for at 16 - 24 h. Following hybridisation, membranes were washed twice in 2 x SSPE, 0.1 % SDS at room temperature for 10 min, once in 1 x SSPE, 0.1 % SDS at 65°C for 15 min and once in 0.1 % SSPE, 0.1 % SDS at 65°C for 15 min. Autoradiography was done at -70°C using two Hi-Speed X-ray intensifying screens (X-OGRAPH Ltd) and Fuji RX-100 or Kodak X-AR X-ray film. Blots were stripped of their radiolabelled probes by the addition of 300 ml of boiling 0.5% SDS, and could be re-used at least 15 times without a noticeable loss of hybridisation as detected by signal intensity.

2.11 Linkage analysis.

Linkage analysis was performed using the LINKAGE (V5.03) package of programmes (Lanthrop *et al.*, 1984), modified by L. Sandkuyl to accommodate the very large pedigree used. The programme was run on a Dell (system 325) PC-compatible computer with a 80387 mathematics co-processor chip. Wordstar v5.5

was used to enter the linkage data

2.12 Pulsed field gel electrophoresis.

2.12.1 Preparation of inserts.

a) Using isolated nuclei.

Nuclei were extracted from *P. infestans* by a modification to the method of Garber & Yoder (1983). 15 g of fresh mycelium was ground under liquid nitrogen, mixed by stirring vigorously with 50 ml ice cold extraction buffer (0.35 M sucrose, 50mM EDTA, 10 mM Tris-Cl pH 7.4)., and centrifuged for 10 min at 750 *g*, 4°C. The supernatant was removed, clarified by pouring through two layers of chesecloth, and was centrifuged for 30 min at 53 000 *g*, 4°C in a SW28 rotor. The resulting pellet, which contains the organelles, was resuspended gently in 1 ml extraction buffer using a rubber policeman. 1 ml of 2% LMT agarose (Beckman) was added, and the mixture transferred to an insert mould and chilled at 4°C for 15 min. Inserts were pushed out into 4 ml ESP (0.5 M EDTA pH 9.0 - 9.5, 1% lauroyl sarcosine, 1 mg/ml proteinase K - incubated at 37°C, 2 h, and frozen in aliquots), and left at 50°C with gentle shaking for two days (Smith *et al.*, 1988).

b) Using protoplasts.

Protoplasts were produced using a procedure based on that of Howlett (1989). 5 day old mycelial mats were washed with 0.4 M mannitol, 0.1 M CaCl₂, 0.05 M MES, and were incubated in 20 ml of the above solution, to which 100 mg Novozyme (Novo Biochemicals Ltd.) and 100 mg Driselase (Sigma) had been added. Incubation was at 30°C, for 40 min to 3 h, until the majority of the mycelium had disappeared. Protoplasts were then separated from mycelial debris by pouring the solution through Miracloth (Cambridge Bioscience), and were concentrated by centrifugation at 500 *g* for 5 min at 4°C. The protoplasts were resuspended in 5 ml of 1 M sorbitol pH 8.0, and were washed 2 times by repeating the centrifugation step.

Resuspension was finally done to achieve a range of concentrations from 10^5 to 10^8 protoplasts/ml. An equal volume of 2% LMT agarose was added to each protoplast suspension and the mixtures transferred to insert moulds. Inserts were incubated in ESP as described above.

2.12.2 Electrophoresis conditions.

Electrophoresis was done using the Biorad CHEF system. Gels were made with low endo-osmosis (LE) agarose (Beckman) and 0.5 x TBE, and were electrophoresed in 0.5 x TBE. Agarose concentration, voltage, pulse times and run times varied according to the size of chromosomes being separated. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomal DNA standards were purchased from Biorad.

2.13 Nomenclature.

Recombinant clones produced from the first (random) cloning were designated pIN1 to pIN300. Those that were produced using size-selected 0.5 kb *Hind*III fragments of *P. infestans* DNA were designated pIN5-1 to pIN5-725. pSTA99 was constructed at St. Andrews University, Dundee, and received its name there.

Polymorphic loci detected by the recombinant clones are assigned the same numerical designation, but pIN is replaced by L, so that pIN2 becomes L2; pIN5-655 becomes L5-655, etc. The mitochondrial locus detected by pSTA99 is designated ML99.

Potato differentials are named according to the standardised nomenclature of Black *et al.*, 1953. Avirulence genes are assigned the same numerical designation as the potato differentials with which they are complementary, but are prefixed by Avr in place of R. Virulence genes are likewise prefixed avr. Thus avr1 is capable of overcoming resistance in the potato differential R1 and is allelic with Avr1, etc. In general, it is the avirulence gene locus (Avr) that is referred to.

CHAPTER 3 : RFLPs IN MITOCHONDRIAL DNA.

3.1 Choice of isolates.

All isolates were obtained from the collection of Drs. D. Shaw and R. Shattock, Bangor University. 16 isolates were chosen from diverse global locations, and a further 8 from various sites in the U.K. The selection included both the A1 and the A2 mating types, as well as one self fertile isolate. Most of these isolates had been included in other studies, and a considerable amount of information on their ploidy, isozyme profiles and virulence phenotypes was available. Details of these are presented in Table 3.1.

3.2 DNA isolation.

Good yields of reasonably pure mtDNA were obtained using the CTAB DNA extraction procedure, followed by CsCl-bisbenzimidazole density gradients. Figure 3.1 shows the mitochondrial and nuclear DNA bands on a CsCl-bisbenzimidazole gradient.

The CTAB method was preferred over other procedures developed to extract fungal DNA (eg. Raeder and Broda, 1985) as it resulted in less contamination by *P. infestans* polysaccharides. These polysaccharides cause high background fluorescence in the presence of bisbenzimidazole, and tend to obscure the DNA bands on the CsCl-bisbenzimidazole gradients. Polysaccharides can also interfere with subsequent restriction enzyme digestion and ligation of the DNA.

Around 5 - 10 µg of mtDNA was obtained from 10g of lyophilised mycelium.

Table 3.1 : Properties of the different global isolates of *P. infestans*.

ISOLATE	ORIGIN (HOST) ¹	MATING TYPE	GPI ²	PEPTIDASE ²	PLOIDY ³	VIRULENCE ⁴
Ca65	USA (California) (T)	A1	100-100	92-100	2C	1,4,7,11
E13a	Egypt (P)	A2	100-100	100-100	2C	1,3,4,7,8,10,11
550	Mexico (S)	A2	86-86	100-100	2C	1,2,3,4,5,7,8,10,11
533	Mexico (P)	A2	122-122	92-100	2C	1,2,3,4,7,10,11
7-85	Sweden (P)	A1	100-100	100-100	NT	1,2,11
WF127	USA (New York) (P)	A1	86-100	92-100	4C	2,3,4,7
83-5	UK (Gwynedd) (P)	A1	NT	NT	4C	1,2,3,4,7,10,11
86-126-1A	UK (Cambridge) (P)	A1	86-100	92-100	3C	NT
K1067	Ireland (P)	A1	86-100	92-100	3C	NT
86-127-2	UK (Gwynedd) (P)	SF	100-100	92-100	NT	NT
86-150-3	UK (S.Wales) (P)	A2	100-100	100-100	2C	NT
87-182-9	UK (Norfolk) (P)	A2	100-100	100-100	2C	NT
E14C2	Egypt (P)	A2	100-100	100-100	2C	1,4,7,10,11
565	Mexico (P)	A1	100-122	92-100	2C	2,3,4,7
Ismailia B	Egypt (P)	A1	100-100	100-100	2C	1,4,7,10,11
84-19	Spain (P)	A1	100-100	92-100	4C	1,3,4,7,8,10,11
K1098	Netherlands (P)	A1	100-100	100-100	2C	3,4,7
NR-132	Switzerland (P)	A2	86-100	100-100	NT	NT
CG3	New Zealand (P)	A1	100-100	100-100	NT	1,2,3,4,7,11
12-87	Brazil (P)	A1	86-100	92-100	2C	3,4,7
644-86	Brazil (P)	A2	100-100	100-100	NT	1,3,4,5,7,11
87-34-4	UK (Dyfed) (P)	A1	100-100	100-100	NT	NT
87-244-1	UK (Avon) (T)	A1	100-100	100-100	NT	NT
87-205-2	UK (Cornwall) (P)	A2	100-100	100-100	NT	NT

¹Host plants from which isolate was obtained. P = potato, T = tomato, S = *Solanum* spp.

² Alleles at glucose phosphate isomerase (*Gpi-1*) and peptidase loci from Tooley *et al.* (1985) and unpublished results of D.S.Shaw and R.C.Shattock. NT = not tested.

³ Ploidy data from Tooley & Therrien (1987) and Whitticker *et al* (1990).
2C = diploid; 3C = triploid; 4C = tetraploid.

⁴ Virulence, as assayed by ability to grow on potatoes carrying the appropriate resistance genes (D.S.Shaw and R.C.Shattock, unpub).

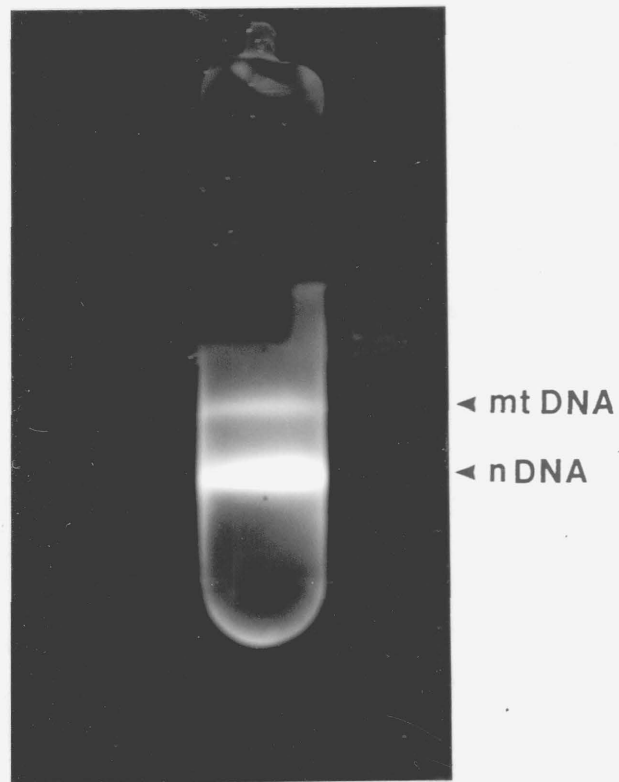


Figure 3.1 : CsCl-bisbenzimidide density gradient of extracted *P. infestans* DNA showing nuclear and mitochondrial bands.

3.3 Restriction endonuclease digestion and RFLP analysis.

Restriction digests were initially performed using *Hind*III, which is an inexpensive and generally reliable enzyme. When mtDNA from each of the 24 isolates under study was fully restricted with *Hind*III and electrophoresed on agarose gels, it appeared to group into two types. Figure 3.2 shows the *Hind*III restriction profiles produced for each isolate. Restriction fragment sizes were obtained by comparison with *Hind*III-digested lambda size standards, and are shown in Table 3.2. 19 of the 24 mtDNAs (designated Type I) had a total genome size of 36.7 kb, consistent with the estimate by Klimczac & Prell (1984) of 36.2 kb. The remaining 5, however, had lost one band of a doublet at 2.1 kb, but had gained two new fragments of 2.3 and 1.65 kb. This produces an overall larger size for these mitochondrial genomes (designated Type II) of 38.5 kb, indicating that an insertion of just under 2 kb has occurred in them. The inserted sequence presumably carries a *Hind*III site, causing the 2 new fragments to be generated from the original one of 2.1 kb.

Further digests using *Bcl*I, *Bgl*II, *Cla*I and *Eco*RI (Figs. 3.3 - 3.6; Table 3.2) located the approximate region of insertion on the restriction map of *P. infestans* DNA developed by Klimczac & Prell (1984) (Fig. 3.7).

In the *Bcl*I digests (Fig. 3.3), the largest (11.7kb) fragment has increased in size by approximately 2 kb, indicating simple insertion within it.

*Bgl*II digests, shown in figure 3.4, reveal loss of the 4.6 kb fragment in Type II mtDNAs, which have two new bands of 3.0 and 3.6 kb. This indicates that the inserted sequence introduces a new *Bgl*II site.

The results of digestion with *Cla*I (Figs. 3.5 & 3.7), in which fragment 2 (6.4 kb) and 3 (4.2 kb) are replaced with a single larger fragment of 12.7 kb following insertion, suggest that the insertion event occurred at or near the *Cla*I site between the two original fragments, and resulted in the destruction

Table 3.2 Restriction fragments obtained with different *P. infestans* mtDNA types.¹

Size of fragments (in Kb)

<i>Hind</i> III		<i>Bcl</i> I		<i>Bgl</i> III		<i>Cla</i> I		<i>EcoR</i> I		<i>Msp</i> I			<i>Hha</i> I		
I(a+b)	II(a+b)	I(a+b)	II(a+b)	I(a+b)	II(a+b)	I(a+b)	II(a+b)	I(a+b)	II(a+b)	Ia	Ib	II(a+b)	I(a+b)	IIa	IIb
7.0	7.0	11.7	14.0	19.0	19.0	23.0	23.0	-	15.5	10.9	10.9	-	-	14.8	-
6.7	6.7	10.2	10.2	8.35	8.35	-	12.7	7.9	-	-	-	6.6	-	-	9.6
4.3	4.3	9.1	9.1	5.3	5.3	6.4	-	-	7.0	5.9	-	5.9	7.5	-	-
2.6 ²	2.6	3.1	3.1	4.6	-	4.2	-	6.6	-	5.35	5.35	5.35	7.3	-	-
2.6	2.6	1.3	1.3	-	3.6	1.65	1.65	5.9	-	-	5.35	-	-	-	5.2
-	2.3	1.1	1.1	-	3.0	1.1	1.1	4.7	4.7	-	-	5.2	5.1	5.1	5.1
2.1	2.1	0.9	0.9	-	-	-	-	4.4	4.4	4.3	4.3	4.3	4.5	4.5	4.5
2.1	-	-	-	-	-	-	-	3.4	3.4	-	-	4.2	-	4.2	4.2
2.0	2.0	-	-	-	-	-	-	2.9	2.9	3.3	3.3	-	3.7	-	-
1.95	1.95	-	-	-	-	-	-	1.5	1.5	-	-	2.8	3.3	3.3	3.3
1.7	1.7	-	-	-	-	-	-	-	-	2.2	2.2	2.2	3.3	3.3	3.3
-	1.65	-	-	-	-	-	-	-	-	1.3	1.3	-	1.5	1.5	1.5
1.5	1.5	-	-	-	-	-	-	-	-	1.3	1.3	-	-	1.3	1.3
0.7	0.7	-	-	-	-	-	-	-	-	1.1	1.1	1.1	0.68	0.68	0.68
0.55	0.55	-	-	-	-	-	-	-	-	-	0.6	-	0.58	0.58	0.58
0.52	0.52	-	-	-	-	-	-	-	-	0.58	0.58	0.58	-	-	-
0.34	0.34	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36.7	38.5	37.4	39.7	37.3	39.3	36.4	38.5	37.3	39.4	36.2	36.3	38.2	37.5	39.3	39.3

¹The mtDNA type of each *P. infestans* isolate is shown in Table 3.3.

²Two fragments of approximately the same size are indicated by a closely spaced doublet or a band of double the intensity.

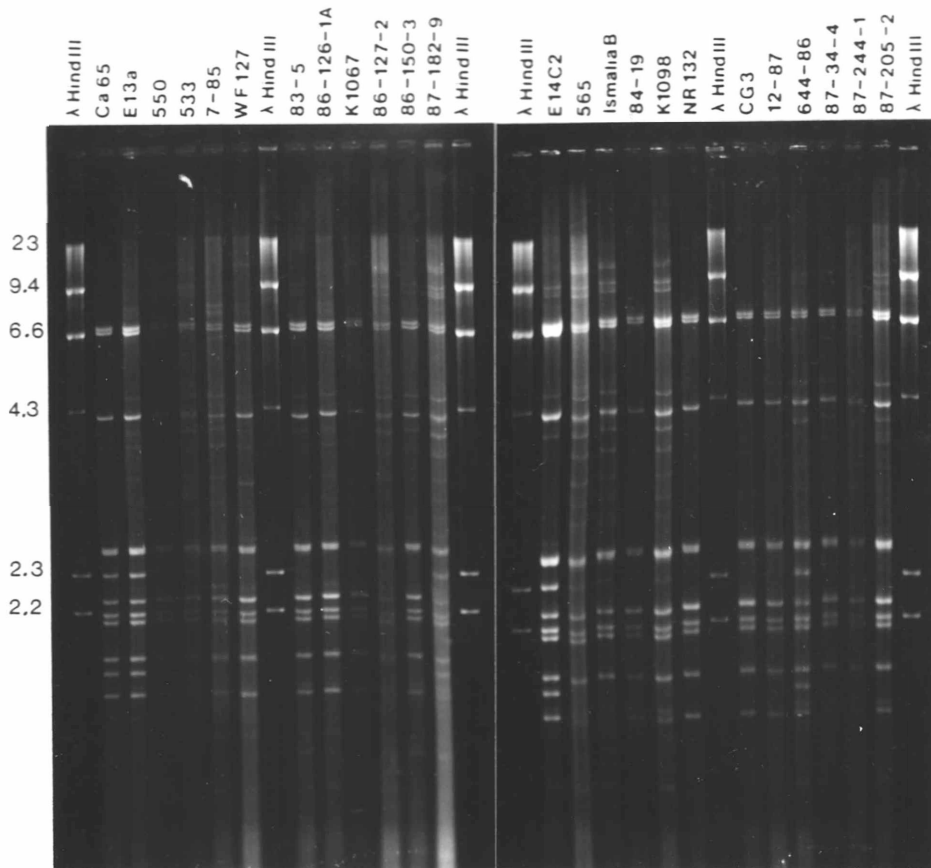


Figure 3.2 : Agarose gel electrophoresis of mtDNA from *P. infestans* digested with *HindIII*

Type I : 550, 533, 7-85, WF127, 83-5, 86-126-1A, K1067, 86-127-2, 86-150-3, 565, Ismailia B, 84-19, K1098, NR132, CG3, 12-87, 87-34-4, 87-244-1, 87-205-2.
 Type II: Ca65, E13a, 87-182-9, E14C2, 644-86.

The sizes (in kb) of marker DNAs (phage lambda digested with *HindIII*) in this and subsequent figures are given on the side of the gel. On this gel, the 0.7 kb and smaller fragments have migrated off the end of the gel.

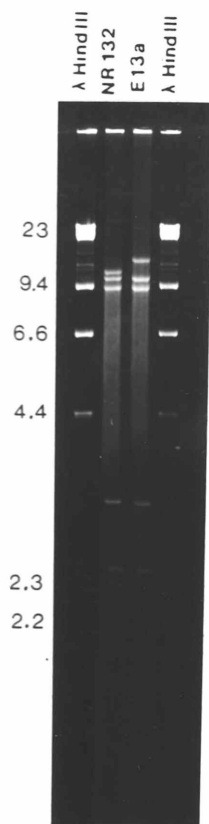
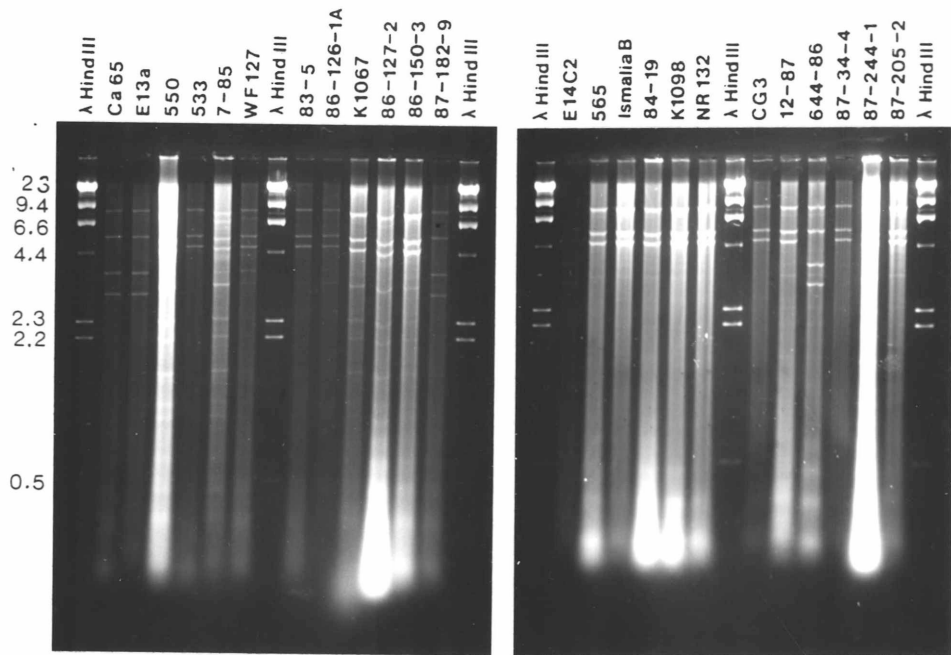


Figure 3.3 : Agarose gel electrophoresis of mtDNA from *P. infestans* digested with *BclI*.

Type I : NR132
Type II : E13a.

Figure 3.4 : Agarose gel electrophoresis of mtDNA from *P. infestans* digested with *BglIII*.

Type I : 550, 533, 7-85, WF127, 83-5, 86-126-1A, K1067, 86-127-2, 86-150-3, 565, Ismailia B, 84-19, K1098, NR132, CG3, 12-87, 87-34-4, 87-244-1, 87-205-2.
Type II: Ca65, E13a, 87-182-9, E14C2, 644-86.



of that site. This is consistent with the results from the *Bcl*I and the *Bgl*II restriction digests, as the junction between *Cla*I fragments 2 and 3 lies within the fragments altered by insertion in these two digests (Fig 3.7).

However the *Eco*RI digests (Fig 3.6 & 3.7), in which fragments 1 (7.9 kb), 2 (6.6 kb) and 3(5.9 kb) of the Type I mtDNA have been replaced by 16.0 and 7.0 kb fragments in Type II mtDNAs indicates that sequence alterations in addition to the insertion have taken place. The simplest interpretation is that the insertion caused sequence alterations resulting in loss of both the *Cla*I site between *Cla*I fragments 2 and 3 and the *Eco*RI site between *Eco*RI fragments 1 and 3. A further sequence rearrangement, possibly an inversion, near the junction of *Eco*RI fragments 2 and 3 could result in displacement of the *Eco*RI site at this junction by about 0.3 kb clockwise (Fig 3.7). Hence the 16.0 kb fragment in Type II mtDNA would consist of *Eco*RI fragment 1 (7.9 kb) plus the 2 kb insertion plus *Eco*RI fragment 3 (5.9 kb) minus 0.3 kb. The 7.0 kb *Eco*RI fragment of Type II mtDNA would consist of *Eco*RI fragment 2 (6.6kb) plus 0.3 kb.

The *Eco*RI restriction pattern appears identical to that described by Goodwin (1990) who reported a 1.7 kb insertion accompanied by rearrangement in a number of global isolates of *P. infestans*.

Further analysis of the insertion and the sequence alterations accompanying it will require nucleotide sequence comparisons of Type I and Type II mtDNA in this region.

None of the above enzymes yielded additional polymorphisms to differentiate mtDNAs within the Type I and Type II groups from each other. Further polymorphisms were therefore sought using additional enzymes, in particular using enzymes that recognise 4 or 5 base pair sequences. These should cut the mtDNA genomes more frequently, therefore should be more likely to reveal RFLPs. *Av*all, *B*scl, *E*coO, *H*incII, *H*paI, *M*luI, *M*spl and *R*saI were all tried on mtDNA from isolate 87-182-9 (not shown). The majority of the

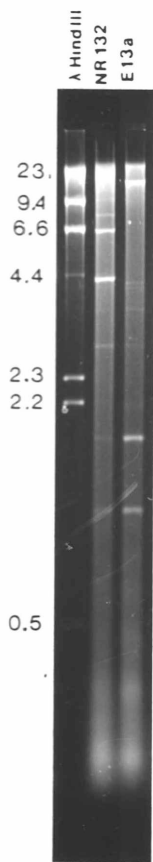
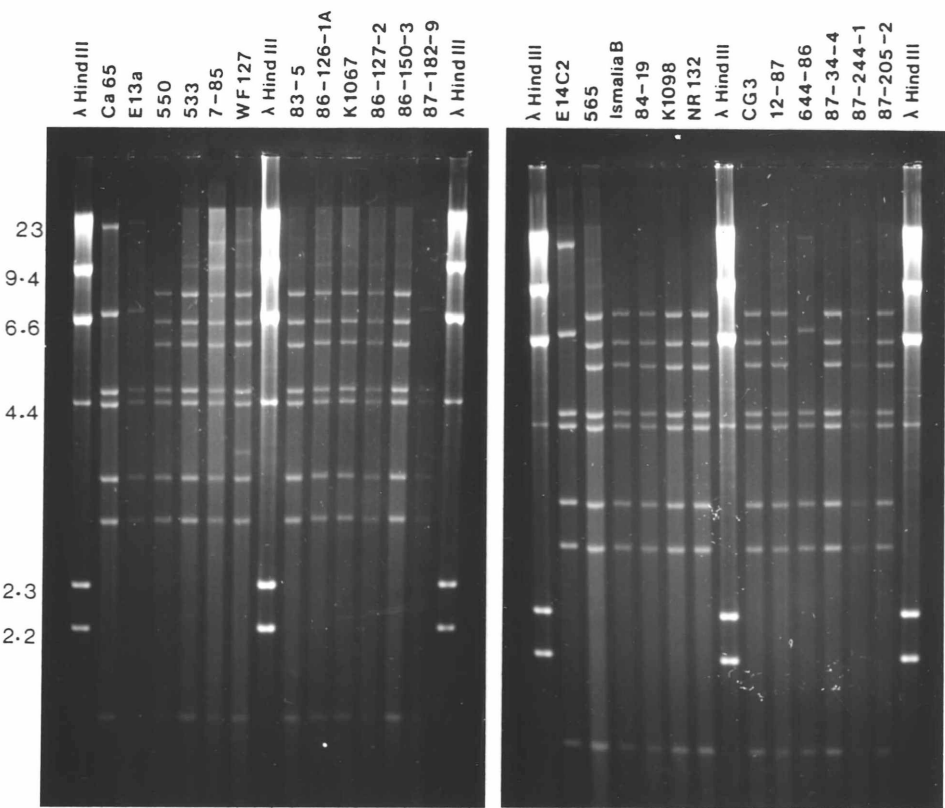


Figure 3.5 : Agarose gel electrophoresis of mtDNA from *P. infestans* digested with *Cla*I.

Type I : NR132
Type II : E13a.

Figure 3.6 : Agarose gel electrophoresis of mtDNA from *P. infestans* digested with *Eco*RI.

Type I : 550, 533, 7-85, WF127, 83-5, 86-126-1A, K1067, 86-127-2, 86-150-3, 565, Ismailia B, 84-19, K1098, NR132, CG3, 12-87, 87-34-4, 87-244-1, 87-205-2.
Type II: Ca65, E13a, 87-182-9, E14C2, 644-86.



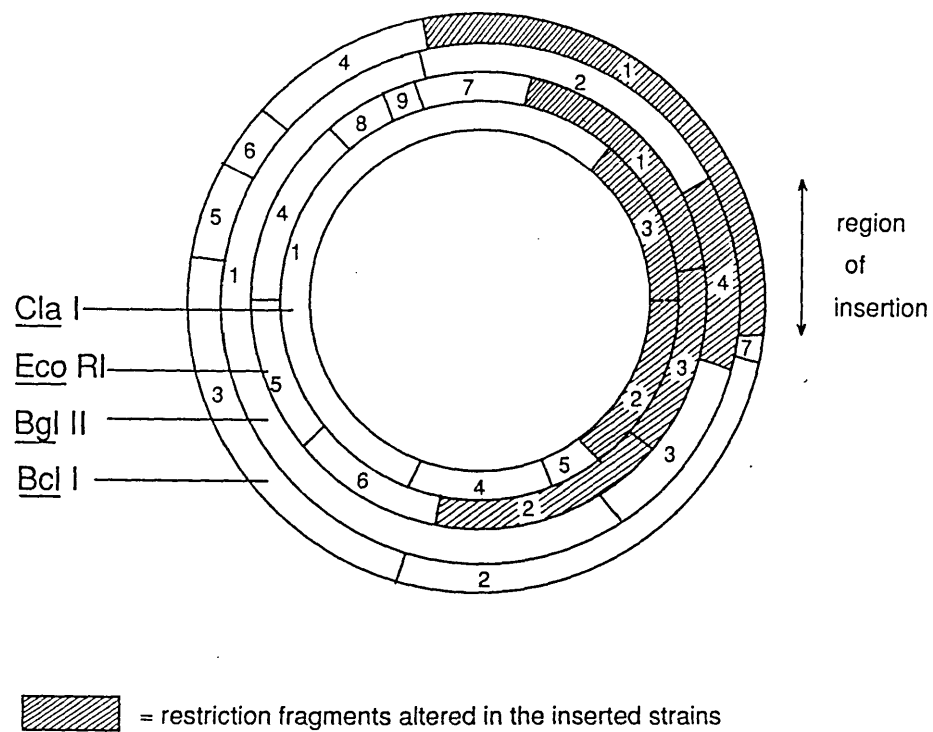


Figure 3.7 : Restriction map of *P. infestans* Type I mtDNA, adapted from Klimczac & Prell (1984) to indicate the region of the 2 kb insertion in Type II mtDNA.

enzymes tried produced fragments that were too small or too numerous to differentiate clearly between them. *Avall* and *MspI* gave reasonably clear restriction profiles, and were chosen to digest the 24 mtDNAs.

Avall produced a range of fragments from 0.5 to 8.0 kb, but did not reveal polymorphisms additional to the 2 kb insertion (Fig 3.8). *MspI* digests, however, revealed a new polymorphism within the Type I mtDNAs (Fig 3.9; Table 3.2). Fifteen of the mtDNA genomes in this group contained a 5.9 kb fragment, which was replaced by two fragments of 5.35 and 0.6 kb in the remaining 4 mtDNAs. This polymorphism therefore results from the presence or absence of an *MspI* site within the 5.9 kb fragment, and probably arose by point mutation. MtDNAs lacking or containing this *MspI* site are designated Type Ia and Type Ib respectively. It is noteworthy that the isolates containing Type Ib mtDNA (12-87, 86-126-1A, K1067 and WF127) share the same alleles at Gpi-1 and Pep, and have very similar virulence phenotypes (see Table 3.1). This point will be discussed in more detail later.

During the time that this work was being undertaken, digests of nuclear *P. infestans* DNA from the same 24 isolates were also being done to produce Southern blots for the analysis of RFLPs in nuclear DNA. In *MspI* digests of nDNA, strong bands of greater size than the majority of the digested DNA were produced, and these bands varied between some of the isolates (Fig 3.10). Initially, these were thought to be satellite sequences, which are seen in all *P. infestans* nDNA digests but are usually conserved between the isolates. A comparison of profiles with the *MspI* digests of mtDNA however, revealed that the strong bands in the nDNA digests were identical in size with those produced by digesting mtDNA alone with *MspI*, and are therefore probably due the presence of mtDNA within the nDNA samples. This was further confirmed by hybridising the nDNA digests with radiolabelled mtDNA (Fig 3.11). This observation was rather surprising given that all the nDNA samples used in

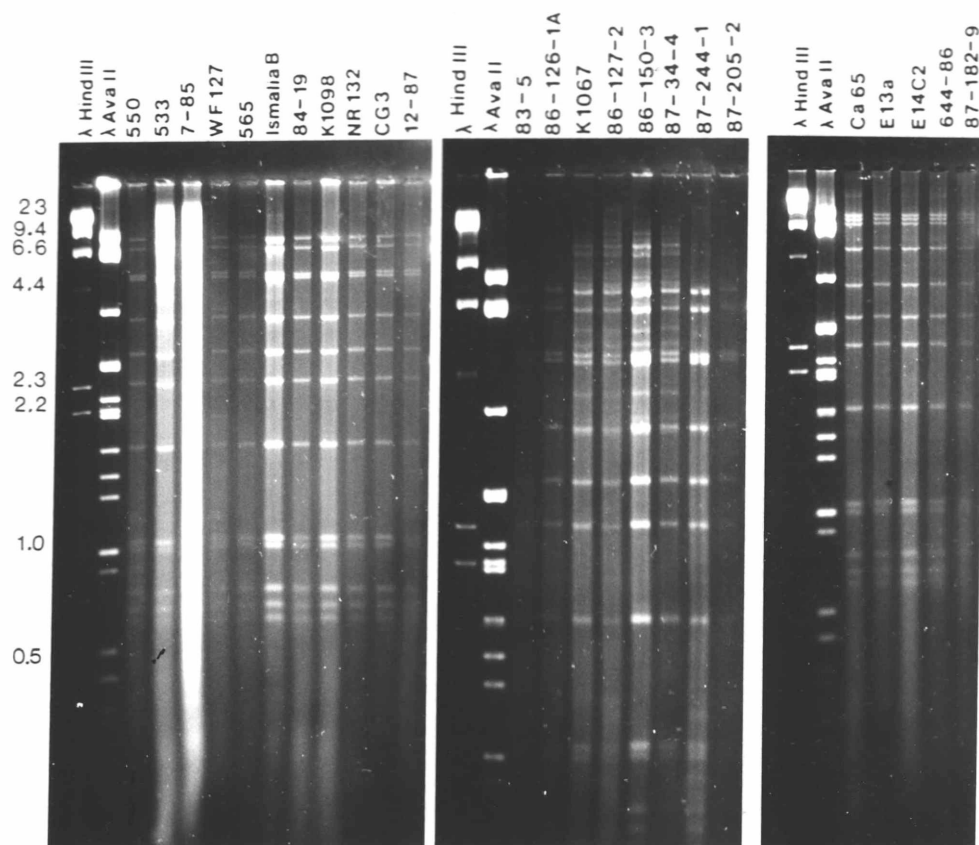


Figure 3.8 : Agarose gel electrophoresis of mtDNA from *P. infestans* digested with *Ava*II.

Type I : 550, 533, 7-85, WF127, 83-5, 86-126-1A, K1067, 86-127-2, 86-150-3, 565, Ismailia B, 84-19, K1098, NR132, CG3, 12-87, 87-34-4, 87-244-1, 87-205-2.
 Type II: Ca65, E13a, 87-182-9, E14C2, 644-86.

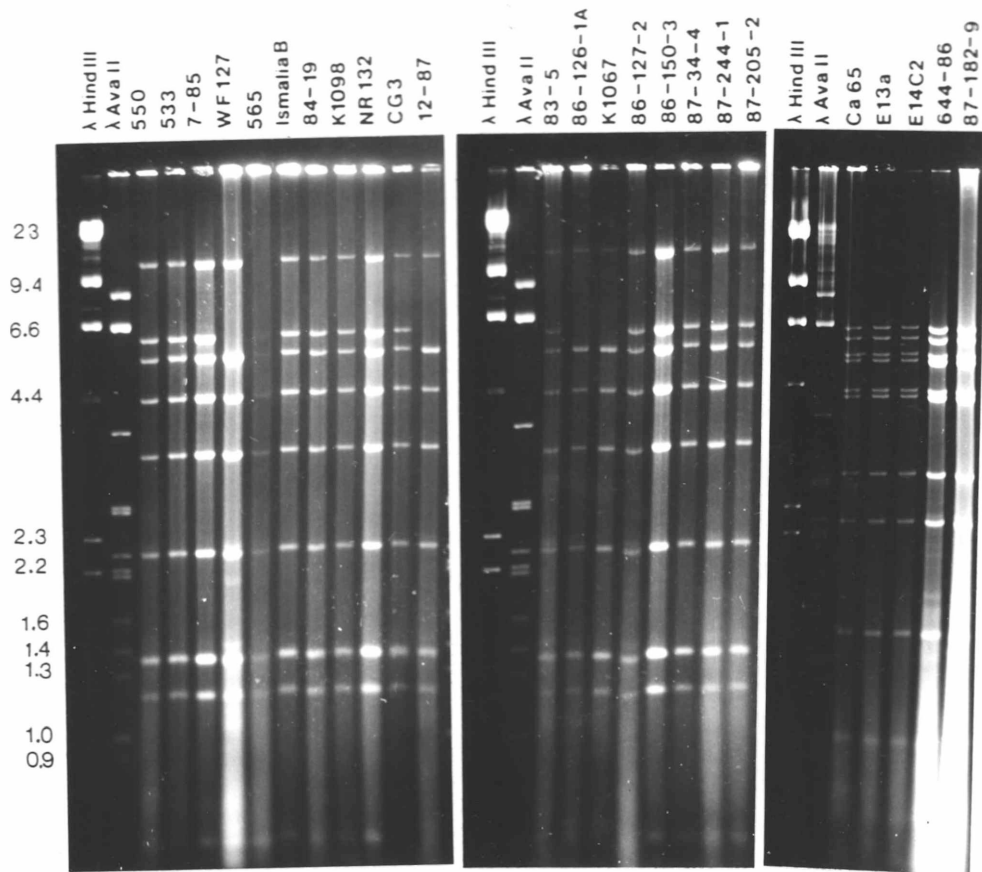


Figure 3.9 : Agarose gel electrophoresis of mtDNA from *P. infestans* digested with *MspI*.

Type Ia : 550, 533, 7-85, 83-5, 86-127-2, 86-150-3, 565, Ismailia B, 84-19, K1098, NR132, CG3, 87-34-4, 87-244-1, 87-205-2.

Type Ib : WF127, 86-126-1A, K1067, 12-87.

Type II : Ca65, E13a, 87-182-9, E14C2, 644-86.

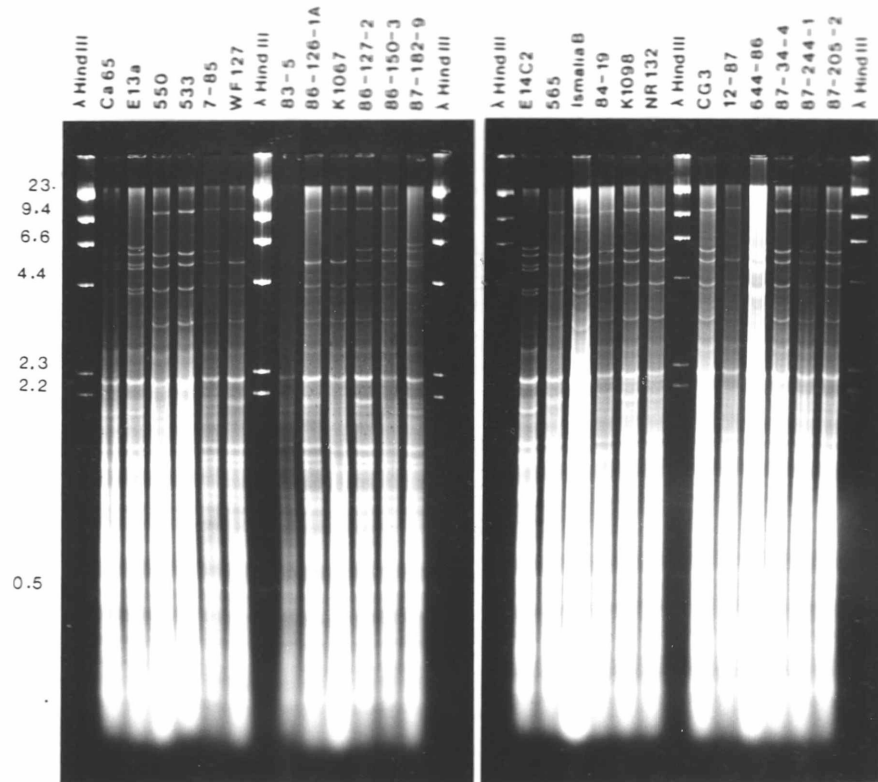


Figure 3.10 : Agarose gel electrophoresis of nDNA from *P. infestans* digested with *MspI*, showing mtDNA bands visible above the bulk of digested nDNA.

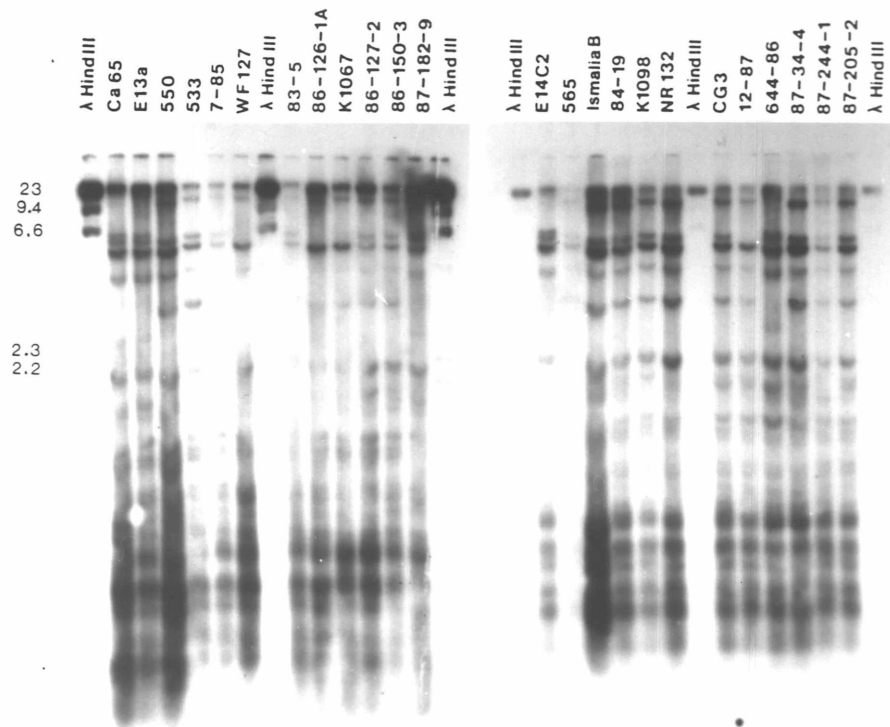


Figure 3.11 : Southern blot of nDNA from *P. infestans* digested with *MspI* and hybridised with mtDNA

these digests were taken from CsCl-bisbenzimidide gradients from which the mtDNA had been extracted. It did however mean that mtDNA RFLPs using certain enzymes could be screened for initially using samples of nDNA, allowing purified mtDNA to be conserved. MtDNA fragments produced by digestion with *MspI* are larger than the majority of *MspI*-digested nuclear DNA because of the AT-rich nature of mtDNA. *MspI*, which recognises the sequence CCGG, cleaves the mtDNA less frequently than would be expected for a restriction enzyme with a recognition site of only 4 base pairs. Other restriction enzymes recognising exclusively G and C residues should therefore also produce visible mtDNA bands in nDNA digests. Restriction digests of nDNA samples were therefore performed using *HhaI* (GCGC), *HaeIII* (GGCC) and *BstU II* (CGCG). *HaeIII* and *BstU II* did not reveal RFLPs additional to the 2 kb insertion within the mtDNA bands visible. Digestion with *HhaI* however revealed a new polymorphism within the Type II mtDNAs (Fig 3.12). 4 of the 5 Type II mtDNAs (from isolates E13a, E14C2, 644-86 and 87-182-9) produced a 14.8 kb fragment, which was replaced by fragments of 9.6 and 5.2 kb in the single other Type II mtDNA (from isolate Ca65). This was confirmed by using purified mtDNA from representative isolates (Fig 3.13). All other *HhaI* restriction fragments were conserved in all the Type II mtDNAs (Table 3.2). This polymorphism therefore represents the presence or absence of a *HhaI* site within the 14.8 kb fragment. mtDNAs lacking or containing this site are designated Type IIa or Type IIb respectively.

Finally, Type II mtDNAs were digested with *RsaI* (Fig 3.14). This enzyme cuts the genome into numerous small fragments, which were separated using gels containing 3% NuSieve GTG agarose (FMC BioProducts). NuSieve agarose has a low viscosity, and can therefore be used to produce highly sieving gels capable of separating very small DNA fragments. No further RFLPs were revealed between the 5 Type II mitochondrial genomes, which are probably

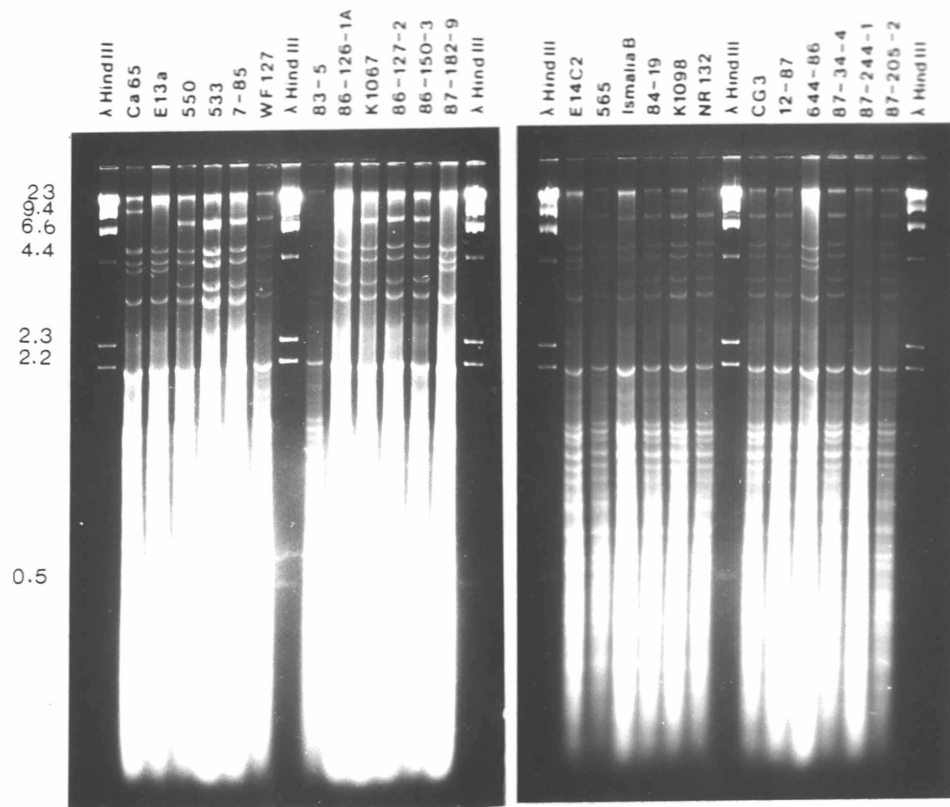
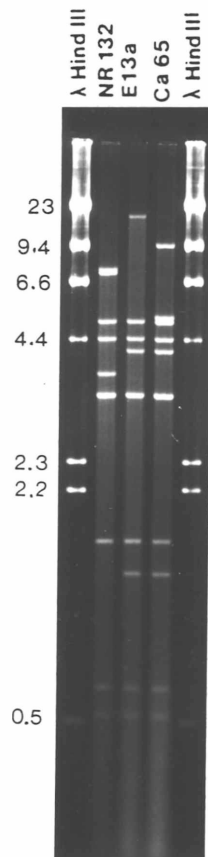


Figure 3.12 : Agarose gel electrophoresis of nDNA from *P. infestans* digested with *HhaI*. MtDNA bands are visible above the bulk of digested nDNA.



Type Ia : 550, 533, 7-85, 83-5,
86-127-2, 86-150-3, 565, Ismailia B, 84-19,
K1098, NR132, CG3, 87-34-4, 87-244-1,
87-205-2.
Type Ib : WF127, 86-126-1A, K1067, 12-87.
Type IIa : E13a, 87-182-9, E14C2, 644-86.
Type IIb : Ca65.

Figure 3.13 : Agarose gel electrophoresis of mtDNA from *P. infestans* digested with *HhaI*.

Type Ia : NR132
Type IIa : E13a
Type IIb : Ca65.

very similar in sequence organisation. Unfortunately, insufficient mtDNA was left to perform *RsaI* digests on the Type I mtDNAs. Isolates containing the various mtDNA types are summarised in Table 3.3.

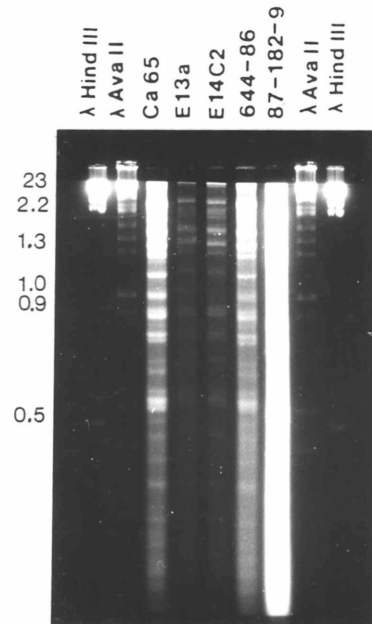


Figure 3.14 : Agarose gel electrophoresis of *RsaI*-digested Type II mtDNA from *P. infestans* using 3% NuSieve agarose.

Table 3.3 : mtDNA types in the different isolates of *P. infestans*.

<u>Type I :</u>	<u>a</u>	<u>b</u>
	550, 533, 7-85, 83-5 86-127-2, 86-150-3, 565, Ismailia B, 84-19, 87-34-4, 87-244-1 87-205-2.	WF127, K1067 86-126-1A, 12-87.
<u>Type II :</u>	<u>a</u>	<u>b</u>
	E13a, E14C2, 644-86, 87-182-9.	Ca65.

3.4 Discussion of Chapter 3.

The main polymorphism detected in the *P. infestans* mitochondrial genome was the presence (Type II) or absence (Type I) of a 2 kb insertion sequence. This appears to be identical to the insertion reported by Goodwin (1990), who called his inserted and uninserted mtDNAs A and B forms, respectively. The site of insertion, within *Bgl*II fragment 4 (Fig. 3.3), appears to be the same in all five isolates with Type II mtDNA, indicating Type II mtDNAs share a common origin. The results of digestion with *Eco*RI and *Cla*I indicate that sequence alterations in addition to the insertion have occurred. Since these occur in all five Type II mitochondrial genomes, and in all of Goodwin's B forms of mtDNA, it is likely that these rearrangements occurred at the same time as, or very soon after, the insertion.

Goodwin went on to map sites for additional restriction endonucleases in this region in the uninserted mitochondrial genome. This map was compared with a consensus map of the region encompassing the small rRNA gene in the mitochondrial genomes of *P. megasperma* and *P. parasitica* (Forster *et al.*, 1988). A very similar arrangement of restriction sites was indicated in both mtDNA genomes. This led Goodwin to postulate that the small rRNA gene lies in this region (within *Bgl*II fragment 4, Fig. 3.7). The large rRNA gene, which is adjacent to the small rRNA gene in *P. megasperma* and *P. parasitica*, was positioned within *Cla*I fragment 3 and *Bgl*II fragment 4. It is curious, however, that these genes, which appear so conserved in arrangement between the different *Phytophthora* species, lie within the area that is rearranged by the insertion in Type II mtDNA from *P. infestans*.

Type II mitochondrial genomes were found in isolates from Brazil, Egypt, the U.K., and the U.S.A., and were also found by Goodwin in isolates from Northeast and Northwest Mexico, Poland, the Netherlands, Israel and Japan, suggesting a

global distribution. The insertion appeared to have some correlation with mating type in the isolates included in my study, occurring in four out of eight isolates of A2 mating type, but in only one out of 15 isolates of A1 mating type. However Goodwin, who examined considerably more isolates, found the inserted mitochondrial genome to occur equally in both mating types.

Croft (1987) found that the major difference in mtDNA genomes of closely related *Aspergillus* spp. in the "nidulans" group was due to the presence or absence of optional introns in certain regions of the genome. Optional introns are likewise found in the mitochondrial genomes of *Neurospora* (Collins & Lambowitz, 1983). It will be interesting to determine if the insertion found in Type II *P. infestans* mtDNA also represents an optional intron.

Additional polymorphisms revealed by *MspI* (in Type I mtDNA) and *HhaI* (in Type II mtDNA) are probably due to single base changes, or to very small insertions or deletions that do not significantly alter the overall mtDNA genome size. It is noteworthy that the *MspI* polymorphism in Type Ib mtDNA was not found in any of the Type II mtDNAs, suggesting that the insertion occurred in a Type Ia mtDNA. Analysis of the mitochondrial bands in *MspI* digests of genomic DNA from 67 additional isolates, obtained from Wales, California, Egypt, Italy and the Netherlands, has not revealed the *MspI* polymorphism within any of the 19 Type II mtDNAs found in this survey (R. Folkertsma, pers. comm.).

All four of the isolates with Type Ib mtDNA were of A1 mating type, and had the same alleles at the Gpi-1 and Pep loci. Furthermore, for the two tested, there was only one difference in virulence profiles. This suggests that these four isolates, although obtained from different countries (Brazil, Ireland, U.K. and U.S.A.) are closely related and may be sexually isolated from other strains. Analysis of nuclear RFLPs supports this suggestion, and this will be discussed further in Chapter 4.

The Type IIb polymorphism was found only in the Californian isolate, Ca65. Further work on^{an} additional 11 isolates from California indicated that all have mitochondrial genomes with this polymorphism (R. Folkertsma, pers. comm.). Isolates of *P. infestans* from this region thus appear to be clonal in origin. Type IIb mtDNA was not found in any of an additional 56 non-Californian global isolates tested, indicating that the *HhaI* polymorphism probably arose in California, and that the export of *P. infestans* from this region has been minimal.

Two additional polymorphisms were found by Goodwin (1990), who labelled the mtDNAs containing them as C and D forms. The C form was found in a single Peruvian isolate. This appeared to have an insertion of about 1.5 kb in *EcoRI* fragment 5 (see Fig. 3.7), but was otherwise identical to the A (uninserted) form of mtDNA. The D form appeared to be a combination of the A and C forms, and contained two additional bands of unknown origin that were not found in any other isolates. Goodwin was unsure as to whether these bands were of mitochondrial origin or whether they were attributable to plasmids with some similarity to mitochondrial sequences. The D form was found in a number of isolates from central Mexico and Peru.

The degree of polymorphism found in the mtDNA of *P. infestans* appears to be intermediate between that found for two other Oomycetes, *Bremia lactucae* and *P. megasperma*. Mitochondrial polymorphisms were only rarely observed in a survey of 25 isolates of *B. lactucae* representing diverse geographical origins (Hulbert & Michelmore, 1988). Twenty-two of the isolates from Europe and the U.S.A. exhibited the same pattern in each of eight different restriction endonuclease digests. A second pattern was shown by two Australian isolates, and a third pattern in an isolate from Japan. In contrast, among 43 isolates of *P. megasperma*, mainly from the U.S.A and Canada, 12 different mtDNA pattern types were observed using six restriction endonucleases (Forster *et al.*, 1989). This probably reflects the greater

diversity of isolates currently classified as *P. megasperma*. The mtDNA profiles clearly distinguished *P. megasperma* f. sp. *glycinea* and *P. megasperma* f. sp. *medicaginis* and suggested that several additional subgroups, possibly biological species, are present in the *P. megasperma* complex.

These results, and those of Goodwin (1990), suggest that mtDNA polymorphisms could be useful for epidemiological studies. A total of six types of polymorphisms have been found, and it is likely that further polymorphisms will be identified with additional restriction endonucleases. Preparation of mtDNA is laborious, involving growth of large amounts of mycelium and density gradient separation. Attempts to minimise the amount of mtDNA used per restriction analysis by end-filling with ^{32}P -dCTP (Maniatis, Sambrook & Frisch, 1982) were unsuccessful due to contamination of the mtDNA sample with nDNA (results not shown). Likewise, hybridisation of digested genomic DNA from *P. infestans* with purified radiolabelled mtDNA did not give clear bands because of contaminating radiolabelled nDNA (eg. see Fig. 3.12. Only the mtDNA bands that are larger than the bulk of digested nDNA are clear). By cloning regions of the mtDNA known to be polymorphic, however, specific probes could be produced to detect mtDNA polymorphisms in small scale preparations of genomic DNA. PCR amplification using oligonucleotide primers flanking the polymorphic regions could likewise be used to screen for these RFLPs. The Type Ia, Ib IIa and IIb mtDNAs can all also be distinguished by *MspI* or *HhaI* digests of genomic DNA, although any additional polymorphisms may go unnoticed in such crude preparations.

CHAPTER 4 : RFLPs IN NUCLEAR DNA.

4.1 DNA isolation and restriction endonuclease digestion

Nuclear DNA from the 24 global isolates was obtained from CTAB extractions followed by CsCl-bisbenzimidazole density gradients, and was readily digested with a number of restriction endonucleases. Figure 4.1 shows the digestion profiles produced by restriction with *Hind*III. Banding patterns seen in the digested DNA lanes are conserved among all the isolates and are due to highly repetitive DNA sequences.

4.2 Production and analysis of recombinant clones.

Ligation of *Hind*III-digested nDNA from isolate 550 into the *Hind*III site of pUC19 resulted in recombinants with inserts ranging from 500 bp to 7 kb. 160 of these were dot-blotted onto Hybond N+, denatured, and probed with radiolabelled *P. infestans* nDNA. The resulting autoradiograph is shown in Figure 4.2. Clones producing an intense signal following autoradiography are likely to contain inserts of repetitive DNA, whereas those producing a very faint signal should have inserts that occur only once or a few times within the *P. infestans* genome. The copy number of insert sequences in the recombinant clones that produced faint signals on the dot-blot was further determined by hybridising a blot of *Hind*III-digested nDNA from isolate 550 with these clones. As the insert sequences do not contain *Hind*III sites (established by digesting the clones with *Hind*III) they should detect a single band if they are single copy and 550 is homozygous at this locus.

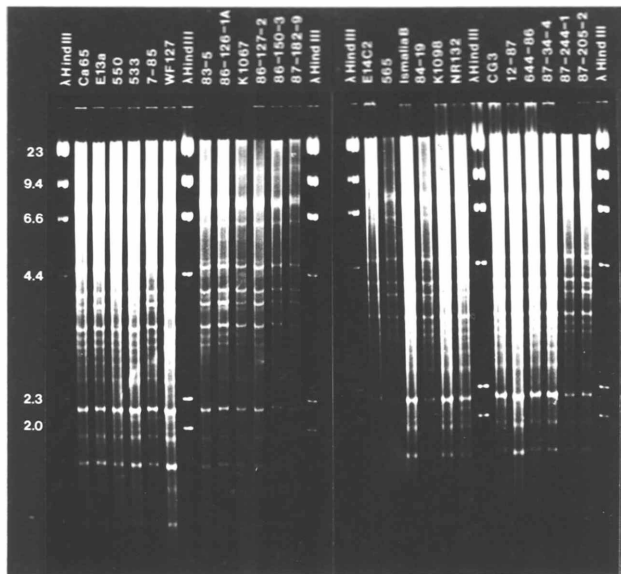


Figure 4.1 : Agarose gel electrophoresis of nDNA from *P. infestans* digested with *HindIII*.

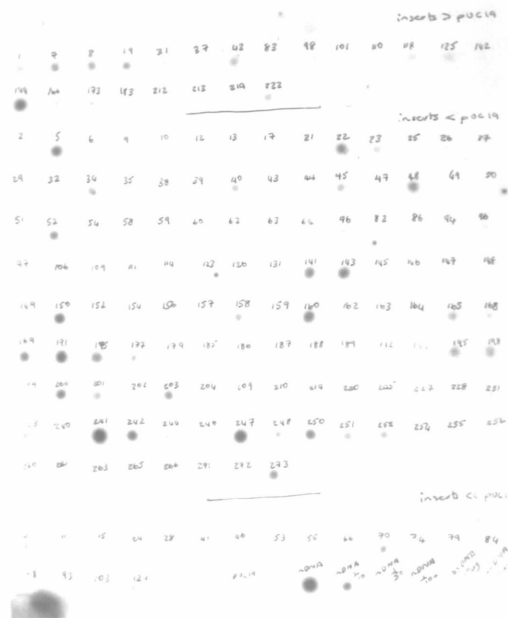


Figure 4.2 : Dot-blot of *P. infestans* clones, hybridised with radiolabelled nDNA.

4.3 RFLP analysis of isolates.

pIN1, which has an insert size of 3.5 kb and gave a faint signal on the dot-blot autoradiograph, was initially selected to screen for polymorphisms among the 24 isolates. The resulting autoradiograph is shown in Figure 4.3, where a considerable number of bands have hybridised with the cloned sequence, indicating that it contains DNA of a moderately repetitive nature. This clone reveals a high degree of nuclear DNA polymorphism between the 24 isolates which could be useful for "typing" closely related isolates or for assessing variation within a limited geographical area.

Less complex hybridisation patterns were sought using low copy number or single copy sequence probes. pIN41, which contained a 0.5 kb sequence, gave a limited number of bands when hybridised with *MspI* digested nDNA and allowed a preliminary grouping of the isolates based on shared hybridisation patterns (Figure 4.4, Table 4.1).

More informative than the low copy number probes, however, were those that detected only a single sequence within the *P. infestans* genome. These enabled a further grouping of the isolates and allowed putative genotypes at the hybridising locus to be constructed. They also provided information on the level of heterozygosity and on the existence of polyploidy or heterokaryosis among the isolates.

pIN2 contains a 1.8 kb insert of single copy DNA, and revealed a number of polymorphisms when hybridised with *MspI* or *BglII* digests of DNA from the 24 isolates (Figures 4.5 & 4.6). Hybridisation with *MspI*-digested nDNA (Figure 4.5a) gave three main bands, each of which could exist either independently or in combination. An explanation for the generation of these bands in terms of different combinations of *MspI* sites is given in Figure 4.5b. Possible bands with their sizes in kilobases are given on the left, and

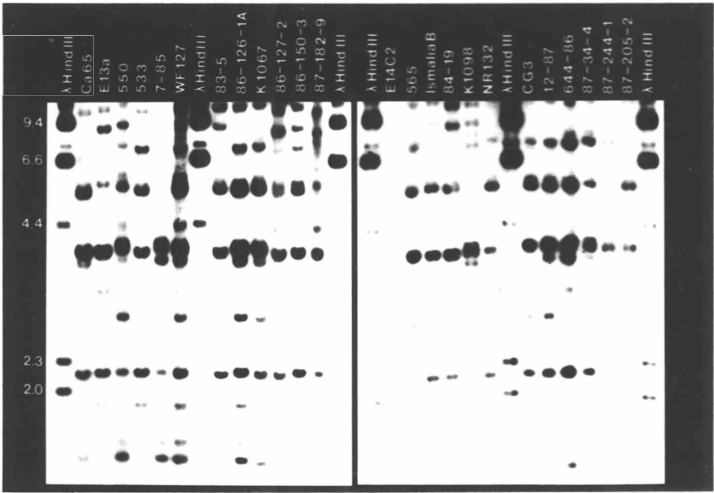
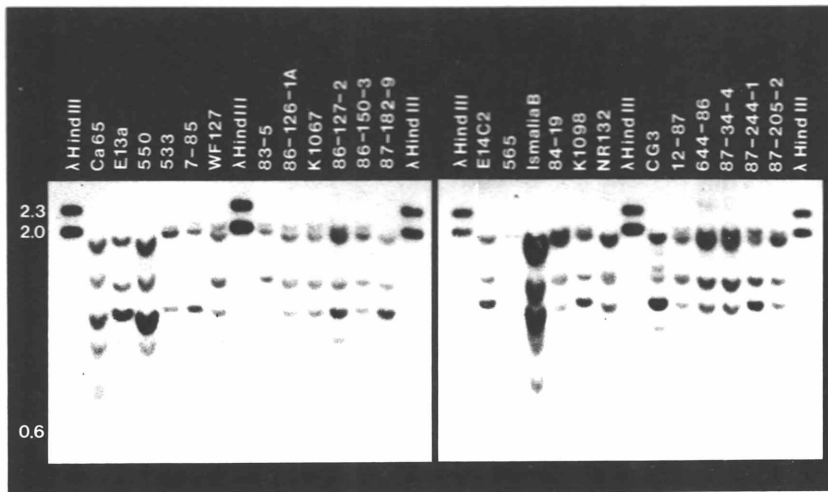
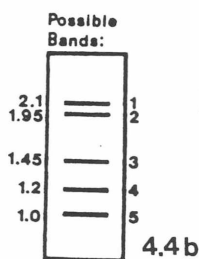


Figure 4.3 : Southern blot of *Bgl*II-digested nDNA from *P. infestans* isolates, hybridised with recombinant clone pIN1.



4.4a



4.4b

Figure 4.4 : a) Southern blot of *MspI*-digested nDNA from *P. infestans* isolates, hybridised with recombinant clone pIN41.
b) Diagram showing size and numbering of bands detected in a).

Table 4.1 : Grouping of isolates according to *MspI* fragments which hybridise with pIN41.

¹ HYBRIDISING BANDS	ISOLATES
2 + 4 + 5	Ca65, E13a, 550, 87-182-9, E14C2, 565, Ismailia B
1 + 2 + 4 + 5	86-126-1A, K1067, 86-127-2 86-150-3, 84-19, K1098, 12-87 644-86, 87-34-4, 87-244-1 87-205-2, WF127
1 + 2 + 3 + 4	83-5
2 + 3 + 4 + 5	NR132, CG3
1 + 2 + 3 + 4 + 5	533, 7-85

¹ The bands are numbered as in Figure 4.4b. Only the first five higher molecular weight bands have been considered.

are numbered from the largest (slowest running on the gel) to the smallest (fastest running). The region of the DNA with which the clone hybridises is shown schematically in the centre of the diagram. The open box represents the region on the chromosome homologous to the pIN2 insert sequence. The polymorphic *MspI* sites within and flanking this sequence are designated M1 to M4. Band 3 is 1.7 kb, slightly smaller than the clone 2 insert which must therefore contain at least one *MspI* site. The remaining 0.1 kb probably hybridises with fragments too small to blot efficiently, as only faint bands were seen at the bottom of the autoradiograph. In band 2, the M2 site has been lost and the radiolabelled insert now detects a fragment of 2.1 kb. Likewise the band 1 phenotype of 2.7 kb is seen when both the M2 and M3 sites are lost.

Genotype designations for each of the band phenotypes are given on the right of the diagram, expressed as the presence (+) or absence (-) of the *MspI* sites. Sites outside the region hybridising with the radiolabelled probe which cannot be determined are designated $\pm$.

As *P. infestans* is a diploid organism, isolates can give rise to a single band (homozygous) or two bands (heterozygous) in any combination. All possible combinations of the bands are seen among the 24 isolates (see Table 4.2). Isolate 533 gives rise to band 2 plus a unique band slightly larger than band 1, which could be the result of loss of the M4 site in one chromosome of this isolate. Three of the isolates produce all three bands on the autoradiograph, indicating that they have more alleles at this locus than would be expected for a normal diploid organism. Ploidy data, (see Table 1), obtained by DAPI staining of nuclei and fluorometric comparisons (Whitticker *et al*, 1990), indicate that 83-5 and 84-19 are tetraploid, which explains the presence of the extra bands. Ismailia B, however, appears to contain nuclei of a normal, diploid content. This isolate may contain nuclei from more than one source and be functioning as a stable heterokaryon. Alternatively, it may be aneuploid and contain extra chromosomal material encompassing this locus, but of

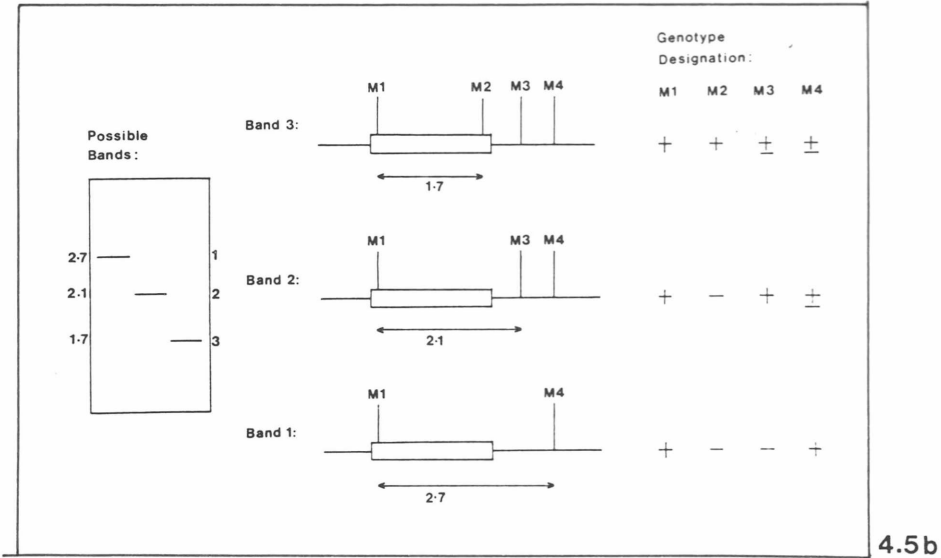
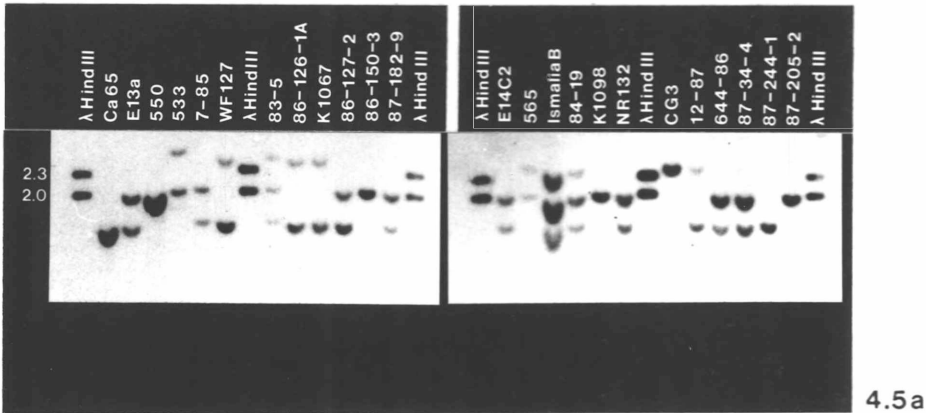


Figure 4.5 : a) Southern blot of *MspI*-digested nDNA from *P. infestans* isolates, hybridised with recombinant clone pIN2.
b) Diagrammatic model to explain the formation of bands detected in a).

Table 4.2 : Grouping of isolates according to *Msp*I fragments which hybridise with pIN2.

HYBRIDISING BANDS	ISOLATES	PROPOSED GENOTYPE
HOMOZYGOUS		
1	CG3	+ - - + / + - - +
2	550, 86-150-3, K1098, 87-205-2	+ - + + / + - + +
3	Ca65, 87-244-1	+ + + + / + + + +
HETEROZYGOUS		
1 + 2	565	+ - - + / + - + +
1 + 3	WF127, 86-126-1A, K1076, 12-87	+ - - + / + + + +
2 + 3	E13a, E14C2, 7-85, 86-127-2, 87-182-9, NR132, 644-86, 87-34-4	+ - + + / + + + +
2.8 kb band + 2	533	+ - - - + / + - + +
HYPERPLOID		
1 + 2 + 3	83-5, Ismailia B, 84-19	+ - - + / + - + + / + + + +

insufficient quantity to be noticeable by fluorimetry.

When pIN2 was used as a probe against *Bgl*II-digested nDNA from the 24 *P. infestans* isolates a more complex pattern resulted. The autoradiograph and a possible explanation are given in Figure 4.6. Isolates are again grouped according to hybridisation patterns and shown in Table 4.3. In this model, there is a *Bgl*II site (B4) which is conserved among all the 24 strains. The observed bands can be generated by polymorphisms at sites B2, B3 and B5. Isolates have been designated homozygous when both chromosomes give the same banding pattern with the pIN2 probe. Again heterozygosity predominates over homozygosity for this clone/enzyme combination. 533 and E14C2 each have a novel band larger than band 1, which probably result from polymorphism at and beyond B1 respectively. Isolates Ismailia B, 84-19 and 83-5 again demonstrate increased ploidy at this locus, as does WF127, which has also been shown previously to be tetraploid (see Table 3.1).

The results of hybridising *Msp*I-digested nDNA with two other single-copy clones, pIN29 and pIN11, are shown in Figures 4.7 and 4.8. Several bands were detected by pIN29, suggesting that the insert in this clone contains a number of *Msp*I sites. When hybridised with pIN11, seventeen of the 24 isolates gave a single band indicating that they were homozygous at this locus. The remaining seven isolates (Ca65, 533, 565, 86-126-1A, K1067, 12-87 and WF127) appeared to be heterozygous, with an additional band of 1.6 kb (1.1 kb in the case of 565). Isolates are grouped according to common banding patterns for pIN29 and pIN11, and are shown in Tables 4.4 and 4.5 respectively.

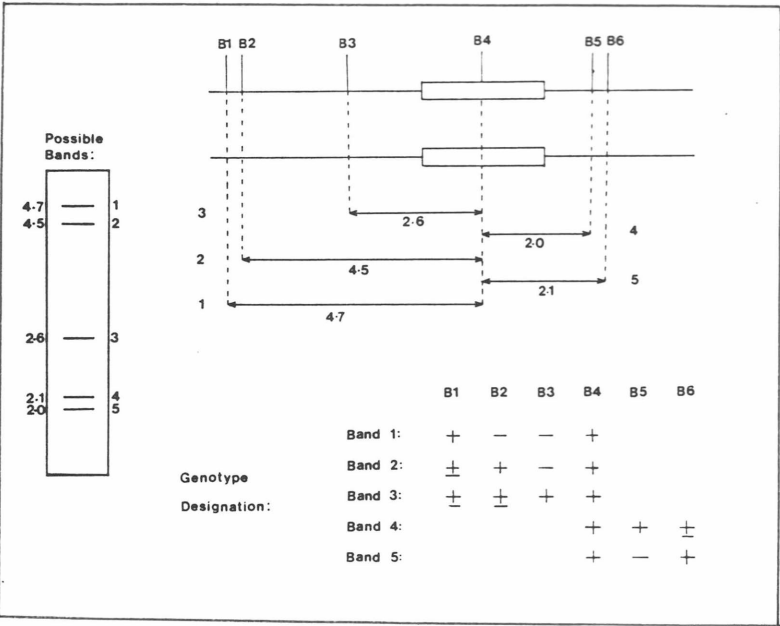
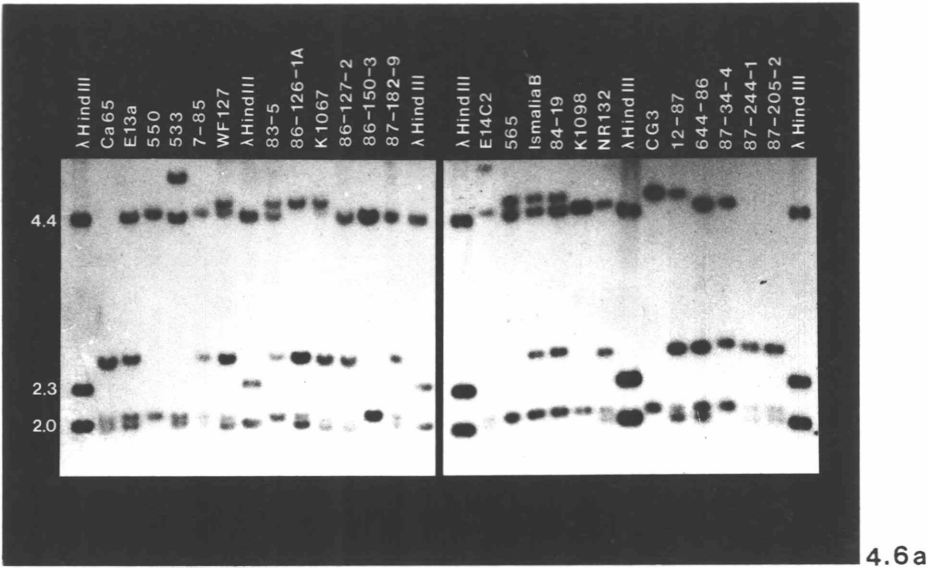


Figure 4.6 : a) Southern blot of *Bgl*II-digested nDNA from *P. infestans* isolates, hybridised with recombinant clone pIN2.
b) Diagrammatic model to explain the formation of bands detected in a).

Table 4.3 : Grouping of isolates according to *Bgl*II fragments which hybridise with pIN2.

HYBRIDISING BANDS	ISOLATES	PROPOSED GENOTYPE ^{1,2}
HOMOZYGOUS		
1 + 4	CG3	+ - - + + + / + - - + + +
2 + 4	550, 86-150-3, K1098	+ + - + + + / + + - + + +
HETEROZYGOUS		
3 + 4 + 5	Ca65, 87-244-1, 87-205-2	+ + + - - + / + + + - - +
1 + 2 + 4	565	+ - - + + + / + + - + + +
2 + 3 + 4	87-34-4	+ + - + + + / + + + - - +
1 + 3 + 4 + 5	12-87, K1067, 86-126-1A	+ - - + - + / + + + - - + OR + - - + + + / + + - + - +
2 + 3 + 4 + 5	E13a, 7-85, 86-127-2, NR132 87-182-9, 844-86	+ + - + + + / + + + - - + OR + + - + - + / + + + - - +
5.4Kb band + 2 + 4 + 5	533	(+) - - + + + / + + - + - +
5.6 Kb band + 2 + 4 + 5	E14C2	(+) - - + - + / + + - + + +
HYPERPLOID		
1 + 2 + 3 + 4	Ismailia B, 84-19, 83-5	+ - - + + + / + + - + + + / + + + + + +
1 + 2 + 3 + 4 + 5	WF 127	+ - - + - + / + + - + - + / + + + + + + / + + + + + +

¹For isolates 533 and E14C2, (+) indicates that site B1 has been displaced upstream of the region shown in Fig. 4.6b.

²There are several possibilities for the genotype of isolate WF127. This one is proposed on the relative intensities of the bands (Fig. 4.6a).

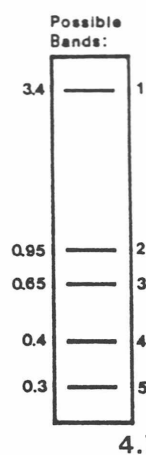
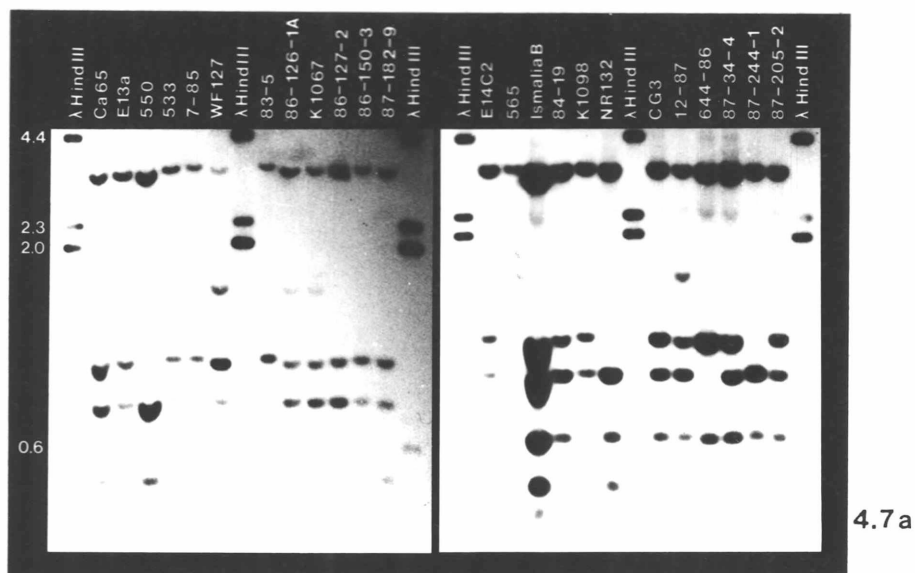


Figure 4.7 : a) Southern blot of *Msp*I-digested nDNA from *P. infestans* isolates, hybridised with recombinant clone pIN29.
b) Diagram showing size and numbering of bands detected in a).

Table 4.4 : Grouping of isolates according to *Msp*II fragments which hybridise with pIN29.

¹ HYBRIDISING BANDS	ISOLATES
1 + 3 + 4 + 5	Ca65, E13a, 533, 7-85, 83-5 86-127-2, 86-150-3, 565 87-182-9, E14C2, Ismailia B 84-19, K1098, CG3, 87-34-4 87-205-2
1 + 4 + 5	550, NR132, 87-244-1
1 + 3 + 5	644-86
1 + 2 + 3 + 4 + 5	WF127, 86-126-1A,K1067,12-87

¹Bands are numbered in order of decreasing size (see Fig. 4.7b).

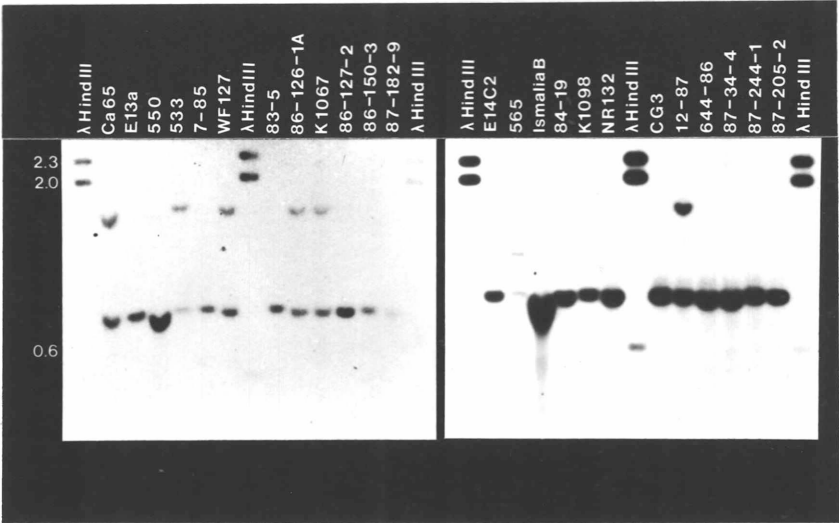


Figure 4.8 : Southern blot of *MspI*-digested nDNA from *P. infestans* isolates, hybridised with recombinant clone pIN11.

Table 4.5 : Grouping of isolates according to *MspII* fragments which hybridise with pIN11.

<u>HYBRIDISING BANDS</u>	<u>ISOLATES</u>
3	E13a, 550, 7-85, 83-5, 86-127-2 86-150-3, 87-182-9, E14C2, Ismailia B 84-19, K1098, NR132, CG3, 644-86 87-34-4, 87-244-1, 87-205-2
1 + 3	Ca65, 533, WF127, K1067, 86-126-1A 12-87
1 + 2	565

4.4 Discussion of Chapter 4.

The degree of nDNA polymorphism detected using these five different recombinant clones is sufficient to distinguish the majority of isolates from one another. 18 of the 24 isolates have unique combinations of RFLP loci. The remaining six fall into two groups. E13a (from Egypt) and 87-182-9 (from the U.K.) share identical alleles at all the loci tested. These isolates are also of the A2 mating type, are 100/100 at Gpi-1 and Pep, and share the relatively rare Type IIa mtDNA. They are obviously very closely related and probably share a common origin despite the diverse locations from which they were obtained. This emphasises the likelihood of spread of *P. infestans* through the import and export of infected tubers. It is noteworthy that Egypt obtains much of its seed potato from the U.K.

The other group of isolates that are largely indistinguishable from one another contains 86-126-1A, K1067, 12-87 and WF127. 86-126-1A, K1067 and 12-87 are identical at all the RFLP loci tested, and often share RFLPs not found in any other isolate, eg. a 1.5 kb band in the *MspI*-digested nDNA detected by pIN29 (Fig. 4.7). These isolates also contain Type Ib mtDNA and possess a combination of isozyme profiles (86-100 for Gpi-1 and 92-100 for Pep) that are also not seen in any of the other isolates included in this study. WF127, the only other isolate studied which has Type Ib mtDNA and Gpi-1: 86-100; Pep: 92-100 isozyme profiles, also has almost identical nDNA RFLP loci to the other three isolates. It differs from the other three only by an nDNA polymorphism detected with pIN2 (Fig 4.6, Table 4.3). It is likely therefore to be derived from the other isolates in this group by mutation, rather than by recombination with a different strain.

The members of this group were isolated from diverse locations (Brazil, Ireland, the U.K. and the U.S.A.) Spielman (1990), in a survey investigating

isozyme diversity found isolates with the genotype: A1, Gpi-1 86-100, Pep 92-100 among isolates from Eastern and Western Europe, Britain, the U.S.A., Peru, and Japan. It would be interesting to determine whether these also possess Type Ib mtDNA and share nDNA RFLPs with 86-126-1A, K1067, 12-87 and WF127. Interestingly, Spielman(1990) found isolates with these profiles appeared to predominate in early European isolates. New isolates, characterised by different isozyme genotypes now appear to be replacing them. In some countries, in particular Britain and the Netherlands, this replacement appears almost complete. Spielman hypothesised that a migration event, which may also have introduced the A2 mating type, was responsible for this shift in population structure. If the isolates of genotype: A1, Gpi-1 86-100, Pep 92-100 can be assumed to be similar to those characterised by Type Ib mtDNA and unique nDNA RFLPs, this supports the notion that they appear to be a sexually isolated group that are not capable of recombining with other *P. infestans* strains.

There is one important difference between 12-87, 86-126-1A, K1067 and WF127. This is their ploidy levels, which are 2C, 3C, 3C and 4C respectively. As the increase in chromosomal content does not appear associated with the introduction of new alleles (with the exception of the additional band detected by pIN2 in WF127) the higher ploidies probably result from incomplete separation following mitosis. Variation in ploidy can be seen most clearly on the Southern blot of *MspI*-digested nDNA hybridised with pIN2 (Fig. 4.5). Band 3 in 86-126-1A and K1067 is of considerably higher intensity than band 1, indicating that two of the chromosomes in these isolates have *MspI* sites M1 and M2, and the third chromosome has sites M1 and M4 and lacks sites M2 and M3. Interestingly, the signal intensity of band 3 in WF127 appears to be three times that of band 1. WF127 thus appears to contain three chromosomes with *MspI* sites M1 and M2 and one chromosome with sites M1 and M4. This indicates that tetraploidy at this locus is not due to

the presence of two identical diploid pairs of chromosomes.

Analysis of Figure 4.5 indicates unequal signal intensity between the bands of a number of other isolates. Most notable is the greater intensity of band 2 compared to band 3 in 644-86 and 87-34-4. Less obvious differences also occur in NR132 and 86-127-2, which may or may not be significant. This is probably not surprising as ploidy values greater than 2C are common in non-Mexican isolates (Tooley & Therrien, 1987; Whitticker *et al*, 1990). No ploidy data is yet available for these isolates. It would be interesting to combine a study on ploidy levels with investigation of nuclear RFLPs. This may help elucidate the mechanisms responsible for the generation of higher ploidy levels; whether by the fusion of two unlike nuclei following sexual recombination without subsequent meiotic segregation (as may have occurred in 84-19 and 83-5) or by failure of mitotic segregation causing one of the original chromosomes to be doubled or even tripled (as may have occurred in 86-126-1A and K1067, and in WF127, respectively). The presence of additional alleles in apparently diploid nuclei, together with the inheritance of cytoplasmic markers such as mtDNA, may also be used to demonstrate aneuploidy or the existence of heterokaryosis (which may be present in Ismailia B).

CHAPTER 5 : DNA MARKERS FOR GENETIC MAPPING.

5.1 Choice of parents

The ultimate aim of genetic mapping in *P. infestans* is the localisation and eventual isolation of the avirulence genes. Parents for the cross were therefore chosen with as diverse virulence phenotypes as possible : Ca65 (A1) which is virulent on differentials r0, R1, R4, and R11, and 550 (A2) which is virulent on differentials r0, R1, R2, R3, R4, R5, R7, R10 and R11 (C. Fininsa, pers. comm.). If the assumption that avirulence is dominant and is encoded heterozygously in these isolates is correct (Al-Kherb, 1988, C. Fininsa, pers. comm.) the progeny from such a cross should yield segregation information on the avirulence genes 2,3,5,7,8 and 10. Ca65 and 550 had also been used in my previous work on RFLPs in global isolates (Chapter 4) and appeared to differ at most of the loci tested. They have been found previously to generate viable progeny in large numbers (Al-Kherb, 1988) and are both known to be diploid (Tooley & Therrien, 1987, Whitticker, Shattock & Shaw, 1990). The cross and all virulence work was done in Bangor by C. Fininsa. Virulence data for the parents and all progeny used subsequently by myself are given in Table 5.1.

Note : Potato differentials R6 and R9 are no longer available, thus Avr6 and Avr9 cannot be scored.

5.2 DNA extraction.

Extraction of DNA on a small scale from the *P. infestans* progeny was complicated by an apparent co-extraction of polysaccharide material. This interfered with some restriction enzymes, especially *HindIII*, reducing and in some cases completely preventing DNA restriction. Polysaccharide contamination also made the DNA difficult to dissolve, and the contaminated DNA

Table 5.1 Segregation of avirulence and virulence in *P. infestans* hybrid F1 progeny of Ca65 x 550 to nine potato differentials.

<u>Parents and segregation</u>	<u>Potato Differentials.</u>								
	R1	R2	R3	R4	R5	R7	R8	R10	R11
Parents									
Ca65/550	+ / +	- / +	- / +	+ / +	- / +	- / +	- / -	- / +	+ / +
No. Avirulent progeny (-)	1	21	19	3	18	19	25	8	4
No. Virulent progeny (+)	40	19	22	38	21	22	14	15	27
Expected ratios	0:1	1:1	1:1	0:1	1:1	1:1	3:1	1:1	0:1
χ^2 (- : +)	0.20	0.10	0.23	0.22	0.23	0.23	2.4	0.61	0.13
Probability	>0.6	>0.6	>0.6	>0.6	>0.6	>0.6	>0.05	>0.4	>0.6

appeared prone to degradation despite storage at -20°C . CsCl density gradients, which had yielded pure and readily digested DNA following mtDNA extraction, were therefore employed. Co-precipitation of any remaining polysaccharides with DNA that had been removed from the gradient and dialysed was prevented by the use of 1 volume of isopropanol in the place of ethanol (Raeder & Broda, 1985). Approximately 25 - 50 μg of genomic DNA was thus produced from a single Petri dish of mycelia (1 - 2 g wet weight).

A gel containing DNA from each of the 80 progeny and from Ca65 and 550 digested with *MspI* is shown in Figure 5.1a. MtDNA bands, showing the profiles of Type Ia (from 550) and Type IIb (from Ca65) mitochondria can be seen faintly at the top of each lane. Hybridisation of a blot containing *BglII* digested DNA from each of the progeny and the two parents with radiolabelled pSTA99 likewise shows the segregation of the different mtDNA types (Figure 5.1b). This recombinant plasmid contains an *EcoRI* insert of mtDNA from *P. infestans* of approximately 7 kb, and hybridises with the third and fourth bands of *BglII* digested Type I mtDNA. The insert therefore probably comprises the third largest *EcoRI* mtDNA fragment (see figures 3.6 and 3.7). Band 4 is replaced in Type II mtDNA by two smaller bands. Both of these bands are detected by pSTA99, although the larger of the two hybridises weakly, indicating pSTA99 contains only a small amount of DNA with which this band is homologous.

The hybridisation profiles obtained with pSTA99 indicate that 47 of the progeny contain Type Ia mtDNA, and 33 contain Type IIb. None of the progeny appear to contain both mtDNA types. This indicates that *P. infestans*, like most other organisms studied to date, receives its mitochondria (and presumably the rest of its cytoplasmic components as well) from one parent only. There is also no evidence, on the blot or on the gel in figure 5.1, of any recombination or alteration to any of the mitochondrial genomes. Again this is

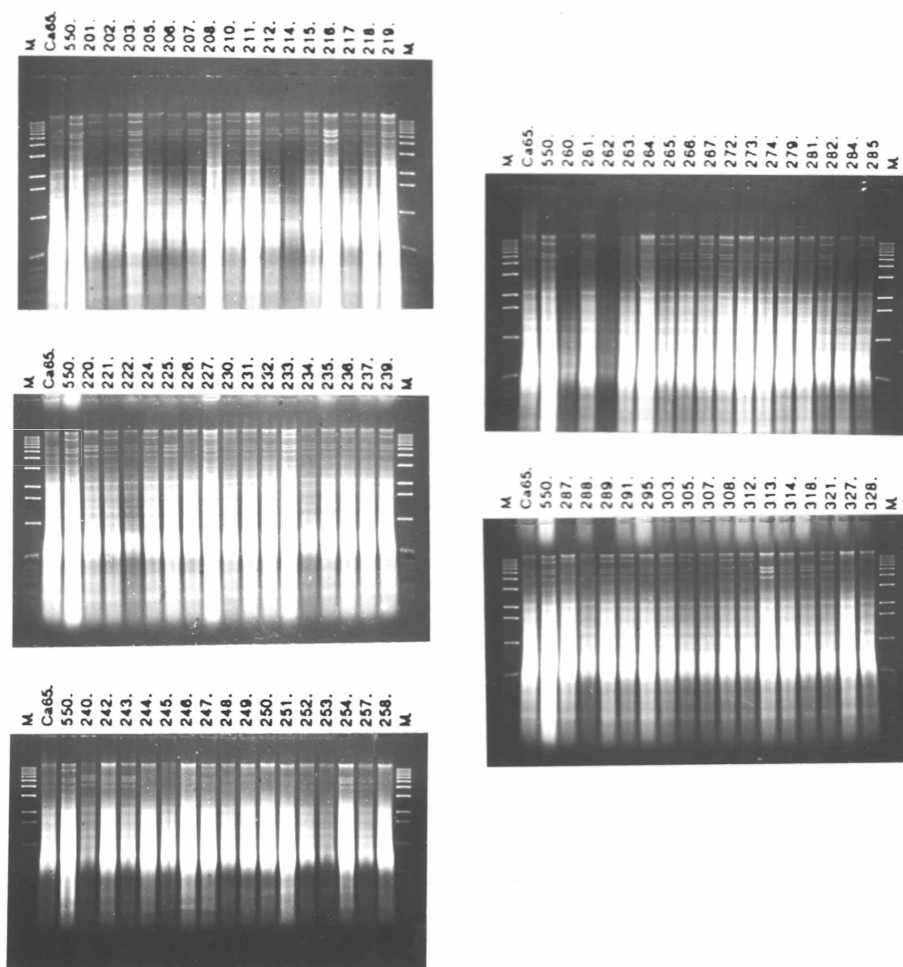


Figure 5.1a: Agarose gel electrophoresis of DNA from Ca65, 550 and 80 progeny from Ca65 x 550 cross digested with *Msp* I. MtDNA bands are visible above the bulk of digested nDNA.

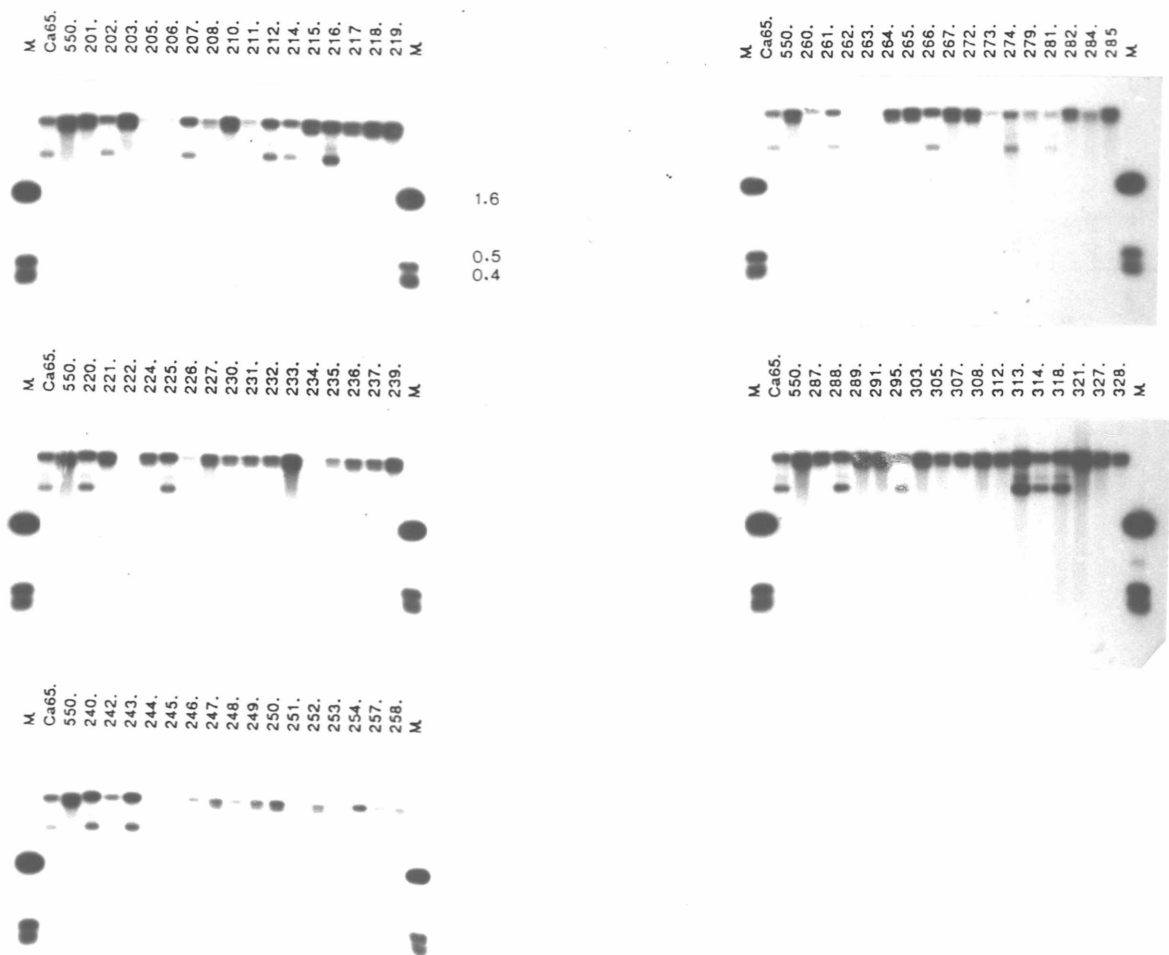


Figure 5.1 b : Southern blot of *Bgl*II- digested DNA from Ca65, 550 and 80 progeny, hybridised with pSTA99.

consistent with mitochondrial inheritance observed in most other organisms, where mtDNA is inherited as a single intact unit.

5.3 Screening for RFLP recombinants.

A new series of recombinant clones was constructed for the genetic mapping work. The clones previously constructed mostly contained inserts of 1 - 3 kb, of which the majority hybridised with repetitive DNA sequences. It was expected that smaller inserts would be less likely to contain repetitive sequences, therefore *Hind*III fragments of 0.5 kb from isolate 550 were selected and ligated into pUC19. Figure 5.2 shows a gel containing digests of the first 95 clones. A total of 725 recombinant plasmids were prepared in this manner, and were labelled pIN5-1 to pIN5-725.

A large number of these recombinants still however contained repetitive DNA. Highly repetitive clones were screened by hybridising dot-blot of the clones with radiolabelled nDNA as previously described (see Chapter 3). Subsequently, screening was done by hybridising southern blots of the undigested clones with radiolabelled nDNA. This had the advantage that the signal on the autoradiograph could be compared directly with a photograph of the original gel to ensure that a low hybridisation signal was not merely due to low concentration of plasmid DNA. An example of such an autoradiograph is shown in Figure 5.3. From this it is obvious that some recombinants contained DNA of an extremely repetitive nature. Only those that produced very faint hybridisation signals were selected for subsequent testing to find single copy, polymorphic and heterozygous RFLPs.

Establishing that the recombinant clones contained single copy sequence *P. infestans* DNA was done by hybridising the radiolabelled insert to a blot containing DNA from isolate 550 fully digested with *Hind*III. Ca65 DNA digested with *Hind*III, as well as DNA from both 550 and Ca65 digested with

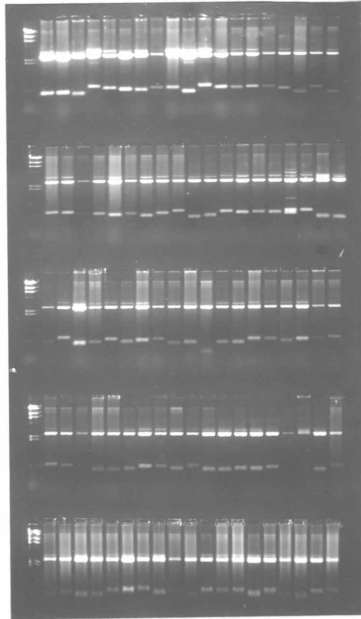


Figure 5.2 : Agarose gel electrophoresis of 95 pUC19 recombinant clones containing 0.5 kb inserts of *P. infestans* DNA from isolate 550. First lane of each row contains size standards (lambda phage DNA digested with *Hind* III).

Row 1 : pIN5-1 - pIN5-19
 Row 2 : pIN5-20 - pIN5-38
 Row 3 : pIN5-39 - pIN5-57
 Row 4 : pIN5-58 - pIN5-76
 Row 5 : pIN5-77 - pIN5-95

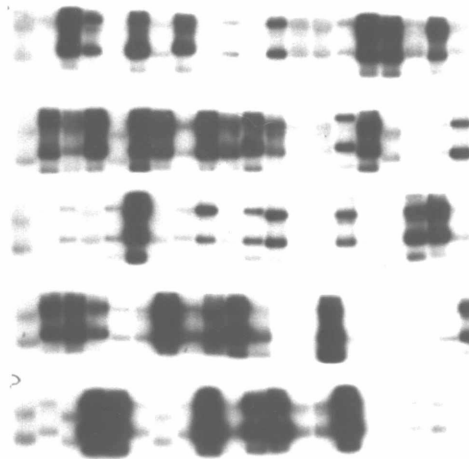


Figure 5.3 : Southern blot of undigested recombinant clones containing 0.5 kb inserts of *P. infestans* DNA from isolate 550 hybridised with radiolabelled *P. infestans* DNA. The first lane in each row contains 0.25 ug of undigested pUC19 DNA.

Row 1 : pIN5-520 - pIN5-531; pIN5-533 - pIN5-539
 Row 2 : pIN5-540 - pIN5-546; pIN5-548 - pIN5-559
 Row 3 : pIN5-560 - pIN5-573; pIN5-575 - pIN5-579
 Row 4 : pIN5-580 - pIN5-598
 Row 5 : pIN5-599 - pIN5-601; pIN5-603 - pIN5-618

*Bgl*II and *Msp*I were also included on this blot. This allowed concomitant screening for polymorphisms between the two parents with these three enzymes. Typical autoradiographs of some of these "test blots" are shown in Figure 5.4. pIN5-6 contains a single copy DNA sequence, as it produces a single 0.5 kb band with *Hind*III. It appears to be polymorphic with *Bgl*II. pIN5-13 likewise contains single copy DNA and is polymorphic with *Msp*I. pIN 5-14 contains single copy DNA but is not polymorphic with any of these enzymes. The insert sequence in pIN5-7 has hybridised with a number of bands in the *Hind*III lanes and therefore contains DNA of a moderately repetitive nature.

A considerable number (around 40%) of the recombinants containing single copy DNA detected RFLPs between the two parent isolates. Non-polymorphic single copy inserts were further tested using *Eco*RV and *Taq*I. In only a single case, however, was a polymorphism revealed in the *Eco*RV digest using a recombinant that was not polymorphic with *Hind*III, *Bgl*II or *Msp*I. *Taq*I, like *Msp*I contains a CpG residue in its cutting site which is prone to C to T transitions following methylation (Coulondre, 1978). This enzyme is used extensively in RFLP studies in humans, but did not reveal any additional polymorphisms in the *P. infestans* genome. *Taq*I also cleaved the *P. infestans* DNA into very small fragments which did not appear to blot efficiently. Only *Hind*III, *Bgl*II and *Msp*I were therefore used in the subsequent production of blots for RFLP mapping.

All the progeny used in this genetic mapping work were of F1 generation, therefore as well as being polymorphic at least one of the parents must also be heterozygous at the RFLP locus under study. Sometimes this could be determined from hybridisation intensities on the test blots, for example pIN5-13 in Figure 5.4. Other hybridisation patterns were less obvious however, and in this case the recombinant clone was first tested on a small number of

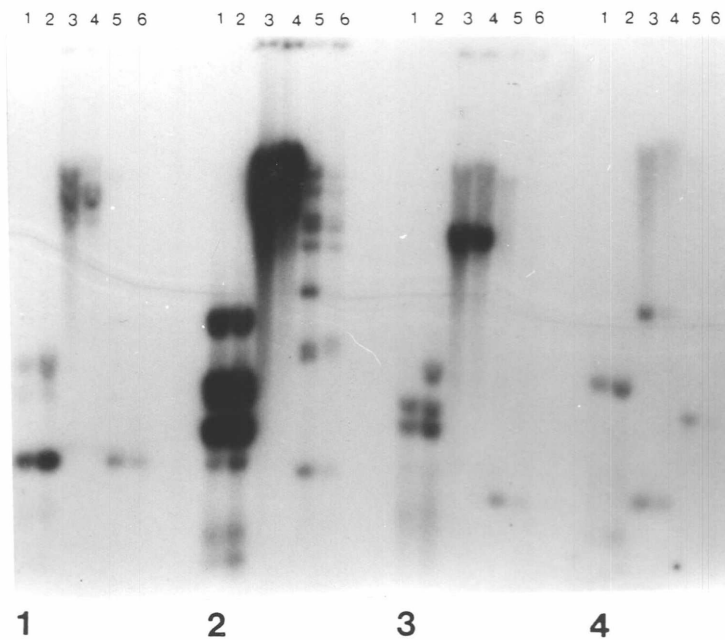


Figure 5.4 : Southern blots of DNA from *P. infestans* isolates Ca65 and 550 digested with *Msp* I, *Bgl* II and *Hind* III. These "test blots" were developed to screen for single copy sequence inserts in the recombinant clones, as well as testing for polymorphism between the parental isolates. The lanes on the four blots are the same :

Lane 1 : 550 DNA digested with *Msp* I
 Lane 2 : Ca65 DNA digested with *Msp* I
 Lane 3 : 550 DNA digested with *Bgl* II
 Lane 4 : Ca65 DNA digested with *Bgl* II
 Lane 5 : 550 DNA digested with *Hind* III
 Lane 6 : Ca65 DNA digested with *Hind* III

Blots have been hybridised with different radiolabelled *P. infestans* recombinant clones :

Blot 1 : pIN5-6
 Blot 2 : pIN5-7
 Blot 3 : pIN5-13
 Blot 4 : pIN5-14

progeny before being applied to the whole sample. pIN5-611 is an example of a "null" allele, being present in 550 and entirely absent in Ca65. If this allele is encoded homozygously in 550, all progeny will receive a single copy of it and will therefore all be identical. When hybridised with a progeny blot, it appeared to occur in some of the progeny but not in others (Figure 5.5). This RFLP thus occurs heterozygously, it segregates in the progeny, and it can therefore be used as a marker in linkage analysis. As noted in Chapter 3, heterozygosity appears to predominate in *P. infestans* isolates, and the majority of clones that were polymorphic were also heterozygous with one or other, or occasionally both, parents.

The amount of repetitive DNA in the *P. infestans* genome appears to be extremely high. These results indicate it to be in the order of a least 90%, which is even greater than the 80% reported in *Bremia* (Hulbert & Michelmore, 1988). Perhaps this is not surprising, given the very high DNA content of *P. infestans*. This, combined with the requirement for polymorphism and heterozygosity meant that only 14 of the 725 recombinant clones gave ideal RFLPs for genetic mapping. A further three clones contained insert DNA of a moderately repetitive nature, but appeared to produce RFLPs that segregated to single loci. Two clones, pIN11 and pIN 29, were isolated previously (see Chapter 3). This gave a total of 19 RFLP markers to be used in addition to 6 avirulence genes in the construction of a genetic linkage map of *P. infestans*.

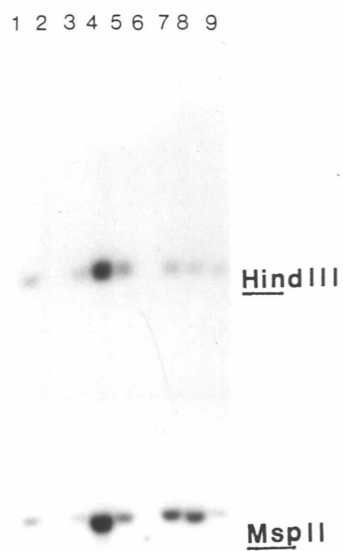


Figure 5.5 : Southern blot containing progeny from the cross between 550 and Ca65, digested with *Hind* III (top) and *Msp* I (bottom) and hybridised with recombinant clone pIN5-611.

Lane 1 : 281
Lane 2 : 282
Lane 3 : 284
Lane 4 : 285
Lane 5 : 287
Lane 6 : 288
Lane 7 : 289
Lane 8 : 291
Lane 9 : 295

5.4 Constructing the linkage map.

5.4.1 Autoradiograph of the RFLPs.

Figures 5.6 - 5.21 show the autoradiographs obtained when blots containing restriction endonuclease digested DNA from the parents and progeny were hybridised with the RFLP-detecting sequences. Autoradiographs of clones pIN5-65, pIN5-599, pIN5-611 and pIN5-725 were too poor to reproduce well in a photograph and have been omitted. Data for each RFLP against each parent is given in Appendix 2, expressed as an 0 when the band is absent and a 1 when the band is present. Bands in the figures are encoded numerically, from the largest (slowest migrating on the gel) to the smallest (fastest migrating).

The autoradiograph in Figure 5.6 was produced by hybridising a blot of *MspI* digested DNA with pIN2, a recombinant plasmid which detects single homozygous bands of different sizes in Ca65 and 550 (see Figure 4.5). Each of the 80 progeny should therefore receive one allele from each parent and be heterozygous at this locus. While this is true for the majority of progeny, 222, 272 and 307 appear to have received only a single band. Isozyme data previously established that 222 was the product of self-fertilisation of the Ca65 parent, as it produced a Gpi-1 profile of 100-100. 272 and 307 however both had hybrid Gpi-1 profiles of 86-100. Gpi-1 analysis was conducted a considerable time before DNA isolation. It is possible, therefore, that these progeny were originally a mixture of two different mycelia, or were a single mycelium containing more than one type of nucleus, with different Gpi-1 alleles specified in the different component mycelia or nuclei. During subculture, these may have segregated, so that one mycelial or nuclear type became dominant and eventually replaced the other.

222 and 307 are identical to Ca65 and 550 respectively at every other RFLP locus examined, therefore do not appear to have undergone recombination following self-

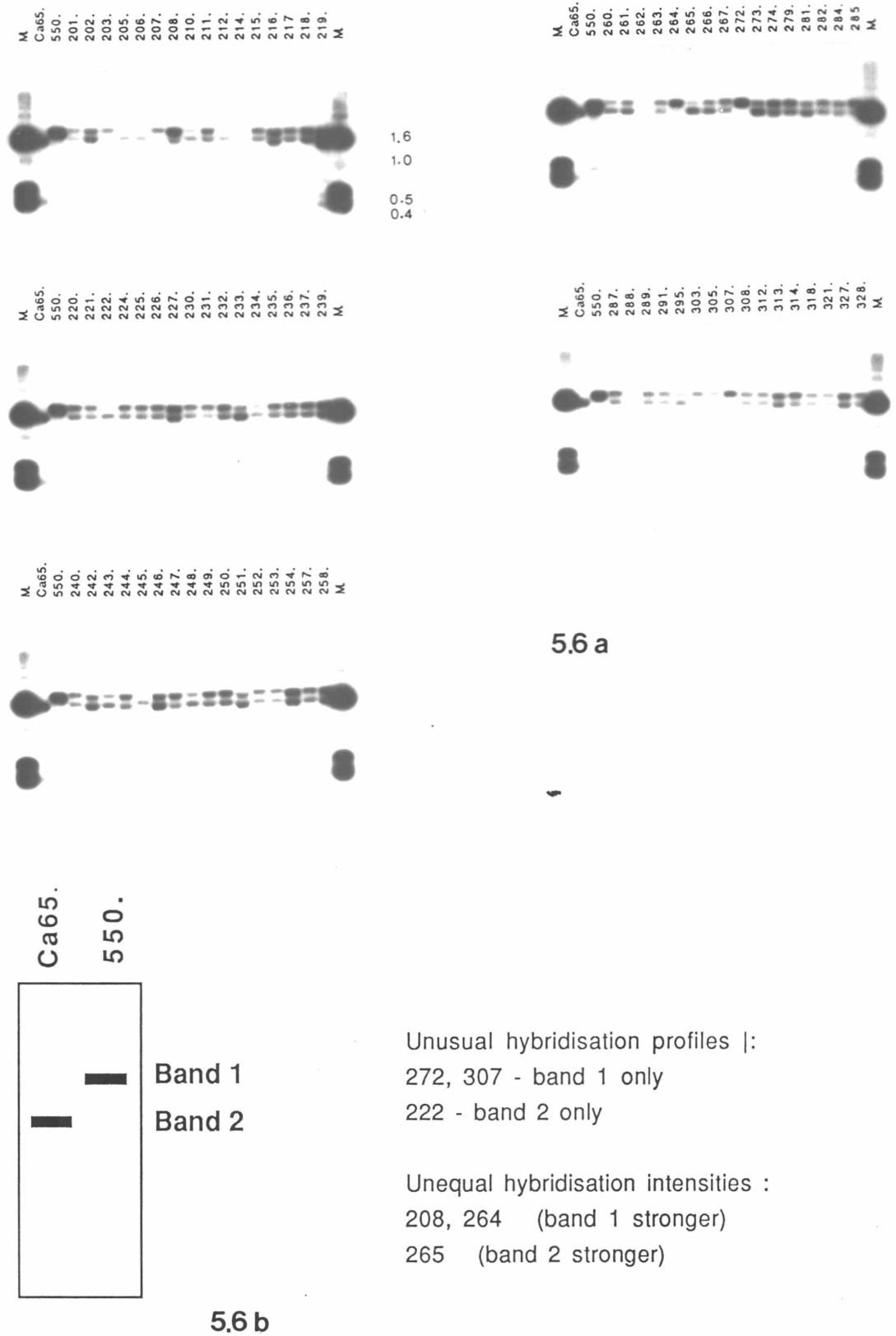


Figure 5.6 : a) Southern blot of *Msp*I-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN2.
 b) Diagram showing numbering of bands detected in a).

fertilisation and have merely regenerated their respective parental types. The exception to this is the avirulence of 307 on potato differential R11. A number of other progeny are also avirulent at this locus, which is unexpected due to the virulent phenotypes of both parents. The avirulence of 307 therefore more likely reflects the instability of virulence at R11, rather than a genetic recombination event.

Unlike 222 and 307, 272 is homozygous at a number of loci at which 550, the parent with which it is identical at L2, is heterozygous. 272 thus appears to be the product of "true" self-fertilisation, resulting from the fusion and recombination of haploid oogonial and antheridial nuclei from 550.

Also apparent on the autoradiograph in figure 5.6 are a few progeny in which one band is of considerably greater signal intensity than the other, most notably 208 and 264, where band 1 is stronger, and 265 where band 2 is stronger. A number of other progeny have slightly unequal intensities in their two bands, which may or may not be significant. These unequal inheritance ratios are unexpected in normal meiotic fusion of single male and female gametic nuclei, and indicate that an extra dosage of the L2 alleles may exist at this locus in these progeny. This could occur locally, by a duplication event, or more generally via aneuploidy or even polyploidy. Progeny exhibiting significantly altered inheritance ratios both with pIN2 and with RFLP-detecting probes used subsequently have been omitted from the linkage analysis. These unequal inheritance profiles do however provide an interesting insight into the nature of meiotic recombination in *P. infestans*. This feature will be discussed more fully later.

Figures 5.7 to 5.21 show the autoradiographs of probe hybridisation at most of the polymorphic loci that were informative for genetic linkage. These are ordered according to the number of alleles at each locus that were detected by the radiolabelled probe.

In Figure 5.7, pIN5-199 hybridises with two bands that are shared by both

parents. Analysis of the inheritance of these bands in the progeny indicates that they are heterozygous alleles at a single locus. Heterozygous progeny are uninformative genetically as the origins of each of their two bands cannot be ascertained, and only homozygous progeny are useful with this probe for mapping analysis.

In Figures 5.8 to 5.16, two alleles are again detected at each polymorphic locus, but one of the parents is homozygous and the other is heterozygous. All progeny are informative for these RFLPs, as the inheritance of the specific band phenotypes from each parent is clear and unambiguous.

With these types of RFLPs, all progeny should be identical to one or other of the two parents. While this is generally the case, some anomalies do occur, and the progeny expressing these are listed alongside the figures. Generally, anomalies are seen as the predominance of one of the two bands over the other (for example, band 1 in the lanes containing DNA from progeny numbered 243 and 251 when hybridised with pIN11; Figure 5.8). Hybridisation with recombinant clone pIN5-362, however, appears to result in a band in four of the progeny (201, 295, 303 and 312) which does not occur in either of the parents (Figure 5.16). These new bands are slightly weaker than the band below them (band 2) which the four progeny also share. It is unlikely, however, that they result from incomplete digestion of the DNA as no corresponding anomalies occurred when the same blot was probed with different clones. It is also not very likely that the four progeny share a new RFLP resulting in this new band. In case other less obvious anomalies occur, these four progeny have all been omitted from the genetic analysis.

In Figures 5.17 to 5.20, the cloned DNA fragments now detect three alleles at each single locus. In Figures 5.17 and 5.18, Ca65 is homozygous and 550 heterozygous at loci L5-65 and L5-216 respectively, so that all progeny receive the Ca65 alleles and receive one or other allele from 550. pIN5-65 again shows significant inequalities in hybridisation in some of the progeny between the bands inherited from Ca65 and those from 550.

Hybridisation with pIN5-216 produces a number of bands, most of which are

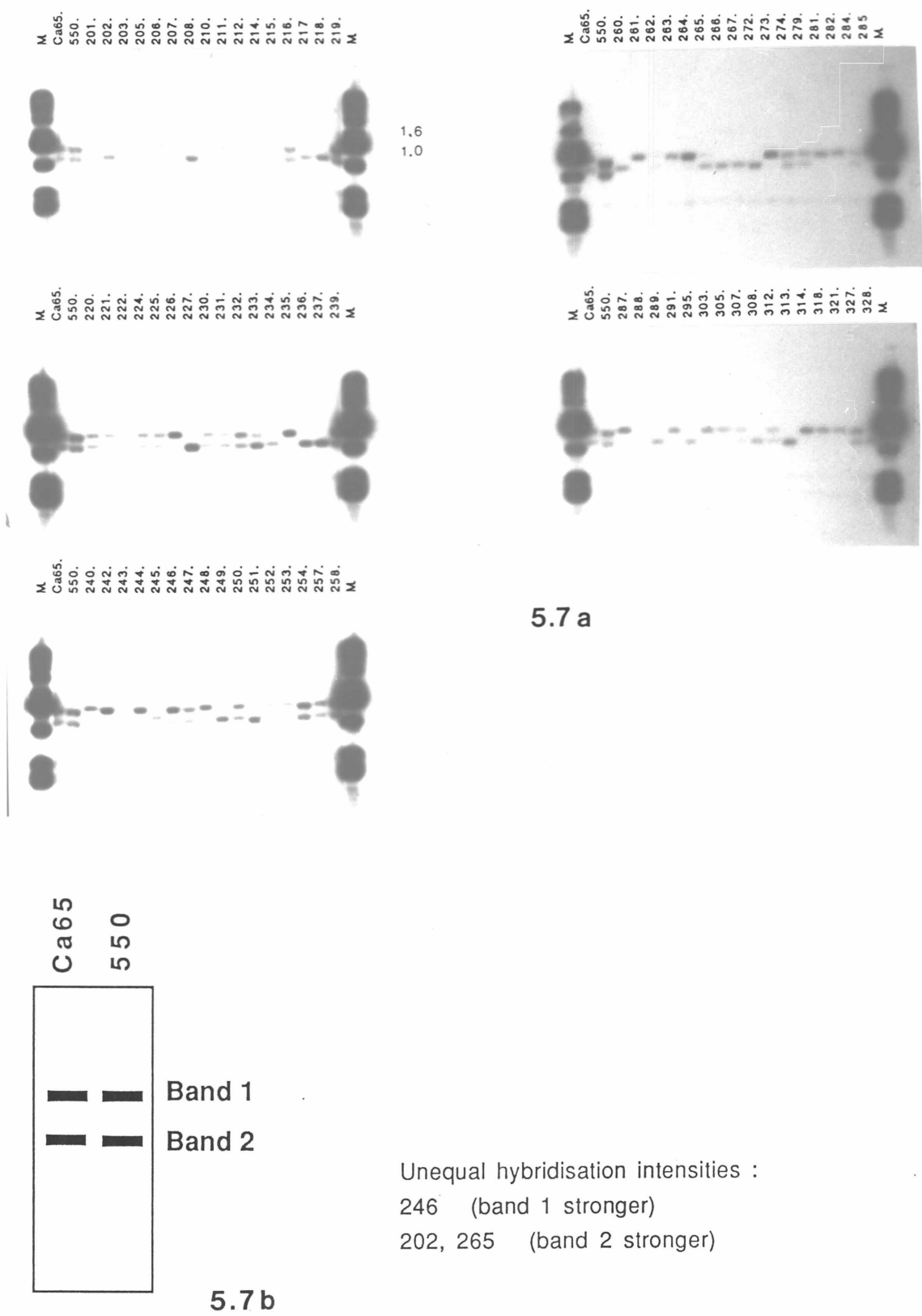
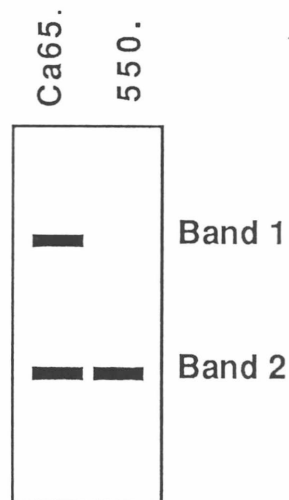
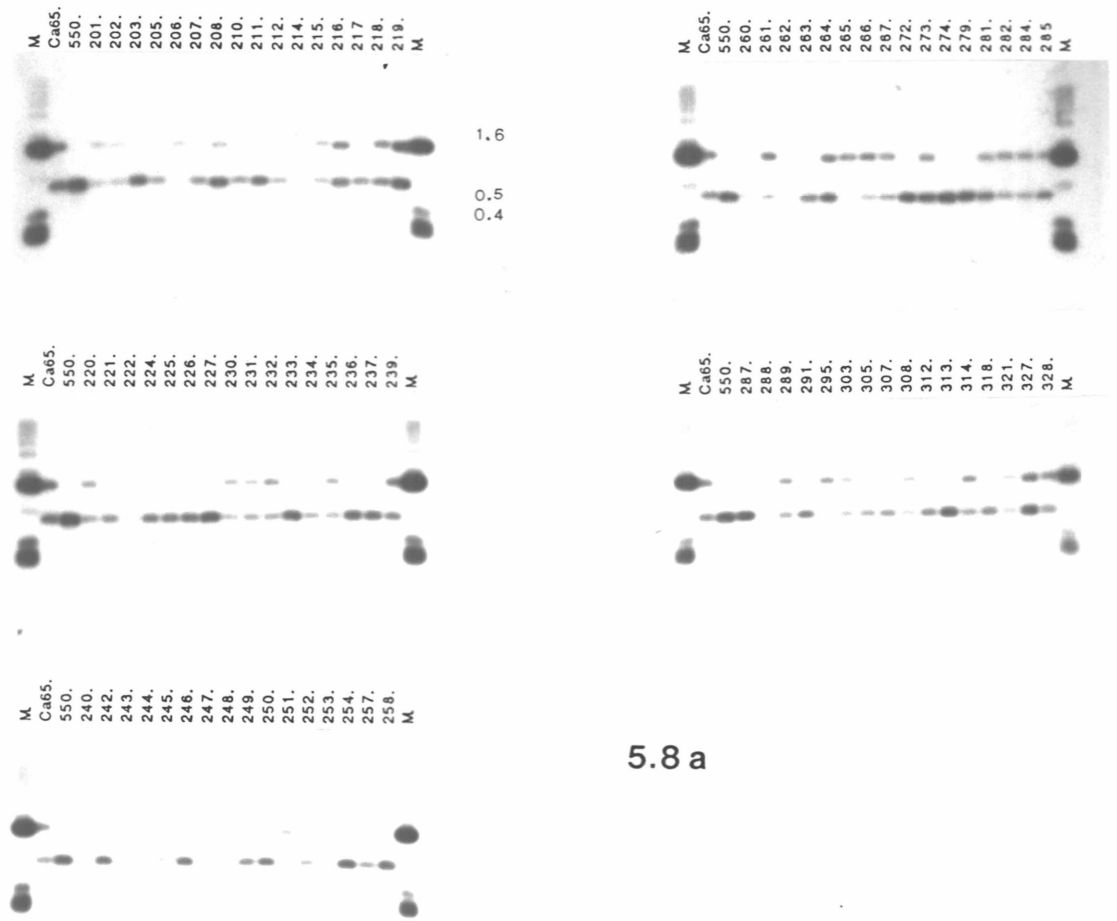


Figure 5.7 : a) Southern blot of *Msp*I-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-199.
b) Diagram showing numbering of bands detected in a).

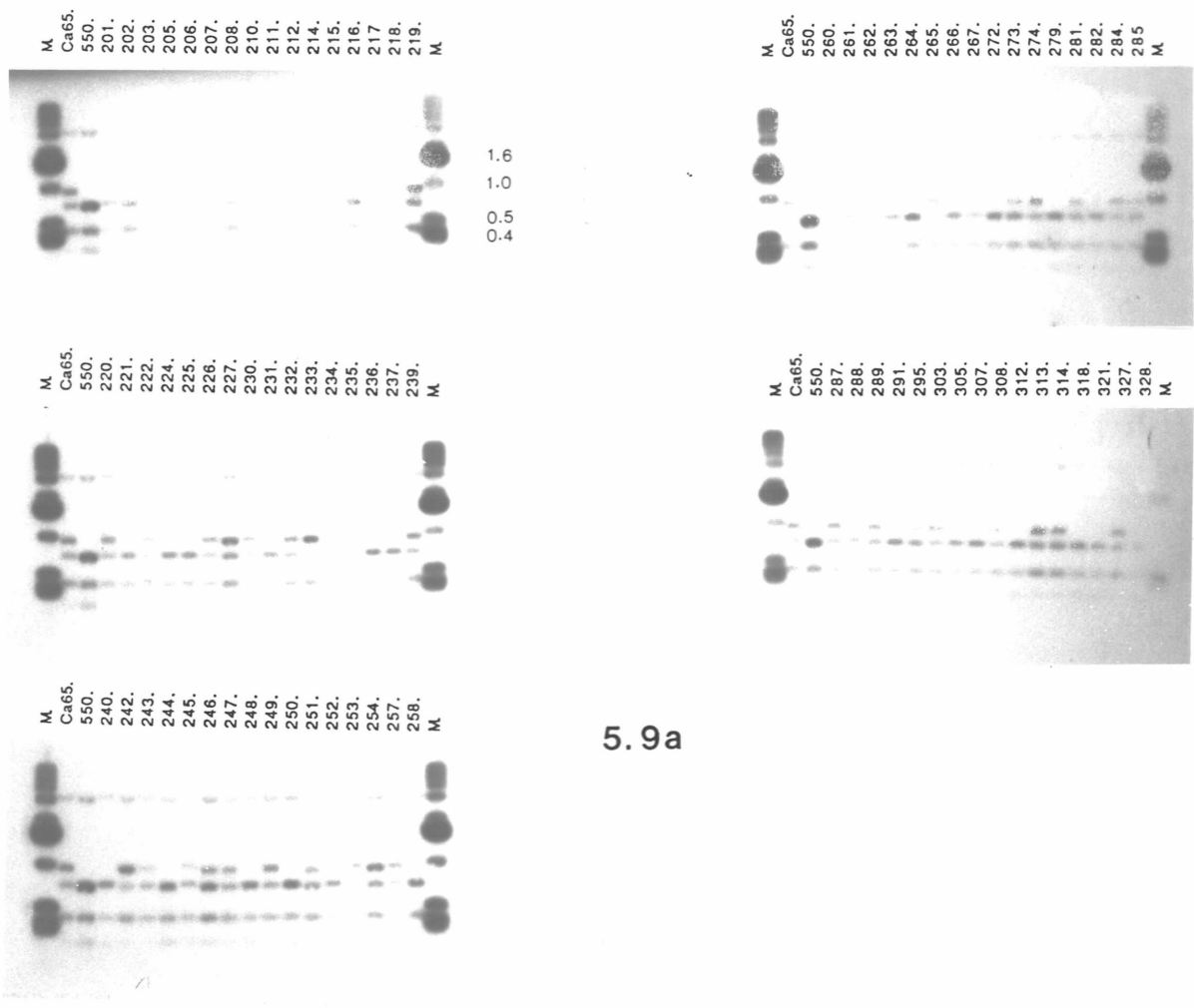


Unequal hybridisation intensities :

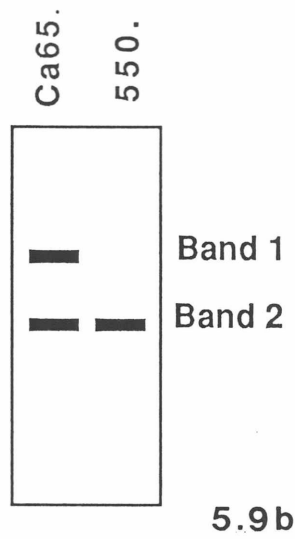
261, 265 (band 1 stronger)

5.8 b

Figure 5.8 : a) Southern blot of *Msp*I-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN11.
b) Diagram showing numbering of bands detected in a).



5.9a



Unequal hybridisation intensities :
233, 242, 265 (band 1 stronger)

5.9b

Figure 5.9 : a) Southern blot of *Msp*I-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN29.
b) Diagram showing numbering of polymorphic bands detected in a).

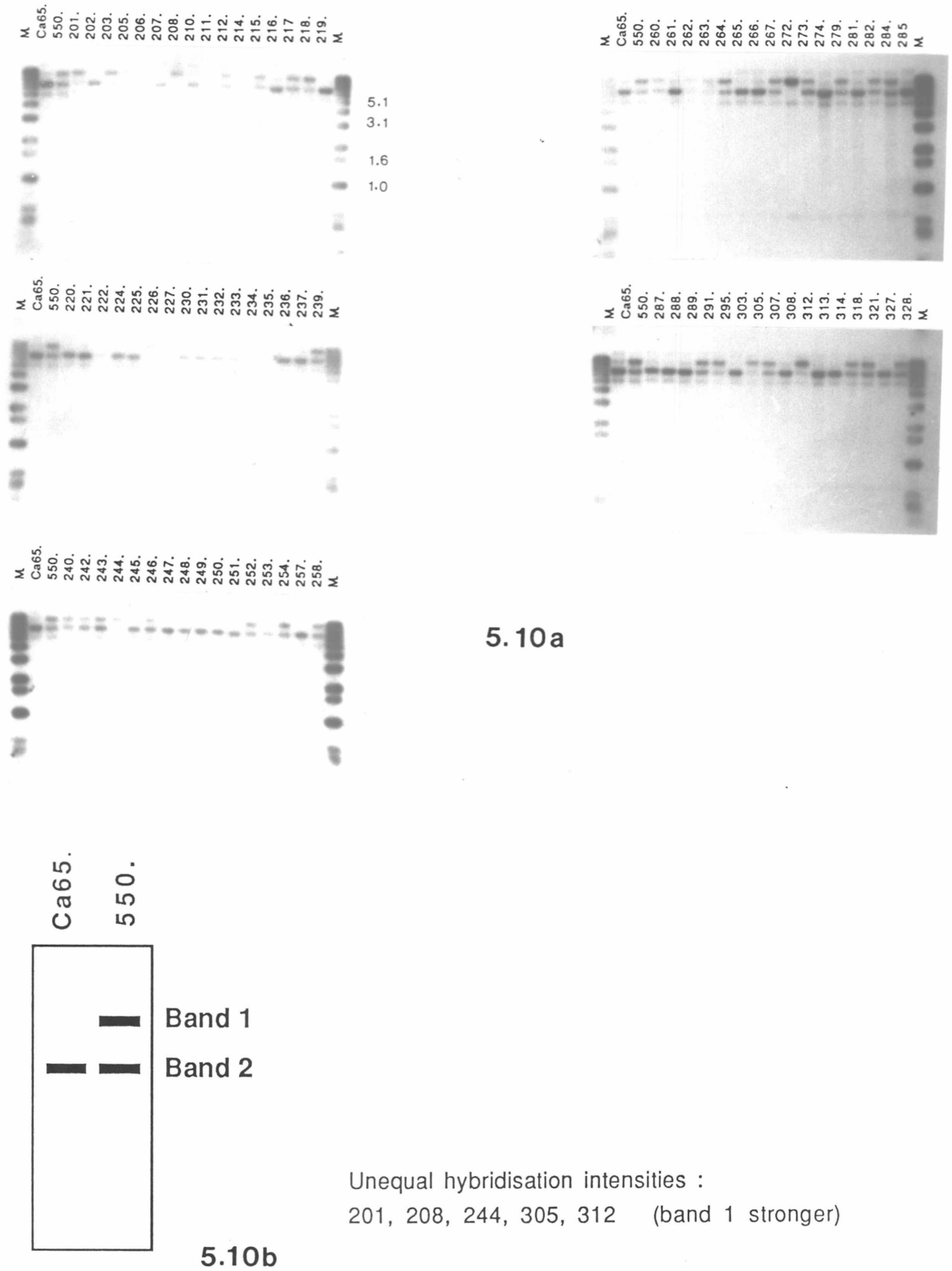
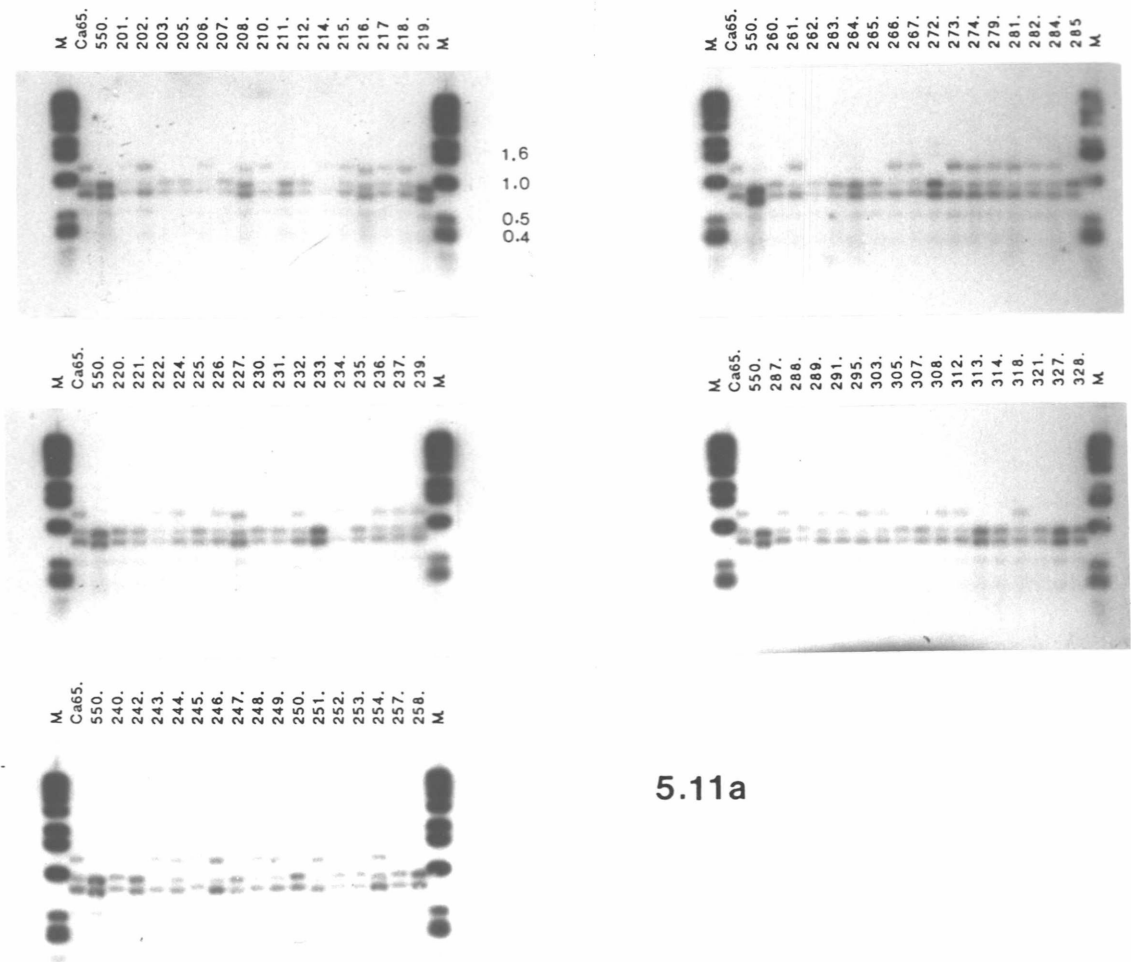
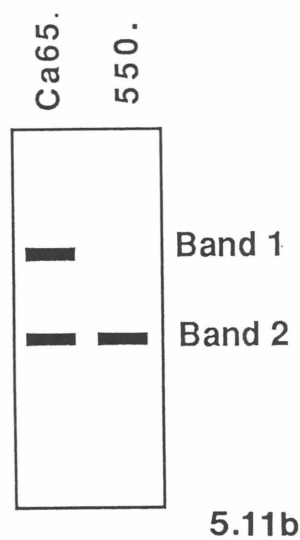


Figure 5.10 : a) Southern blot of *Bgl*II-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-6.
b) Diagram showing numbering of polymorphic bands detected in a).



5.11a



5.11b

Figure 5.11 : a) Southern blot of *MspI*-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-13.
b) Diagram showing numbering of polymorphic bands detected in a).

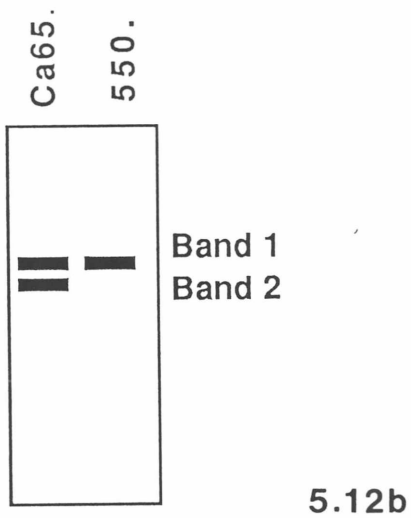
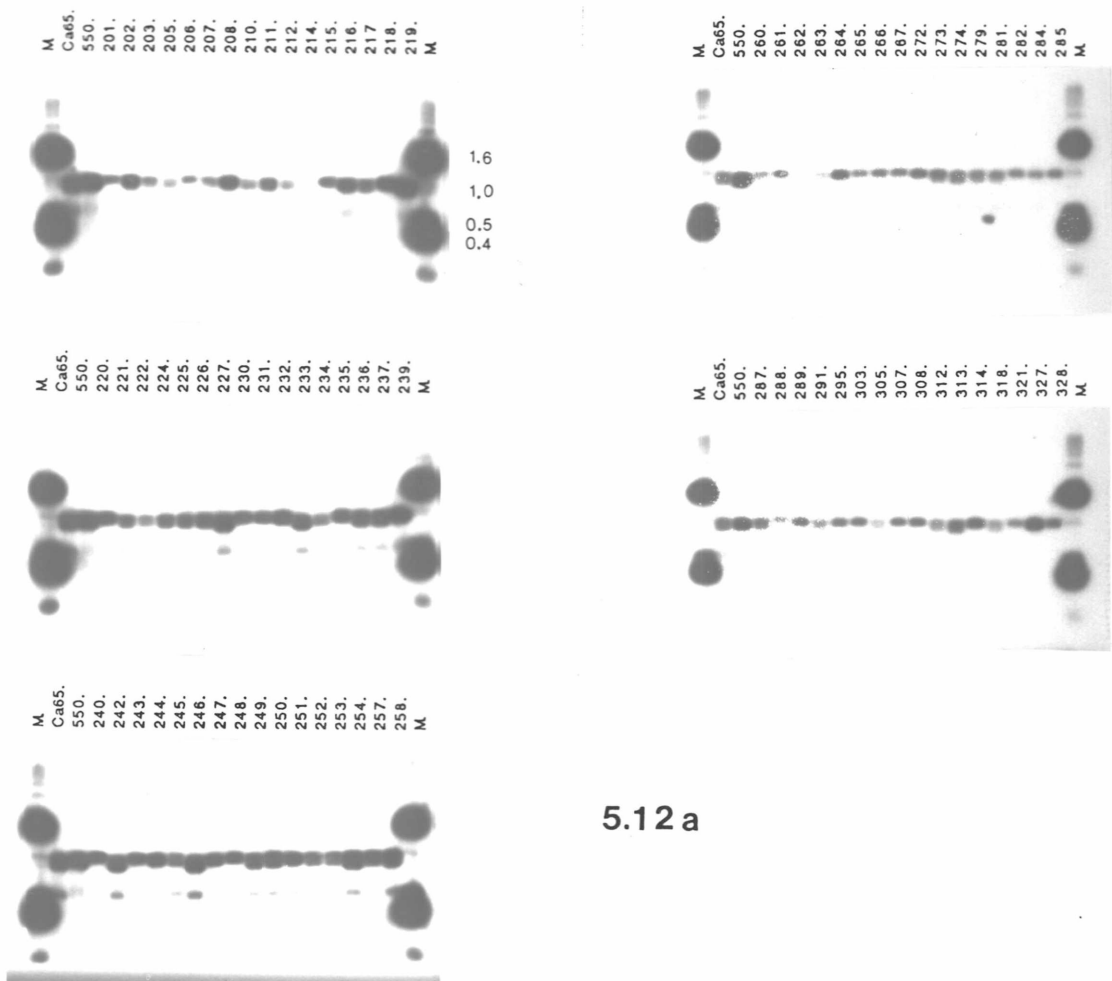


Figure 5.12 : a) Southern blot of *Msp*I-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-26.
b) Diagram showing numbering of bands detected in a).

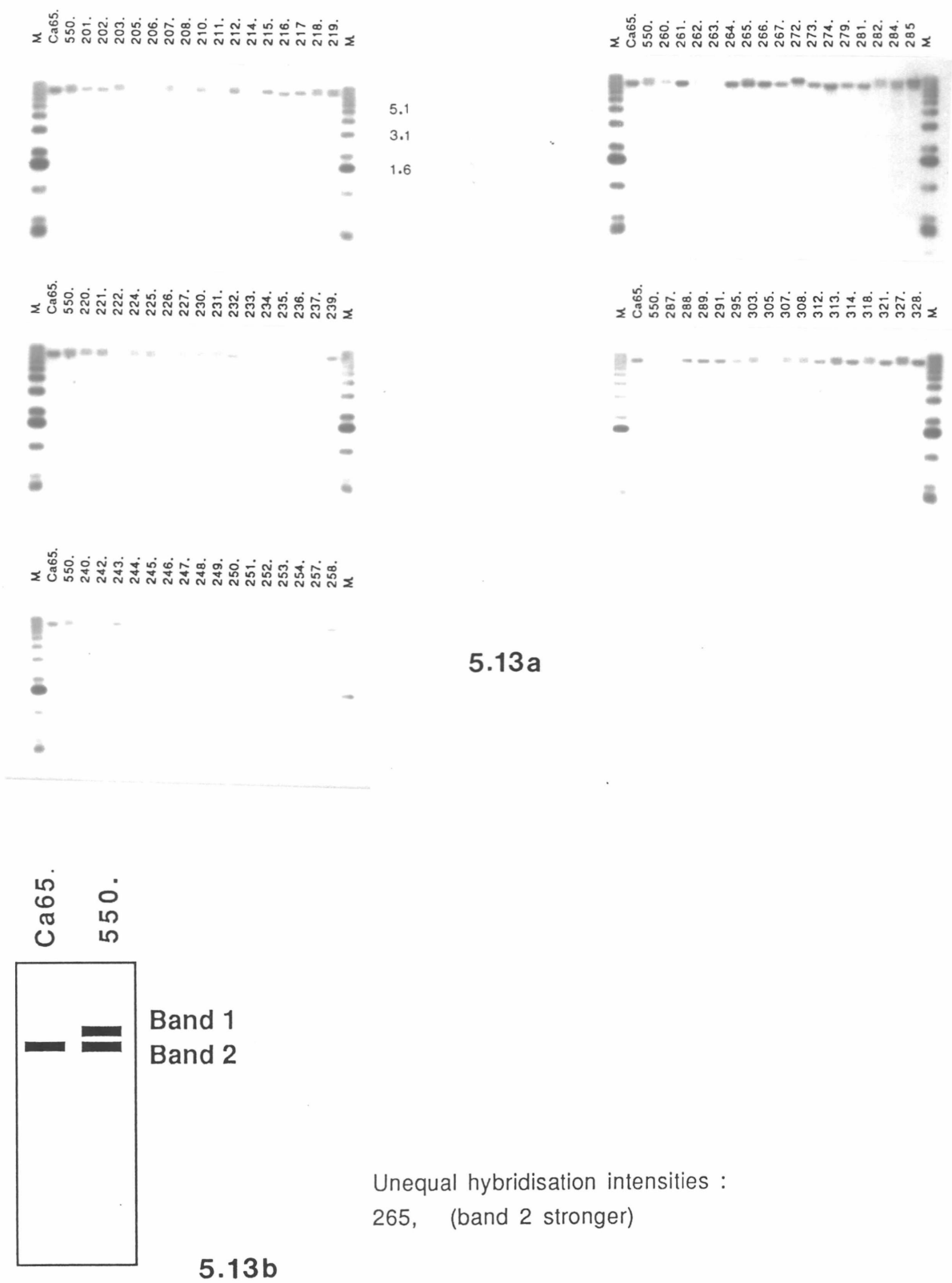
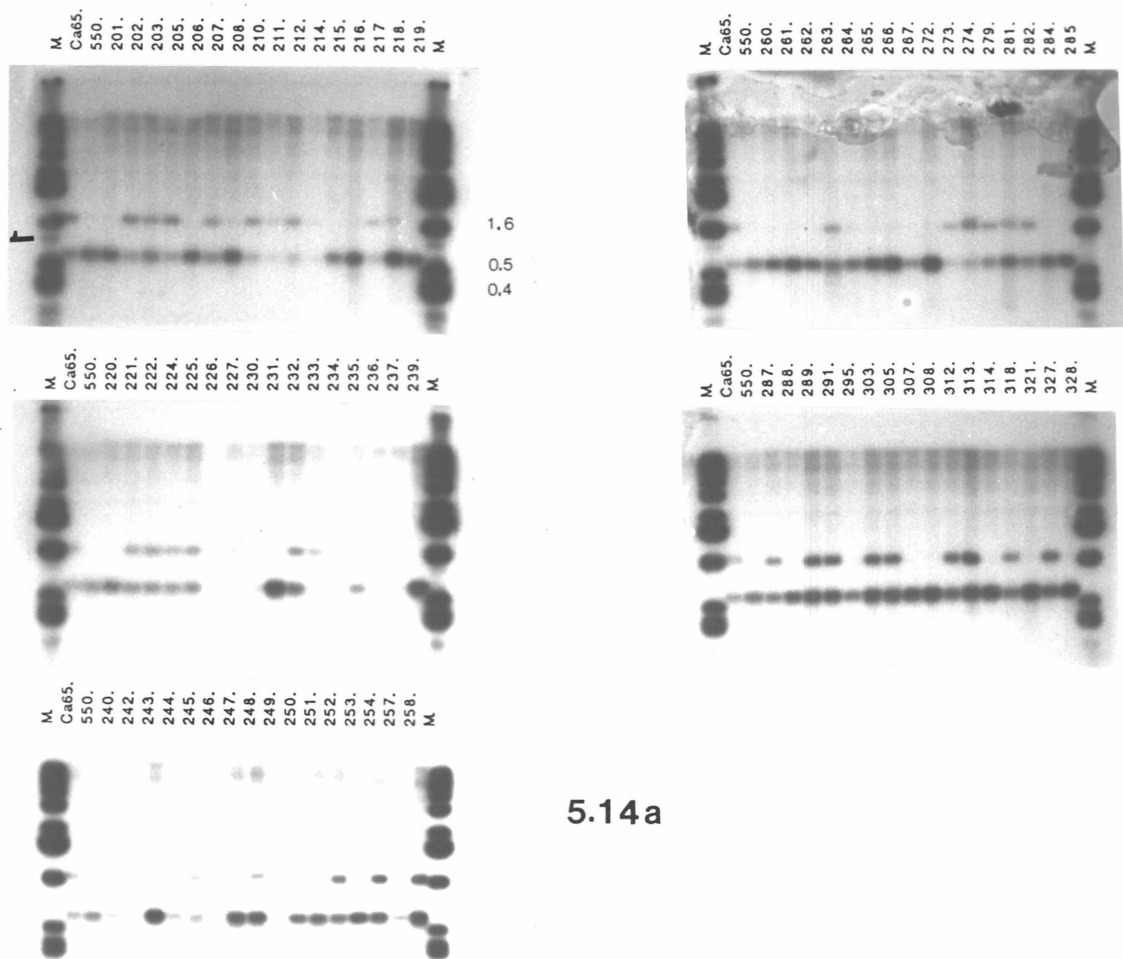
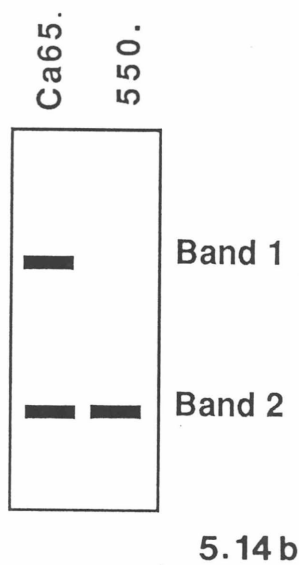


Figure 5.13 : a) Southern blot of *Bgl*II-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-174.
b) Diagram showing numbering of bands detected in a).

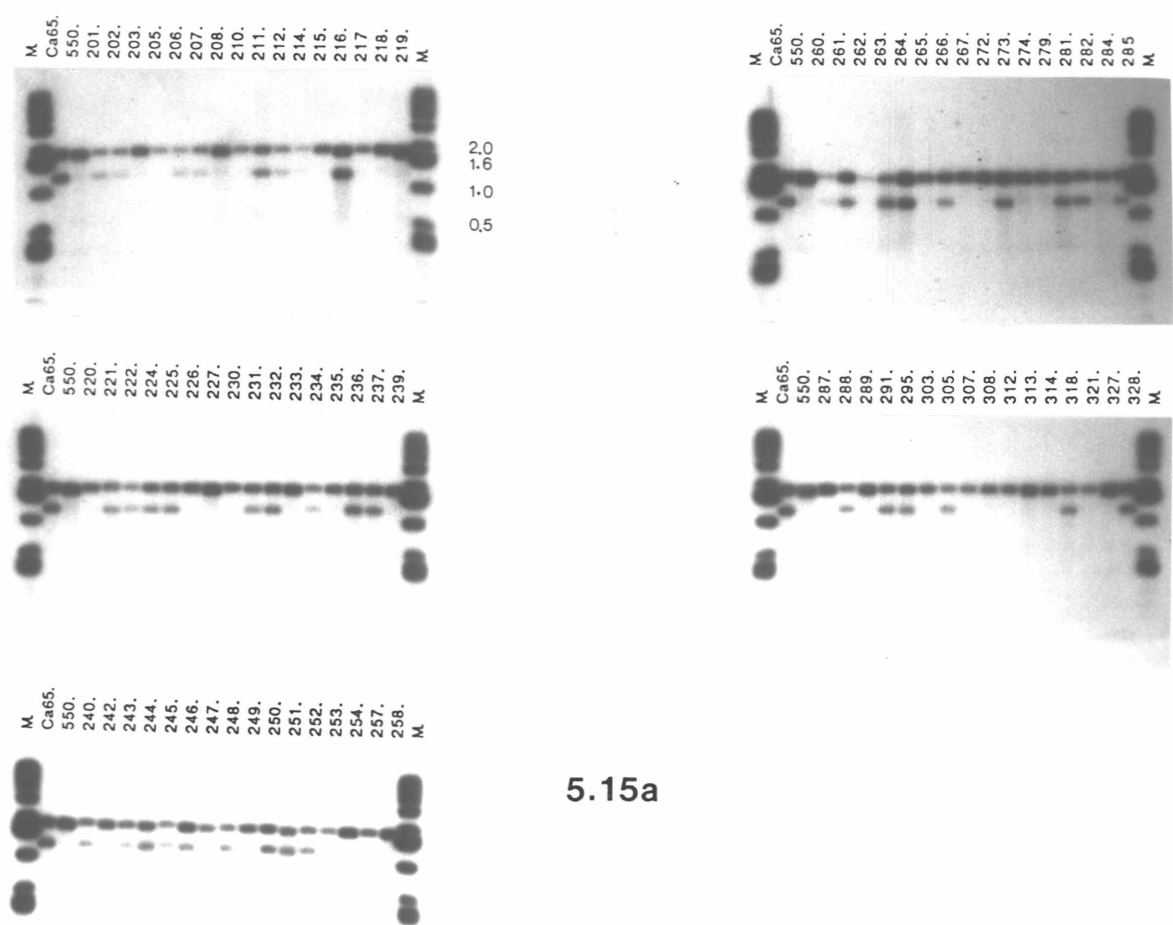


5.14a

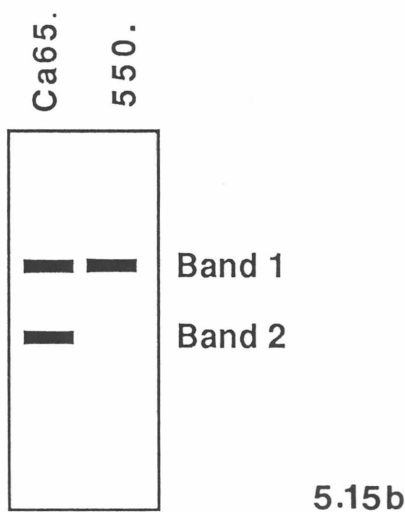


Unequal hybridisation intensities :
218, 247, 248, 251 (band 2 stronger)

Figure 5.14 : a) Southern blot of *Hind*III-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-296.
b) Diagram showing numbering of bands detected in a).

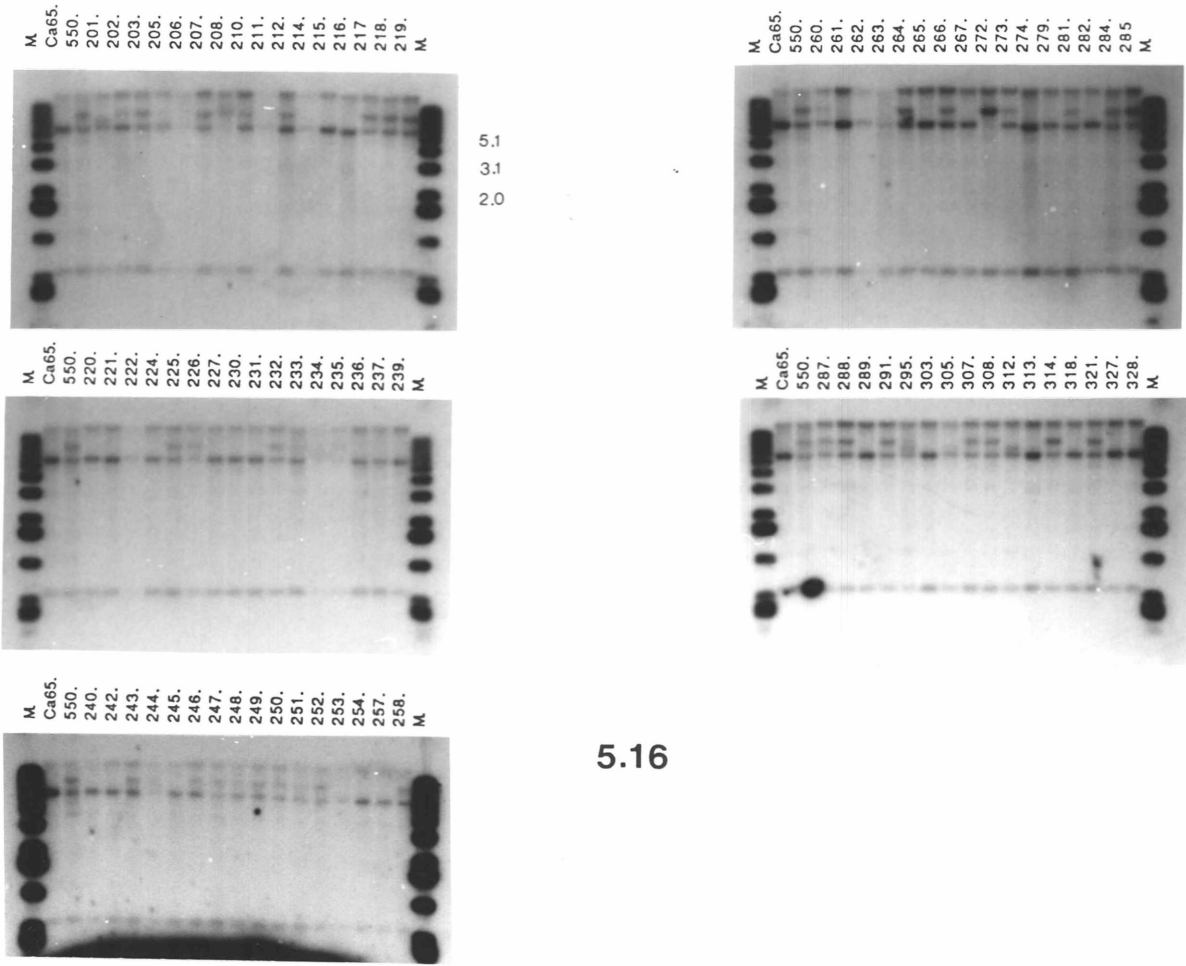


5.15a

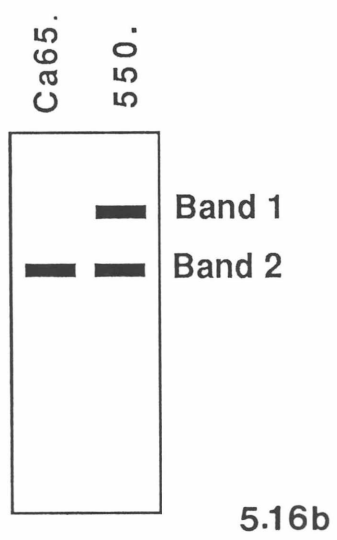


5.15b

Figure 5.15 : a) Southern blot of *Msp*I-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-313.
b) Diagram showing numbering of bands detected in a).



5.16



5.16b

Band not present in either parent :
201, 295, 305, 312

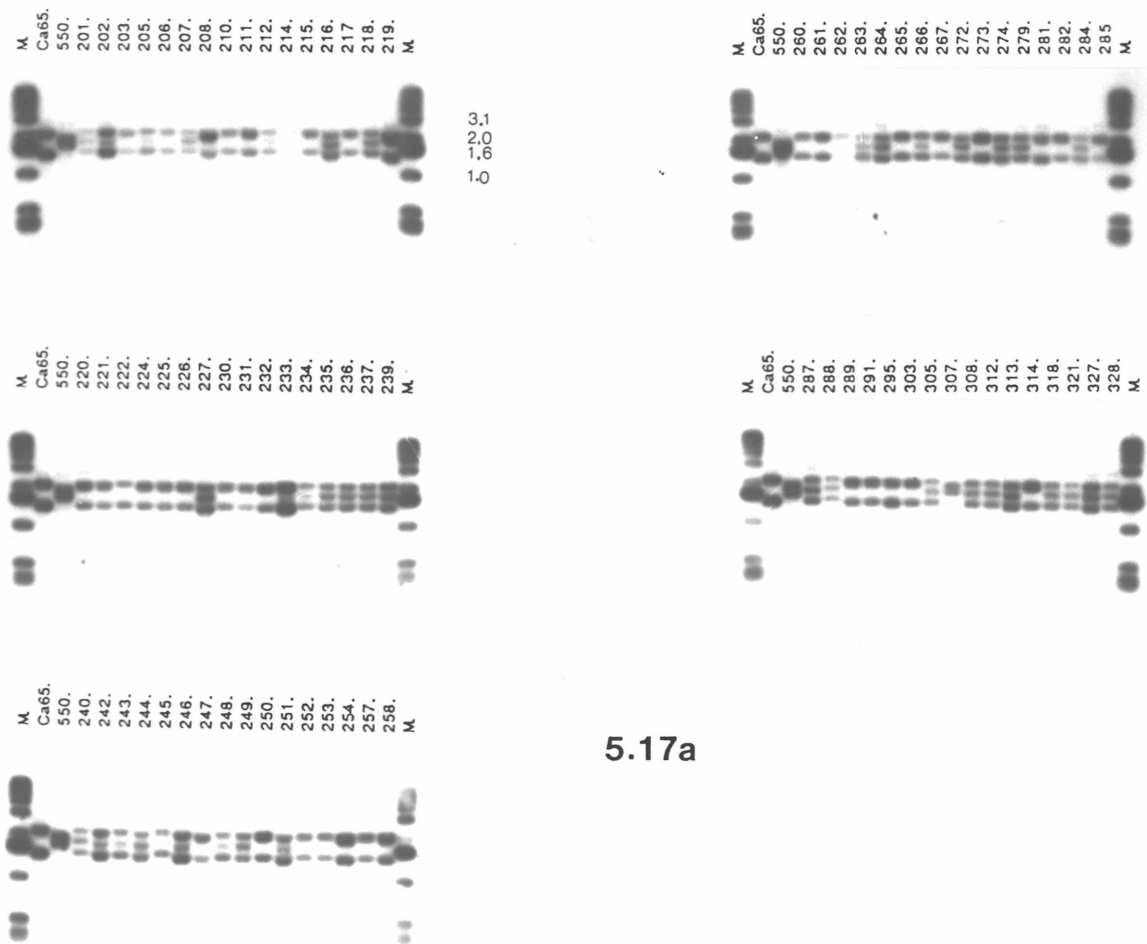
Unequal hybridisation intensities :
208, (band 1 stronger)

Figure 5.16 : a) Southern blot of *Bgl*II-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-362.
b) Diagram showing numbering of polymorphic bands detected in a).

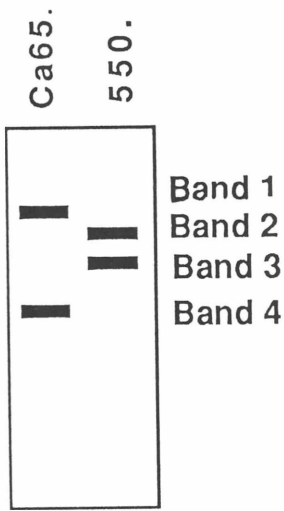
conserved between the parents and all of the progeny. Allelism of polymorphic bands was deduced by their inheritance patterns in the progeny; that is, progeny generally inherited either bands 1 and 5 or bands 2 and 3 from 550, and all inherited band 2 from Ca65. Again, a number of anomalous patterns are seen, especially with respect to hybridisation intensities. Some other more striking peculiarities are also obvious, however. Progeny 262 appears to be completely lacking a 1 kb band which is conserved in both parents and in all other progeny. 239 also lacks the band 2 allele from Ca65. As it is highly unlikely that Ca65 and 550 are heterozygous for the 1 kb band (since all other progeny possess it), it is probable that 239 has a deletion encompassing this region, which could also be responsible for the absence of the band 2 allele from Ca65.

Progeny number 272, the apparent product of self-fertilisation of 550, likewise has an unusual hybridisation phenotype with pIN5-216. Unlike 550, 272 is lacking Band 1 but has Bands 3 and 4 (it is difficult to ascertain whether Band 5 is present or not). This is consistent with the meiotic segregation of alleles following self-fertilisation which is proposed to have occurred in 272. However, 272 also has a new band not present in 550. This band migrates slightly faster than the normal 550 Band 1 allele. The most likely explanation for its occurrence is the presence of a new *Bgl*II restriction site, or a deletion, within the band 1 sequence, causing a smaller band to now hybridise with pIN5-216.

Progeny 295 is also curious in its hybridisation with pIN5-216. All other progeny receive either bands 1 and 5 or bands 2 and 3 from 550, but 295 appears to receive bands 1 and 3 only. A possible explanation for this is that the hybridising sequences producing the two bands of each pair are not contiguous, but are sufficiently close together on the chromosome that they are almost always inherited together. A cross-over between the regions specifying these hybridising sequences could reassort them to allow bands 1 and 3 and bands 2 and 5 to now occur together, giving rise to the hybridisation profile seen for progeny 295. A further cross would



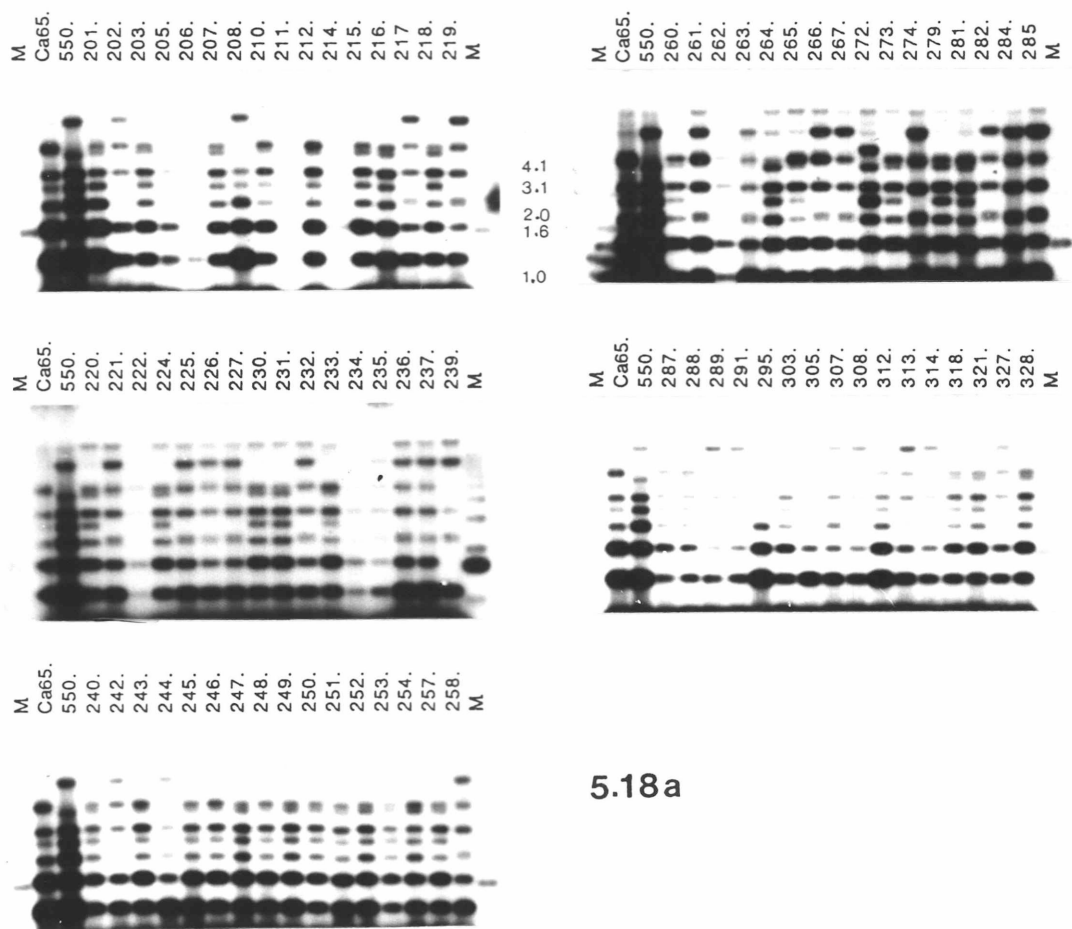
5.17a



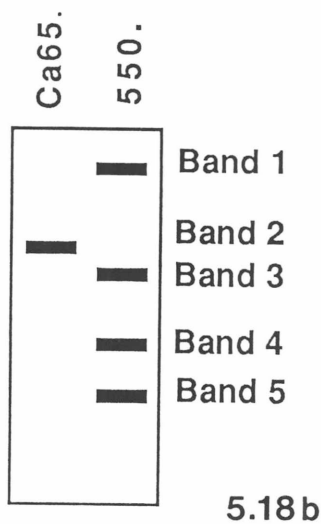
5.17b

Unequal hybridisation intensities :
202, 233, 242 (bands 1 & 4 stronger)
208 (band 2 stronger)

Figure 5.17 : a) Southern blot of *Msp*I-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-62.
b) Diagram showing numbering of bands detected in a).



5.18a



Bands not present in either parent :
272, 305

Unequal hybridisation intensities :
208, 239, 264 (bands 1 & 5 stronger)
233, 265 (band 2 stronger)

Reassorted alleles?
295

Deletion?
239

Figure 5.18 : a) Southern blot of *Bgi*II-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-216.
b) Diagram showing numbering of polymorphic bands detected in a).

be necessary to establish that this pair of bands is now inherited as a unit.

Like 272, progeny 305 appears to have a new RFLP affecting the length of band 1, but this time making it slightly larger. Apparently, one of the original *Bgl*II sites flanking this band is now missing, or there is an insertion of DNA between the two sites.

It is curious that so many band differences occur using this clone, and these results question the validity of using clones containing repetitive sequences in genetic analysis. The anomalies are interesting, however, from the viewpoint of DNA sequence divergence, and this clone could be very informative in the analysis of closely related *P. infestans* populations. All progeny exhibiting unusual banding patterns at L5-216 have been omitted from genetic analysis at this locus.

Clones pIN5-304 and pIN5-317 (Figures 5.19 & 5.20 respectively) also detect three alleles at their respective polymorphic loci, but both parents are heterozygous at these loci so that there are 4 possible combinations of banding phenotypes in the progeny. There are again some cases of unequal band intensities in a few of the progeny. Also, one of the progeny in the blot hybridised with pIN5-317 (progeny number 251) and 8 in the blot hybridised with pIN5-304, appear to contain all three alleles. This is no doubt equivalent to unequal hybridisation intensities, but extra chromosomal dosage is now showing up more clearly as the presence of additional bands. Interestingly, in all cases where three alleles are present the hybridisation intensities of all bands appear to be very equal. This indicates that some form of trisomy, either localised or more extensive, has produced the extra alleles.

pIN5-304 is an interesting clone as it appears to hybridise with tandemly repeated DNA (Figure 5.19b). Hybridisation with Ca65 and 550 DNA digested with *Hind*III produces a series of bands of increasing size, apparently related in size in a linear way, which would be expected if each band contained an increasing number of copies of the repeat unit. *Bgl*II appears to cut outside the repeat unit, but one or both of

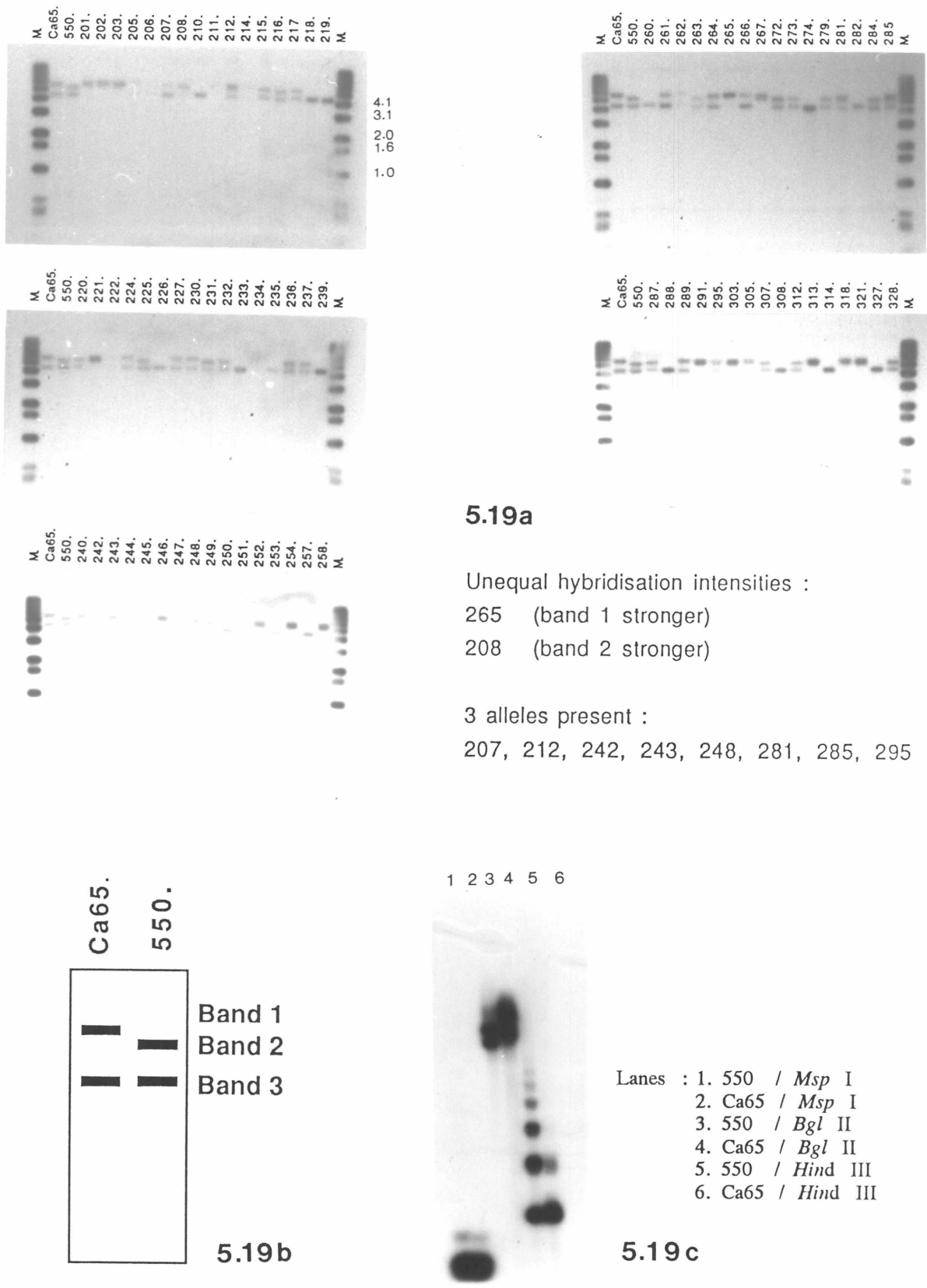
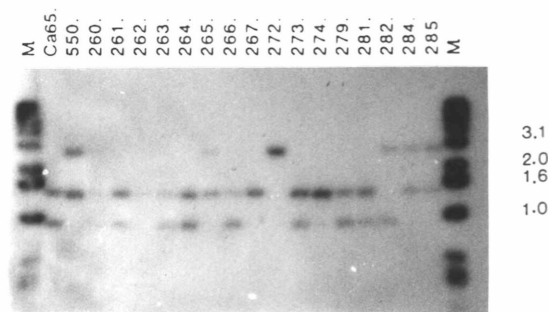
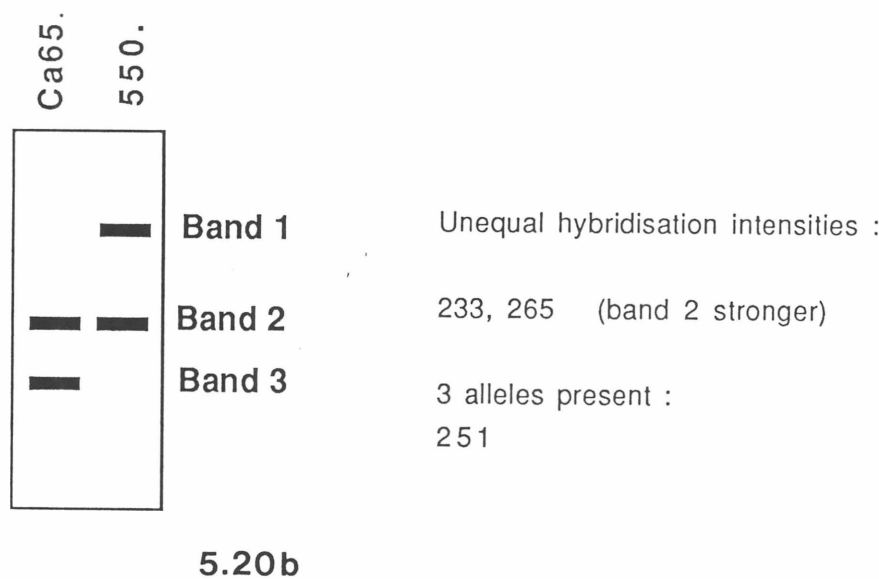


Figure 5.19 : a) Southern blot of *Bgi*II-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-304.
b) Diagram showing numbering of bands detected in a).
c) Original "test blot" showing hybridisation profile on Ca65 and 550 DNA digested with *Msp*I, *Bgl*II and *Hind*III.



5.20a



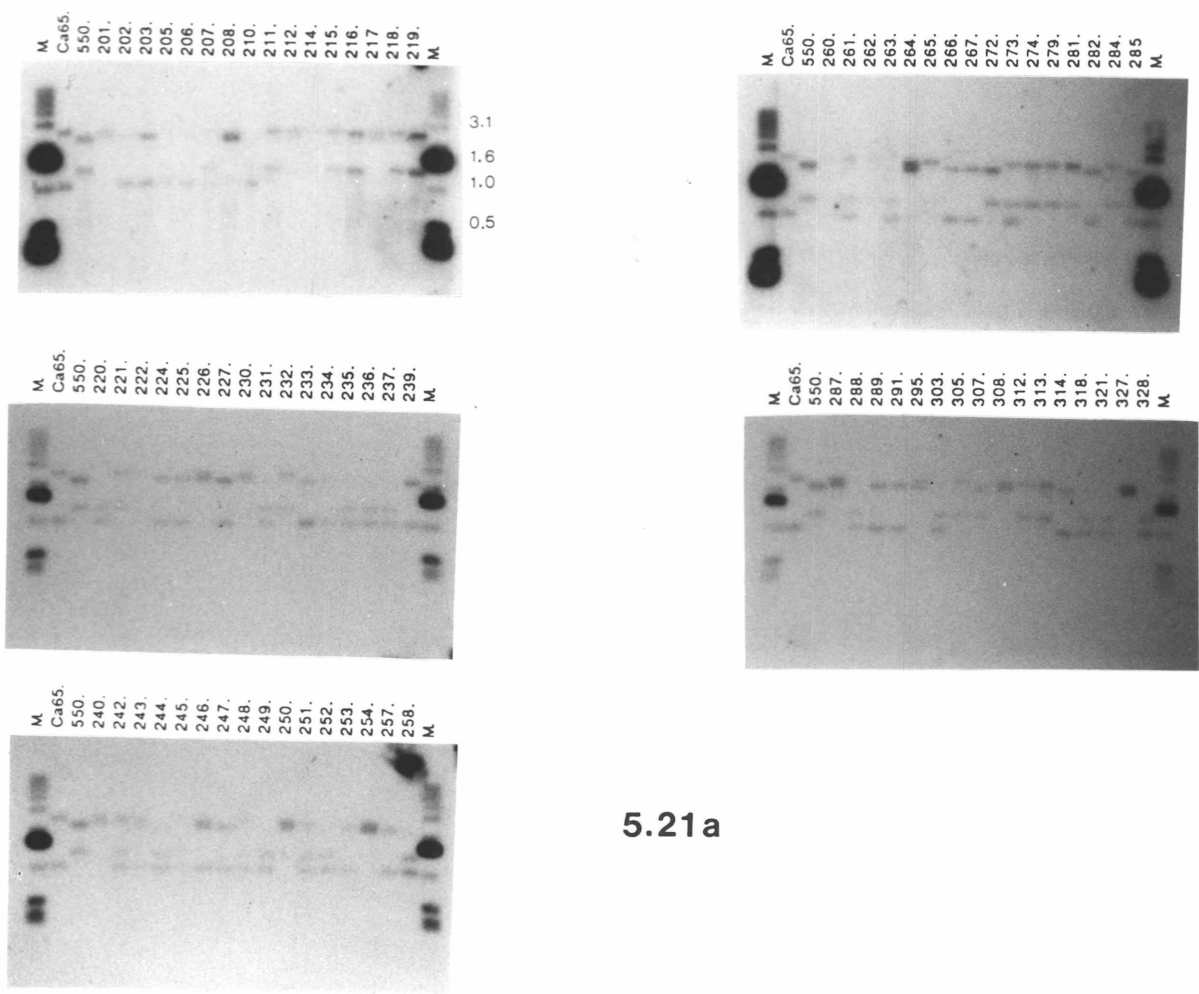
5.20b

Figure 5.20 : a) Southern blot of *Msp*I-digested DNA from Ca65, 550, and progeny numbers 260 - 285, hybridised with pIN5-317 (other autoradiographs were too poor to reproduce well in a photograph.. b) Diagram showing numbering of bands detected in a).

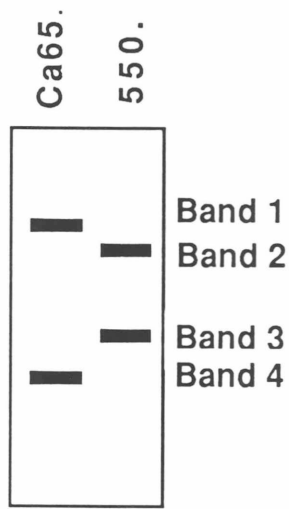
these flanking sites are polymorphic, so that the radiolabelled probe hybridises with bands of three different sizes. *MspI* presumably cleaves within each repeat unit, reducing the tandem to its component units and resulting in an intense band of small size (the slight band above this could be due to the loss of one such site). This was the only obvious case of tandemly repeated DNA found with any of the recombinant clones tested, although more may have been present among the clones containing more highly repetitive DNA.

The final autoradiograph (Figure 5.21) was produced by hybridisation of *MspI*-digested DNA with the clone pIN5-655. This clone detects 4 different alleles at its hybridising locus L5-655. Ca65 and 550 each possess two unique heterozygous alleles, so that there are four possible combinations of band phenotypes in the progeny, none of which are the same as the parental phenotypes. This is the most useful type of RFLP for genetic analysis of the progeny, as inheritance of each band from each parent is unequivocal. There are two obvious cases of unequal hybridisation between the bands detected by this probe, and 8 of the progeny appear to contain three alleles. As with L5-304, the three alleles at this locus, when occurring together, appear to be present in equal ratios. None of the progeny contain all four alleles.

All the progeny that gave unexpected hybridisation profiles at any of the RFLP loci tested were omitted from genetic analysis. This was considered necessary as it is possible that anomalies at other loci, in particular the avirulence loci, would go unnoticed but could produce misleading results. Out of the 77 hybrid progeny, 28 were omitted on the basis of unequal hybridisation intensities in allelic bands. 303 was also omitted because of the unusual band seen following hybridisation with pIN5-362 (201, 295 and 312, which also had this unusual band were previously excluded due to unequal hybridisation intensities.



5.21a



5.21b

Unequal hybridisation intensities :

- 265 (band 1 stronger)
- 208 (band 2 stronger)
- 233 (band 4 stronger)

3 alleles present :

- 242, 243, 245, 246, 248, 251, 261,
- 273, 285 (bands 1, 3 & 4)
- 244 (bands 2, 3 & 4)

Figure 5.21 : a) Southern blot of *Msp*I-digested DNA from Ca65, 550, and 80 progeny hybridised with pIN5-655.
b) Diagram showing numbering of bands detected in a).

5.4.2 Linkage analysis of the polymorphic loci.

Linkage analysis was performed on an Dell (system 325) PC-compatible computer using a modified version of the LINKAGE pedigree analysis programme (Lanthrop *et al.*, 1984). This programme was written to analyse linkage in human pedigrees, so is compatible with obligately outcrossing species like *Phytophthora*. Adaptations were made by L. Sandkuyl in order to accommodate the very large pedigree used in this work.

The linkage groups thus obtained are shown in Table 5.2, along with the most likely order in which the different loci occur with respect to each other. All other pairs of markers not listed did not show linkage.

$\theta_{\max}$ expresses the recombination between the pairs of alleles, and can otherwise be expressed as centiMorgans (cM), so that a $\theta_{\max}$ of 0.9 between L5-317 and L29, for example, is equivalent to a genetic distance of 9 cM. How this relates to the physical distance separating the loci is dependent on the rate of recombination between homologous chromosomes during meiosis, which is not known for *P. infestans*.

Z_{max} values represent the LOD score at $\theta_{\max}$. LOD scores express the probability of the hypothesis of linkage of two alleles against the probability that the two alleles are unlinked, that is, they express likelihood ratios. Generally, when a likelihood ratio for a given hypothesis exceeds 1000 : 1 (that is, the likelihood of this hypothesis is 1000 fold greater than the likelihood of the alternative hypothesis), then the hypothesis is accepted in favour of its alternative. LOD scores express the $\log_{10}$ of these likelihood ratios. For linkage, a LOD score of 1-2 is usually considered "interesting", 2-3 is "suggestive", and 3 or more "proof" that the two loci under consideration are linked. For non-linkage, a LOD score of less than or equal to -2 is considered significant (Botstein *et al.*, 1985). With the present set of data, however, far more information is available than in human pedigree analyses (owing

Table 5.2. Linkage analysis of RFLP and avirulence gene loci.

<u>Marker Pair</u>	<u>$\theta_{\max}$</u>	<u>$Z_{\max}$</u>	<u>Most likely order</u> <u>"by eye".</u>
<u>Linkage Group I:</u>			
L5-317 - L5-174	0.00	15.35	29----317/174----313
- L29	0.09	8.72	
- L5-313	0.18	4.72	
L5-313 - L29	0.17	5.80	
<u>Linkage Group II:</u>			
L5-296 - L11	0.18	5.36	?65<-----296----11-26
- L5-65	0.29	1.85	
- L5-26	0.24	3.19	
L11 - L5-26	0.05	11.75	
<u>Linkage Group III:</u>			
L5-599 - L5-655	0.13	6.05	599---655
<u>Linkage Group IV:</u>			
L5-216 - L5-725	0.30	1.56	216-----725
<u>Linkage Group V:</u>			
<u>Avr3</u> - <u>Avr7</u>	0.17	4.54	<u>Avr7</u> --- <u>Avr3</u> ---- <u>Avr8</u> (--ML99)
- <u>Avr8</u>	0.13	1.66	
¹ (<u>Avr8</u> - ML99	0.20	1.91)	

¹"Linkage" of Avr8 with ML99 is shown in bracket as these two loci are associated with one another but cannot be physically linked as pSTA99 is a mitochondrial clone.

to the much larger pedigree size) and a LOD score of 2 or more may be considered as significant evidence for linkage (L. Sandhuyl, pers. comm.). Hence the inclusion in Table 5.2 of pairs L5-296 and L5-65 (LOD 1.85), L5-216 and L5-725 (LOD 1.56), Avr-3 and Avr-8 (LOD 1.66) and Avr-8 and ML99 (LOD1.91). These are all below the LOD score threshold of 2, but are sufficiently high to be considered interesting, especially as the LOD scores for all other pairs of loci were negative or only just positive.

They may reflect distant linkage of the loci on a single chromosome, or in the case of Avr-8 and ML99, some sort of inheritance association.

The ratios of inherited phenotypes for each RFLP were calculated for all progeny used in the genetic analysis. No significant deviation from Mendelian inheritance was found among these progeny for any of the RFLP markers.

5.5 Discussion of chapter 5.

Of the 25 loci analysed, 15 have been shown to be linked with at least one other locus, resulting in the establishment of five small linkage groups. A preliminary linkage map for *P. infestans* has thus been established, although a considerable amount of further work is obviously necessary to achieve anything approaching saturation. The high LOD scores obtained between the linked markers are encouraging however, and indicate that the quality of information obtained by RFLP analysis is good. The high proportion of loci showing linkage also indicates that the rate of recombination in *P. infestans* is not so high as to make genetic mapping impractical.

The very high LOD scores between L5-317 and L5-174, with 0% recombination between them indicates that the two recombinant plasmids pIN5-317 and pIN5-174 probably contain the same DNA sequence. As pIN5-174 was hybridised with DNA digested with *Bgl*II, which produced two alleles at L5-174, and pIN5-317 was hybridised with an *Msp*I digest revealing three alleles at L5-317, the identity of the two recombinant clones was not established prior to linkage analysis. Their identity has since been confirmed by hybridising *Bgl*II-digested DNA with pIN5-317, and *Msp*I-digested DNA with pIN5-174 (not shown).

An interesting result on Table 5.2 is the highly significant linkage between Avr-3 and Avr-7. Generally, fungal avirulence genes are thought to be unlinked (Pryor, 1987) In *Bremia lactucae*, the only other oomycete in which the genetics of virulence has been extensively studied, all 8 avirulence genes appear to be unlinked (Michelmore *et al.*, 1984).

A similar result for Avr-3 and Avr-7 was, however, obtained by Al-Kherb (1988), in a cross between Ca65 and E13a. Coinheritance of virulence or avirulence to potato differentials R3 and R7 exceeded the inheritance to one but not the other by a factor of 94 : 24 in 109 progeny examined. Al-Kherb also found significant evidence of linkage of Avr-3 and Avr-7 with Avr-11 in the Ca65 x E13a

cross. Unfortunately, as 550 and Ca65 are both virulent at this locus, the current cross is uninformative for R11.

A loose association between Avr-3 and Avr-8 can be seen in the linkage data in Table 2. No linkage information was available in Al-Kherbs work for these two loci. Establishing whether the LOD score obtained, which at 1.66 is at the boundary of what may be considered significant, reflects true linkage between these two genes requires further work. The inheritance of virulence phenotypes is less clear-cut than RFLP inheritance, and is complicated by the occasional reversion to avirulence in what should be virulent progeny (as can be seen in some of the progeny on differentials R1, R4 and R11). Weakly pathogenic, intermediate reactions may also occur, which are difficult to score. These problems may skew data, and could mean that higher LOD scores are necessary to establish true linkage.

Probably the most surprising result in Table 5.2 is the apparent "linkage" seen between Avr-8 and ML99, the locus detected by the mitochondrial clone, pSTA99. Again, this could merely reflect a poorer quality of information for Avr-8 inheritance. The inheritance ratios at the Avr-8 locus among the progeny tested are just within the confidence limits for those expected for heterozygous avirulent parents ; that is, 3 : 1 of avirulent to virulent progeny (see Table 5.1). This slight deviation from Mendelian inheritance could have another interpretation, however. Small chromosomes, known as "B" chromosomes, have been found to occur in some organisms. These do not observe Mendelian inheritance, and appear associated with some unusual phenotypes (Nuret *et al.*, 1988). If they carry avirulence gene(s) and their inheritance is correlated to some extent with maternal (oogonial) or paternal (antheridial) sexual structures, one might expect to see a correlation between the inheritance of a specific avirulence phenotype with the inheritance of a certain mtDNA type. Intriguing in the light of this theory is the apparent cross-hybridisation of pSTA99 with a small, possibly chromosomal, band from *P. infestans* following pulsed field gel electrophoresis (see Chapter 6). There is

obviously a lot more work to be done before any firm conclusions can be drawn regarding the association of ML99 with Ayr-8.

The anomalies seen in hybridisation intensities and in the number of alleles at a single locus, while a nuisance for genetic mapping work, are very interesting in their own right and may help explain some of the difficulties encountered in the past in the genetic analysis of *P. infestans*. Indeed, it appears that not only *P. infestans* is plagued by such inconsistencies. RFLP work on *P. parasitica* has likewise revealed unusual inheritance of band phenotypes, apparently to an even greater extent than in *infestans* (H. Forster, pers. comm.). RFLP analysis of *Bremia lactucae*, however, did not reveal a significant number of progeny with unusual inheritance phenotypes (R. Michelmore, pers. comm.).

Unequal hybridisation intensities of allelic bands, and the occurrence of more than two alleles at a single locus in individual progeny, both reflect an increased dosage of the locus in question. This could occur locally, through a duplication event, or more generally via aneuploidy, or even triploidy. Given the range of ploidies at which *P. infestans* can occur naturally, it is perhaps not surprising that it can so easily accommodate such duplications.

Tabulating the progeny expressing an increased number of alleles alongside each polymorphic locus reveals a preponderance of some progeny to contain extra alleles (see Table 5.3). Progeny enumerated 208, 233 and 265 all appear to consistently contain more than two alleles at each locus examined for which they are heterozygous. These offspring quite possibly contain a third copy of the entire haploid genome. Indeed, for 265, often the hybridisation intensity of one band appears to be more than twice that of the other, thus its chromosomal complement may be more than $n = 3$. Further confirmation of higher ploidy in these three offspring will require cytofluorimetric comparisons with diploid standards.

When information regarding the parent from which the extra allele is derived (where possible to ascertain) is tabulated for each affected progeny alongside the

Table 5.3. Progeny with more than 2 alleles at RFLP loci.

<u>RFLP Locus.</u>	<u>Progeny number.¹</u>
L2	208 (550), 264 (550), 265 (Ca65)
L5-199	202, 246, 265
L5-6	201 (550), 208 (550), 244 (550), 305 (550), 312 (550)
L11	261 (Ca65), 265 (Ca65)
L5-13	234 (Ca65), 246 (Ca65)
L29	233 (Ca65), 242 (Ca65), 265 (Ca65)
L5-65	233 (Ca65)
L5-174	265
L5-296	218, 247, 248, 251
L5-362	208 (550)
L5-599	208, 248 (Ca65), 251 (Ca65), 260 (Ca65), 265 (Ca65)
L5-62	202 (Ca65), 208 (550), 233 (Ca65), 242 (Ca65)
L5-216	208 (550), 233 (Ca65), 239 (550), 264 (550) 265 (Ca65)
L5-305	207 (Ca65), 208 (550), 212 (Ca65), 242 (Ca65), 248 (Ca65), 265 (Ca65), 281 (Ca65), 285 (Ca65), 295 (Ca65)
L5-317	233, 265, 251
L5-655	208 (550), 242 (Ca65), 243 (Ca65), 244 (550), 245 (Ca65), 246 (Ca65), 248 (Ca65), 251 (Ca65), 265 (Ca65), 273 (Ca65), 285 (Ca65)

1. Parent contributing the extra alleles, where discernible, is shown in brackets alongside the progeny.

origin of their mtDNA, an interesting pattern emerges. In the majority of cases, the extra alleles are derived from the same parent that contributed the mtDNA; that is, the maternal, or oogonial parent. This information is summarised in Table 5.4.

A plausible explanation for this result could be the meiotic nondisjunction of the oomycete nucleus prior to fertilisation by the antheridial nucleus. The majority of progeny inheriting extra alleles from the maternal parent do not appear to have extra alleles at all RFLP loci tested; the one exception being 208. This is consistent with the idea of meiotic nondisjunction, rather than the fusion of two haploid oogonial nuclei along with a haploid antheridial nucleus. The latter may have occurred in 208, however, thus forming a triploid offspring.

Interestingly, progeny 233 and 265, which like 208 are both postulated to be triploid (or possibly polyploid in the case of 265) receive their extra alleles from the antheridial parent. The most likely cause of this is the introduction of two nuclei via the antheridial tube into the oogonium, both of which fuse with the oogonial nucleus. It is worth noting that in hybridisation with pIN5-655, only 208, 233, and 265 have unequally hybridising bands. This is consistent with inheritance of three distinct, separate nuclei. Receipt of three different alleles could also occur from three single nuclei, but this can also result from the fusion of two nuclei, one of which contains both parental alleles at this locus.

244 and 285 also contain extra alleles derived from the antheridial parent, but unlike 233 and 265 show normal inheritance of alleles at the majority of RFLP loci for which this can be discerned. Meiotic nondisjunction of the antheridial nucleus could be responsible for this apparent aneuploidy.

233, 244, 265 and 285 all derive their extra alleles from Ca65 as the antheridial parent. No progeny appeared to possess extra alleles from 550 when it functioned as the antheridial parent, although less of the progeny have 550 as the antheridial parent in this cross. There appeared to be fewer cases of progeny with extra alleles from 550 on the whole. Possibly meiosis in 550 is regulated more precisely than in Ca65, so that disjunction occurs with less frequency. Alleles from 550 could also be

Table 5.4 Mitochondrial inheritance in progeny with extra alleles.

	<u>mtDNA from Ca65:</u>	<u>mtDNA from 550:</u>
<u>Extra alleles</u> <u>from Ca65:</u>	202, 207, 212, 234, 242, 243, 245, 246, 248, 251, 260, 261, 273, 281, 295.	233, 244, 265, 285.
<u>Extra alleles</u> <u>from 550:</u>		201, 208, 239, 264 305, 312.

more lethal when inherited together via two haploid nuclei.

It was hoped that unequal hybridisation intensities at some of the loci would support the information on linkage between these loci. On the whole, however, the most obvious differences in hybridisation intensities of alleles did not occur among those loci showing close linkage. Slight hybridisation differences, which occur in a considerable number of progeny, are difficult to judge objectively and have generally been omitted, as restriction digestion and blotting of the DNA should be repeated to ensure they do not merely result from problems in either of these. Progeny were also often homozygous at loci linked to those exhibiting increased allele dosage, and any increase of these alleles would go unnoticed.

Within linkage group I, progeny 248 and 251 did show significant differences in intensities when hybridised with pIN11 and pIN5-296, although these differences were much weaker with the former. This could be due to the two different blots used, as the blot containing DNA digested with *HindIII*, against which pIN5-296 was tested, is very blurred and DNA transfer may not have been very efficient. Also, restriction endonuclease digestion of the DNA with *HindIII* did not appear to work as thoroughly as digestion with the other enzymes. Curiously, progeny 247, which appeared to have an increased complement of alleles at L5-296, appeared normal at L11. Again, this could be the result of using different blots, and the blotting and hybridisation procedures should be repeated before any firm conclusions are drawn. Alternatively, 247 may contain a short duplication at this locus, and may not be trisomic across the entire chromosome. It did not show significant hybridisation inequalities at any of the other loci.

Within linkage group III, most of the loci exhibiting more than two alleles at L5-655 were either homozygous at L5-599, or gave banding patterns that were very faint or contained slight additional bands not present in either parent (possibly due to incomplete digestion of the DNA, which, like that probed with pIN5-296, was restricted with *HindIII*). Progeny 248 and 251, which both possess an extra allele

at L5-655 from the Ca65 parent appeared to have a higher dosage of Ca65 alleles at L5-599 compared to the 550 allele. Progeny 244, however, appeared heterozygous and normal at L5-599, although the bands were very faint making this difficult to judge subjectively.

There was no evidence in any of the progeny for the existence of heterokaryosis (with the exceptions of 272 and 303, the two "selfs" with hybrid Gpi-1 profiles, which may have segregated before RFLP analysis was conducted). With the possible exception of 265, none of the progeny appeared to possess more than three alleles at any of the loci analysed. MtDNA data is supportive of the absence of heterokaryosis, as no progeny containing mitochondrial genomes from both parents were seen. It appears, therefore, that at least as far as this Ca65 x 550 cross is concerned, sexual recombination is the sole means of recombining phenotypes from both parents.

CHAPTER 6 : PULSED FIELD GEL ELECTROPHORESIS.

6.1 Preparaton of nuclei and protoplasts.

As I did not initially have access to a suitable protoplasting technique for *P. infestans*, chromosomes were prepared by isolation of intact nuclei from mycelium using the method of Garber & Yoder (1983), but, omitting any proteinase K treatment until after the nuclei were embedded in agarose. The most critical step in this method appeared to be resuspension of the organelles following centrifugation at 53 000 *g*, as the resulting pellet often became compacted and sticky. A better way of concentrating the organelles could be to band them on a sucrose density gradient.

Protoplasting was achieved by a modification to the method of Howlett (1989). The combined use of Novozyme 234 (Novo Biolabs, Ltd.) and Driselase (Sigma Biochemicals, Ltd) completely protoplasted 5 day old mycelium within 1 - 2 hours. The protoplasts appeared to be fairly robust and survived several washes in 2M Sorbitol before being embedded in low-melting agarose.

6.2 Electrophoresis of whole chromosomes.

Electrophoresis conditions were adjusted according to the anticipated size of the chromosomes being separated. Both the *S. cerevisiae* and *S. pombe* chromosomal standards separated well using the appropriate conditions supplied by BioRad (30 min switch interval, 200 volts for 24 hours for *S. cerevisiae*; 60 min switch interval, 50 volts for 6 days for *S. pombe*).

The high DNA content of *P. infestans* relative to that of other fungi indicated that the chromosomes would probably be fairly large. Conditions for separating *S. pombe* were therefore initially applied to the agarose inserts containing intact *P. infestans* nuclei (Figure 6.1; protoplasts had not yet been prepared at this stage).

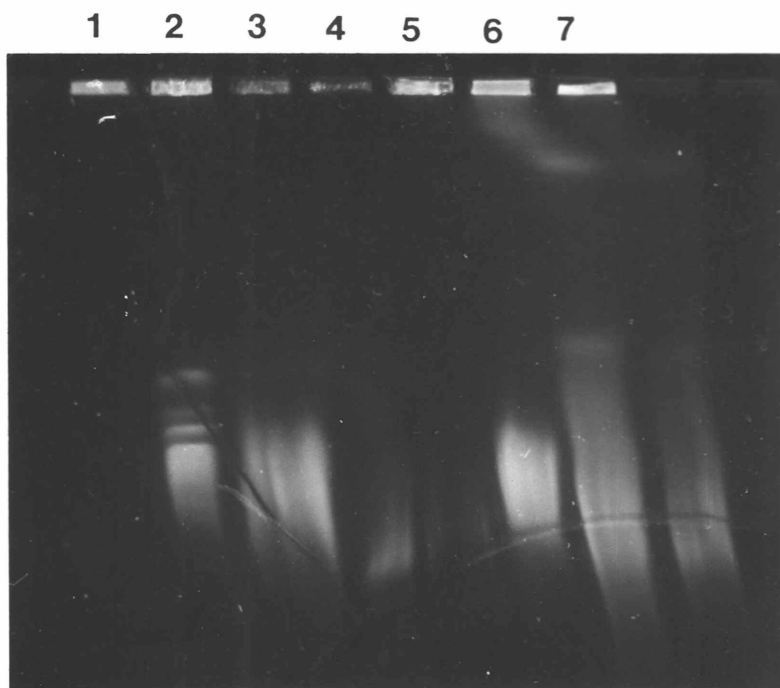


Figure 6.1 : Pulsed field gel electrophoresis of *Saccharomyces cerevisiae* chromosomes, *Scit^hzosaccharomyces pombe* DNA(degraded), and DNA prepared from intact nuclei of *P. infestans*.
Electrophoresis conditions : 50 volts; 60 min switch time; 144 h..
Low Endo-osmosis Agarose (Beckman).

Lanes : 1. *S. cerevisiae*
2-5. *S. pombe*
6-7. *P. infestans* (nuclei)

This gel ran badly due to buffer exhaustion, and in subsequent gels the buffer was changed daily. However, there did appear to be a band in the *P. infestans* lane of about 3 Mb. The strong bands at the top of the *P. infestans* lanes are presumably due to unresolved DNA.

Electrophoresis was repeated using Chromosomal Grade Agarose (BioRad Labs, Ltd.). This agarose has been specifically designed for pulsed field gel electrophoresis work, and produces a gel matrix with large pores and a high gel strength, allowing very low percentage gels to be prepared. This gave good separation of the *S. pombe* chromosomes, and a band of approximately 3 Mb was again seen in the *P. infestans* lane (Figure 6.2). Two lanes of *P. infestans* DNA prepared from protoplasts were also included on this gel, but contained little or no DNA.

A considerable amount of fluorescent material was present below the 3 Mb band in the *P. infestans* lane. This could be due to *P. infestans* chromosomes that are not being resolved under these electrophoresis conditions. Conditions for the separation of *S. cerevisiae* chromosomes, which range from 250 bp to approximately 2.6 Mb, were therefore employed. The resulting gel is shown in Figure 6.3. Some faint bands appear to be present in the *P. infestans* lane, however bands of the same size appear to occur in the lane containing *S. pombe* standards. As these certainly do not correspond to chromosomes in the *S. pombe* sample, they are probably artifacts. They could be due to polysaccharide material, which can move through an agarose gel under an electric current and can also bind ethidium bromide to a limited extent. Like the *S. pombe* chromosomes (and the larger chromosomes in the *S. cerevisiae* sample), *P. infestans* DNA appears as a broad, unresolved band at the top of the gel.

In an attempt to resolve the chromosomes in the range of 2 - 4 Mb, conditions were employed that were used by Howlett (1989). This involved varying the switching time from 30 to 7 minutes during 84 hours (Figure 6.4 a). The strong band of 3 Mb is no longer apparent on this gel, but there does appear to be a faint band of

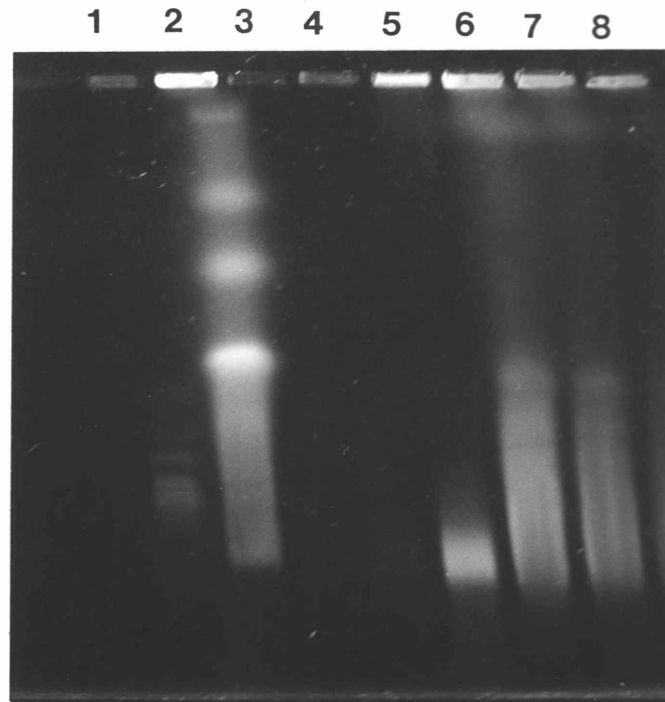


Figure 6.2 : Pulsed field gel electrophoresis of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomes, and DNA prepared from protoplasts and intact nuclei of *P. infestans*.
Electrophoresis conditions : 50 volts; 30 min switch time; 72 h, Chromosomal Grade Agarose (Biorad).

Lanes : 1. *S. cerevisiae*
2. *S. pombe*
4-5. *P. infestans* (protoplasts)
6-7. *P. infestans* (nuclei)

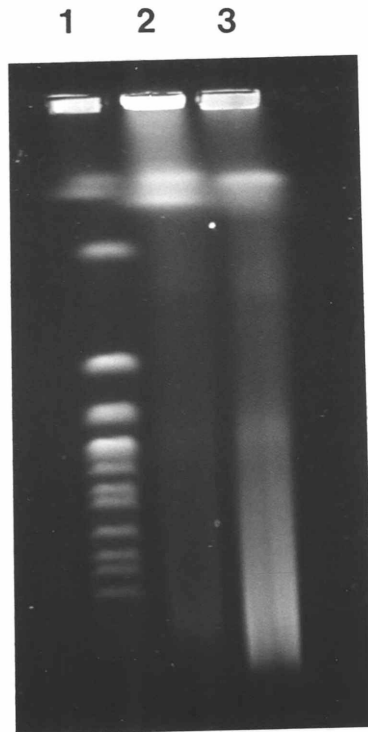


Figure 6.3 : Pulsed field gel electrophoresis of *Saccharomyces cerevisiae* and *Scitzosaccharomyces pombe* chromosomes, and DNA prepared from intact nuclei of *P. infestans*.
Electrophoresis conditions : 200 volts; 60 second switch time; 24 h,
Low Endo-osmosis Agarose (Beckman)

Lanes : 1. *S. cerevisiae*
2. *S. pombe*
3. *P. infestans* (nuclei)

approximately the same size as the largest *P. infestans* chromosome, with possibly two other bands below it. Again, faint bands of similar sizes occur in the lane containing DNA from *S. pombe*, and it was initially thought that these bands were also merely artifacts. Evidence that the bands may contain DNA, however, was obtained by hybridising a Southern blot of this gel with radiolabelled DNA from *P. infestans* (Figure 6.4 b). Considerable hybridisation occurred in the region of the *P. infestans* lane in which the bands occur, as well as with the band of unresolved DNA at the top of the lane. Unfortunately, DNA transfer above this band appears to have been impaired, possibly due to an air bubble trapped between the gel and the membrane during blotting. Hybridisation also occurred with material in the *P. infestans* lane of smaller size than the smallest *S. cerevisiae* chromosome. This may represent degraded nuclear DNA, or mitochondrial DNA. Some cross-hybridisation is apparent with the largest *S. cerevisiae* chromosome and with the 3.5 Mb chromosome of *S. pombe*, which is probably due to conserved ribosomal sequences. This was confirmed by hybridising the blot with a recombinant plasmid containing an insert of ribosomal DNA from *Drosophila melanogaster*(Figure 6.4 c). The plasmid hybridises strongly with the largest *S. cerevisiae* chromosome, and with the 3.5 Mb chromosome from *S. pombe*, and to a lesser extent with the unresolved DNA band in *P. infestans*, reflecting a much lower concentration of DNA in this sample.

In Figure 6.4d, the blot has been hybridised with the recombinant clone, pSTA99. Originally, this clone was thought to contain an insert of single copy sequence nDNA from *P. infestans*. It was later established, however, that the insert was of mitochondrial DNA (see Chapter 5). Very strong hybridisation occurred with the low molecular weight DNA present in the *P. infestans* sample. This is consistent with the pulsed field gel electrophoresis of mtDNA reported by Skelly and Maleszka, 1989, who isolated intact mtDNA from *Torulopsis glabrata* and *S. cerevisiae* from a pulsed field gel. The mtDNA was likewise found to migrate ahead of the smallest *S. cerevisiae* chromosome.

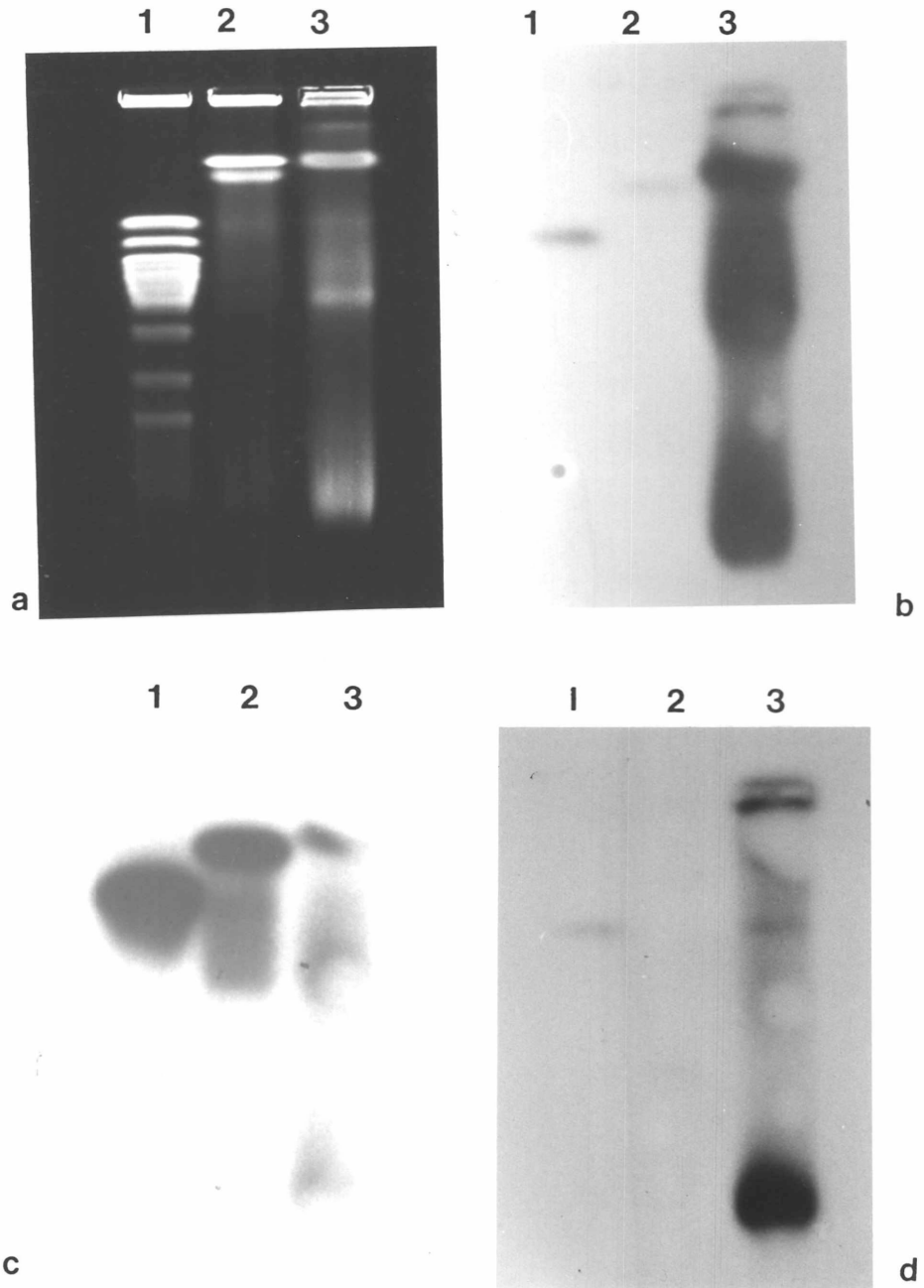


Figure 6.4a) : Pulsed field gel electrophoresis of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomes, and DNA prepared from intact nuclei of *P. infestans*.

Electrophoresis conditions : 50 Volts, 30 min switch interval decreasing to 7 min over 84 h.

Low Endo-osmosis Agarose (Beckman)

b) : Southern blot of a) hybridised with total radiolabelled *P. infestans* DNA.

c) : Southern blot of a) hybridised with radiolabelled rDNA from *Drosophila melanogaster*.

d) : Southern blot of a) hybridised with radiolabelled pSTA99, a recombinant clone containing an insert of *P. infestans* mtDNA.

Lanes : 1. *S. cerevisiae*; 2. *S. pombe*; 3. *P. infestans* (nuclei).

Curiously, hybridisation also occurred with a band in the *P. infestans* lane of the same size as the largest *S. cerevisiae* chromosome. Slight cross-hybridisation with the 2.6 Mb *S. cerevisiae* chromosome also occurred, and in the original autoradiograph there also appeared to be a very faint cross-hybridisation with the largest *S. pombe* band. pSTA99 does not contain any nuclear sequences (see Figure 5.1b). Hybridisation with these chromosomes is therefore probably due to the presence of related sequences that are somewhat conserved in the different fungal species.

In the final Figure (6.5), electrophoresis conditions were employed in the hope of separating very large *P. infestans* chromosomes. Orbach *et al* (1988) used a very long switch time (180 min) and a low field strength 1.3 V cm^{-1} for 192 hours to separate the two largest chromosomes from *Neurospora crassa*, these being estimated at 9.1 and 12.6 Mb in size. These conditions were therefore applied to the *P. infestans* samples, although the switch time was reduced to 165 min, which is the maximum time possible using the BioRad CHEF system. The figure indicates considerable movement of all *S. pombe* chromosomes under these conditions, but still no resolution of the DNA banding just below the wells in the *P. infestans* samples. Insufficient time was available to ascertain, via blotting and hybridisation, whether any chromosomes too faint to be detected by gel electrophoresis and ethidium bromide staining, had been resolved using these conditions.

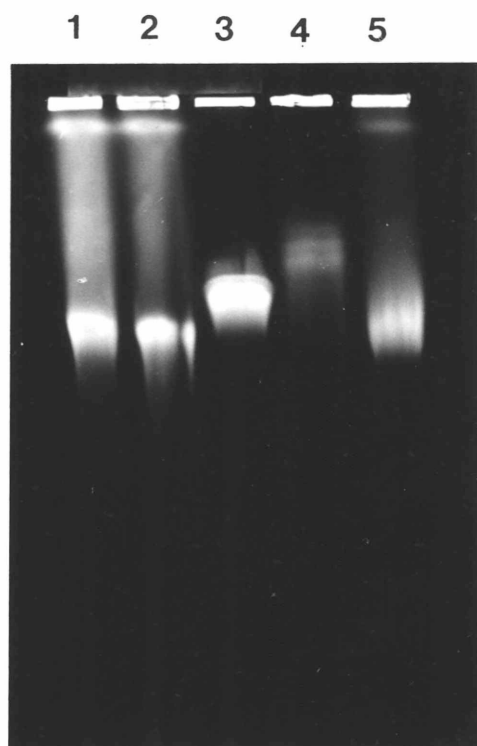


Figure 6.5 : Pulsed field gel electrophoresis of *Saccharomyces cerevisiae* and *Scitizosaccharomyces pombe* chromosomes, and DNA prepared from protoplasts and intact nuclei of *P. infestans*.
Electrophoresis conditions : 30 Volts, 165 switch interval, 192 h.
Low Endo-osmosis Agarose (Beckman)

Lanes : 1-2. *P. infestans* (protoplasts)
3. *S. cerevisiae*
4. *S. pombe*
5. *P. infestans* (nuclei)

6.3 Discussion of Chapter 6.

A number of groups have attempted the separation of *P. infestans* chromosomes by pulsed field gel electrophoresis, but to date without success. Protoplasting, using mycelium, sporangia or zoospores from *P. infestans* can be carried out simply and reliably (Campbell *et al.*, 1989) and is generally the procedure favoured in the preparation of fungal chromosomes. Pulsed field gel electrophoresis of agarose plugs containing *P. infestans* protoplasts prepared either by myself or by colleagues at Bangor University, who are very experienced at handling protoplasts, did not, however produce any evidence of intact *P. infestans* chromosomes in these plugs. They appeared to either contain little or no DNA (as in Figure 6.2), or produced a broad smear of staining material with a narrow band of possibly unresolved DNA just below the wells (as in Figure 6.5). As both the quality and quantity of protoplasts prior to embedding in agarose appeared satisfactory, this must be due either to incomplete lysis of the protoplasts during incubation with ESP, or to degradation of the DNA during incubation or storage of the agarose plugs. The broad smear of staining material seen in Figure 6.5 may be due to release of cell-soluble material following protoplast lysis.

The results presented here suggest that intact nuclei may be more suitable in the preparation of material from *P. infestans* for pulsed field gel electrophoresis. This may be due to the smaller size of nuclei relative to protoplasts, allowing a higher concentration of DNA in the agarose plugs. Alternatively, the nuclei may lyse more readily with ESP than the protoplasts, with less resulting cell debris present to obscure bands or impede their movement into the agarose gel.

The results of hybridisation of the gel in Figure 6.4 are encouraging, although further work is obviously necessary to establish that chromosomal bands from *P. infestans* are being separated. The apparent cross-hybridisation of pSTA99 with a sharp band in the lane containing *P. infestans* DNA is particularly interesting,

especially as this recombinant plasmid cross-hybridises with what are definitely chromosomes in the *S. cerevisiae* and *S. pombe* lanes, and also because of the apparent linkage of ML99 with avirulence gene Av8 shown in Chapter 5.

A curious feature of the gel in Figure 6.4a is the absence of the 3 Mb band seen so clearly in the *P. infestans* samples in Figures 6.1 and 6.2. The same nuclear preparation was used for all these gels, although it is possible that some degradation of DNA in the agarose inserts has occurred, as the gel in Figure 6.4 was run some time before those in Figures 6.1 and 6.2. Indeed, the apparent "bands" in the *P. infestans* lane in Figure 6.4 may all be artifacts, with hybridisation in Figure 6.4d occurring with DNA that is of too low concentration to be seen by ethidium bromide staining and UV. It was very unfortunate that insufficient time was available to hybridise this blot with other clones containing single copy sequence nuclear DNA from *P. infestans*.

An interesting future step would be the selected cloning of DNA from the region of the *P. infestans* lane in which the apparent bands occur. This could be achieved by electrophoresis of the DNA followed by excision (without ethidium bromide staining) of the areas in which the bands are present. DNA in the gel fragments could then be digested with a restriction endonuclease and ligated into a plasmid vector. If recombinant clones can be constructed in this manner, they will prove conclusively that chromosomal DNA from *P. infestans* is being banded in this area, and they can be used to generate specific linkage groups for these chromosomes. Further linkage analysis with the phenotypic markers, if conclusive, may allow some of these markers to be assigned to these chromosomes, thereby aiding their eventual isolation.

Hybridisation of the pulsed field gel with the mitochondrial clone pSTA99, also illustrates the feasibility of using pulsed field gel electrophoresis to isolate mitochondrial DNA. Skelley & Maleszka (1989) obtained intact mitochondrial DNA in this manner from *Torolopsis glabrata* and *S. cerevisiae*, which could be restricted and analysed for RFLPs in the normal manner. This eliminates the need

for costly CsCl-bisbenzimidazole ultracentrifugation. It may be possible to produce mtDNA adequately from protoplasts, as the preparation of nuclei still requires ultracentrifugation. Figures 6.2 and 6.5 both indicate considerable staining material in the region expected for mtDNA (that is, ahead of the smallest *S. cerevisiae* chromosome) in the lanes containing DNA prepared from protoplasts of *P. infestans*.

All the electrophoresis conditions tried produced a strongly fluorescing band just below the wells in the lanes containing DNA preparations from *P. infestans*. This banded in the same region as the large *S. pombe* chromosomes when electrophoresis conditions were used that resolve DNA of a much smaller size (for example, in Figure 6.3). This, and the strong hybridisation of this band in the *P. infestans* lane with radiolabelled nDNA from *P. infestans* (Figure 6.4b), suggests that the band may contain unresolved *P. infestans* chromosomes. Tooley & Therrien (1987) in cytological staining comparisons with chicken erythrocyte standards, estimated the mean DNA content of a diploid *P. infestans* nucleus to be 0.52 pg. This is equivalent to approximately 4.7×10^8 base pairs per diploid genome. If it is assumed that *P. infestans* has twelve pairs of chromosomes (Whitticker, Shattock & Shaw, 1990), this makes the average length of a *P. infestans* chromosome in the order of 20 Mb. This obviously places the majority of chromosomes in *P. infestans* outside the resolving limits of the current pulsed field gel systems. It will probably be necessary to restrict the DNA embedded in agarose using infrequent-cutting restriction endonucleases such as *NotI* to electrophoretically resolve the DNA from these very large chromosomes. Unfortunately, this may make the physical mapping of *P. infestans*, and the assignment of RFLP markers directly to *P. infestans* chromosomes considerably more difficult.

Chromosomal fragments may still be used, however, to aid linkage studies. Evidence of co-linkage of RFLPs to phenotypic markers, and the construction of specific libraries from the chromosome fragments, may be used to track down the

genes of interest, in this case the avirulence genes. Alternatively, large recombinant molecules, such as YACs (Yeast Artificial Chromosomes; Burke *et al.*, 1987) could be constructed from restriction endonuclease-digested DNA from *P. infestans*, and screened with RFLP markers showing linkage with avirulence genes. Contiguous mapping may then be used to order the genes and the RFLPs on the *P. infestans* chromosomes.

Pulsed field gels may also provide information on the nature of the chromosomes in *P. infestans*. Analyses of other plant pathogenic fungi indicate considerable heterogeneity between isolates in their electrophoretic karyotypes. Kinscherf and Leong (1988), in a study on strains of *Ustilago maydis*, found that although the general features of the chromosome banding patterns were conserved between strains, no two strains had completely identical karyotypes. As well as providing information on the population dynamics and the relatedness of isolates, differences in number or electrophoretic mobility of chromosomes (or chromosome fragments) may help explain the unusual inheritance profiles often observed in sexual progeny from *P. infestans* (see Chapter 5). Hybridisation of blotted gels with RFLP-detecting sequences could be especially informative, where the observation of an increased number of alleles at a locus may be correlated directly with aneuploidy of all or part of the chromosome on which the locus in question resides.

CHAPTER 7 : OVERALL DISCUSSION AND SUMMARY.

This is the first report of the extensive use of RFLPs in the analysis of the population biology and genetics of *P. infestans*. Although work here is still at a very preliminary stage, the potential offered by DNA markers in furthering our understanding of this difficult, but extremely important organism can be seen.

Analysis of mitochondrial and nuclear polymorphisms among global isolates of *P. infestans*, and among the less dispersed population from around the UK, indicates that the degree of diversity between isolates at the DNA level is considerable. Polymorphism in nuclear DNA is somewhat greater than in mtDNA, allowing individuals to be distinguished from one another using only a small number of probes. Nuclear DNA polymorphisms would therefore probably be best suited for studies on the diversity and spread of natural *P. infestans* populations. Analysis of polymorphism in the mitochondrial genomes may then be correlated with the nDNA results to provide information on the level of sexual recombination occurring in the field, and on the ancestral lineages of *P. infestans* isolates.

Most of the recently isolated, single copy nDNA clones can be expected to be useful in population studies. As demonstrated in Chapter 4, clones hybridising with single copy sequences are easier to analyse and interpret than those that detect repetitive sequences, and allow additional information on ploidy and heterozygosity levels to be obtained

Clone pIN5-216, although detecting a number of different bands, produced a fairly clear, simple hybridisation profile. This clone uncovered a number of apparent RFLPs within the very closely related sample of progeny from the Ca65 x 550 cross, which could indicate a considerable amount of activity at the different loci with which it hybridises. If so, it may be useful in studies of very closely related populations, for example within a limited area, or at the initial stages of an epidemic, to analyse the divergence of isolates from one another.

Probably the biggest obstacle in the use of RFLPs in studies of *P. infestans* populations is the considerable amount of work involved in obtaining isolates in pure culture, growing sufficient mycelium for DNA extraction, and performing the extraction procedure itself. The recently developed polymerase chain reaction (PCR) offers considerable promise in alleviating at least the first two of the problems, and is expected to be of great benefit to studies of natural populations.

PCR allows the amplification of very small amounts of target sequence DNA by successive rounds of chain elongation followed by thermal denaturation. This relies on the use of specific oligonucleotide primers (usually of around 20 nucleotide residues each), which flank the target sequence, one homologous to the + strand on one side of the target site, and the other homologous to the - strand on the other side of this site. Within a heterogenous population of DNA, only sequences flanked by these specific primers will be amplified. Potentially then, this could allow the detection and analysis of *P. infestans* in DNA extracts from material present within an infected lesion, or in crude samples such as soil, water and plant tissue. As only the sequences present in *P. infestans* should be homologous with the oligonucleotide primers, only *P. infestans* DNA will become amplified, and as this is an amplification procedure very small amounts of *P. infestans* material may be detected. This very sensitive detection procedure may also allow assessment of the infective potential of the different components of the environment with which a potato crop may come into contact

As well as population biology and genetics, there are other features of the biology of *P. infestans* that have traditionally been difficult to analyse and may be aided by the use of RFLPs. Polyploidy, aneuploidy and the occurrence of heterokaryosis have all been referred to in this study, and some information has been obtained about each of these already. Parasexuality, or the ability of different *P. infestans* isolates to apparently recombine phenotypes without sex, is another curious feature about which little is known genetically or at the molecular level.

RFLPs in nuclear genomes allow the movement of DNA between isolates to be followed directly. Different, doubly homozygous or doubly heterozygous RFLPs (like those detected by pIN2 and pIN5-655 respectively in the Ca65 x 550 cross) would be particularly useful in this analysis. Similarly, exchange of cytoplasm can be monitored by the analysis of mitochondrial DNA polymorphisms, which can be easily detected by hybridisation with clones containing polymorphic mtDNA sequences such as pSTA99. Work is currently being initiated at Bangor University, North Wales, to investigate the nature of parasexuality using RFLP markers.

The development of a genetic map, and the localisation by linkage with RFLPs of the avirulence genes appears to be a feasible prospect for *P. infestans*. The fact that 15 out of the 25 markers analysed showed some linkage, and that highly significant LOD scores were found for 9 of these indicates that the recombination rate during meiosis in *P. infestans* is not especially high. It would be expected, therefore, that the majority of additional RFLPs found will be linked to one of the five established linkage groups or with one of the 9 loci which are not as yet linked with anything else.

The main problem hindering further development of the genetic map is the difficulty of obtaining clones hybridising to single loci. In this study, approximately 1000 recombinant clones were screened to find the 18 single locus RFLPs eventually used for genetic mapping, representing an inefficient use of time and resources. With the high rate at which anomolous hybridisation profiles occur in *P. infestans*, multilocus probes (eg. pIN5-216) do not appear to be very satisfactory. The construction of a cDNA library will probably be the necessary next step in producing additional single locus RFLPs at a satisfactory rate. Hulbert & Michelmore (1988) produced cDNA clones for RFLP mapping of *Bremia lactucae*, but found the complexity of the cDNA library to be considerably lower than that of the corresponding genomic library of *B. lactucae*. The authors stated, however, that this could be due to the necessity of producing the library from

spores (as *B. lactucae* is an obligate biotroph), which may possess less complex mRNA populations than actively growing mycelia. This problem may not, therefore, be so great in *P. infestans*.

An interesting alternative approach to developing more single locus RFLPs is the removal of repeated sequences from hybridisation probes by the pre-reassociation of these probes with excess denatured genomic DNA (Sealey, Whitacker & Southern, 1985). Reassociation is performed to a final Cot value of just less than $Cot\ 1/2$ of the sequence of interest, so that repetitive copy sequences form duplexes with complementary sequences present in the denatured genomic DNA, and are effectively removed from the hybridisation probe, but single copy sequences do not. Sealey *et al.* used this technique to successfully distinguish single copy sequences in mixtures of different plasmids containing single copy and repeated sequences, and in clones in which single copy and repetitive DNA were present within the same plasmid. In the absence of reassociation, broad smears without any visible bands were produced on the resulting autoradiographs. By reassociating to between Cot_{100} and Cot_{1000} (where $Cot = \text{driver concentration (mg/ml)} \times \text{time in minutes}$), however, distinct bands were produced corresponding to those expected for the single copy portions of the recombinant clones. It may be worth testing this technique on some of the many clones already obtained which contain repetitive sequences of *P. infestans* DNA.

An alternative use of this procedure would be the creation of a single copy sequence-enriched library, by reassociation followed by elimination of repetitive double stranded sequences. Further reannealing of the remaining single stranded DNA may then allow it to be used to produce recombinant clones in the normal manner.

In human genetic mapping, where the search for RFLPs is becoming increasingly intense as the human genome mapping project is initiated, ingenious new techniques are being developed to uncover polymorphic loci with greater ease.

Some of these are applicable to organisms like *Phytophthora*. One such technique is the analysis of interspersed tandemly repeated sequences in the form $(dC-dA)_n \cdot (dG-dT)_n$; the so called $(CA)_n$ repeats (Weber & May, 1989). These sequences occur many thousands of times in the human genome, with n being roughly equal to 15 - 30. Like other repeated DNAs (eg. minisatellites, Jeffreys, 1985, and tandem repeats within parasite antigen genes, Kemp *et al.*, 1987), the number of repeats within the $(CA)_n$ blocks varies between members of a species. Thus, variation at these single loci can be used in the same way as RFLPs. This technique again relies on the amplification capabilities of PCR. Cloned DNA in sequencing vectors like M13 is screened for $(CA)_n$ repeats using synthetic radiolabelled poly(dC-dA). poly(dG-dT). Single stranded DNA is then isolated and sequenced from positive clones, and this sequence thus determined is used to synthesise oligonucleotide primers flanking the $(CA)_n$ region. The region can then be amplified by PCR, and variation in repeat number assayed by electrophoresis on standard denaturing polyacrylamide sequencing gels.

Other tandemly repeated simple sequences occur in addition to the $(CA)_n$ repeats. These have been found in human, *Drosophila*, sea urchin, protozoan and yeast DNA sequences (Tautz & Renz, 1984), thus appear to be a ubiquitous component of eukaryote genomes. It appears that they have arisen by slippage replication and unequal crossover, and like the $(CA)_n$ repeats show extensive length polymorphisms and can be exploited in a similar manner (Tautz, 1989).

It appears highly likely that similar simple sequence length polymorphisms (SSLP) occur in the genome of *P. infestans*. These can be easily screened for in the clones already obtained by using synthetic radiolabelled simple sequences as outlined above for the $(CA)_n$ repeats. The disadvantage of this technique is the extra work involved in obtaining sequences for constructing the flanking oligonucleotide primers. However, as most of the simple sequence repeats appear to be polymorphic among individuals in the organisms studied to date, this

probably represents considerably less work than the large number of hybridisation reactions necessary to obtain single copy sequences from genomic *P. infestans* libraries. Also, as PCR amplification is used, only very small amounts of progeny DNA need be prepared. This could allow many more progeny to be processed, thus increasing the likelihood of detecting linkage.

In the light of the above stated frequency of polymorphism in tandemly repeated DNA, it may also be of interest to hybridise some of the available clones at low stringency with pIN5-304. This recombinant clone contained a *P. infestans* sequence which hybridised with tandemly repeated DNA. Other repeats similar in sequence may also occur throughout the *P. infestans* genome. If individuals vary in the number of copies of repeat units contained within the tandemly repeated block these can also be treated as single locus RFLPs (Jeffreys, 1985).

It is unfortunate that more progress has not yet been made in the establishment of an electrophoretic karyotype for *P. infestans*. This would obviously be of great benefit to genetic mapping work, and undoubtedly would uncover additional interesting features in the genomes of different *P. infestans* isolates. Further progress in this area will probably require endonuclease digestion with rare site restriction enzymes to render the DNA a suitable size for PFGE. The resulting fragments may then be inserted into the recently developed YAC vectors (Burke *et al.*, 1988), and the order of cloned sequences on the different *P. infestans* chromosomes may then be ascertained by contiguous mapping of overlapping clones.

Eventually, when reasonable linkage with an avirulence gene is established, a transformation system will probably be necessary to recover a clone containing the avirulence gene sequence. This is because the physical distance represented by a genetic distance that is even as short as 1 cM is probably too great to allow screening of all the genes in this region for the one conferring avirulence. Also, the large amount of repetitive DNA in the *P. infestans* genome makes chromosome walking from an RFLP marker to a linked gene impractical. Transformation of a

genetically defined *P. infestans* host with *P. infestans* DNA, followed by screening of transformants for reversion from virulence to avirulence, would allow the direct isolation of the functional avirulence gene. A promising transformation system was reported by Prell *et al.*, (1990), but no results have yet been published using this vector to introduce and express cloned *P. infestans* genes into a *P. infestans* host. A number of other groups are working to achieve the transformation of *P. infestans* but to date without success. A transformation system will not circumvent the need for RFLP mapping to obtain the avirulence genes however, as the size of the *P. infestans* genome, and the low transformation efficiency generally obtained with fungal systems would make transformation with total *P. infestans* DNA to obtain single genes impractical. Localisation of genes, via RFLP mapping and PFGE or YAC cloning, to certain regions of the *P. infestans* genome could then allow transformation to be performed using DNA of a far more manageable size, and would greatly increase the likelihood of transforming the fungus with the avirulence gene.

P. infestans is a relative new-comer to the field of molecular biology and RFLP analysis. In the field of population biology, RFLPs provide the level of variation which has long been desired to distinguish different isolates of *P. infestans* from one another. Nuclear and mitochondrial RFLPs also provide a means for analysing gene flow in natural populations. Genetic studies likewise benefit from the frequency of RFLPs, and the similar features of *P. infestans* recombination with the human system (ie. diploid, and obligately out-crossing) means data can be analysed using already established systems. New methods developed for human work to increase RFLP detection, as outlined above, may likewise be applicable to *P. infestans*. As RFLP markers are co-dominant, anomalous results which have often plagued *Phytophthora* geneticists may be more directly observable. Unusual progeny are thus less likely to complicate genetic studies.

As the demand for food worldwide increases, and the damage of existing practices

for pathogen control in the form of chemical sprays becomes more obvious, new methods for disease suppression are becoming increasingly necessary. DNA markers, while by no means capable of solving these problems will be a useful tool in furthering our understanding of the biology, ecology, pathology and genetics of *P. infestans*, and undoubtedly of many of the other phytopathogens to which they are currently being applied. It is hoped that this knowledge, combined with that produced in other fields, will eventually allow us to develop methods of control that are not only effective and durable, but also result in minimal damage to the other components of the natural environment.

REFERENCES.

Al-Kherb, S.M. (1988) **The Inheritance of Host-Specific Pathogenicity in *Phytophthora infestans*.** Ph.D thesis. University College of North Wales, Bangor.

Anderson, J.B., Petch, D.M., & Smith, M.L. (1987) **Restriction fragment length polymorphisms in biological species of *Armillaria mellea*.** Mycologia, 79: 69-76.

Awise, J.C., Gidlin-Davidson, C., Laerm, J., Patton, J.C. & Lansman, R.A. (1979) **Mitochondrial DNA clones and matriachial phylogeny within and among geographic populations of the pocket gopher, *Geomys pinetis*.** Proceedings of the National Academy of Sciences, U.S.A., 76 : 6694-6698.

Ball, R.M., Freeman, S., James, F.C., Bermingham, E. & Awise, J.C. (1988) **Phylogenetic population structure of Red-winged Blackbirds assessed by mitochondrial DNA.** Proceedings of the National Academy of Science, U.S.A., 85 : 1558-1562.

Bates, M.R., Buck, K.W. & Brasier, C.M. (1990) **Molecular variation in the Dutch elm disease fungus.** In: Molecular Evolution. UCLA Symposium on Molecular and Cellular Biology, Vol. 122. M.T. Clegg & S.J. O'Brien, Eds. Wiley-Liss, Inc.

Black, W., Mastenbroek, C., Mills, W.R. & Peterson, L.C. (1953) **A proposal for an international nomenclature of races of**

***Phytophthora infestans* and of genes controlling immunity in *Solanum demissum* derivatives.** Euphytica, 2 : 173-178.

Botstein, D., White, R., Skolnick, M. & Davis, R.W. (1980) **Construction of a genetic linkage map in man using restriction fragment length polymorphisms.** American Journal of Human Genetics, 32 : 314-331.

Boyd, D.A., Hobman, T.C., Gruenke, S.A & Klassen, G.R. (1984) **Evolutionary stability of mitochondrial DNA organisation in *Achlya*.** Canadian Journal of Cell Biology and Biochemistry, 62 : 571-576.

Braithwaith, K.S. & Manners, J.M. (1989) **Human hypervariable minisatellite probes detect DNA polymorphisms in the fungus *Colletotrichum gleosporioides*.** Current Genetics, 16 : 473-475.

Burke, D.T., Carle, G.F. & Olson, M.V. **Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors.** Science, 236 : 806-812.

Burke, T. & Bruford, M.W. (1987) **DNA fingerprinting in birds.** Nature, 327 : 149-152.

Cann, R.L., Stoneking, M. & Wilson, A.C. (1987) **Mitochondrial DNA and human evolution.** Nature, 325 : 31-36.

Cantor, C.R. (1984) **Huntington's disease : Charting the path to the gene.** Nature, 308 : 404-405.

Campbell, A.M., Moon, R.P., Duncan, J.M., Gurr, S-J. & Kinghorn, J.R. (1989) **Protoplast formation and regeneration from sporangia and encysted zoospores of *Phytophthora infestans***. Physiological and Molecular Plant Pathology, 34 : 299-307.

Caten, C.E. & Jinks, J.L. (1968) **Spontaneous variability of single isolates of *Phytophthora infestans***. 1. Cultural variation. Canadian Journal of Botany, 49 : 329-348.

Cobianchi, G.F. & Wilson, S.H. (1987) **Enzymes for modifying and labelling DNA and RNA**. In: Guide to Molecular Cloning Techniques. S.L. Berger & A.R. Kimmel, Eds., pp 94-110. Academic Press Inc.

Coffey, M.D. & Wilson, U.E. (1983) **Histology and cytology of infection and disease caused by *Phytophthora***. In : *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology*. D.C. Erwin, S. Bartnicki-Garcia & P.H. Tsao, Eds. pp 289-301. American Phytopathological Society, St. Paul, Minnesota.

Collins, R.A. & Lambowitz, A.M. (1981) **Characterisation of a variant *Neurospora crassa* mitochondrial DNA which contains tandem reiterations of a 1.9 kb sequence**. Current Genetics, 4 : 131-133.

Coulandre, C., Miller, J.H., Farnbaugh, P.J. & Gilbert, W. (1978) **Molecular basis of base substitution hotspots on *Escherichia coli***. Nature, 274 : 775-780.

Croft, J.H. (1987) **Genetic variation and evolution in *Aspergillus***. In : Evolutionary Biology of the Fungi. A.D.M. Rayner, C.M. Brasier & D.

Moore, Eds. Symposium of the British Mycological Society, CUP, Cambridge.
311-324.

Dallas, J.F. (1988) **Detection of DNA "fingerprints" of cultivated rice by hybridisation with a human minisatellite DNA probe.** Proceedings of the National Academy of Science, USA. 85 : 6831-6835.

Deahl, K.L., Goth, R.W., Young, R., Sinden, S.L. & Gallegly, M.E. (1990) **Occurrence of the A2 mating type of *Phytophthora infestans* in fields in the United States and Canada.** Phytopathology (Abstract), (in press)

Evola, S.V., Burr, F.A. & Burr, B. (1986) **The suitability of restriction fragment length polymorphisms as genetic markers in maize.** Theoretical and Applied Genetics, 71 : 765-771.

Feinberg, A.P. & Vogelstein, B. (1983) **A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity.** Analytical Biochemistry. 132 : 6-13.

Flor, H.H. (1954) **Identification of races of flax rust by lines with single rust conditioning genes.** U.S. Department of Agriculture Technical Bulletin, 1087 : 25.

Forster, H. & Coffey, M.D. (1990) **Approaches to the taxonomy of *Phytophthora* using polymorphisms in mitochondrial and nuclear DNA.** In : *Phytophthora* '89 (in press).

Forster, H., Kinscherf, T.L., Leong, S.A. & Maxwell, D.P. (1987) **Molecular analysis of the mitochondrial genome of *Phytophthora***. Current Genetics, **12** : 215-218.

Forster, H., Kinscherf, T.G., Leong, S.A. & Maxwell, D.P. (1989) **Restriction fragment length polymorphisms of the mitochondrial DNA of *Pytophthora megasperma* isolated from soybean, alfalfa and fruit trees**. Canadian Journal of Botany, **67** : 529-537.

Forster, H., Oudemans, P. & Coffey, M.D. (1990) **Mitochondrial and nuclear DNA diversity within six species of *Phytophthora***. Experimental Mycology, (in press).

Garber & Yoder (1983) **Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal and plasmid components**. Analytical Biochemistry. **135** : 416-422.

Garber, R.C. & Yoder, O.C. (1984) **Mitochondrial DNA of the filamentous ascomycete *Cochliobolus heterostrophus***. **Characterisation of the mitochondrial chromosome and population genetics of a restriction enzyme polymorphism**. Current Genetics : **8** : 621-628.

Goodwin, S.B. (1990) **Molecular mapping in *Phytophthora infestans***. In : *Phytophthora* '89 (in press)

Gregory, P.H. (1984) **Some major epidemics caused by *Phytophthora***. In : *Phytophthora : Its Biology, Taxonomy, Ecology and Pathology*.

D.C. Erwin, S. Bartnicki-Garcia & P.H. Tsao, Eds. pp 271-278. American Phytopathological Society, St. Paul, Minnesota.

Grodzicker, T., Williams, J., Sharp, P. & Sambrook, J. (1974) **Physical mapping of temperature sensitive mutations of adenoviruses.** Cold Spring Harbour Symposium on Quantitative Biology. 39 : 439-446.

Gunderson, J.H., Elwood, H., Ingold, A., Kindle, K. & Sogin, M.L. (1987) **Phylogenetic relationships between Chlorophytes, Chrysophytes and Oomycetes.** Proceedings of the National Academy of Science, U.S.A. 84 : 5823-5827.

Hanahan, D. (1985) **Techniques for transformation of *Escherichia coli*.** In: **DNA cloning: a practical approach.** D.M. Glover, Ed. Oxford: IRL Press. Vol. 1, 109-135.

Helentjaris, T., King, G., Slocum, M., Siedenstrang, C. & S. Wegman. (1985) **Restriction fragment polymorphisms as probes for plant diversity and their development as tools for applied plant breeding.** Plant Molecular Biology, 5 : 109-118.

Hoeben, P. & Clark-Walker, G.D. (1986) **An approach to yeast classification by mapping mitochondrial DNA from *Dekkera/Bretanomyces* and *Einiella* genera.** Current Genetics, 10 : 371-379.

Hohl, H.R. & Iselin, K. (1984) **Strains of *Phytophthora infestans* from Switzerland with A2 mating type behaviour.** Transactions of the British

mycological Society, 83 : 529-530.

Howlett, B.J. (1989) **An electrophoretic karyotype for *Phytophthora megasperma***. Experimental Mycology, 13 : 199-202.

Hudspeth, M.E.S. (1985) **Oomycete mitochondrial genomes**. Journal of Cellular Biochemistry suppl. 9C : 187.

Hudspeth, M.E.S., Timberlake, W.E. & Goldberg, R.B. (1977) **DNA sequence organisation in the water mould *Achlya***. Proceedings of the National Academy of Science, U.S.A., 74 : 4332-4336.

Hudspeth, M.E.S., Shumard, D.S., Bradford, C.J.R. & Grossman, L.I. (1983) **Organisation of *Achlya* myDNA : A population with two orientations and a large inverted repeat containing the rRNA genes**. Proceedings of the National Academy of Science, U.S.A., 80 : 142-146.

Hulbert, S.H., Illott, T.W., Legg, E.J., Lincoln, S.E., Lander, E.S. & Michelmore, R.E. (1988) **Genetic analysis of the fungus *Bremia lactucae*, using restriction fragment length polymorphisms**. Genetics, 120 : 947-958.

Hulbert, S.H. & Michelmore, R.W. (1988) **DNA restriction fragment length polymorphism and somatic variation in the lettuce downy mildew fungus *Bremia lactucae***. Molecular Plant Microbe Interactions, 1 : 17-24.

Hutchinson, C. III, Newbold, J., Potter, S. & Edgell, M. (1974) **Maternal**

inheritance of mammalian mitochondrial DNA. Nature, 251 : 536.

Jeffreys, A.J. (1979) **DNA sequence variants in the α g - , Ag - , δ - and β -globin genes of man.** Cell, 18 : 1-10.

Jeffreys, A.J., Wilson, V & Thein, S.L. (1985) **Hypervariable "minisatellite" regions in human DNA.** Nature, 314 : 67-73.

Jeffreys, A.J., Wilson, V., Kelley, R., Taylor, B.A. & Bulfield, G. (1987) **Mouse DNA "fingerprints" : analysis of chromosome localisation and germ line stability of hypervariable loci in inbred strains.** Nucleic Acids Research 15 : 2823-2836.

Kinscherf, T.G. & Leong, S.A. (1988) **Molecular analysis of karyotypes of *Ustilago maydis*.** Chromosoma :

Klimczac, L.J. & Prell, H.H. (1984) **Isolation and characterisation of mitochondrial DNA of the oomycetous fungus *Phytophthora infestans*.** Current Genetics, 8 : 323-326.

Ko, W.H. (1988) **Hormonal heterothallism and homothallism in *Phytophthora*.** Annual Review of Phytopathology, 26 : 57-73.

Kolar, M., Punt, P.J., Cees, A.M., van den Hondel, J.J. and Schwab, H. (1988) **Transformation of *Penicillium chyogenum* using dominant selection markers and expression of an *Escherichia coli* lacZ fusion gene** Gene 62 : 127-134.

Lander, E.S. (1989) **DNA fingerprinting on trial.** Nature, **339** : 501-505.

Landry, B.S., Kesseli, R.V., Farrara, B. & Michelmore, R.W. (1987) **A genetic map of lettuce (*Lactuca sativa* L.) with restriction fragment length polymorphism, isozyme, disease resistance and morphological markers.** Genetics, **116** : 331-337.

Landry, B.S. & Michelmore, R.W. (1985) . **Selection of probes for restriction fragment analysis from plant genomic clones.** Plant molecular Biology Reports, **3** : 174-179.

Lanthrop, G.M., Lalouel, J.M., Julier, C., & Ott, J. **Strategies for multilocus linkage analysis in humans.** Proceedings of the National Academy of Science. USA. **81** : 3443-3446.

Latorre, A., Moya, A. & Ayala, F.J. (1986) **Evolution of mitochondrial DNA in *Drosophila subobscura*.** Proceedings of the National Academy of Science U.S.A., **83** : 8649-8653.

Laviola, C. & Gallegly, M.E. (1983) **Genetic recombination and mode of inheritance of pathogenic characters by *Phytophthora infestans* through sexual reproduction.** In : Durable resistance in crops. F. Lamberti, J.M. Walker, & N.A. Van Der Graaff, Eds., pp 339-345. Plenum Press, New York (US).

Li, W.H., Luo, C.C. & Wu, C.I. (1985) **Evolution of DNA sequences.** In : Molecular Evolutionary Genetics, R.J. MacIntyre, Ed., pp1-94. Plenum, New York/London.

- Malcolmson, J.F. (1970) **Vegetative hybridity in *Phytophthora infestans***. Nature, **225** : 971-972.
- Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982). **Molecular Cloning: A Laboratory Manual**. New York, U.S.A. : Cold Spring Harbour Laboratory Press.
- Manella, C.A., Pittenger, T.H. & Lambowitz, A.M. (1979) **Transmission of mitochondrial deoxyribonucleic acid in *Neurospora crassa* sexual crosses**. Journal of Bacteriology, **137** : 1449-1451.
- McNabb, S.A. & Klassen, G.R. (1988) **Uniformity of mitochondrial DNA complexity in Oomycetes and the evolution of the inverted repeat**. Experimental Mycology, **12** : 233-242.
- Michelmore, R.W., Norwood, J.M., Ingram, D.S., Crute, I.R. & Nicholson, P. (1984) **The inheritance of virulence in *Bremia lactucae* to match resistance factors 3, 4, 5, 6, 8, 9, 10, and 11 in lettuce (*Lactuca sativa*)**. Plant Pathology **33** : 301-315.
- Mitchell, .B. & Mitchell, H.K. (1952) **A case of "maternal" inheritance in *Neurospora crassa***. Proceedings of the National Academy of Science, U.S.A., **38** : 442-449.
- Montagne, C. (1845) **Observations sur la maladie des pommes de terre**. L'Institut. Bulletin de la Societe Philomatique de Paris, **13** : 312-314.

Mosa, A.A., Kato, M., Sato, N., Kobayashi, K. & Ogoshi, A. (1989)

Occurrence of the A2 mating type of *Phytophthora infestans* on potato in Japan. Annals of the Phytopathological Society of Japan, **55** : 615-620.

Mullis, K.B., & Faloona, F.A. (1987) **Specific synthesis of DNA in vitro**

via a polymerase catalysed chain reaction. Methods in Enzymology, **155** : 335-350.

Murrien, F., Holtby, J., Nolan, R.A. & Davidson, W.S. (1986) **The genome of *Entomophaga aulicae* (Entomophthorales, Zygomycetes): Base composition and size.** Experimental Mycology, **10** : 67-75.

Newhook, F.J., Waterhouse, G.M. & Stamps, D.J. (1978) **Tabular key to the species of *Phytophthora* de Bary.** Mycological Paper No. 143, Commonwealth Mycological Institute, Kew, Surrey.

Nur, U., Werren, J.H., Eickbush, D.G., Burke, W.D. & Eickbush, T.H. (1988) **A "selfish" B chromosome that enhances its transmission by eliminating the paternal genome.** Science **240** : 512-514.

Orbach, J.M., Vollrath, D., Davis, R.W. & Yanofsky, C. (1988) **An electrophoretic karyotype of *Neurospora crassa*.** Molecular and Cellular Biology, **8** : 1469 - 1473.

O'Dell, M., Wolfe, M.S., Flavell, R.B., Simpson, C.G. & Summers, R.W. (1989) **Molecular variation in populations of *Erysiphe graminis* on**

barley, oats and rye. Plant Pathology, **38** : 340-351.

Petes, T.K. & Botstein, D. (1977) **Simple Mendelian inheritance of the reiterated ribosomal DNA of yeast.** Proceedings of the National Academy of Science, U.S.A., **74** : 5091- 5095.

Poedinok, N.L. & Dyakov, Y.T. (1981) **Observations of vegetative incompatibility in *Phytophthora infestans* (Mont) de Bary.** Mikologiya i Fitopatologiya **15** : 275-279.

Prell, H.H., Karlovsky, P. & Bahnweg, G. (1990) **Towards transformation in *Phytophthora*.** In : *Phytophthora '89* (in press).

Raeder, U. & Broder, P. (1985) **Rapid preparation of DNA from filamentous fungi.** Letters in Applied Microbiology, **1** : 17-20.

Raeder, U., Thompson, W. & Broda, P. (1989) **RFLP-based genetic map of *Phanerochaete chrysosporium* ME446 : lignin peroxidase genes occur in clusters.** Molecular Microbiology, **3** : 911-918.

Riordan, J.R., Rommens, J.M., Kerem, b., Alon, N., Rozmahel, R., Grezelczac, Z., Zielenski, J., Lok, S., Plavsic, N. & Chou, J.L. (1989) **Identification of the cystic fibrosis gene: cloning and characterisation on complementary DNA.** Science, **245** : 1066-1073.

Rogstad, S.H., Patton, J.C. & Schaal, B.A. (1988) **A human minisatellite probe reveals RFLPs among individuals of two angiosperms.** Nucleic Acids Research, **16** : 11378.

Romero, S. & Erwin, D.C. (1969) **Variation in pathogenicity among**

single-oospore cultures of *Phytophthora infestans*.

59 : 1310-1317.

Rotwein, P., Chyn, R., Chirgwin, J., Cordell, B., Goodman, H.M. & Permutt, M.A. (1981) **Polymorphism in the 5'-flanking region of the human insulin gene and its possible relation to type 2 diabetes.** Science, 213 : 1117-1120.

Rutherford, F.S. & Ward, E.W. (1985) **Estimation of relative DNA content in nuclei of races of *Phytophthora megasperma* f. sp. *glycinea* by quantitative fluorescence microscopy.** Canadian Journal of Genetics and Cytology, 27 : 614-616.

Saiki, R.K., Scharf, F., Faloona, K.B., Mullis, G.T. Horn, G.T., Erlich, H.A. & Anrhein, N. (1985) **Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia.** Science, 230 : 1350-1354.

Sealey, P.G., Whittacker, P.A. & Southern, E.M. (1985) **Removal of repeated sequences from hybridisation probes.** Nucleic Acids Research, 13 : 1905 - 1922.

Sederoff, R.R., Levings III, C.S., Timothy, D.H. & Hu, W.W.L. (1981) **Evolution of DNA sequence in mitochondrial genomes of *Zea*.** Proceedings of the National Academy of Science, U.S.A., 78 : 5953-5957.

Schwartz, D.C. & Cantor, C.R. (1984) **Separation of yeast chromosome-**

sized DNAs by pulsed field gradient electrophoresis. Cell, 37 : 67-75.

Shattock, R.C., Tooley, P.W., Swiegard, J. & Fry, W.E. (1987) **Genetic studies of *Phytophthora infestans***. In : Genetics and Plant Pathogenesis, P.R. Day & G.J. Jellis, Eds., pp 175-185. Blackwell Scientific Publications, Oxford.

Shattock, R.C. & Shaw, D.S. (1976) **Novel phenotypes of *Phytophthora infestans* from mixed cultures of antibiotic resistant mutants**. Transactions of the British mycological Society, 67 : 201-206.

Shattock, R.C., Shaw, D.S., Fyfe, A.M., Dunn, J.R., Loney, K.H. & J.A. Shattock. (1990) **Phenotypes of *Phytophthora infestans* collected in England and Wales from 1985 to 1988 : mating type, response to metalaxyl and isoenzyme analysis**. Plant Pathology, (in press).

Shattock, R.C., Tooley, P.W. & Fry, W.E. (1986a) **Genetics of *Phytophthora infestans* : Characterisation of single-oospore cultures from A1 isolates induced to self by intraspecific stimulation**. Phytopathology, 76 : 407-410.

Shattock, R.C., Tooley, P.W. & Fry, W.E. (1986b) **Genetics of *Phytophthora infestans* : Determination of recombination, segregation, and selfing by isozyme analysis**. Phytopathology, 76 : 410-413.

Shaw, D.S. (1990) **The genetics of *Phytophthora infestans***. Advances

in Plant Pathology. Z (in press).

Shaw, D.S., Fyfe, A.M., Hibberd, P.G. & Abdel-Sattar, M.A. (1985)
Occurrence of the rare A2 mating type of *Phytophthora infestans*
on imported Egyptian potatoes and the production of sexual progeny
with A1 mating types from the U.K. Plant Pathology, **34** : 552-56.

Skelley, P.J. & Maleszka, R. (1989) **Isolation of mitochondrial DNA**
using pulsed field gel electrophoresis. Nucleic Acids Research, **17** :
 7537.

Skolnick, M.H. & Wallace, R.B. (1988) **Simultaneous analysis of**
multiple polymorphic loci using amplified sequence polymorphisms.
Genomics, **2** : 273-279.

Smith, C.L., Kico, S.R. & Cantor, C.R. (1988) **Pulsed field gel**
electrophoresis. In : **Genome Analysis: A Practical Approach.** K.E.
 Davies, Ed. pp41-72. IRL Press.

Smith, M.L. & Anderson, J.B. (1989) **Restriction fragment length**
polymorphisms in mitochondrial DNAs of *Armillaria*: identification of
North American biological species. Mycological Research, **93** : 247-256.

Spielman, 1990. **Isozymes and the population genetics of**
***Phytophthora infestans*.** In : *Phytophthora*'89. (in press)

Spielman, L.J., Swiegard, J.A., Shattock, R.C. & Fry, W.E. (1990) **The**
genetics of *Phytophthora infestans* : Segregation of allozyme
markers in F2 and backcross progeny and the inheritance of

virulence against potato resistance genes R2 and R4 in F1 progeny.

Experimental Mycology, 14 : 57-69.

Tanaka, K., Heath, I.B. & Moens, P.B. (1982) **Karyotype, synaptonemal complexes, and possible recombination nodules of the oomycete fungus *Saprolegnia*.** Canadian Journal of Genetics and Cytology, 24 : 385-396.

Tautz, D. (1989) **Hypervariability of simple sequences as a general source for polymorphic DNA markers.** Nucleic Acids Research 17 : 6463 - 6471.

Tautz, D. & Renz, M. (1984) **Simple sequences are ubiquitous repetitive components of eukaryote genomes.** Nucleic Acids Research, 12 : 4127 - 4138.

Taylor, J.W. (1986) **Fungal evolutionary biology and mitochondrial DNA.** Experimental Mycology 10 : 259-269.

Therrien, C.D., Ritch, D. Davidse, L.C., Jespers, A.B.K. & Spielman, L.J. (1989) **Nuclear DNA content, mating type and metalaxal sensitivity of eighty-three isolates of *Phytophthora infestans* from the Netherlands.** Mycological Research, 92 : 140-146.

Tooley, P.W., Fry, W.E. & Villarreal Gonzalez, M.J. (1985) **Isozyme characterisation of sexual and asexual *Phytophthora infestans* populations.** Journal of Heredity, 76 : 431-435.

Tooley, P.W., Swiegard, J.A. & Fry, W.E. (1986) **Fitness and virulence of *Phytophthora infestans* isolates from sexual and asexual populations.** Phytopathology, 76 : 1209-1212.

Tooley, P.W. & Therrien, C.D. (1987) **Cytophotometric determination of the nuclear DNA content of 23 Mexican and 18 Non-Mexican isolates of *Phytophthora infestans*.** Experimental Mycology, 11 : 19-26.

Tooley, P.W., Therrien, C.D. and Ritch, D.L. (1989) **Mating type, race composition, nuclear DNA content and isozyme analysis of Peruvian isolates of *Phytophthora infestans*.** Phytopathology, 79 : 478-481.

Ullrich, A., Dull, T.J., Gray, A., Brosius, J. & Sures, I. (1980) **Genetic variation in the human insulin gene.** Science 209 : 612-615.

Varma, A. & Kwon-Chung, K.J. (1989) **Restriction fragment polymorphism in mitochondrial DNA of *Cryptococcus neoformans*.** Journal of General Microbiology, 135 : 3353-3362.

Weber, C.A., Hudspeth, M.E.S., Moore, G.P. & Grossman, L.I. (1986) **Analysis of the mitochondrial and nuclear genomes of two Basidiomycetes. *Coprinus cinereus* and *Coprinus stercorarius*.** Current Genetics, 10 : 515-525.

Weber, J.L. & May, P.E. (1989) **Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction.** American Journal of Human Genetics. 44 : 388-396.

Whitticker, S.L., Shattock, R.C. and Shaw, D.S. (1990) **Variation in DNA contents of *Phytophthora infestans* as measured by a microfluorimetric method using the fluorochrome DAPI.** Mycological Research, (in press).

APPENDIX 1

Virulence data for Ca65, 550 and 80 progeny from
Ca65 x 550 cross. (Unpublished results from C.Fininsa)

PROGENY NUMBER	MATING TYPE	DIFFERENTIALS									
		R ₀	R ₁	POTATO R ₂	POTATO R ₃	R ₄	R ₅	R ₇	R ₈	R ₁₀	R ₁₁
201	A1	+	+	-	+	+	-	+	-	+	-
202	A2	-	NT	NT	NT	NT	NT	NT	NT	NT	NT
203	A1	+	+	-	+	+	-	+	+	-	+
205	A1	+	+	+	-	+	-	-	-	-	-
206	A2	+	+	-	-	+	+	-	-	-	+
207	A1	+	+	-	+	+	-	+	-	+	+
208	A2	+	+	+	-	+	+	-	-	+	+
210	A1	-	NT	NT	NT	NT	NT	NT	NT	NT	NT
211	A2	+	+	-	-	+	+	-	-	+	+
212	A2	+	+	+	-	+	-	+	+	+	+
214	A1	+	+	-	-	+	+	+	-	+	+
215	A1	+	+	+	+	+	+	-	+	-	+
216	A1	+	+	+	+	+	+	-	-	+	+
217	A1	+	+	-	+	+	+	+	+	+	+
218	A2	+	+	+	+	+	-	+	+	+	+
219	A1	+	+	-	+	+	+	+	+	+	+
220	A1	+	+	+	+	+	+	+	-	+	+
221	A2	-	NT	NT	NT	NT	NT	NT	NT	NT	NT
224	A1	-	NT	NT	NT	NT	NT	NT	NT	NT	NT
225	A2	+	+	+	-	+	-	-	-	+	+
226	A2	+	+	+	+	+	-	+	-	+	+
227	A2	+	+	-	+	+	+	-	+	+	+
230	A1	+	+	+	+	+	-	+	-	-	+
231	A2	+	+	-	-	+	-	-	+	+	+
232	A2	+	+	+	+	+	-	+	-	+	+
233	A1	+	+	+	+	+	-	+	-	+	+
234	A1	+	+	-	+	+	+	-	-	+	+
235	A1	+	+	+	+	+	-	+	-	+	+
236	A2	+	+	-	-	+	+	-	-	-	+
237	A2	+	+	-	-	+	+	-	-	-	+
239	A1	+	+	-	-	+	-	-	-	-	+
240	A2	+	+	+	+	+	+	-	+	+	+
242	A1	+	-	+	-	+	-	-	-	+	+
243	A1	+	+	-	-	+	-	-	-	-	+
244	A1	+	+	+	+	+	-	+	-	+	+
245	A2	+	+	-	-	+	-	-	-	+	-
246	A1	+	+	-	+	+	+	+	+	+	+
247	A2	+	+	-	-	+	+	-	-	+	+
248	A1	+	+	-	-	+	-	-	-	-	+
249	A1	+	+	+	-	+	+	+	-	+	+
250	A1	+	+	+	+	+	+	+	-	+	+
251	A2	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
252	A2	+	+	+	-	+	-	-	-	NC	-
253	A1	+	+	-	-	+	-	-	-	NC	+
254	A2	+	+	+	+	-	+	+	+	NC	+
257	A1	+	+	-	-	+	-	-	-	NC	-
258	A2	+	+	+	+	+	+	+	+	NC	+
260	A1	+	+	+	-	-	-	-	-	NC	-
261	A1	+	+	+	-	+	-	-	-	NC	+

262	A2	+	+	-	+	+	-	+	-	NC	+
263	A2	+	+	+	+	+	+	+	+	NC	+
264	SF	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
265	A2	-	NT	NT	NT	NT	NT	NT	NT	NT	NT
266	A2	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
267	A1	+	+	-	+	+	-	+	-	NC	+
273	A2	+	+	-	-	+	-	-	-	NT	NT
274	A2	+	+	-	-	+	+	+	+	NT	NT
279	A1	+	-	+	+	+	+	+	+	NT	NT
281	A1	+	+	-	+	+	-	+	-	NT	NT
282	SF	+	+	-	-	+	-	-	-	NT	NT
284	A1	+	+	-	+	+	+	+	+	NT	NT
285	A1	+	+	+	-	+	+	+	+	NT	NT
287	A1	+	+	-	-	-	-	-	-	NT	NT
288	A2	+	+	-	+	-	-	+	-	NT	NT
289	A1	+	+	-	-	+	-	-	-	NT	NT
291	A2	+	+	-	+	+	+	+	+	NT	NT
295	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
303	A2	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
305	A1	+	+	NT	+	+	NT	+	NT	-	-
308	A1	+	+	NT	-	NT	NT	NT	NT	NT	+
312	A1	+	+	NT	+	+	NT	NT	NT	+	+
313	A2	+	+	+	-	+	NT	-	NT	-	-
314	A2	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
318	A1	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
321	A1	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
327	A2	-	NT	NT	NT	NT	NT	NT	NT	NT	NT
328	A1	-	NT	NT	NT	NT	NT	NT	NT	NT	NT

NC = Not Conclusive

NT = Not Tested

+

- = Avirulent (Incompatible)

APPENDIX 2

Presence (1) of absence (0) of alleles at each RFLP and avirulence gene locus for Ca65, 550 and the 77 hybrid progeny.

The following progeny were omitted from the genetic analysis :

201, 202, 207, 208, 212, 218, 233, 234, 329, 242, 243, 244, 245, 246,
247, 248, 251, 260, 261, 264, 265, 273, 281, 285, 295, 303, 305, 312.

Progeny
Designation

RFLP loci

Avirulence gene loci

	174	29	6	304	317	313	13	362	11	269	99	61	62	655	725	599	65	26	216	199	Avr-2	Avr-3	Avr-5	Avr-7	Avr-8	Avr-10
Ca65	10	11	10	101	011	11	11	10	11	11	101	10	001	1001	01	11	11	11	001	11	01	01	01	01	01	01
550	11	10	11	011	110	10	10	11	10	10	110	11	110	0110	11	01	01	10	110	11	11	11	11	11	01	11
201	10	10	00	110	011	11	11	00	11	10	110	11	011	1100	11	11	11	10	011	11	11	01	11	01	11	01
202	10	11	10	110	011	11	11	11	11	11	101	11	000	0101	01	01	01	11	101	11	00	00	00	00	00	00
203	11	11	11	110	110	10	10	11	10	11	110	10	101	0101	01	01	11	11	011	01	11	01	11	01	01	11
205	10	11	11	101	010	10	10	11	10	11	101	11	011	0011	11	01	01	11	011	11	01	11	11	11	11	11
206	00	11	10	101	000	11	11	11	11	10	101	00	101	0101	11	01	11	01	101	10	11	11	11	11	11	11
207	11	11	10	101	110	11	10	11	10	11	101	11	011	0011	01	01	01	11	011	11	11	01	11	01	11	01
208	10	11	00	000	010	10	11	11	11	10	110	11	101	1100	11	00	01	00	101	01	01	11	01	11	11	01
210	11	11	10	001	000	10	11	11	10	11	110	11	101	0011	01	01	11	11	011	11	00	00	00	00	00	00
211	00	10	00	101	101	11	10	10	10	11	110	11	101	1010	11	00	11	11	000	11	11	11	01	11	11	01
212	11	11	11	000	000	11	10	11	10	11	101	10	011	1100	01	11	01	11	011	10	01	11	11	01	01	01
214	00	11	00	000	000	11	11	10	10	11	101	10	011	0000	00	11	00	00	000	10	11	11	01	01	11	01
215	10	11	11	011	010	10	11	10	11	10	110	10	101	1010	01	11	01	10	011	11	01	01	01	11	01	11
216	10	10	10	011	011	11	11	10	11	10	101	10	011	1010	11	11	01	10	011	11	01	01	01	11	11	01
217	10	11	11	011	010	10	11	11	10	11	110	11	101	1100	00	11	01	11	101	01	11	01	01	01	01	01
218	11	11	11	001	110	10	11	11	11	00	110	10	011	1010	11	01	01	10	011	01	01	01	11	01	01	01
219	11	11	10	001	110	10	10	11	11	10	110	10	101	1010	11	11	01	11	101	11	11	01	01	01	01	01
220	11	11	10	011	110	10	10	10	11	10	101	11	101	0011	01	01	11	10	011	11	01	01	01	01	11	01
221	11	10	10	110	101	11	10	10	10	11	110	11	101	1010	11	11	11	11	101	11	00	00	00	00	00	00
224	11	10	10	101	101	11	11	10	10	11	110	10	101	0101	01	01	11	11	011	11	00	00	00	00	00	00
225	11	10	10	011	110	11	10	11	10	11	101	11	101	0101	01	01	11	11	101	11	01	11	11	11	11	01
226	11	11	11	001	110	10	11	11	10	00	110	10	101	1100	01	00	01	11	101	10	01	01	11	01	11	01
227	11	11	11	101	110	10	11	10	10	11	110	11	011	0101	11	00	11	11	101	01	11	01	01	11	01	01
230	11	11	10	101	110	10	10	10	11	10	110	11	101	1100	01	00	01	10	011	11	01	01	11	01	11	11
231	11	10	10	011	101	11	10	10	11	10	110	10	101	0011	01	01	01	10	101	11	11	11	11	11	01	01
232	10	11	10	011	010	11	11	11	11	11	110	10	101	1010	01	01	01	10	011	11	01	01	11	01	11	01
233	10	11	00	001	110	10	10	10	11	10	110	10	000	0101	11	01	11	11	000	11	01	01	11	01	11	01
234	00	11	00	000	011	11	11	00	10	11	101	11	011	0101	01	00	01	11	011	01	11	01	01	11	11	01
235	10	11	11	001	010	10	10	00	11	10	110	11	011	0011	01	00	01	10	101	10	01	01	11	01	11	01

Progeny
Designation

RFLP loci

Avirulence gene loci

	174	29	6	304	317	313	13	362	11	269	99	61	62	655	725	599	65	26	216	199	Avr-2	Avr-3	Avr-5	Avr-7	Avr-8	Avr-10
236	11	10	10	011	110	11	11	10	10	11	110	10	011	0011	11	00	11	11	101	01	11	11	01	11	11	11
237	11	10	10	011	110	11	11	10	10	11	110	10	011	0011	11	00	11	11	101	01	11	11	01	11	11	11
239	10	11	11	001	010	10	11	10	11	10	110	10	011	0101	11	01	11	00	000	11	11	11	11	11	11	11
240	10	10	11	110	011	11	10	10	11	11	101	11	011	1100	01	11	11	10	011	10	01	01	01	11	01	01
242	11	11	11	000	110	10	10	10	10	11	101	10	011	0000	01	01	01	11	101	10	01	11	11	11	11	01
243	10	11	11	000	011	11	11	11	11	10	101	10	011	0000	01	01	11	10	011	11	11	11	11	11	11	11
244	10	10	11	011	011	11	11	11	11	10	101	11	011	0000	11	11	11	11	101	10	01	01	11	01	11	01
245	10	11	10	000	011	11	11	10	10	11	101	00	101	0011	11	01	01	11	011	01	11	11	11	11	11	01
246	10	11	11	110	011	11	11	11	10	11	101	10	011	0000	01	00	01	11	011	11	11	01	01	01	01	01
247	11	11	10	101	101	10	10	11	11	11	110	10	101	0101	01	01	11	10	011	11	11	11	01	11	11	01
248	10	10	10	000	011	11	11	11	11	11	101	11	011	0000	11	11	01	10	011	10	11	11	11	11	11	11
249	10	11	10	011	010	10	11	11	10	11	110	11	011	0011	11	00	11	11	011	01	01	11	01	01	11	01
250	11	10	10	001	101	11	10	11	10	10	110	11	101	1100	11	11	11	11	011	11	01	01	01	01	11	01
251	11	11	10	101	000	11	11	11	00	11	101	11	011	0000	01	11	01	11	011	01	00	00	00	00	00	00
252	10	10	11	110	011	11	10	11	10	11	110	11	101	0011	01	01	01	11	011	11	01	11	11	11	11	00
253	10	11	10	001	010	10	10	10	10	10	101	11	101	0101	01	01	01	11	011	11	11	11	11	11	11	00
254	10	11	11	110	010	10	11	10	10	11	101	11	101	1100	11	11	01	11	011	11	01	01	01	01	01	00
257	10	11	10	001	010	10	10	10	10	10	101	11	101	0101	01	01	11	11	011	11	11	11	11	11	11	00
258	10	10	11	110	011	10	10	11	10	11	110	10	101	0011	11	01	01	11	011	11	01	01	01	01	01	00
260	11	11	11	001	000	11	10	11	11	10	101	00	101	0000	01	11	11	11	011	01	01	11	11	11	11	00
261	10	11	10	101	011	11	11	10	11	10	101	10	101	0000	01	01	00	10	101	10	01	11	11	11	11	00
262	10	11	11	101	00	10	10	10	11	10	110	00	101	1010	00	11	11	10	011	11	11	01	11	01	11	00
263	10	10	11	011	011	11	10	10	10	11	110	10	011	0011	11	01	01	11	101	10	01	01	01	01	01	00
264	10	10	11	101	011	11	10	11	11	10	110	10	011	1100	01	11	11	10	000	10	00	00	00	00	00	00
265	11	00	10	000	110	10	10	10	00	10	110	11	101	0000	01	00	01	10	000	00	00	00	00	00	00	00
266	10	10	10	101	011	11	11	11	11	10	101	10	011	0101	11	01	01	10	101	01	00	00	00	00	00	00
267	10	11	11	110	010	10	11	10	11	10	110	11	101	0101	11	11	11	10	101	01	11	01	11	01	11	00
273	10	11	11	011	011	11	11	11	11	11	101	11	101	0000	01	01	01	11	011	10	11	11	11	11	11	00
274	10	11	10	001	010	10	11	10	10	11	101	11	011	1010	11	00	11	11	101	11	11	11	01	01	01	00
279	10	10	11	011	011	10	11	10	10	11	110	11	011	1010	01	11	11	11	011	11	01	01	01	01	01	00

Progeny
Designation

RFLP loci

Avirulence gene loci

	174	29	6	304	317	313	13	362	11	269	99	61	62	655	725	599	65	26	216	199	<u>Avr-2</u>	<u>Avr-3</u>	<u>Avr-5</u>	<u>Avr-7</u>	<u>Avr-8</u>	<u>Avr-10</u>
281	10	11	10	000	011	11	11	11	11	11	101	11	101	1010	01	00	01	11	011	10	11	01	11	01	11	00
282	11	10	11	001	101	11	11	10	11	11	110	10	101	0101	11	01	01	10	101	10	11	11	11	11	11	00
284	11	11	11	011	110	10	11	11	11	10	110	11	011	1010	01	11	01	10	101	11	11	01	01	01	01	00
285	11	11	10	000	110	11	10	11	11	10	110	11	101	0000	01	11	01	11	101	10	01	11	01	01	01	00
287	11	11	10	011	110	10	11	11	10	11	110	11	011	1100	01	11	01	11	011	10	11	11	11	11	11	00
288	10	10	10	001	011	11	10	11	11	10	101	10	011	0011	11	01	01	10	011	11	11	01	11	01	11	00
289	10	11	10	101	010	10	11	10	11	11	110	11	101	0101	11	01	11	10	101	01	11	11	11	11	11	00
291	10	10	11	110	011	11	11	11	10	11	110	11	101	0101	11	01	11	11	101	10	11	01	01	01	01	00
295	10	11	11	000	011	11	11	00	11	10	101	11	101	1100	01	00	01	10	000	01	00	00	00	00	00	00
303	11	11	10	110	110	10	11	10	11	11	110	10	101	0011	01	01	11	10	011	10	00	00	00	00	00	00
305	11	10	11	110	101	11	10	00	10	11	110	10	011	1010	11	11	11	11	101	10	00	01	00	01	00	11
308	11	11	10	001	110	10	11	11	11	10	110	10	011	1100	11	11	01	10	101	01	00	11	00	00	00	00
312	10	10	00	011	011	10	11	00	10	11	110	11	011	1010	01	11	11	11	011	11	00	01	00	00	00	01
313	11	11	10	110	110	10	10	10	10	11	101	10	011	1010	01	11	01	11	101	01	01	11	00	11	00	11
314	10	11	10	001	010	10	10	11	11	10	101	10	101	0101	11	11	01	10	101	10	00	00	00	00	00	00
318	11	10	11	110	101	11	11	10	10	11	101	11	011	0011	01	11	11	11	011	10	00	00	00	00	00	00
321	10	10	11	110	011	10	10	11	11	10	110	10	011	0011	01	11	01	10	011	10	00	00	00	00	00	00
327	11	11	10	001	110	10	10	10	11	11	110	11	011	1100	01	11	11	10	101	11	00	00	00	00	00	00
328	10	10	11	011	011	11	10	10	11	10	110	11	011	0011	01	01	01	10	011	01	00	00	00	00	00	00