MOLECULAR CHARACTERISATION OF *Peronospora parasitica* INFECTING *Brassica* SPECIES

by

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Submitted in partial fulfilment of the requirements for the degree Doctor of Philosophy in the Department of Microbiology in the Faculty of Science and Engineering at the University of Durban-Westville, Durban, South Africa.

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Date submitted: June 2002

DECLARATION

The Registrar (Academic)
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Dear Sir

I, Maneshree Jugmohan, Reg. No., 9509660, hereby declare that the thesis entitled: Molecular Characterisation of *Peronospora parasitica* infecting *Brassica species*, is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other University.

Maneshree Jugmohan

June 2002

Dedicated to the memory of my father

Mr Jugmohan Ramdan

for his inspiration and altruism

CONTENTS

PREFACE

Aspects of the thesis which have been presented and/or published:

1. **Jugmohan, M., Achar, P.N., Lucas, J.A. and P.K. Pius.** 1999. A PCR-based assay for the phylogenetic analysis of *Peronospora parasitica* infecting *Brassica* species, Proceedings: 37th Conference of the South African Society for Plant Pathology, University of Natal, Pietermaritzburg.
2. **Jugmohan, M., Achar, P.N., Lucas, J.A. and K.J. Edwards.** 2000. Preparation of a genomic DNA Library of *Peronospora parasitica* enriched for microsatellites, Proceedings: BioY2K - South African Society for Plant Pathology (January 2000), Rhodes University, Grahamstown, South Africa.
3. **Jugmohan, M., Achar, P.N., Lucas, J.A. and K.J. Edwards.** 2000. Isolation of microsatellites from *Peronospora parasitica*, a biotrophic fungal pathogen of crucifers. Canadian Phytopathological Society and the American Phytopathological Society. June 18-21, 2000, Victoria, Canada.
4. **Achar, P.N., Jugmohan, M. and Xia, Z.** 2001. Molecular characterisation of *Peronospora parasitica* in brassicas and *Stenocarpella maydis* in maize. Invited Speaker, University of Wisconsin, Milwaukee, U.S.A.
5. **Jugmohan, M., Achar, P.N., Lucas, J.A. and K.J. Edwards.** 2002. The preparation of a genomic DNA library of *Peronospora parasitica* enriched for microsatellites (manuscript in preparation).
6. **Jugmohan, M., Achar, P.N. and J.A. Lucas.** 2002. Molecular phylogeny of pathotypes of *Peronospora parasitica* (manuscript in preparation).
7. **Jugmohan, M., Achar, P.N., Lucas, J.A. and K.J. Edwards.** 2002. Assessment of microsatellite diversity in pathotypes of *Peronospora parasitica* (manuscript in preparation).

ACKNOWLEDGEMENTS

The author acknowledges the following:

My mother, Mrs Shama Ramdan for her support and encouragement;

Professor P.N. Achar, for her supervision, encouragement and for making this study possible;

Professor John A. Lucas, Institute of Arable Crops Research (IACR) - Long Ashton and Rothamsted, University of Bristol, UK, co-promoter, for his invaluable advice, encouragement, and dedication to this project;

Professor Keith J. Edwards, University of Bristol, UK, for his support and input with the preparation of the microsatellite library and for provision of laboratory facilities;

Mr Richard Parker (Top Crop Nurseries, Wartburg) and Mr Ian Sumner (Sunshine Seedling Services, Cramond), for generously supplying field isolates of *P. parasitica*;

Dr Nash Nashaat, Institute of Arable Crops Research (IACR) - Rothamsted, UK, for supplying isolates and for his invaluable advice on single spore isolates and maintenance of *P. parasitica*;

Dr Drissa Silué, Bretagne Biotechnologië Vegétalé, France for his input in the project and for providing isolates;

Dr Angela Karp, Institute of Arable Crops Research (IACR) - Long Ashton Department of Agricultural Sciences, University of Bristol, UK, for provision of laboratory facilities for microsatellite screening;

Mr James Townsend, Institute of Arable Crop Research (IACR) - Rothamsted, UK, for his assistance with import of viable isolates;

Dr Uli Hannappel, GarstSeedCo, USA, for his support and input in the project;

Mr Steve Hanley, Institute of Arable Crops Research (IACR) - Long Ashton, Department of Agricultural Sciences, University of Bristol, UK for his assistance and input in the project;

The National Research Foundation and the University of Durban-Westville for financial assistance, and UNESCO for the awarding of a three month Internship at IACR-Long Ashton, UK;

Dr Gillian M. Arnold, Institute of Arable Crops Research (IACR) - Long Ashton, University of Bristol, UK, for her assistance with statistical analysis of microsatellite data;

Mr Claus Hallwirth, Nelson R. Mandela School of Medicine, for his assistance with phylogenetic analysis;

Mr Asok Rajh, University of Durban-Westville for his support and assistance with photography;

Dr Raffaella Carzaniga, Dr Richard o' Connell, Dr Andy Bailey and Mr John Keon, Members of staff, Department of Cell Biology, Institute of Arable Crops Research (IACR) - Long Ashton and Rothamsted, University of Bristol, UK, for their support and assistance;

My colleagues, Department of Haematology, Nelson R. Mandela School of Medicine, Durban, for their encouragement and support;

Dr Olumede Latunde-Dada, IACR-Rothamsted, UK, for his friendship, encouragement and support;

Miss Veloshinie Govender, Miss Ravini Pillay and Miss Sandhya Kanaye, research assistants of the Downy Mildew Project in the Department of Microbiology, UD-W;

Dr Jenitha Badul, for her friendship, encouragement, support and input in this project;

Mrs Chezlyn Chetty for her friendship, encouragement and input in the project;

My sister Kajal and my brother Rookesh for their encouragement and technical support.

ABSTRACT

Keywords: *Peronospora parasitica* (Pers. ex. Fr.) Fr., pathotype, single spore isolate, *Brassica* spp., downy mildew, internally transcribed spacers, ITS1, 5.8S, ITS2 sequence, radial phylogram, phylogeny, PCR, microsatellite markers, SSRs, DNA library, microsatellite primer sequences, allele, geographic and pathotype diversity, polyploidy

The downy mildew disease caused by *Peronospora parasitica* (Pers. ex. Fr.) Fr. infects approximately 100 plant species in the family Cruciferae. It is primarily a foliage blight which causes 60-70% seedling losses to nurserymen and rural subsistence farmers in Kwazulu-Natal, South Africa who depend on this crop to maintain a healthy cash flow not provided by other products. The disease is also frequent in India, France, Portugal and in the UK. The pathogen is especially debilitating in winter when low temperatures and high humidity favour infection and spread of the disease. Control of the disease with the use of fungicides is inefficient since *P. parasitica* has developed resistance to metalaxyl-based fungicides. There is a need to establish patterns of genetic diversity in order to address the ambiguities surrounding species definition of *P. parasitica.*

The present study focused on clarifying the relationships between different pathotypes of *P. parasitica* found on various *Brassica* species viz. *B. oleracea*, *B. juncea*, *B. napus*, *B. rapa*, as well as the *Arabidopsis thaliana* pathotype, a wild host of *P. parasitica.* Co-evolution with plant hosts over long periods was suggested to have lead to the divergent forms of this pathogen adapted to different host taxa. Genetic analysis of host specificity was investigated, based on sequence analysis of intergenic spacer regions of ribosomal DNA and on microsatellite markers. The current study provides the first comprehensive ITS-based phylogeny of pathotypes of *P. parasitica.* Based on ITS1, 5.8S and ITS2 sequences all pathotypes of *P. parasitica* from *Brassica* species (viz. *Brassica oleracea*, *Brassica napus*, *Brassica juncea* and *Brassica rapa*) were monophyletic. Based on ITS1 sequences, the pathotype of *P. parasitica* from the wild host, *Arabidopsis thaliana,* was found to be significantly different from the *Brassica* pathotypes (i.e. cultivated host pathotypes). Furthermore the genetic distance between the genus *Peronospora* and *Phytophthora* was closer compared to other taxa such as *Pythium*, supporting the observation that the downy mildews have derived from a *Phytophthora* ancestor. rDNA sequence analysis was unable to differentiate *Brassica* pathotypes of *P. parasitica.*

The development of a powerful class of genetic markers known as microsatellites (SSRs) in the present study provided greater insight into the relationships between *Brassica* pathotypes of *P. parasitica*. A genomic DNA library highly enriched for various microsatellites was prepared and a large number of potential SSRs consisting primarily of dinuceotide repeats $(CA)_n$ and $(CT)_n$ were obtained. Sequence analysis of 351 clones yielded 120 clones containing SSR loci, and 29 (24%) potentially useful SSRs from which primers could be designed were identified. PCR amplification with radiolabelled probes at 8 loci yielded useful polymorphisms across 27 isolates representing four pathotypes of *P. parasitica*. The alleles showed pathotype specific diversity since isolates of the same pathotype could be grouped together. The value of microsatellites as a strong discriminatory tool for intraspecies variation was further demonstrated by the ability to significantly separate isolates of the *Brassica oleracea* pathotype by their geographic origin and even field population in some instances. Several monoconidial lines from the same field population were grouped together and in some instances were found to be alike. The large number of alleles observed per genotype in the present study suggests that *P. parasitica* is polyploid and more complex than previously known. This parallels similar observations in other downy mildews such as *Phytophthora*, the closest relative of *Peronospora*. Future work involving sequencing at microsatellite loci would provide more insight into the exact ploidy, as well as the mapping of characterised single nucleotide polymorphisms (SNPs) which may be useful in specific identification of pathotypes. The primers developed for *P. parasitica* were able to amplify genomic DNA of *Bremia lactucae* indicating the value of the library developed in this study in comparative diversity studies with other Oomycetes.

ITS-PCR and microsatellites markers provide useful tools for improved classification and diagnosis of downy mildew diseases. Furthermore, knowledge on the genetic diversity in *P. parasitica* together with the wealth of information now available about *RPP* genes of *P. parasitica* in *Arabidopsis thaliana* provides many exciting avenues for a complete understanding of the genetics of host specificity.

LIST OF ABBREVIATIONS

@ - at

AFLP(s) amplified fragment length polymorphism (s)

bp - basepairs

cDNA - copy DNA

°C – degrees celcius

DNA - deoxyribonucleic acid

ESTs - expressed sequence tags

Fig. - Figure

g – grams

gr – gravity

hr(s) – hour(s)

ITS1 - internally transcribed spacer 1

ITS2 - internally transcribed spacer 2

kbp - kilobasepairs

kPa - kiloPascals

ml – millilitres

min. – minute(s)

MWM - molecular weight marker

% - percentage

PCR- polymerase chain reaction

PNK - polynucleotide kinase

PHYLIP - phylogenetic inference package

RAPD(s) - random amplified polymorphic DNA(s)

rDNA - ribosomal DNA

RFLP(s) - restriction fragment length polymorphism

RNA - ribonucleic acid

rpm – revolutions per minute

RPP - recognition of *P. parasitica*

RT - room temperature

SDW - sterile distilled water

sec - seconds

SSR(s) - simple sequence repeats

STR(s) - short tandem repeats

STS - sequence tagged site

µC - microCuries

µl – microlitres

$\mu Es^{-1}m^{-2}$ – microEinsteins per second per metres squared

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CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Plants are vital for human sustenance, including food and comfort. In nature, plants encounter several biotic and abiotic stresses that drastically affect their yield. The burgeoning world population will put considerable pressure on agriculture since the gap between demand and supply of food and other commodities is continuously widening (Rao et al. 1999). To bridge this gap efforts are underway to increase yields by development of highly yielding varieties and hybrids or alternatively to protect crops from yield losses due to biotic and abiotic factors.

Plant pathogens cause considerable yield losses in all important crop plants (Table 1.1). In consequence much effort is expended in disease prevention and control. Plant pathology has evolved rapidly in recent years, extending our knowledge of genetic and biochemical aspects of plant-pathogen interactions (Rao et al. 1999). This has lead to the control of disease through application of fungicides and development of disease-resistant varieties. Recently, methods based on systemic-acquired resistance (SAR) (Alexander et al. 1993, Delaney 1997, Lucas 1999, Uknes et al. 1992) biological control (Lucas et al. 1998) are gaining popularity in many plant species.

Despite these efforts, our understanding of various pathosystems remains incomplete. Plant pathologists have realised that more knowledge about the genetic structure of populations of plant pathogens is needed to implement effective control strategies. "Genetic Structure" refers specifically to the amount and extent of genetic variation within and among populations (Jones et al. 1997). In the last few decades, molecular biology has developed many techniques for analysing genetic structure of plant pathogens and has succeeded in addressing important questions on population diversity.

Table 1.1: Geographical distribution of the most serious plant diseases caused by fungi and Oomycetes (Rao et al. 1999)

Disease	Location	Comments
Cereal Rusts	World-wide	Frequent Severe epidemics, huge annual losses
Cereal Smuts	World-wide	Poisonous to human and animals
Ergot of rye and wheat	World-wide	Continuous loss to all grains
Late blight of potato	Cool, humid climates	Annual epidemics, e.g. Irish famine (1845-46)
Brown spot of rice	Asia	Epidemics, e.g. the great Bengal famine (1943)
Southern corn leaf blight	US	Epidemic 1970, $1 billion lost
Powdery mildew of grapes	World-wide	Europeans epidemics (1840s and 50s)
Downy mildew of grapes	US, Europe	European epidemics (1870s and 80s)
Downy mildew of tobacco	US, Europe	European epidemic 1950s-60s) epidemics in North America (1979)
Chestnut blight	US	Destroyed almost all American chestnut trees
Dutch elm disease	US, Europe	Destroying all American elm trees (1930 to date)
Coffee rust	US, Europe	Destroyed all coffee in South East Asia (1870s –80s), Since 1970 present in South and Central America
Banana leaf spot or Sigatoka disease	World-wide	Great annual losses
Rubber leaf blight	South America	Destroys rubber tree plantations

1.1.1 Downy mildew of crucifers

The downy mildews are primarily foliage blights that attack and spread rapidly in young, tender green tissues including leaves, twigs, and fruit. True downy mildews are caused by a group of Oomycete pathogens that belong to the family Peronosporaceae (Agrios 1997). All species of this family are obligate parasites of higher plants and cause downy mildew diseases on a large number of cultivated grain crops, vegetables, and many field crops, ornamentals, shrubs, and vines.

The development and severity, in areas where susceptible hosts and the respective downy mildew pathogens are present, depend greatly on the presence of a film of water on the plant tissues and on high relative humidity in the air during cool or warm, but not hot periods. The reproduction and spread of the downy mildew pathogens are rapid and these diseases cause heavy losses in short periods of time. Some of the most common or most serious downy mildews and the diseases they cause are listed in Table 1.2.

Table 1.2: Downy mildews and their hosts (Agrios 1997, Lucas 1995, 1998, Mathews 1981)

Genus	Species	Host
Peronospora	*P. parasitica*	Cruciferae
	P. farinosa	Chenopodiaceae
	P. hyoscyami/P. nicotianae	Tobacco
	P. manshurica	Soybean
	P. destructor	Onions, *Allium* spp.
	P. statices	*Limonium sinuatum*
	P. viciae	Peas
	P. antirrhini	Snapdragon
	P. schachtii	Beetroot
	P. trifoliorum	Alfalfa and Clover
Bremia	*B. lactucae*	Lettuce
Plasmopara	*P. viticola*	Grape
Pseudoperonospora	*P. cubensis*	Cucurbits
	P. humuli	Hops
Sclerospora	*S. graminicola*	Grasses, Millets
	S. macrospora	Cereals, grasses
	S. maydis	Corn
	S. oryzae	Rice and Corn
	S. philippinensis	Sugarcane and Corn
	S. sorghi	Sorghum and Corn
	S. sacchari	Sugarcane and Corn

1.2 Peronospora parasitica

Peronospora parasitica (Pers. ex. Fr.) Fr. is a biotrophic Oomycete pathogen which causes downy mildew disease in crucifers. The pathogen has a wide host range, affecting approximately 100 plant genera and species in the family Cruciferae. These include the cultivated Brassicas (*Brassica oleracea* L. var. *capitata* (cabbage), var. botrytis (cauliflower), var. *gemmifera* (Brussels sprouts), *B. rapa* (turnip), *B. napus* (oilseed rape), *B. juncea* (mustard)), *Raphanus sativus* (radish), ornamentals and wild crucifers such as *Arabidopsis thaliana* (wild cabbage) (Lucas 1995, 1998).

P. parasitica occurs world-wide (Channon 1981), and large crop losses have been reported especially at the seedling stage of growth. In KwaZulu Natal, South Africa, approximately 60-70% of brassica crop losses are due to this disease (Brophy and Laing 1992). This causes concern for a number of reasons: 1) Brassicas are important in the cropping patterns of Natal as there are few economically viable crops which can be grown in winter and 2) Many poor subsistence farmers in KwaZulu Natal depend on the production of vegetable *Brassica* spp. particularly cauliflower and cabbage to maintain a healthy cash flow not provided by other products (Beukes 1996).

1.2.1 Symptoms

The attack of *P. parasitica* on seedlings of all common brassica hosts results initially in the development of discoloured spots on the surface of the cotyledons which may then turn yellow and shrivel and die (Channon 1981). At such an early stage of growth, loss of the cotyledons may be fatal. Before the seedling dies, sporulation occurs resulting in a fine loose carpet of conidiophores and conidia mainly on the undersurface of the cotyledons (Channon 1981) (Fig. 1.1).

The disease is most severe at the seedling stage since older plants usually have a waxy outer layer that protects them, however, occasionally adult plants may exhibit a compatible interaction (Jensen et al. 1999). This was evident in cauliflower and broccoli where infection extended to the curds (Lund and Wyatt 1978, Jensen et al.

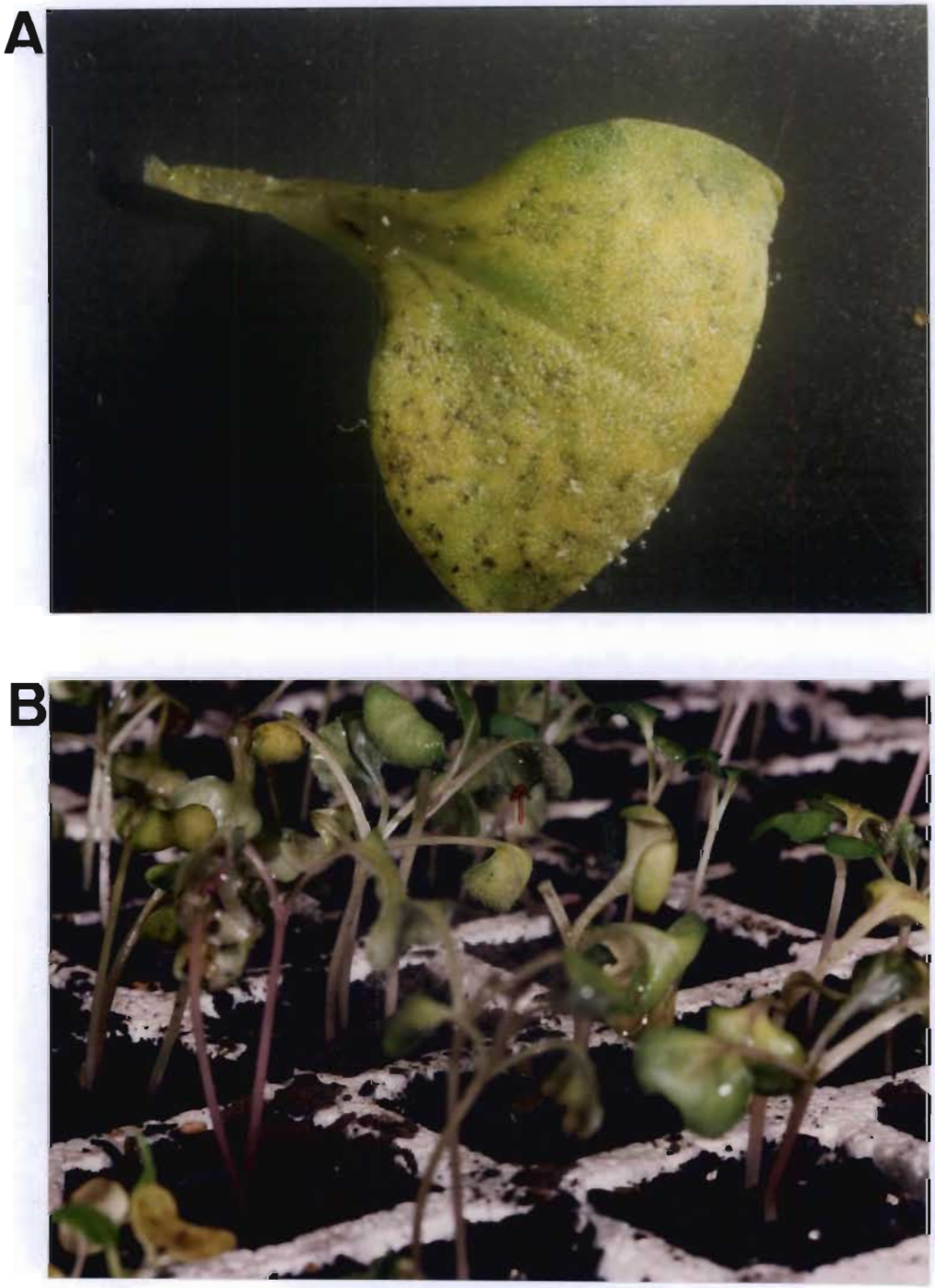

Fig. 1.1: Symptoms of *Peronospora parasitica* infection on brassica seedlings. A: leaf necrosis. B: sporulation on undersurface of cotyledons.

1999). Histological examination of sectioned embedded curd material revealed intercellular hyphae and haustoria typical of *P. parasitica* (Jensen et al. 1999). Pale brown or greyish discolouration of the surface of the curd and greyish or blackish spots and streaks also appear on the stem of the immature heads. This leads to down-grading of cauliflower in the market and secondary rotting of the tissues by bacteria results in destruction of the curd (Channon 1981).

1.2.2 Morphology and life cycle

Based on morphological correlation and physiology, the categorisation of 3 genera of downy mildews and many species have been described (Channon 1981). Downy mildew fungi produce conidia on conidiophores that are distinct from the mycelium in their way of branching. The conidia are located on the tips of the branches. Each genus of the downy mildew fungi has its own distinctive type of branching of its conidiophores and this is used for its identification.

P. parasitica has a coenocytic mycelium which ramifies in the intercellular spaces of the host and forms haustoria which penetrates the cells of infected tissues (Channon 1981). Infection of the host plant can either occur asexually (from conidia borne on definite conidiophores) (Tommerup 1981) or sexually (from oospores in the soil) (McMeekin 1960) (Fig. 1.2).

1.2.2.1 Asexual Reproduction

During suitable environmental conditions (high humidity and low temperatures) conidia landing on the surface of a susceptible host form germ tubes from which appressoria develop (Fig. 1.2). In *P. parasitica* on brassicas, appressoria are invariably formed in the trough between epidermal cells, directly above the anticlinal cell wall. Electron microscope studies have indicated that the contents of the conidium pass into the appressorium from which an infection hypha develops (Chou 1970). This hypha may occasionally enter the tissue through the stoma but usually penetrates directly when it breaks a hole (4-5 μm in diameter) through the cuticle which then fits closely around the infection hypha. After entering the host the hypha

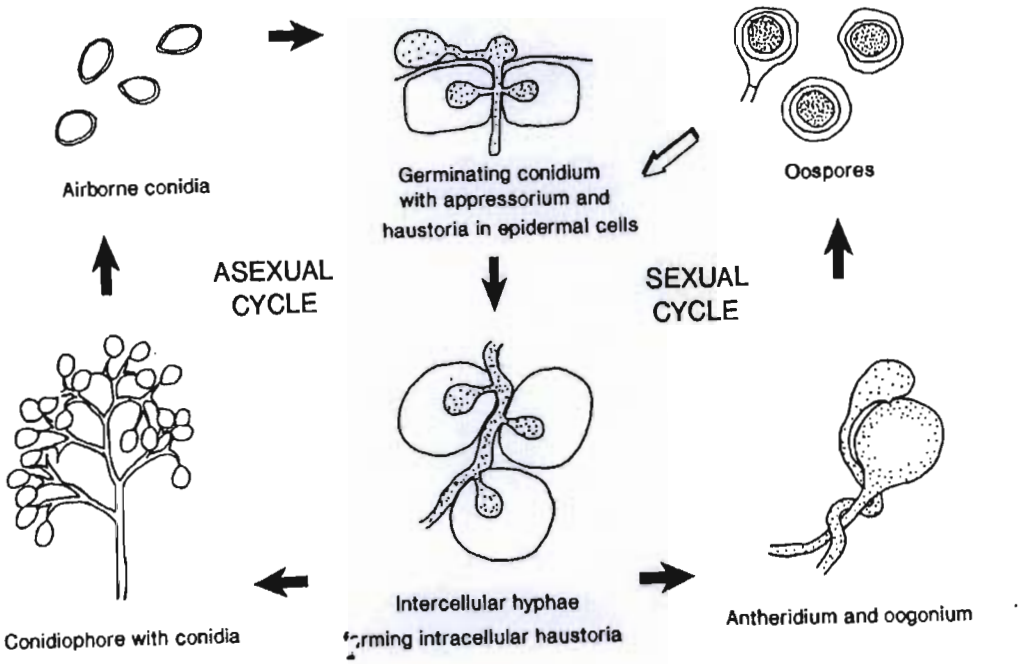

Fig. 1.2: Diagrammatic life-cycle of *Peronospora parasitica* showing asexual and sexual cycles (Lucas et al. 1994).

expands to a diameter of 7-8 µm and grows initially in the region of the middle lamella between the anticlinal walls of the epidermal cells. Subsequent penetration is via the anticlinal wall, with the first haustoria penetrating adjacent epidermal cells (Chou 1970) (Fig. 1.2). The size and shape of haustoria may vary. In cabbage, some haustoria are large, irregular vesicles while others are bilobed and regular in shape whereas the haustoria of cauliflower are single globose and uniform in size (Fraymouth 1956). Following penetration of the epidermal cells, the haustorium enlarges, invagination of the host plasmalemma occurs and a sheath, possibly of callose, forms round the intrusive fungal feeding organ.

The infection hypha continues its growth between the cells of the host tissue branching in all directions and varying in diameter and form according to the size and shape of the intercellular spaces (Tommerup 1981). The intercellular hyphae differentiate to form the conidiophores (200-500 µm in length) which project singly or in groups through the stomatal opening. The conidiophores bear primary, secondary and tertiary branches, which bifurcate to form pairs of finely pointed and in-curved sterigmata, bearing single terminal conidia (Davison 1968b) (Fig. 1.3). These spores are spherical at first but become ellipsoidal (24-27 x 12-22 µm) and are delimited from the sterigma by cross walls at maturity. At 8°C, the rate of elongation

of the condiophore reaches 100-200 μm h^{-1}, and the whole process from emergence to spore formation takes approximately 4-6 hrs (Davison 1968b). Spore release is mainly by hygroscopic twisting and untwisting in response to change in atmospheric humidity (Pinckard 1942).

1.2.2.2 Sexual reproduction

Peronospora parasitica is an oomycetous pathogen in which sexual reproduction involves fertilisation of an oogonium by passage of nuclear material from an antheridium, leading to the formation of an oospore (Sheriff and Lucas 1989b). The oogonia of *P. parasitica* are spherical whereas the antheridia are tendril-like (McMeekin 1960) (Fig.1.2). Prior to fertilisation, the protoplast of the oogonium becomes differentiated into a central vacuolated ooplasm and a peripheral multinucleate granular periplasm. At the point of contact between the antheridium and the oogonium, a thin receptive papilla forms. A fertilising tube then grows from the antheridium through this receptive papilla towards the "central body" in the ooplasm and there discharges a single "male" nucleus. Meanwhile, a single "female" nucleus detaches itself from the periplasm and also migrates towards the central body (Wager 1900). The mature oospore is thick-walled, yellowish-brown and spherical and usually measures 26-45 μm in diameter (Wager 1900, Jugmohan 1997) (Fig. 1.4).

The frequency of oospore formation has not been systematically evaluated. Oospores have been found in abundance in field-grown *Brassica napus* (oilseed rape) (Kluczewski and Lucas 1983), *Brassica oleracea* var. *italica* (broccoli), var. *botrytis* (cauliflower) and var. *capitata* (cabbage) (McMeekin 1960). Oospore formation is favoured by conditions which induce senescence of the host tissues, such as deficiency of nitrogen, phosphorus or potash (McMeekin 1960). In consequence, oospores were found abundantly in necrotic or chlorotic leaves rather than in green tissues, and occur more frequently in cotyledons than true leaves since the former senesce more rapidly (McMeekin 1960). Oospores remaining in host debris were suggested to be a source of primary infection for subsequent crops (McMeekin 1960). Jang and Safeeulla (1990b) reported that oospores of *P. parasitica* stay viable and infective in the soil for two to three years. Therefore, oospores constitute an important means of survival of *P. parasitica* during unfavourable conditions.

Fig. 1.3: Conidiophore (c) and hyaline conidia (s) of *Peronospora parasitica* viewed with a stereomicroscope. 40X Magnification.

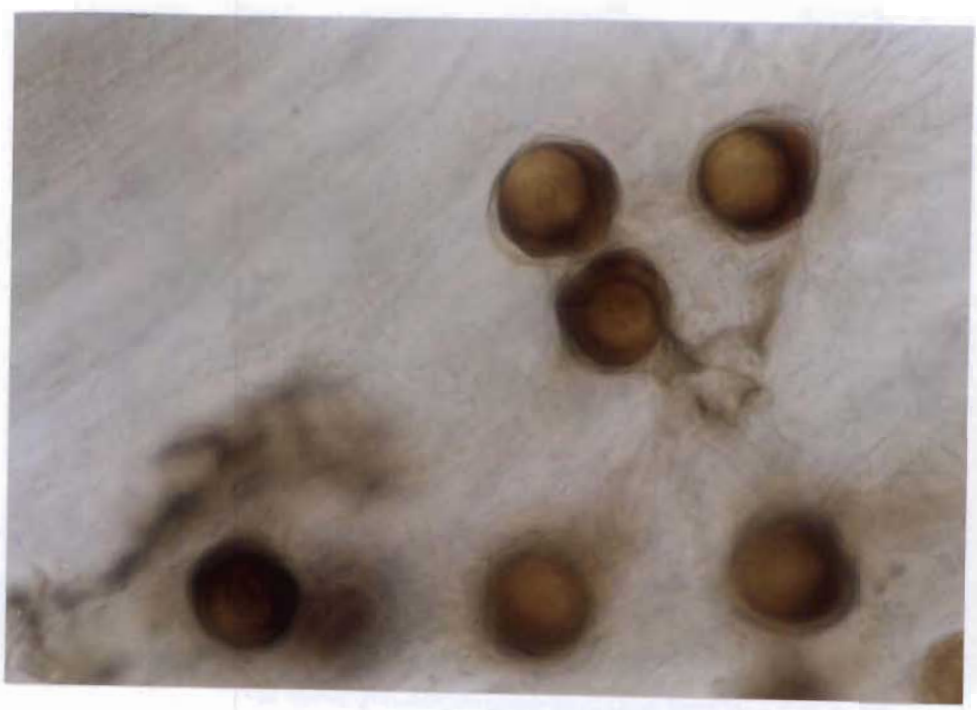

Fig. 1.4: Illustration of oospores of *P. parasitica* viewed with the light microscope. 40X Magnification.

In most downy mildews, induction of the sexual process requires the presence of two strains of opposite mating type to form oospores (Michelmore 1981). Early observations of De Bruyn (1937) have shown that *P. parasitica* is apparently heterothallic since antheridia and oogonia arose from different hyphae. The existence of heterothallism, in *P. parasitica*, was confirmed by McMeekin (1960) and Sheriff and Lucas (1989b). Furthermore, the pattern of oospore production suggested the existence of two mating types, thus illustrating the heterothallic nature of a portion of the *P. parasitica* population. Two mating-type designations were proposed for the heterothallic isolates viz. P1 and P2.

In contrast, evidence in support of homothallism (self-fertility) comes from single spore isolates (monoconidial lines) of *P. parasitica* (Sheriff and Lucas 1989b). Low percentages of spores from them reproduced sexually and so exhibited a homothallic capacity. It was further proposed by Sheriff and Lucas (1989b) that self-fertility could due to (a) isolates were mixed populations of the two heterothallic mating types, (b) the isolates were heterokaryotic and contained both mating types in different nuclei, (c) apparent homothallism may be induced by some environmental factor, or (d) the isolates were genuinely homothallic. However, the possibility of a mixed population was rejected since repeated derivation and subculture of single spore isolates from self-fertile isolates showed that oospores were still produced. The question of heterokaryosis in *P. parasitica* could not be easily resolved, by analysis of single-spore isolates that have originated, because each conidium is multinucleate and contains up to 30 nuclei (Davison 1968a). In comparison, transitory heterokaryosis has been suggested to occur in *Bremia lactucae* (Michelmore and Ingram 1982) since a small proportion of the monoconidial lines derived from *Bremia lactucae* segregated and were self-sterile and of opposite compatibility type. The majority of lines, however, retained self-fertility, indicating that homothallism was a relatively stable property.

A cytogenetic study of heterothallic and homothallic isolates of *P. parasitica* (Sheriff and Lucas 1989a) has shown that at metaphase I of meiosis, a ring of four chromosomes was found in all isolates. By comparison with similar observations in other Oomycete fungi such as *Bremia lactucae* (Michelmore and Ingram 1982), this ring of four was interpreted as a reciprocal translocation complex between chromosomes carrying the mating type alleles (Sheriff and Lucas 1989a). In homothallic isolates a

fifth chromosome was associated with the ring of four. The self-fertility of these isolates was found to be due to the presence of a third mating type allele on the fifth chromosome, a condition known as secondary homothallism (Sheriff and Lucas 1989a). The gametangia of heterothallic and homothallic mating in *B. lactucae* are however, alike (Tommerup et al. 1974).

Little information is available on host factors influencing sexual reproduction in the downy mildews. Complete susceptibility of a host to a pathogen was not a prerequisite for oospore formation (Kluczewski and Lucas 1983). The comparatively restricted development of one isolate in conjunction with the extensive host-cell necrosis typical of less compatible reaction types was not considered to be a serious obstacle to sexual reproduction. However, differences in the numbers of oospores produced observed in different hosts (Sheriff and Lucas 1989b) were suggested to be related to the relative susceptibility of the hosts used. Analysis of isolates collected from different brassica hosts showed that the large majority were heterothallic with the exception of isolates collected oilseed rape (de Bruyn 1937, Sheriff and Lucas 1989b). In comparison all single spore isolates from cabbage (*B. oleracea*) were homothallic (Jugmohan 1997). All isolates collected from the wild crucifer *Arabidopsis thaliana* were also apparently homothallic (Holub et al. 1994).

Homothallism in *P. parasitica* may have significant effects on the epidemiology of the pathogen on its hosts. Homothallism was suggested to increase the frequency and extent of oospore formation, as sexual reproduction does not rely on simultaneous infection by opposite mating types. The field survey (McMeekin 1960) confirmed that oospores are common in the crop, especially in cotyledons and first leaves which on abscission will enter the soil. Homothallism may also reduce the amount of variation in the population, although this will have less impact on a diploid organism or polyploid organism than on a haploid organism (Sheriff and Lucas 1989b). The dual set of genes in diploid organisms provide a wider range of accommodative responses to unfavourable conditions, due to the greater extent of heterozygosity, and will thus increase variation. These effects may be significant since oospores are an important inoculum source and the homothallic condition was found to be a stable character.

1.2.3 Taxonomy

True downy mildews are classified in the group of Oomycetes of the family Peronosporaceae (Agrios 1997). *Peronospora parasitica* (Pers. ex. Fr.) Fr. belongs to the order Peronosporales, in the class Oomycetes. The Oomycetes were originally classified within the Mastigomycotina in the Kingdom Fungi (Agrios 1997). More recently, however, there has been debate as to whether the Oomycetes should be included in a new Kingdom, the Protoctista, separate from that of the Fungi (Whitaker and Margulis, 1978) and the Oomycetes be placed in a Phylum of their own (Dick 1990).

Traditionally, generic delimitation in the family Peronosporaceae was based on morphological characters such as sporangiophore and spore type as well as different modes of germination, germ tube or zoospore. A downy mildew pathogen was reported to infect approximately 50 genera and more than 140 different species of the family Cruciferae. Originally Gaumann (1918), on the basis of conidial measurements and cross- inoculation tests, recognised 52 species of *Peronospora* on crucifer hosts. Later Yerkes and Shaw (1959) concluded that there were no morphological criteria for distinguishing *Peronospora* isolates from different host species and all collections of downy mildew from the Cruciferae have been grouped into a single aggregate species *Peronospora parasitica*.

P. parasitica is very specific in its host range, however, it also has the ability to colonise heterologous host species to different extents i.e. hosts different to those from which it was derived. Several studies based on the differential response test have shown that isolates are more virulent on their species of origin (Nashaat and Awasthi 1995, Silué et al. 1996, Jugmohan 1997) and the varying abilities to infect other hosts was estimated using a disease index (DI). The overall results support the idea that host adaptation exists at the species level. Co-evolution with plant hosts over long periods was suggested to have led to divergent forms of the pathogen adapted to different host taxa (Lucas et al. 1994). This dependence of downy mildews on their hosts further suggested that forms of the pathogen found on closely related plants share some common phylogeny. However, host specificity may be an unreliable indicator of the evolutionary history of downy mildews (Lucas et al. 1994).

The taxonomy of Oomycetes has been the subject of much controversy and has been reorganised several times. Based on sequence analysis of the small subunit (SSU) ribosomal coding regions Oomycetes were found to be closely related to Chrysophytes (golden-brown algae) (Gunderson et al. 1987, Förster et al. 1990). These findings supported the theory of Cavalier-Smith (1986) that Oomycetes, Chrysophytes and diatoms are members of the plant Kingdom Chromista derived from a common ancestor. Furthermore, based on DNA analysis of the large ribosomal subunit (LSU rDNA) *Peronospora* was found to be closely related to *Phytophthora* (Cooke et al. 2000, Peterson and Rosendahl 2000). *Peronospora* was suggested to be derived from a *Phytophthora* that has lost the ability to produce zoospores and has become an obligate biotroph. Rehmany and co-workers (2000) have further suggested a divergence between *P. parasitica* isolates from *Arabidopsis thaliana* and *Brassica oleracea*. The above findings are discussed in more detail in Chapter Four.

1.3 HOST SPECIFICITY AND HOST RESISTANCE

1.3.1 Host specificity

Crucifer downy mildew provides an interesting case study of specificity, as the pathogen occurs on a wide variety of hosts as well as on species cultivated in agriculture and horticulture (Lucas et al. 1994). Important hosts include the brassica crops (Table 1.3) as well as the wild crucifer *Arabidopsis thaliana* which serves as a model system for genetic analysis.

As obligate biotrophs, downy mildews are highly host-dependent pathogens (Lucas et al. 1994). Isolates of *P. parasitica* exhibit considerable specialisation within the Cruciferae and are usually only completely compatible with host genotypes of the species from which the isolate was derived (Sheriff and Lucas 1990). Studies have shown that isolates from different *Brassica* spp. are most virulent on their species of origin, however, they may also grow to a varying lesser extent on other *Brassica* spp. (Kluczewski and Lucas 1982, 1983, and Sheriff and Lucas 1987, 1990). For example, isolates from cauliflower (*B. oleracea* var. *botrytis*) and another from

oilseed rape (*B. napus*) were capable of infecting either host, but there were marked contrasts in the time course and extent of mycelial development, the amounts of associated host-cell necrosis, and the intensity of sporulation (Kluczewski and Lucas 1982). Cluster analysis revealed that the different pathotypes/host-adapted species could be grouped together on the basis of this host-specificity (Sheriff and Lucas 1990).

Recently the differential response test was employed to determine the severity of infection in *Brassica juncea* (Nashaat and Awasthi 1995), *B. rapa* (Moss et al. 1991), *B. napus* ssp. *oleifera* (Nashaat and Rawlinson 1994), *B. oleracea* (Silué et al. 1996) and *Arabidopsis thaliana* (Koch and Slusarenko 1990). The differential response test of *P. parasitica* to *Brassica* spp. is based on phenotypic observations in the host and disease indices then calculated for each host-isolate pair. The severity of infection by an isolate on a host (interaction phenotype or IP) for approximately 25 replicates of seedlings was first scored on a scale of zero to nine, zero being resistant and nine being susceptible. The statistical mean of these interaction phenotypes for a set of 25 replicates yielded the disease index (DI) of the host-isolate pair. The DI reflects the resistance or susceptibility of the host cultivar to an isolate and was proposed as a measure of the differential response (Sheriff and Lucas 1990, Nashaat and Awasthi 1995, Silué et al. 1996). The differential response test was found to be useful in identifying new genes/sources of resistance to *Peronospora parasitica* in oilseed rape Nashaat et al. 1997). Recent studies play an important role in breeding for resistance (Silué et al. 1995).

Different pathotypes of *P. parasitica* have been realised on the basis of host specificity and based on DI the hosts could be categorised. The virulence frequencies of pathotypes of *P. parasitica* to a *Brassica* spp. was found to be influenced by the genetic relationships between the *Brassica* spp and the host origin of the pathotype (Sheriff and Lucas 1987, Silué et al. 1996). Significant variation occurred in the response of *Brassica oleracea* accessions to *P. parasitica* isolates derived from *B. oleracea*, *B. napus* and *B. juncea* (Nashaat and Rawlinson 1994, Nashaat and Awasthi 1995, Silué et al. 1996). For example, an isolate of *P. parasitica* occurring on *B. oleracea* var. *capitata* hosts was found to be more virulent on other accessions of *B.*

oleracea var. *capitata*, however, it also infected other hosts such as *B. oleracea* var. *botrytis* (cauliflower) or *italica* (broccoli) to different extents (Silué et al. 1996).

Furthermore, within the brassicas, virulence frequencies obtained for certain accessions were more similar than others (Silué et al. 1996). For example, virulence frequencies of *P. parasitica* isolates derived from *B. napus* on *B. oleracea* accessions are not much different and in some instances were the same as those derived from *B. oleracea* accessions. Comparatively, the virulence frequencies of *P. parasitica* derived from *B. juncea* on *B. oleracea* was markedly less than those isolates derived from *B. napus* and *B. oleracea*. These results were similar to observations of Sheriff and Lucas (1987) which confirmed host adaptation in *P. parasitica*.

The above observations were partly interpreted in terms of the genetic relationships between *Brassica* species. *B. campestris, B. nigra* and *B. oleracea* are diploid with the genomic designations AA, BB and CC (Fig. 1.5). *B. juncea and B. napus* and *B. carinata* are amphidiploid derivatives with genomic designation AABB, AACC and BBCC respectively, indicating their cytogenetic origin (U, 1935, Neiuwof 1969, Sheriff and Lucas 1987). Fungal isolates found on *B. oleracea* and *B. napus* isolates, adapted to the C genome that both of these hosts share, are therefore likely to be more specialised than isolates from *B. juncea*, which would have adapted to both A and B genomes (Sheriff and Lucas 1987, Silué et al.1996). The differential response test was also used to determine the relation between numerous ecotyopes of *Arabidopsis* and several isolates of *P. parasitica* (Koch and Slusarenko 1990).

Certain accessions of *B. oleracea* var. *capitata, italica* and *botrytis* may exhibit a heterogenous reaction to some isolates of *P. parasitica* (Silué et al. 1996). These observations were attributed to lack of consideration given to resistance to downy mildew during their breeding. During seed multiplication, a combination of self-pollination and cross-pollination was suggested to contribute additional variation in the host response.

Table 1.3: The most important species of the genus *Brassica* (Neiuwof 1969)

Species	Cultivated types	No. of chromosomes (n)	Constitution of genome
B. nigra	Black mustard	8	B
B. oleracea	Cole crop	9	C
B. campestris (rapa)	Turnip group	10	A
B. carinata	Abyssinian mustard	17	Bc
B. juncea	Indian (brown) mustard	18	Ab
B. napus	Swede Group	19	Ac

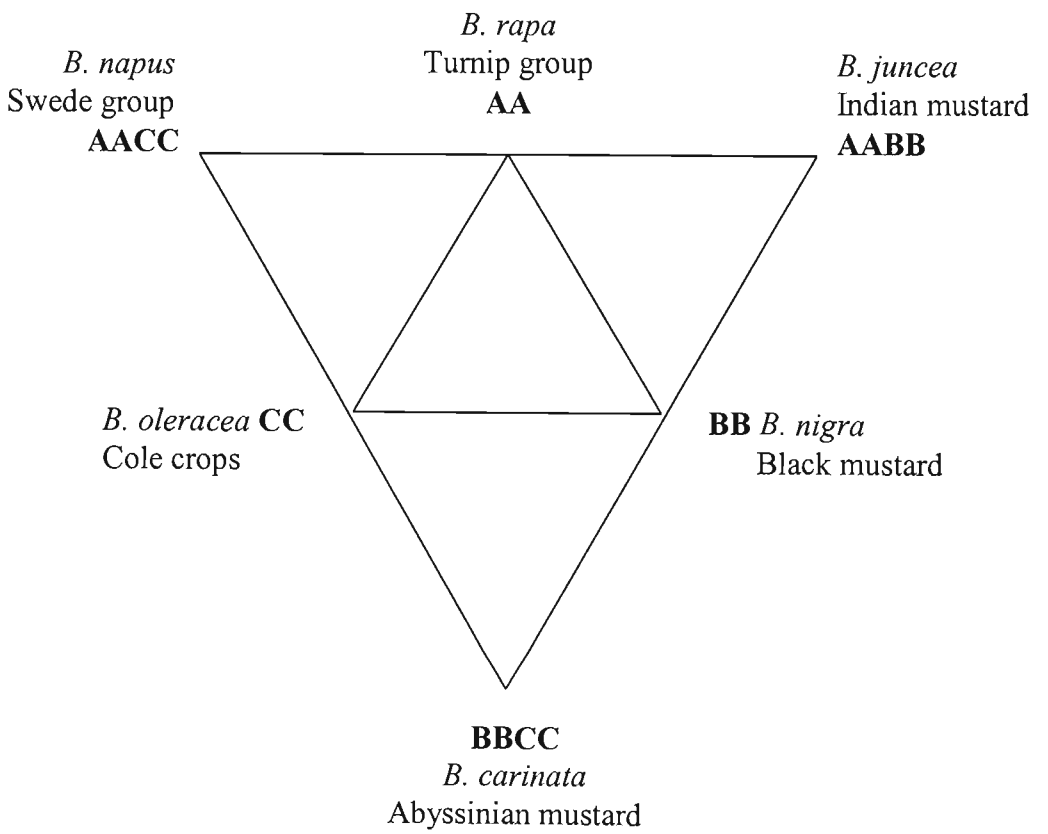

Fig. 1.5: Diagrammatic representation of the genetic relationships between the different *Brassica* species (U, 1935).

Cole crops: cabbage, cauliflower, kale, broccoli, cauliflower

Swede group: swede, oilseed rape

Turnip group: turnip, oilseed rape.

1.3.2 Host resistance

1.3.2.1 Plant defence mechanisms

Plants are continuously exposed to insects, nematodes and other potentially damaging pests, as well as to a wide variety of parasitic micro-organisms. Since plants nevertheless remain healthy most of the time, plants must possess highly effective mechanisms for preventing parasitism and predation or at least limit their effects (Lucas 1998). Plant defence against pathogens has been extensively reviewed by Agrios (1997) and Lucas (1998). Plant defence systems may be passive (constitutive) or active (inducible) depending upon whether they are pre-existing features of the plant, or are switched on after challenge.

Passive defence mechanisms serve as a first line of defence and include preformed inhibitiors and secondary metabolites that are toxic to potential pests. Compounds such as phenols, alkaloids, glycosides, saponins, tannins, and resins all possess antibiotic properties and may merely contribute to resistance. Inhibitors in plant tissues often occur as non-toxic precursors, which are converted to active forms following cell damage or exposure to enzymes. Brassica crops such as cabbage are rich in sulphur-containing mustard oils, which degrade to form volatile isothiocyanates inhibitory to several fungal pathogens (Lucas 1998). In wild and cultivated *Brassica* lines, a correlation between high levels of flavour volatiles (allylisothiocyanate) released by tissue damage, and limitation of fungal growth was observed (Greenhalgh and Dickinson 1975, Greenhalgh and Mitchell 1976). This resistance took the form of necrosis of host tissues associated with limited mycelial spread and much reduced sporulation of the fungus. It was further suggested that in cultivated *Brassica* spp. breeding has resulted in reduced levels of flavour volatiles with a consequent reduction in their general resistance to *P. parasitica*.

Several types of plant proteins have been found to serve a defensive function (Lucas 1998, Shewry and Lucas 1997) (Table 1.4). Other proteins may specifically inhibit microbial enzymes (for example pectolytic enzymes such as polygalacturonases) produced during tissue colonization. Proteins present in the cell walls of many plants, for example hydroxyprolin-rich glycine protein (HRGPs)

Table 1.4: Some plant proteins that confer resistance to pests and pathogens (after Lucas 1998)

TYPE	EXAMPLE	BIOLOGICAL ACTIVITY
Hydrolases	Chitinase	
	Glucanase	Digest fungal cell walls
	Lysozyme	Lyses bacterial cells
Enzyme inhibitors	Polygalacturonase-inhibiting proteins(PGIPs)	Inhibit fungal pectinases
	Protease inhibitors	Inhibit insect digestive enzymes
	Amylase inhibitors	
Chitin-binding proteins	Hevein (in latex from rubber)	Inhibits growth of fungi
	Lectins	Inhibits growth of insects and fungi
Antifungal peptides (AFPs)	Plant defenins	Inhibits growth of fungi
Thionins	Hordothionin (from barley)	Inhibits growth of fungi and bacteria
Ribosome-inactivating proteins(RIPs)	Pokeweed antiviral protein (PAP)	Inhibits mechanical transmission of viruses

(Showalter 1993), may contribute to resistance by trapping pathogen cells or acting as structural barriers and sites for lignin deposition (Lucas 1998). Other proteins may inhibit the action of polygalacturonases produced by fungi, and thus interfere with the infection process. Such proteins not only enhance the resistance of plant cell walls to enzymatic attack, but also influence the nature of any end-products released. Rather than being digested completely to monomers, pectic substrates are partially degraded to oligomers, many of which were active inducers of plant defence responses. These inhibitor proteins contributed to an early-warning system whereby plant cells could detect the presence of an invading fungus.

Active defence responses are induced once penetration of the host begins. When a plant is infected with a pathogen (viral, bacterial or fungal) to which it is resistant, a wide variety of defence responses are rapidly induced. Active defence mechanisms in plants have been reviewed by Hutcheson (1998). Active defence mechanisms include the hypersensitive response (HR), synthesis of antimicrobial

compounds like phytoalexins and hydrolytic enzymes (chitinases and β-1,3 glucanases), the deposition of lignin, callose and related wall-bound phenolics, and re-inforcement of cell walls surrounding the infected proteins by cross-linking of cell-wall proteins. In an incompatible interaction, resistance is accompanied by restriction of pathogen growth without spreading beyond the site of inoculation (Buffard et al 1996). In a compatible reaction, when a plant is infected with a pathogen to which it is susceptible, the pathogen is able to spread throughout the plant, causing disease development (Buffard et al. 1996).

The hypersensitive response involves localised cell death resulting in tissue necrosis at the site of infection (Kunkel 1996). Such an interaction is incompatible. In Peronosporaceous fungi, the hypersensitive response is a common feature associated with the expression of incompatibility (Ingram 1981). The hypersensitive response may occur at different rates and to differing extents depending on the host-parasite association in question (Dickinson and Greenhalgh 1977). Cell death may be an integral component of resistance, a consequence of resistance or an event which is associated with but irrelevant to resistance (Ingram 1981).

Pathogenesis related proteins (PR proteins) are relatively small, stable proteins which accumulate in the intercellular spaces of plant tissues. Their appearance was demonstrated by gel electrophoresis of tissue extracts. PR proteins which have been characterised include proteins which have antifungal, β-1,3 Glucanase and Chitinase activity. The substrates β-1,3 glucan and chitin do not occur naturally in plants and are also the main polymers found in the cell walls of many fungi, therefore a direct role in defence against pathogens seems likely. Deposition of lignin in host cell walls of radish following infection by *P. parasitica* has been demonstrated by Ohguchi and Asada (1975).

Unlike animal immunization, systemic acquired resistance (SAR) is non-specific. This form of protection has been demonstrated in *Arabidopsis*. Acquired resistance was found to be effective against a broad range of pathogens and the degree of protection was usually high, especially under field conditions. In SAR, primary inoculation involved a chemical or biological agent which induced necrosis in the

treated tissues (Delaney 1997, Lucas 1998). Active defense systems in the surrounding cells were then triggered. Other parts of the plant receive a signal which primes unexposed tissue against subsequent challenge. The changes induced in distant tissues are not fully understood, although alterations in the structure and chemistry of the cuticle and cell wall have been detected and defense related genes such as those encoding PR proteins were expressed. Upon challenge the full repertoire of the active defense mechanism was activated more quickly and plants were sensitized to subsequent exposure to the pathogens.

An example of an active chemical inducer of SAR is salicylic acid (Yalpani et al. 1991). Expression of increased levels of pathogenesis related protein (PR-1a) has been demonstrated to result in increased tolerance to two Oomycete pathogens from tobacco (*Peronospora tabacina* and *Phytophthora parasitica* (Alexander et al. 1993). Plants which express salicylate dehydrogenase, the enzyme that converts salicylic acid to catechol, are unable to accumulate salicylic acid and thus unable to activate SAR genes to develop resistance against the pathogens (Kunkel 1996, Delaney 1997).

1.3.2.2 Resistance genes

Disease resistance in plants is an economic trait. Defenses in plants are mediated through gene-for-gene systems in which the plant carrying a particular resistance (R) gene allele responds to pathogens carrying a matching avirulence (avr) gene (Flor 1971). Whether or not infection by a particular pathogen on a particular host leads to disease depends on many factors, including the activation of host defenses and the production of appropriate virulence factors by the pathogen (Crute et al. 1994a). Studies in biochemistry and molecular biology have made it possible to identify and clone genes associated with pathogenesis-related proteins, antifungal proteins and compounds, phytoalexins, enzymes controlling oxidative burst etc. This wealth of information has given us the chance to devise strategies to confer disease resistance to pathogens (Rao et al. 1999).

Several studies focussed on the genetics of host resistance to *P. parasitica*. In *B. oleracea*, genotypes exhibiting race-specific resistance to pathotypes adapted to

this species have been reported (Natti et al. 1967, Lucas et al. 1988). The inheritance of race 1, (the predominant physiological race of *P. parasitica* pathogenic to all types of *B. oleracea* grown in New York) was found to be governed by one dominant gene. Such resistance was further found to be independent of foliage wax. A physiological race of *P. parasitica* pathogenic to plants resistant to race 1 was designated race 2. Resistance to both races was found to be inherited independently (Natti et al. 1967). A study of Lucas and co-workers (1988) indicated that an oilseed rape cultivar, Cresor was resistant to 14 isolates of *P. parasitica* derived from *Brassica napus* in the U.K. Segregation for resistance to one isolate among the F_2 and F_3 progeny of crosses between Cresor and the susceptible cultivars, Victor and Jet Neuf, indicated that resistance in *B. napus* was also controlled by a single gene (Lucas et al. 1988). Similar observations were made by Jensen et al. (1999) who demonstrated dominant inheritance of resistance to *P. parasitica* in *B. oleracea* var. *botrytis* (lines K107 and K102) as well as indicating control of resistance by a single gene. Similarly inheritance of resistance in other downy mildews such as *Sclerospora graminicola* on pearl millet, was found to be controlled by a single dominant gene (Singh and Talukdar 1998). Lucas and co-workers (1988) suggested that genetic background and the environment could influence the phenotypic expression of resistance in *P. parasitica*. Furthermore, two sexual progeny isolates derived from a homothallic isolate of *P. parasitica* avirulent on Cresor were completely virulent on this cultivar. This suggested that the parental isolate was heterozygous at a matching locus or loci for avirulence and demonstrated the race-specific nature of the resistance. The differential response test (described earlier) has been a useful tool for identifying new sources of resistance among hosts of *P. parasitica* (Nashaat and Rawlinson 1994, Nashaat and Awasthi 1995, Silué et al. 1996)

A detailed review of Bent (1996) provides an overview of the structure of proteins encoded by *R* genes. Resistance genes from diverse plant species encode structurally similar proteins, which therefore suggested a high degree of mechanistic conservation among the pathways that trigger defense responses (Bent 1996). The structural domains of resistance genes include serine-threonine kinases, leucine rich repeats (LRRs), nucleotide binding sites (NBS), transmembrane receptors kinases and leucine zippers (LZ) (Bent 1996). NBS is a common protein motif in all organisms, occurs in numerous structural forms and functions to bind ATP or GTP (Traut 1994).

Many resistance genes that encode LRRs, also encode NBS and/or leucine zippers and the Toll/interleukin 1 domain at the N terminus of the protein. LRRs are multiple, serial repeats of a motif (approximately 24 amino acids in length) confined predominantly to eukaryotes and are involved in specific protein-protein interactions (Jones and Jones 1997). LRRs contain leucines or other hydrophobic residues at regular intervals and can also contain spaced prolines and asparagines (Bent 1996). One of the first resistance genes (encoding LRR proteins) to be isolated was the tomato *Cf*-9 gene for resistance to *Cladosporium fulvum*, which causes leaf mould in tomato (Jones et al.1994, cited in Jones and Jones 1997). This gene encodes an 863 amino acid, membrane-anchored, extracytoplasmic, glycoprotein containing 27 imperfect LRRs, averaging 24 amino acids in length.

Genetic studies have shown that many different plant resistance genes occur in clusters and two distinct arrangements may exist, either single genes encoding multiple alleles encoding different resistance specificities or a series of tightly linked genes forming complex loci. In lettuce, resistance clusters incorporate a family of resistance gene candidates (RGC2), which encode an NBS and an LRR region. The RGC2 genes span at least 3.5 Mb and at least 10 *Dm* genes map to this cluster (Meyers et al. 1998). The tomato *Cf* genes conferring resistance to *Cladosporium fulvum* encodes a largely extracytoplamic proteins with different *Cf* genes containing varying numbers of LRR repeats (Dixon et al. 1998).

The wild crucifer *Arabidopsis thaliana* is a useful system for basic studies in plant molecular genetics due to its relatively small genome size, small amounts of dispersed repetitive DNA and rapid generation time (Crute et al. 1994a). These attributes have made *Arabidopsis* an attractive model system for the analysis of genome organization and the development and use of technology to identify and clone resistance genes. One powerful application of the tools available to *Arabidopsis* researchers is in dissecting the processes by which plants recognise and respond to microbial pathogens (Dangl et al. 1992). The associations between *A. thaliana* and its bacterial and fungal pathogens provide opportunities to answer some intriguing questions about host-pathogen relationships from the level of interactions between molecules to population biology. Of all plant pathogens, fungi and Oomycetes are unquestionably the most important. Specialised biotrophic pathogens of this wild

crucifer include: *Peronospora parasitica* (downy mildew), *Erysiphe cruciferarum* (powdery mildew), *Albugo candida* (white blister) and *Plasmodiophora brassicae* (clubroot) (Crute et al. 1994a, Dangl et al. 1992).

Research on *Arabidopsis* has produced many inroads to understanding the basis of the gene-for gene interactions between plant pathogens and their hosts. The use of *Arabidopsis* for genetic dissection of plant defense responses has been extensively reviewed by Glazebrook et al. (1997). Interest in this study was spurred by initial observations of the considerable diversity in the interactions between *A. thaliana* and *Peronospora parasitica* (Koch and Slusarenko 1990). Subsequently Holub et al. (1994), identified corresponding loci for genotype specificities in the host and parasite, and numbered them according to the hypothesis of recognition. Thus, specificity loci of *A. thaliana* have been named *RPP* loci (abbr. of "recognition of *P. parasitica*") and were numbered accordingly.

Combining phenotypic observations to isolates of *P. parasitica* with segregation data for *Arabidopsis* accessions (Holub et al. 1994), the inheritances of the different interaction phenotypes were investigated. Segregation ratios were used to predict the allele/s involved with each phenotype. In addition, advanced generation recombinant inbred lined were used to confirm the identity of loci (Holub et al. 1994). RFLP and RAPD probes were then used to determine the specific map positions of three loci of *A. thaliana* associated with isolate specific recognition of *P. parasitica* (Table 1.5) (Tör et al. 1994). In the last decade studies on the interaction between *Arabidopsis thaliana* and *P. parasitica* has advanced rapidly and several *RPP* genes have been cloned and characterised (Crute et al. 1994a, Holub et al.1994, Tör et al. 1994, Reignault et al. 1996, Parker et al. 1997, McDowell et al. 1998, Botella et al. 1998, Cooley et al. 2000) (Table 1.5). Furthermore, genes other than those at *RPP* loci may be involved in the determinations of the interaction phenotype (Crute et al. 1994b).

Table 1.5: *Peronospora parasitica* recognition (*RPP*) genes in *Arabidopsis thaliana*

RPP Loci	Reference	Chromosome Location
RPP6, *RPP9*	Crute et al. 1994b	I
*RPP7*A, *RPP7*B	Tör et al. 1994	I
RPP1	Crute et al. 1993, Botella et al. 1998	III
RPP10	Crute et al. 1994b	III
RPP11	Joos et al. 1996, Crute et al. 1994b	III
RPP13	Bittner-Eddy et al. 1999, 2000	III
RPP14.1-3	Reignault et al. 1996	III
RPP2, *RPP4E*^{c}, *RPP4C*^{c}	Crute et al.1993, Tör et al. 1994	IV
RPP5	Parker et al. 1993, 1997 Reignault et al. 1996	IV
RPP12	Crute et al. 1994b	IV
*RPP8*A, *RPP8C*, *RPP8E* & *RPP8M*	McDowell et al. 1998, Cooley et al. 2000	V
RPP3	Crute et al. 1993	Not known

In December 2000, the sequencing of *Arabidopsis thaliana*, the first plant genome, was completed (Walbot 2000). The *Arabidopsis* consortium has sequenced 115.4 megabases of the 125 megabase genome extending into centromeric regions as well (The *Arabidopsis* Genome Initiative 2000). The genome contains 25.478 genes encoding proteins from 11 000 families similar to the function diversity of *Drosophila* and *Caenorhabditis elegans* and other sequenced multicellular eukaryotes. Resistance gene evolution may involve duplication and divergence of linked families, however most (46) resistance genes are singletons. Resistance genes are unevenly distributed between chromosomes with a large proportion on chromosome 1 (*Phytophthora* Genome Initiative 2000). With the genome in hand, the next challenge will be to investigate the roles of the individual *Arabidopsis* proteins (Bevan et al. 2000).

Two related structural *R*-gene types have been isolated from *Arabidopsis* (Phytophthora Genome Initiative 2000, Ellis et al. 2000). They encode modular proteins with a central NBS and a carboxy-terminal LRR. However, they differ substantially in their amino-termini which carry a Toll/interleukin homology region (TIR) domain, or a putative coil (CC). Thus two classes of *R*-genes viz. CC-NBS-LRR and TIR-NBS-LRR are found. There are 85 TIR-NBS-LRR resistance genes at 64 loci, and 36 CC-NBS-LRR resistance genes at 30 loci. Some NBS- LRR genes expressed neither obvious TIR nor CC domains. However, other TIR-NBS genes that lack an LRR at 10 loci were often found adjacent to full TIR-NBS-LRR genes. Intriguingly, two TIR-NBS-LRR genes that carry a WRKY domain, found in transcription factors, were implicated in plant defence, and one of these encoded a protein kinase domain (Phytophthora Genome Initiative 2000).

R-genes of the TIR-NBC-LRR structural type which confer resistance to *Peronospora parasitica* include *RPP5* (Parker et al. 1997) and *RPP4* (van der Biezen et al. 2002). Further investigations revealed *RPP5* and *RPP4* to be the only functional members of a complex multi-gene family that spans approximately 100 kb on the lower arm of chromosome IV. In contrast the *RPP1* complex locus on the lower arm of chromosome III of Arabidopsis accession Wassileskija possesses at least three functional *R*-genes that specify recognition of four distinct *Peronospora parasitica* isolates (Botella et al. 1998).

The RPP13 locus in *Arabidopsis* encodes and NBS-LRR type R protein with a leucine zipper (LZ). Comparison of three RPP13 alleles revealed a high rate of amino acid divergence within the LRR domain. Furthermore, an amino acid sequence (LLRVLDL) identical among these alleles was found to be conserved in other LZ NBS-LRR type proteins, suggesting functional significance (Bittner-Eddy et al. 2000). The RPP8 genes were found to be different in resistant Landsberg erecta (Ler-0) and susceptible Columbia (Col-0). RPP8-Ler was found to encode an NBS-LRR protein with a putative N-terminal Leucine zipper and was more closely related to previously cloned R genes that confer resistance to bacterial pathogens than it was to other known RPP genes. RPP8 haplotype in Ler-0 contains the functional RPP8-Ler gene and a non-functional homolog RPH8A. In contrast, RPP8-Col contained a single chimeric gene, which was likely to be derived from unequal

crossing over between RPP8-Ler and RPH8A. These observations suggested that NBS-LRR molecular evolution was driven by the same mechanisms that promote rapid sequence diversification among other genes involved in non self recognition (McDowell et al. 1998).

More recently investigations are focusing on how the respective *R*-proteins function within a disease resistant signalling pathway (Aarts et al. 1998b, Feys et al. 2001, Muskett et al. 2002, Varett et al. 2002). Some of these pathways are triggered by specific recognition between plant resistance (*R*) gene and pathogen avirulence (*avr*) gene encoded products. Other pathways exert low level (basal) resistance against virulent pathogens. The TIR-NBS-LRR type *R*-genes were found to be strongly dependent on the *EDS1* and *PAD4* genes but do not require *NDR1*, a different resistance signalling component (Aarts et al. 1998b, Feys et al. 2001). Conversely most *R*-genes of the CC-NBS-LRR type were found to depend strongly on *NDR1* but not *EDS1* or *PAD4* (Feys et al. 2001). These observations suggested that different *R*-proteins were products of distinct resistance pathways. However, the observation that *RAR1* and *SGT1* were required by both NBS-LRR classes indicated that resistance signalling was much more complex (Austin et al. 2002). Aspects of new genes with potential role/s in resistance signalling have recently been described (Varett et al. 2002, Muskett et al. 2002). LRR repeats evolve at unusually high rates (Bergelson et al. 2001) and modification of the length of the LRR may be an important contributor to *R*-gene diversification (Ellis et al. 2000). The evolutionary dynamics of *R*-genes are discussed further by Bergelson et al. (2001).

1.4 APPLICATION OF MOLECULAR TECHNIQUES FOR POPULATION DIVERSITY ANALYSIS

Plant pathogens are continuously evolving; Pathogen populations must constantly adapt to changes in their environment to survive. Many factors contribute to evolution within populations. These include mutation, mating systems, gene flow or migration, population size and selection (McDonald 1997). Furthermore, in

agricultural systems, environmental changes may include resistant varieties, applications of fungicides and fertilizers, irrigation and crop rotation.

Traditionally, studies on pathogen variation focused on correlating morphological and physiological characters with pathogenicity (Duncan 1994). For example, *P. parasitica* isolates from different hosts were found to be morphologically similar (Yerkes and Shaw 1959, Jugmohan 1997) but vary in host range (Sheriff and Lucas 1990, McMeekin 1969). Different pathotypes of *P. parasitica* have been distinguished on the basis of this host specificity (Sheriff and Lucas 1990) but such tests are time consuming and may not reveal the full extent of the variation present. Other phenotypic markers such as sexual compatibility type (Illot et al. 1987, Michelmore and Ingram 1980) or fungicide sensitivity (Crute 1987) can be assessed but they provide limited information for epidemiological studies or genetic analysis. Thus, there was a need for alternative markers to further define variation in the pathogen (Michelmore and Hulbert 1987).

Defining the genetic structure of populations is a first logical step in studies of fungal population genetics because the genetic structure of a population reflects its evolutionary history and its potential to evolve (McDonald 1997). Genetic markers that are selectively neutral, highly informative and relatively easy or inexpensive to assay are preferred for population genetic studies. Advances in molecular techniques have provided a new set of tools for analyzing genomic variation and host-pathogen interactions. DNA polymorphisms are a rich source of molecular markers for genetic analysis and are also of potential value as diagnostic characters discriminating between species or pathotypes (Lucas et al. 1994).

The development of molecular techniques has led to increasing use of whole protein profiles, isozymes and now variation in the fungal DNA to characterise pathovars or races (Duncan 1994). Isozymes are differently charged protein molecules that can be separated by electrophoresis. Isozymes have been used in population genetic studies of *Puccinia graminis* (Burdon and Roelfs 1985), *Phytophthora infestans* (Fry et al. 1991) and for characterising host-specialised variants of *Leptographium wageneri* (Zambino and Harrington 1989). Isozymes, however have a number of limitations. These include: a) the limited number of usable

enzymes systems in any one species, b) the genic diversity of a population of isozymes is often underestimated because only one third of amino acid substitutions are electrophoretically detected (Lewontin 1974, cited by McDermott and McDonald 1993), and c) isozymes are subject to post-translational modification (Staub et al. 1982, cited by Staub et al. 1996).

In the 1980s, restriction fragment length polymorphisms RFLPs became popular as a method for the construction of genetic linkage maps. RFLP analysis is based on two techniques widely used in modern molecular biology: restriction endonuclease digestion of DNA and the transfer of DNA fragments to a filter onto which can then be hybridised a labelled DNA fragment (Southern 1975). RFLPs are detected by the use of restriction enzymes that cut genomic DNA at specific nucleotide sites (restriction sites) thereby yielding variable size DNA fragments (Staub et al. 1996). Size fractionation to distinguish a fragment of interest from all other fragments of similar size is achieved by gel electrophoresis. Individual fragments are then picked out from the mass by hybridising them to an appropriately labelled DNA probe (usually 10-20 bases), homologous to the entire fragment or some part of it (Southern 1975, Beckman and Soller 1986, Brettschneider 1998). Genetic variation in DNA nucleotide sequence between individuals (i.e. polymorphism at the DNA level) means that the specific distribution of cleavage sites along their respective DNA molecules will also differ, resulting in a different mix of restriction products. Furthermore RFLP markers are co-dominant. RFLPs have been used in the analysis of variation of Oomycetes including *Phytophthora infestans* (Whisson et al. 1992) and the downy mildew *Bremia lactucae* (Hulbert et al. 1988, Hulbert and Michelmore 1988). RFLPs have been used to effectively discriminate different clones in a population of *Septoria tritici* (McDonald and Martinez 1991). *S. tritici* which causes leaf blotch disease on wheat is the imperfect stage of the wheat pathogen *Mycophaerella graminicola*. *S. tritici* does not possess clearly defined virulence genes or stable morphological variants that can be used as genetic markers. Multiclocus RFLP probes enabled the differentiation of haplotypes of *S. tritici*. While the number of RFLP markers can be effectively unlimited, detection of RFLPs by Southern blot hybridisation is laborious and relatively costly (Williams et al. 1990).

The development of the polymerase chain reaction (PCR) is a milestone in genome analysis. PCR is a rapid procedure for *in vitro* enzymatic amplification of a specific target segment of DNA (Mullis 1990, White et al.1989). PCR involves three steps. The DNA is first denatured (i.e. the strands separated) then cooled to allow annealing of the primers. PCR utilises short oligonucleotide primers that are able to bind to the genome close to the target region thus flanking the region. These primers hybridise to opposite -strands of the DNA and are orientated with their 3' ends facing each other so that in the final step, synthesis by a thermostable DNA polymerase (which catalyzes growth of new strands in a 5' to 3' direction) extends across the segments of DNA between them. The extension products can serve as templates for the other primer, therefore the whole process can be continued in a cyclical fashion, resulting in exponential amplification (Coen 1990, White et al. 1989). PCR provides a simple technology that can be used to rapidly acquire sequence information on large numbers of species and individuals within populations including rare and extinct organisms (Arnheim et al. 1990). It has greatly increased the efficiency of genome mapping in a number of organisms. PCR has also been used for the detection and analysis of a wide variety of plants, animal and fungi such as *Fusarium monoliforme* from infected maize (Murillo et al. 1998), *Fusarium oxysporum* f. sp. *dianthi* from carnation (Manulis et al. 1994), and the Oomycete *Peronospora sparsa* from cultivated arctic bramble (Lindqvist 1998).

Polymorphisms based on PCR require target DNA sequence information for the design of amplification primers (Williams et al. 1990). The time and cost of obtaining this sequence information is prohibitive for many large-scale mapping applications. A number of variations of the PCR technique or methods based on PCR have thus been developed. These include the use of random amplified polymorphic DNA (RAPDs) and sequence characterised amplified regions (SCARs), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs or microsatellite) markers.

Random amplified polymorphic DNA (RAPD) analysis is a simple technique, which is based on the PCR amplification of genomic DNA with a single primer of arbitrary nucleotide sequence. The polymorphisms thus detected are inherited in a Mendelian fashion and have been used to construct genetic linkage maps of a variety

of species (Williams et al. 1990, Welsh and McClelland 1990). RAPD markers are dominant, as DNA segments of the same length are amplified from one individual but not from another. Of greater importance to plant pathologists, RAPDs can be assayed using very small amounts of fungal biomass, making them an ideal tool for obligate biotrophs such as rusts and mildews (McDonald, 1997). Like most molecular markers, the information content of individual RAPD markers is very low. It is only when many of these anonymous markers are used to define a genome that they begin to have utility (Williams et al. 1990). The RAPD technique has been successful in the identification and characterisation of strains/isolates of fungi, bacteria, plants and animals (Welsh and McClelland 1990, Ouellet and Siefert 1993, Permaul et al. 1996).

RAPD analysis is particularly useful in the examination of genetic variation in pathotypes and/or races of culturable fungi such as *Leptosphaeria maculans* (Goodwin et al. 1991) and *Fusarium oxysporum* spp. (Manulis et al. 1994, Mighelli et al. 1998, Woo et al. 1996). RAPDs have been used extensively to describe intraspecies variation in various *Fusarium* spp. on the basis of pathogenicity, race, compatibility and geographical origin (Manulis et al. 1994, Woo et al. 1996, Mighelli et al. 1998). RAPD generated by each of 22 primer sets could clearly distinguish between 58 pathogenic and non-pathogenic isolates of *Fusarium oxysporum* f. sp. *dianthi* infecting carnation (Manulis et al. 1994). Further analysis by Mighelli et al. (1998) showed that various races of *Fusarium oxysporum* f. sp. *dianthi* could be distinguished using RAPDs. In addition isolates of *F. redolens* different geographical locations including Japan, Italy, Israel and Netherlands were clearly distinguishable according to their RAPD fingerprint (Mighelli et al. 1998). Furthermore, RAPD probes for the specific detection of race 2 of *Fusarium oxysporum* f. sp. *dianthi* (Manulis et al. 1994) was possible. Isolates of *Mycophaerella brassicicola* which causes ringspot disease of crucifers could be categorised into two groups on the basis of RAPD markers (Lesur et al. 1998).

RAPDs have proved useful in non-culturable pathogens including Oomycetes. RAPDs and SCARs were useful for the development of reliable markers linked to downy mildew resistance genes in lettuce (Paran and Michelmore 1993). RAPDs were valuable in the early detection of the blue mould pathogen, *Peronospora tabacina* that causes downy mildew of tobacco (Wiglesworth et al. 1994). RAPD

fingerprints have been obtained from a range of *P. parasitica* isolates from different *Brassica* species (Tham et al. 1994). Using two primers B12 and B17, reproducible polymorphisms were found between pathotypes of *P. parasitica*. In addition, different isolates within a pathotype of *P. parasitica* could be distinguished. Comparing fingerprints of all isolates, some bands were seen to be common to all isolates while others were pathotype or isolate specific. Disregarding the common bands, there were three or four bands that were able to distinguish between the *B. napus* and *B. oleracea* pathotypes. Furthermore, sufficient genetic variation was present to permit complete differentiation between the two *Brassica* pathotypes of *P. parasitica* and this illustrated the potential of RAPDs for detecting polymorphism between isolates of a non-culturable pathogenic fungus. Polymorphisms were also detected between different field isolates of the same pathotype. Labelled diagnostic bands could then be hybridised to genomic fungal DNA i.e. the cloned amplified fragments and may also serve as conventional RFLP probes (Tham et al. 1994). In contrast, RAPD analysis of *Sphaerotheca fuliginea*, the causal agent to powdery mildew of cucurbits, revealed a low level of polymorphism (Bardin et al. 1997). Using 22 primers which produced reproducible patterns, cluster analysis did not separate groups within the species of *S. fuliginea*.

RAPDs have several disadvantages. They are difficult to reproduce between sometimes within laboratories (Jones et al. 1998). Some of these technical limitations can be overcome by proper controls such as replicate DNA preparations, Southern analysis and the conversion of RAPD amplicons into sequence characterised amplified regions (SCARS). There are also analytical problems associated with RAPDs. RAPDs have two alleles (amplification or non-amplification) for each amplicon locus. Although this is ideal for genetic mapping, it is a drawback for measures of genetic diversity affected by the number of alleles at a locus. RAPDs are dominant, so they cannot differentiate homozygotes and heterozygotes without a progeny test. This is not an issue with haploid fungi, but it can be a problem with many basidiomycetes and Oomycetes that are heterokaryons, diploid or polyploid (McDonald 1997). PCR based genetic markers that can detect more than two alleles and that exhibit codominance, such as SCARS and microsatellites are likely to replace RAPDs as studies of fungal population genetics become more sophisticated.

The AFLP technology developed by Keygene (Vos et al. 1995) is a useful tool for analysing population diversity. The AFLP technique is based on the principle of selectively amplifying a subset of restriction fragments from a complex mixture of DNA fragments after digestion of genomic DNA with restriction endonucleases. Polymorphisms are then detected by differences in the length of the amplified fragments after polyacrylamide gel electrophoresis (PAGE) (Matthes et al. 1998) Basically, the first step in the generation of AFLPs is to double digest genomic DNA with two restriction enzymes. Next, a specific short DNA sequence is linked to one end of the fragment, and a different sequence added to the other. These sequences, together with the adjacent restriction sites serve as binding sites for PCR primers. The primers are designed to match the two different added sequences, and they also carry short extensions of 1-3 nucleotides to bring about selective amplification of those fragments with complementary 1-3 nucleotide sequence (Jones et al. 1997). The AFLP system is technically difficult and expensive to set up, but it detects a large number of loci, reveals a great deal of polymorphism and produces high complexity DNA fingerprints which can be used for identification and for high resolution mapping and marker assisted cloning (Jones et al. 1997).

AFLPs have found wide use in plants (Winfield et al. 1998) and in the Oomycetes *Phytophthora infestans* and *Peronospora parasitica* (Van der Lee et al. 1997, Rehmany et al. 2000). AFLPs were used to develop the first comprehensive genetic linkage map of *Phytophthora infestans* (Van der Lee et al. 1997) as well as to demonstrate heterokaryosis in *Thanatephorus cucumeris* (*Rhizoctonia solani*) (Julian et al. 1999). AFLP fingerprints generated from single zoospore progeny and 73 F1 progeny from two parental isolates of *P. infestans* revealed parental isolates to be homokaryotic and diploid. A total of 183 AFLP and 7 RFLP markers were mapped in *Phytophthora infestans*; the linkage map comprises 10 major and 10 minor linkage groups covering a total of 827 cM (Van der Lee et al. 1997). This map will be useful for analysing the inheritance of (a) virulence phenotypes in *P. infestans* and to clone avirulence genes. Van der Biezen et al. (2000) developed a c-DNA AFLP technique to detect *P. parasitica* genes expressed during infection of *Arabidopsis thaliana*. This technique has useful application in the development of EST libraries. Rehmany and co-workers (2000) used AFLPs together with ITS1 sequence analysis to demonstrate

the divergence between *P. parasitica* isolates from *Arabidopsis thaliana* and *B. oleracea*.

A new class of markers known as microsatellites or simple sequence repeats (SSRs) have recently been described as a powerful tool for analysis of eukaryote diversity. Microsatellites occur as tandem repeats of di-, tri-, tetra- and penta-nucleotides and are ubiquitous in eukaryotic genomes (Epplen 1988, Gross and Garrard 1986, Ostrander et al. 1992). They are usually less then 100 bp long and can occur to a lesser extent in prokaryotes or eubacteria (Tautz 1989). The repeat sequences are thought to arise by slippage-like events during DNA replication. Of the most studied repeats $(GT)_n$, $(GA)_n$, $(GATA)_n$ and $(GACA)_n$, the copy number of $(GT)_n$ per haploid genome varies from 100 copies in yeast to 100 000 copies in the mouse genome (Stallings et al. 1991).

In general, SSRs do not have a defined function in the genome but may reflect the occurrence of genetic "change" via the production or deletion of these sequences. However, various functional roles have been attributed to SSRs; for example, hot spots of recombination (Kabori et al. 1986, Bullock et al. 1986) and the regulation and expression of genes (Hamada et al. 1984). Being much smaller than 1 kb and varying over a narrow size range microsatellites can be readily analysed by PCR and electrophoresis, circumventing the need for large quantities of DNA and Southern blotting. Allelic variation at these loci also derives from variation in the number of tandem repeats, and hence in the length of the region (Armour and Jeffreys 1992). In comparison to isozymes, RFLPs, and RAPD markers, microsatellite markers can be analysed with considerably less material, even if it is old and partially degraded (Groppe et al. 1995). Furthermore microsatellites are more reproducible than RAPDs because they use longer primers (Barroso et al. 2000). The mutation rate of microsatellites is estimated to be between 5×10^{-4} and 5×10^{-5}, which is low enough to permit their use in linkage studies, as probes for linkage disequilibrium with disease mutations in population studies, and in forensic applications (Dietrich 1992, cited by Hearne et al. 1992).

The elucidation of microsatellites as a valuable tool in molecular biology took the form of database searches (Field and Wills 1996, Groppe et al. 1995),

oligonucleotide fingerprinting with microsatellite probes (Meyer et al. 1991, DeScenzo and Harrington 1994, Groppe et al. 1995), random amplified microsatellites (Couto et al. 1996, Hantula et al. 1996, Hantula and Müller, 1997, Longato and Bonfante 1997, Müller and Hantula 1998, Geistlinger et al. 1997b), or more recently the generation of genomic libraries enriched for microsatellites (Burgess et al. 2001, Edwards et al. 1996, Kölliker et al. 2001, Mba et al. 2001).Genomic libraries enriched for microsatellite diversity provide a more practical reflection of the diversity of repeat sequences in the genome of various organisms. Microsatellites have been used in the detection of polymorphism in humans (Armour and Jeffreys 1992, Brook et al. 1992, Hearne et al. 1992, Litt and Luty 1989), plants (Kijas et al. 1994, Lagercrantz et al. 1993), various fungi (Bucheli et al. 2001, Couto et al. 1996, Hantula and Müller 1997, Kistler et al. 1991, Levy et al. 1991, Sastry et al. 1995) as well as downy mildew pathogen *Sclerospora graminicola* (Sastry et al. 1995). Furthermore various haplotypes or pathotypes have been distinguished based on microsatellites (Bucheli et al. 2001, Levy et al. 1991, Sastry et al. 1995). A complete description of microsatellites, their evolution and applications, are provided in Chapters Four and Chapter Five.

1.5 SCOPE OF PRESENT STUDY

Many *Brassica* crops are of growing importance in rural communities of Southern Africa. Crops such as broccoli, cauliflower, cabbage, and turnip, form part of the staple vegetable diet. Downy mildew disease, caused by *Peronospora parasitica* is one of the most serious pathogens infecting vegetable *Brassica* species and almost 60-70% of total crop losses to nurserymen and farmers is due to infection by *P. parasitica*. Attempts to control the disease using fungicides have been unsuccessful since *P. parasitica* has developed resistance to metalaxyl-based fungicides (Brophy and Laing 1992, Vishunavat et al.1998). Similar observations were recorded for other downy mildews such as *Plasmopara halstedii* (Albourie et al. 1998). The induction of systemic acquired resistance (SAR) by various chemicals as a means to control disease are being investigated. *Arabidopsis* plants treated with chemical inducers such as 2.6-dichloroisonicotinic acid have been found to result in inhibited growth of *P. parasitica* and other pathogens (Uknes et al. 1992), however the biochemical mechanisms of SAR is not understood. Pathogenesis-related (PR) proteins are good

markers of induced resistance. *Brassica oleracea* var. *botrytis* (cauliflower) seedlings treated with DL- β-amino-*n*-butanoic acid (BABA) and challenged with *P. parasitica* resulted in accumulation of the PR protein PR-2 (Silué et al. 2002). Despite the recent efforts at biological control (Wilson 1997) few commercial products have reached the marketplace.

It is imperative to look for alternative control methods for the disease not only to protect crops but also to ensure a safe and clean environment. Genetic engineering of plants with tolerance to fungal pathogens holds potential in this respect. The coevolution of interacting plants and microbes has given rise to a diverse array of exchanged signals and responses. Rapid defense responses in plants are controlled by resistance (R) genes and much effort has been focussed on identification and characterisations of resistance genes (Bent 1996). Most strategies for engineering fungal resistance in plants have involved manipulation of genes regulating PR proteins, antimicrobial proteins, enzymes controlling oxidative burst and phytoalexins (Rao et al. 1999). Transgenics known to confer resistance include enzymes such as chitinase and glucanase transgenics, PR-1a protein, osmotin, lysozyme and thionins. Significant inroads have been described on the identification of resistance genes in *Arabidopsis thaliana*, the wild host of *P. parasitica*. *A. thaliana* has been found to be a model system for cloning resistance genes against *P. parasitica* (Crute et al. 1993, 1994a, Tör et al. 1994, Reignault et al. 1996, Parker et al. 1997, McDowell et al. 1998, Botella et al. 1998, Cooley et al. 2000). The function of R-genes is dependent on the genotype of the pathogen (Keen 1990). A greater understanding of plant pathogen interactions is dependent on genetic studies in both the plant and the pathogen.

Until recent years studies on *P. parasitica* have been hindered by the biotrophic nature of the pathogen. Intensive work initiated on host specificity of various isolates/pathotypes on each other have only hinted at the extent of variation present (Nashaat and Rawlinson 1994, Nashaat and Awasthi 1995, Silué et al. 1996). To date only two races of *P. parasitica* have been described (Lucas et al. 1988, Natti et al. 1967). However, many pathotypes, including the *Arabidopsis* as well as various *Brassica* pathotypes, are known to exist. Different mechanisms for generating

variation in *Peronosporas* such as sexual recombination and both secondary homothallism and heterothallism (Sheriff and Lucas 1989a,b, Jugmohan 1997) add to the diversity of this pathogen. Furthermore, there is no evidence to substantiate that mitotic crossing-over, polyploidy, aneuploidy and somatic fusion with heterokaryon formation occur in *P. parasitica*. However, it does seem probable that these mechanisms might occur if one considers that every conidium contains up to 30 nuclei (Davison 1968a).

Molecular methods involving the analysis of nucleic acids are now well developed and have been applied to many different fungi (Bruns et al. 1991). PCR-based methods such as RAPDs and RFLPs have been utilised in Oomycetes, *P. parasitica* and *Bremia lactucae* respectively, to design probes for the identification and location of crop pathogens in host tissues but there have been few purely systematic studies (Hall 1996). Early taxonomic studies on the downy mildews were based solely on morphogenetic characters such as hyphal branch or spore type (Yerkes and Shaw 1959). More recently Oomycetes have been suggested to be more closely related to alga or diatoms than to true fungi (Gunderson et al. 1987, Förster et al. 1990). The development of accurate species descriptions in pathogens such as *P. parasitica* is crucial to the work of plant pathologists, because failure to provide them has serious implications for plant quarantine and plant hygiene legislation and the free movement of economically important produce between countries (Hall 1996). This is a further necessity since *P. parasitica* is a seedborne disease (Jang and Safeeulla 1990a) and many of the seeds used locally in South Africa are imported thus introducing other pathogen isolates into the system. The current global interest in biodiversity and, in particular, the search for bio-control agents for crop diseases has further increased the importance of species recognition (Hall 1996).

Molecular markers have been widely used in the analysis of diversity of fungi. These include the application of isozymes, RFLPs, RAPDs and AFLPs (Bruns et al. 1991, Fry et al. 1991, Ouellet and Siefert 1993, Wiglesworth et al. 1994, McDonald 1997, Lesur et al. 1998, Murillo et al. 1998). Despite the availability of these techniques, research on obligate Oomycete pathogens such as *P. parasitica* has lagged behind for a number of reasons: These include a) the large amount of DNA required for RFLP analysis, b) difficulties with reproducibility and RAPDs and c) the high

quality DNA required for AFLPs. Despite these initial drawbacks, research has in recent years advanced rapidly with the utility of both RAPD (Tham et al. 1994) and AFLP (Rehmany et al. 2000) markers in differentiation of pathotypes of *P. parasitica* contributing new information to our understanding of the genetics of *P. parasitica*.

A new class of markers known as microsatellites or simple sequence repeats (SSRs) constitute an important source of genetic markers because in addition to their high polymorphism and wide dispersion in the genome, they are codominant, multiallelic and easily scored. Furthermore microsatellites have been shown to be more reproducible then RAPDs mainly because they use longer primers (Barroso et al. 2000). In comparison to isozymes, RFLPs, and RAPD markers, microsatellite markers can be analysed with considerably less material, even if it is old and partially degraded (Groppe et al. 1995). For studies of plant-associated fungi, microsatellite markers have the added advantage that the analysis can be performed directly in planta if the flanking primers are suficiently specific. Microsatellite markers were found to be very useful in characterising variation in fungi (Meyer et al. 1991, Kistler et al. 1991, Morjane et al. 1994, Hantula et al. 1996, Longato and Bonfante 1997, Müller and Hantula 1998). Furthermore, microsatellite markers were found to be efficient in differentiating pathotypes of fungi (Levy et al. 1991, Bucheli et al. 2000) as well as pathotypes of the downy mildew pathogen *Sclerospora graminicola* (Sastry et al. 1995). In view of the above, microsatellites were thus considered to be the method of choice for analysis of *P. parasitica*. Microsatellites occur abundantly throughout most eukaryotic genomes, are easy to clone and characterise. They display considerable polymorphism due to the variation it the number of repeat units (Hearne et al. 1992) and therefore provide and attractive source of genetic polymorphisms in fungi (Buscot et al. 1996). The development and use of microsatellite markers would be an important contribution to understanding the relationships between different *P. parasitica* isolates, pathotypes and species.

The present study focused on elucidation of relationships between isolates and pathotypes of *P. parasitica* on two levels 1) sequence analysis the internally transcribed regions of the rDNA operon and 2) screening of pathotype diversity of a microsatellite library of *P. parasitica*.

1.6 HYPOTHESIS TO BE TESTED

It is hypothesized that isolates of *Peronospora parasitica* occurring on different *Brassica* species share some common phylogeny. Genetic variability between isolates and pathotypes of *P. parasitica* may be influenced by the relatedness of the host/s infected. Furthermore, resolution of different *Peronospora parasitica* pathotypes is directly influenced by the type of genetic marker applied.

1.7 APPROACHES ADOPTED

The main aim of this study was to analyse isolate and pathotype diversity in *Peronospora parasitica* in South Africa. To achieve this 1) a representative isolate collection and 2) Tools for estimating diversity and relatedness were needed. Simple sequence repeats (SSRs) also referred to as microsatellites, were developed as a new tool for the analysis of pathogen diversity.

Phase I: Establishment of a culture collection of *Peronospora parasitica*

a. To collect and maintain various pathotypes of *Peronospora parasitica* from different geographic areas the UK, France and other areas.
b. To derive single spore isolates and maintain different pathotypes of *Peronospora parasitica* from South Africa.
c. To produce axenic cultures of the pathogen and collect spores for DNA isolation.
d. To isolate good quality DNA for further analysis.

Phase II: To analyse the phylogeny of *Peronospora parasitica* by amplification of rDNA

a. To amplify the intergenic spacer region (ITS1→ITS4) of the ribosomal DNA operon (rDNA) for various pathotypes/isolates of *Peronospora parasitica* using the polymerase chain reaction (PCR).
b. To sequence the ITS-PCR products to elucidate phylogenetic relationships.

Phase III: Use of microsatellites to determine the relationships between *Peronospora parasitica* isolates

a. To construct a genomic DNA library of *Peronospora parasitica* enriched for microsatellites.

b. To screen the above library for microsatellite polymorphism among pathotypes of *P. parasitica* using the polymerase chain reaction (PCR).

CHAPTER TWO

ESTABLISHMENT OF A CULTURE COLLECTION OF *Peronospora parasitica*

2.1 INTRODUCTION

The downy mildew pathogen *Peronospora parasitica* (Pers. ex. Fr.) Fr. occurs world-wide (Channon 1981). Some of the most studied isolates have been in the UK, France, Portugal, India and South Africa. Since *P. parasitica* has a very wide host range (Yerkes and Shaw 1959), several attempts have been made to a) determine virulence on each of the hosts (Kluczewski and Lucas 1982, 1983, Sheriff and Lucas 1987, Koch and Slusarenko 1990, Sheriff and Lucas 1990); b) to determine the relationship between each of the pathotypes (Tham et al. 1994, Silué et al. 1996) and perhaps most importantly; c) to develop methods of culturing this biotrophic pathogen *in vitro* in order to achieve a) and b).

Being an obligate pathogen, *P. parasitica* cannot be cultured on artificial media and relies solely on the host plant for its nutrition (Lucas et al. 1994). Studies on *P. parasitica* require a continuous supply of pathogen material of known origin. Several methods of callus culture of *P. parasitica* have been described (Achar 1995, Ingram 1969). Callus culture has been reported to sustain *P. parasitica* for 21 days, however by this time calluses were senescent and brown in colour although heavily invaded with hyphae (Ingram 1969). Callus culture reduced cross-contamination, however calluses could not be transferred directly. Differences in cell wall structure in callus tissue compared with whole plant cell walls were suggested to affect colonisation and growth of downy mildew (Ingram 1969). Therefore, callus tissue did not provide a means of testing material for resistance since it did not necessarily reflect the response of the whole plant.

Maintenance of *P. parasitica* in controlled environments on appropriate brassica hosts or ideally *in vitro* under the optimum conditions for proliferation was essential. A

simple method for *in vitro* maintenance of *P. parasitica* on living plants in a climatic chamber was developed (Kluczewski and Lucas, 1982, 1983, Sheriff and Lucas 1989 a,b). Young crucifer plants or detached cotyledons on moist filter paper were inoculated, covered to raise the humidity to 100% then incubated. In general, a day night cycle with approximately 16 hours photoperiod and low temperature of 14-20°C was favoured (Channon 1981, Kluczewski and Lucas 1982, 1983, Sheriff and Lucas 1987, 1989, 1990, Nashaat and Awasthi 1995, Silué et al. 1996). Approximately seven days after inoculation the isolate was sub-cultured onto a fresh pot of seedlings. A similar system was optimised in the laboratory at the University of Durban-Westville (Jugmohan 1997). The ideal conditions for maintenance of *Peronospora parasitica* was in a climatic chamber on seven day old seedlings grown in sterile pots with a 12 hr photoperiod at 16°C utilising fluorescent and incandescent light. Control measures to minimise cross-contamination include surface sterilised seed, autoclaved soil and sub-culturing at intervals of seven days.

Field populations of *P. parasitica* are highly variable. The problem of variable field populations is exacerbated by the occurrence of different races or *forma specialis* of *P. parasitica* (Natti et al. 1967, Sheriff and Lucas 1990, Tham et al. 1994) and due to both heterothallic (Kluczewski and Lucas 1983, McMeekin 1960, Sheriff and Lucas 1989a, 1989b) and homothallic (de Bruyn 1937, Jugmohan 1997, Sheriff and Lucas 1989a, 1989b) behaviour. For genetic analysis it was necessary to compare different clones or isolates, each having a uniform genetic makeup. Therefore uniform genetic lines (also referred to as "single spore" isolates or monoconidial lines) were derived from field isolates and such monoconidial lines could be easily compared. This procedure was necessary for experiments on heterothallism, homothallism, differential response and genetic fingerprinting (Nashaat and Awasthi 1995, Sheriff and Lucas 1989a, 1989b, Tham et al. 1994).

The availability of inoculum may be complicated by the viability of conidial/inoculum sources. The survival of conidia depends on the environment in which they are maintained. In Germany, conidia of *P. parasitica*, *P. tabacina* and *P. farinosa*

stored in dry soil or dust or in an open shed were still infective after several weeks in summer and several months in winter indicating that conidia could overwinter in cold barn soils (Kröber 1969). The survival period was however reduced (to a maximum of 22 days) (Kröber 1970) if the storage soil was moist. In Australia, Hill (1966) found that less than one percent of *P. tabacina* conidia were still able to germinate after more than 100 days storage at low humidities and constant temperatures in the laboratory. However observations of McMeekin (1960) and Jang and Safeeulla (1990b) indicate that oospores constitute an important means of survival in cultivated *Brassica* spp. as well as in wild hosts over periods of unfavourable conditions. Oospores have been reported to stay viable and infective in the soil for two to three years. Moss et al. (1994) reported that it was possible to recover and characterise sexual progeny from pairings between isolates specialised to different host species. However, the majority of such isolates sporulated weakly and proved difficult to maintain (Moss et al. 1994).

Several methods exist for long term storage of obligate pathogens (Michelmore and Ingram, 1980). Isolates of *Bremia lactucae* have been stored for extended periods by freezing cotyledons showing pathogen sporulation, contained in sealed plastic culture boxes, at -20°C (Michelmore and Ingram, 1980). Such treatment did not normally result in any major loss of viability, even after one year of storage. Viability was then determined using the fluorescein diacetate test and confirmed by germination in distilled water. Sheriff and Lucas (1989b) reported that isolates of *P. parasitica* could be stored by freezing infected plants at -20°C for up to one year without much loss of viability. This method was frequently used for storing isolates, including single spore isolates. *P. parasitica* isolates have also been restored from -80°C after five years (Jugmohan, unpublished results).

The aims of the present chapter are a) to maintain various pathotypes of *Peronospora parasitica* from different geographic areas including the UK, France, India and South Africa b) to derive single spore isolates and maintain different pathotypes of *Peronospora parasitica* of diverse geographic origins c) to produce axenic cultures of the pathogen and collect spores for DNA isolation and d) to isolate good quality DNA for further genetic analysis.

2.2 MATERIALS AND METHODS

2.2.1 Culturing *Peronospora parasitica* in the laboratory

Seeds of *Brassica oleracea* L., *B. napus*, *B. rapa* and *B. juncea* were tested for seedborne infection of *P. parasitica* and other pathogens. Seeds of cv. Glory of Enkhuizen, cv. Hercules F1 hybrid, cv. Flowers of Spring, cv. Green Coronet F1 hybrid, cv. PPBJ-1, cv. Capricorn, cv. Karoo and cv. Sumo were surface-sterilised with 3% sodium hypochlorite (Merck, South Africa) and grown in pots in autoclaved soil (1 kPa, 121°C for 15 min.) at 25°C in the greenhouse for seven days. Soil consisted of potting medium:sawdust, 3:1 (Growmor, South Africa). Uninoculated seedlings were incubated in a Schnidjers climatic chamber set at the optimum conditions for *P. parasitica* infection. The Schnidjers climatic chamber was programmed with a light dark cycle: 9 hrs dark and 15 hrs light (40% light) at a constant temperature of 16°C. The light cycle had an intensity of 100 $\mu Es^{-1}m^{-2}$ as measured with a light meter. No infection was obtained for all cultivars after three weeks at the optimum growth conditions for *P. parasitica* and the seeds were subsequently used in experiments.

A modified *in vitro* method was used to maintain both field and single spore isolates of *P. parasitica* in the laboratory (Nashaat pers. commun.). South African field isolates of *P. parasitica* of the *Brassica oleracea* pathotype viz. SSH, TC, PPSAM and PPSAR were collected from seedling nurseries in cabbage growing areas in Kwazulu Natal (Table 2.1). Field isolate ACAT was collected from the Eastern Cape, South Africa by Prof. P.N. Achar (UD-W) (Table 2.1). Where the cultivar of origin of the isolate was not available, a susceptible cultivar of the same species/subspecies was used.

Brassica seeds were surface-sterilised for 1 min. with 3% sodium hypochlorite or 0.2% mercuric chloride and sown in pots in autoclaved soil (1 kPa, 121°C for 15 min.) at 25°C in the green house. The above field isolates were sub-cultured onto seven day old seedlings by pipetting a freshly prepared spore suspension (5×10^5 conidia/ml) onto each cotyledon. All inoculated seedlings were covered to maintain humidity at 100%, then

incubated in a Schnidjers Climatic Chamber. The climatic chamber was programmed with a light dark cycle. Different light dark cycles ranging from 8 - 12 hrs dark and 12-16 hrs light within a cycle of 24 hrs were tried. The temperature of was varied from 14-20°C. The light cycle was set at 40% light (Schnidjers Climatic Chamber) which had a intensity of 100 $\mu Es^{-1}m^{-2}$ as measured by a light meter. Isolates were sub-cultured on a laminar flow bench every seven days. To prevent cross-contamination, laminar flow benches were thoroughly swabbed with 70% alcohol and 1% sodium hypochlorite and allowed approximately 20 minutes between subcultures.

Isolates of various pathotypes of *P. parasitica*, including the *Brassica juncea*, *B. napus* and *B. oleracea* were kind gifts from Dr Nash Nashaat (Institute of Arable Crops Research (IACR), Rothamsted, UK), Prof. John Lucas (Institute of Arable Crops Research, Long Ashton) and Dr Drissa Silué (Bretagné Biotechnologie Végetalé, France) (Table 2.1). Additional isolates of the *B. rapa* pathotype from India were obtained from Dr Nashaat. Isolates of the *B. oleracea* pathotype from Portugal and Mozambique were obtained from Dr Silué and Prof. Lucas, respectively. The brassica isolates were maintained on appropriate hosts, sub-cultured regularly and incubated at the optimum maintenance conditions described previously. For long term storage of isolates, intact cotyledons showing pathogen sporulation were snap frozen at -20°C for up to three months or -70°C for up to one year.

For the *Arabidopsis* pathotype, isolates Noks1 (Table 2.1), seeds of Columbia were allowed to swell in sterile distilled water (SDW) for 30 min., then surface sterilised in 1-5% sodium hypochlorite and washed in SDW 4X. Seeds were then dried on filter paper and sprinkled onto MS shoot initiation medium containing 1% sucrose and 1% agar and incubated at 4°C overnight. Seedlings were then transferred to a growth cabinet and incubated with a light-dark cycle, 16 hrs day and 8 hrs night at 22° for two to three weeks. Seedlings were then inoculated and incubated under the conditions described for *P. parasitica* infection. The isolate was sub-cultured onto fresh seedlings every week.

2.2.2 Derivation of single spore isolates of *Peronospora parasitica*

Single spore isolates of *P. parasitica* were derived from South African field isolates by transferring a single conidium to the surface of an excised cotyledon. The main objective was to obtain successful infection and profuse sporulation from the single conidium so that this "line" could then be propagated, and DNA extracted for molecular analyses.

Single spore isolates were derived from field isolates in Kwazulu Natal (Table 2.1) and maintained on the host cultivars utilised for maintenance of the original field isolates. Seven day-old seedlings were excised midway down the hypocotyl and placed in multicompartment boxes (magenta jars modified to contain a holder for seedlings as well as a ready supply of sterile distilled water at its base) (Nashaat pers. commun., Sheriff and Lucas 1989b) The upper surface of each uninfected cotyledon was moistened with 10 µl of sterile distilled water. Seedlings showing fresh pathogen sporulation were used as the source of inoculum; Conidia were tapped gently onto sterile Petri plates containing one ml of sterile distilled water. Spore suspensions were collected and concentrated in 1.5 ml eppendorf tubes (Whitehead, SA) and centrifuged in a benchtop centrifuge at 5000 rpm (2000 x gr) for 2 min. Excess supernatant was removed and a few microlitres of the concentrated spore suspension was spread onto water agar plates (2% tap water agar, 1% activated charcoal) using a mini glass spreader and pre-dried for a few minutes. The activated charcoal permitted easy visualisation of the single translucent conidia with a Nikon microscope (x40 magnification). Individual conidia were removed by cutting out a small agar block beneath each conidium, and the block was then placed spore side down onto the minute droplet of water on the surface of the cotyledon (Sheriff and Lucas 1989b). Multicompartment boxes were incubated in the Schnidjers climatic chamber programmed with a light dark cycle: 9 hrs dark and 15 hrs light (40% light) at a constant temperature of 16°C and a light intensity of 100 $\mu Es^{-1}m^{-2}$. Inoculated cotyledons were carefully monitored for infection. As soon as the first conidiophore was visible (after 2-3 days) individual cotyledons were transferred to moistened filter paper on Petri plates and incubated for a further 2-3 days. Thereafter isolates were subcultured using the method previously described.

2.2.3 Axenic cultures of *Peronospora parasitica*

P. parasitica is an obligate pathogen which survives solely on the living plant host. For molecular analysis, surface sterilisation of the leaf inoculated with *P. parasitica* was necessary. In addition it was important to separate the pathogen from the host plant.

Six day old seedlings were grown in autoclaved potting medium as previously described were inoculated with *P. parasitica* isolates (Table 2.1); the cultivars used for maintenance of the isolates were used in the axenic culture methods. All procedures for this method were performed on a laminar flow bench. Inoculated seedlings were covered and incubated for 24 hrs in a Schnidjers Climatic Chamber (a dark cycle of 9 hrs first at 16 ^{0}C followed by 15 h light at 16 ^{0}C).

Pure ("Axenic") cultures of *P. parasitica* were prepared as follows: excised inoculated cotyledons were sterilised 24 hrs after inoculation by dipping in 70 % ethanol and then in 1 % sodium hypochlorite (or 0.2% mercuric chloride) (Nashaat pers. commun.). Cotyledons were then subjected to three washes in sterile distilled water (SDW) of 5 min. each, dried on sterile filter paper and placed on Murashige and Skoog (1962) medium (excluding growth hormones) or Knops medium (0.5 M calcium nitrate, 0.125M potassium nitrate, 0.125 M potassium dihydrogen orthophosphate, 0.125 M magnesium sulphate, traces of ferric orthophosphate and 10 g/l purified agar) containing 0.1 mg/ml Ampicillin. Care was exercised to avoid contact of the cut end of the cotyledon with the media. The cotyledons were then incubated in the Schnidjers Climatic Chamber under the conditions described previously.

Excellent sporulation was observed after approximately six to seven days of incubation. The cotyledons showing pathogen sporulation, were lifted out gently with tweezers and spores were collected by tapping them gently to collect spores in 1 ml of sterile water containing 0.1 % Tween 20 on glass Petri plates. To avoid host contamination, the

conidial suspension was discarded if cotyledons were accidentally dropped in the water. Approximately 20-30 cotyledons containing the sporulating pathogen were sufficient for a single DNA extraction.

2.2.4 Isolation and purification of genomic DNA from isolates of *Peronospora parasitica*

Total genomic DNA from all isolates of *P. parasitica* was isolated using the modified method of Tham et al. (1994). DNA was extracted from conidia, of 4 to 5 day-old *P. parasitica* axenic cultures sporulating on host cotyledons maintained in a growth room at a light dark cycle (9 hrs dark and 15 hrs light at 16 ^{0}C) and a light intensity of 100 $\mu Em^{-2}s^{-1}$.

Conidia, collected as described (2.2.3), were centrifuged and the conidial pellet washed once in sterile distilled water. Purity of conidial suspension was confirmed by observation with a light microscope (40 x magnification). Clean conidia were then vortexed for 1 min. with a mixture of 1- and 6 mm diameter glass beads (Sigma) in 600 µl lysis buffer (100 mM tris-HCl (pH 7.20, 100 mM EDTA, 10 % (w/v) SDS and 2 % (v/v) 2-mercaptoethanol). DNA was recovered from the suspension of broken conidia using the protocol described by Lee and Taylor (1990). The suspension was incubated at 65 ^{O}C for 1.5 hr and vortexed every 20 min. for 30 sec. To remove the protein and cell debris, 600 µl chloroform:phenol (1:1) was added, vortexed briefly, and microcentrifuged at 10 000 x gr for 15 min. at room temperature or until the aqueous (top) phase is clear. Approximately 300 to 500 µl of the aqueous phase containing the DNA was transferred to a new tube. Precipitation of DNA was achieved using 10 µl 3 M sodium acetate and 0.54 volumes isopropanol and centrifugation at 10 000 X gr for 2 min. DNA pellets were washed in 70 % alcohol and resuspended in 30 µl of TE (10 mM Tris-HCI, 0.1 mM EDTA) buffer. Following resuspension, the concentration and the purity of the DNA was determined and the DNA was stored at -20 ^{0}C.

2.2.5 Extraction and purification of *Brassica* host DNA

Genomic DNA was isolated from seven day old cotyledons (two each) from *Brassica oleracea* (cv. Glory of Enkhuizen), *B. napus* (cv. Capricorn), *B. rapa* (cv. Sumo) and *B. juncea* (cv. PPBJ-1) (Labelled 30-33 – Table 4.1) using the method of Edwards et al. (1991). Cotyledons were ground thoroughly using a pestle and mortar, and then transferred to 1.5 ml eppendorf tubes. 400 µl of pre-warmed (60 °C) Supaquick buffer (200 mM Tris-HCl, pH7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added, mixed gently and incubated at 60 °C in a waterbath for 30 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed well by inverting the tube several times for 5 min. prior to centrifugation at 10 000 gr of 10 min. 350 µl of the supernatant was carefully transferred to a clean microfuge. DNA was precipitated by addition of alcohol and mixed by gently inverting the tubes 50 x; 0.6 volumes (210 µl) of isopropanol (RT) or 2 volumes of ice cold 96 % ethanol (–20 °C for 30 min.). At the end of the incubation, the DNA was pelleted at 10 000 gr for 10 min., the ethanol was aspirated and the pellet washed with 500 µl 70 % ethanol. The ethanol was then removed and the pellet allowed to air-dry before being resuspending in 100 µl TE buffer.

2.3 RESULTS

2.3.1 Representative Culture collection of *Peronospora parasitica*

A representative isolate set consisting of isolates from diverse localities including the UK, India, France, Portugal, and South Africa were obtained (Table 2.1). The majority (22) of these were single spore isolates. Isolates of five pathotypes of *P. parasitica* were represented (Table 2.1), viz. the *Brassica oleracea*, *B. napus*, *B. rapa*, *juncea* and *Arabidopsis thaliana* pathotypes. Isolate IP05B was a single spore isolate of the *Brassica juncea* pathotype from the UK. Isolates IP09 and IP13 were field isolates of the *B. campestris (rapa)* pathotype from India; due to low infection levels derivation of single spore isolates from these field isolates was not possible. Isolate P003 and R1 were

isolates of the *B. napus* pathotype from the UK. The majority (26) of the isolates were of the *B oleracea* pathotype and included isolates from South Africa (KwaZulu Natal and Eastern Cape), Mozambique, France, Portugal, India and UK. Isolates BR1F, Br5A and Pp9 were from France whereas isolates P501 was from Portugal. Isolate P005 was from the UK. Noks1 was an isolate of the *Arabidopsis thaliana* pathotype. The majority of the isolates were from South Africa and included isolates from the Eastern Cape (isolates ACAT2B, ACAT2C, ACAT2D, ACAT2E from a single field population in (ACAT)) and KwaZulu Natal. Isolates from KwaZulu-Natal included isolates SSH1, SSH2, SSH3, SSH4, SSH5, SSH6 from a single field population (SSH), isolate PPSAM1 from PPSAM isolates PPSAR2, PPSAR4, PPSAR5, PPSAR6 from the PPSA field group and isolates TC1 and TC2 from the TC field group.

2.3.2 Maintenance of *Peronospora parasitica* field and "single spore" isolates

Profuse sporulation of *P. parasitica* isolates on their maintenance host was obtained 4-5 days after inoculation (Fig. 2.1). The optimum conditions for maintenance of *P. parasitica* in the Schnidjers climatic chamber was a dark light cycle: 9 hrs dark and 15 hrs light (40% light) at a constant temperature of 16°C and a light intensity of 100 $\mu Es^{-1}m^{-2}$. At these conditions optimal sporulation was observed after approximately six to seven days after inoculation. Thereafter the cotyledons became senescent and began to collapse. Therefore it was necessary to subculture isolates within 5-7 days. Furthermore, optimal sporulation occurred when a) susceptible cultivars were used for maintenance of the isolate b) the cotyledons were incubated immediately in the dark after inoculation c) the inoculum source was relatively free of contaminating bacteria or nematodes d) humidity was 100% and e) the temperature was optimal (14-16°C). At temperatures above 18°C sporulation efficiency decreased. At very low temperatures 4-8°C sporulation was found to found to be delayed; this could be reversed if the temperature was altered to 16 °C.

Fig. 2.1: Profuse sporulation of an isolate of *Peronospora parasitica* 4-5 days after sub-culture on brassica seedlings.

Fig. 2.2: Multi-compartment boxes utilised for derivation of single spore isolates of *Peronospora parasitica*. Excised brassica cotyledons, placed on modified microtitre plates in contact with a 5mm film of water, were inoculated with a single conidium.

All South African single spore isolates represented in Table 2.1 were derived using the single spore technique. Infection rates of between 0-50% was achieved using this method. Successful infection depended on the spore being sandwiched between the block and the leaf. Infection was further enhanced by the addition of a tiny droplet (10 µl) of water to the site of infection. Multicompartment boxes (Fig. 2.2) were highly effective in maintaining the 100% humidity needed for single spore infection and resulted in high rates of infection. Lower infection rates were achieved by single spore inoculations on excised cotyledons placed on moistened filter paper. With both methods infection was achieved 3 to five days after inoculation.

Single spore isolates which showed the first conidiophore emergence were immediately transferred to MS media or moist filter paper in individual Petri plates, then bulked two days later by inoculating a pot of 7 day-old seedlings. Fig. 2.3 shows profuse sporulation of a single spore isolate two days after incubation on moist filter paper (the cotyledon was photographed on a dry Petri plate to enhance visibility). Isolates were grown for several generations by subculturing at regular intervals and axenic culture methods were applied. Conidia were collected approximately 4 days after incubation at the optimal conditions, following axenic culture, when sporulation of *P. parasitica* was optimal. In addition to maintenance at the optimum conditions for proliferation of *P. parasitica*, the success of the axenic culture method depended on the time utilised for the surface sterilisation and the quality of seedlings at the outset. Brassica cotyledons that were left in the 70% alcohol and 1% sodium hypochlorite (or 0.2% mercuric chloride) for longer than a momentary "dip" showed reduced sporulation and in some instances the cotyledons became bleached and senescent and were discarded. Seedlings grown in the greenhouse were adequate for this procedure however seedlings of *Arabidopsis* grown in the growth chamber were not amenable to this method as they did not survive the axenic culture washes in 70% ethanol and 1 % sodium hypochlorite.

Fig. 2.3: Sporulation of a single spore isolate of *Peronospora parasitica* after inoculation with a single conidium.

The purity of conidia collected by the axenic culture method was high. The use of ampicillin in axenic culture methods prevented growth of endogenous bacteria. Light microscope observations (40 x magnification) of spore collections revealed spores to be contaminant free and DNA isolation was possible. However, the concentration of spores collected by tapping onto water, following axenic culture methods, was very low (5×10^4 - 1×10^5 conidia/ml). The addition of Tween 20 during collection assisted spore collection by preventing the spores from adhering to the Petri plates. Higher concentrations of spores were collected on glass Petri plates. Attempts to create an "aspirating spore collector" similar to that described by Silué (pers. commun.) were not successful since most of the spores adhered to the glass Pasteur pipettes used (data not shown). Repeat sub-culturing at regular intervals and axenic culture methods for several generations were necessary to bulk DNA for each isolate.

2.3.3 Genomic DNA Isolation of *Peronospora parasitica*

The generation of axenic culture and bulking of DNA of *P. parasitica* was a repetitive process with pooled DNA representing a number of generations of the same single spore isolate. Final *P. parasitica* DNA concentrations were estimated in 2 ways a) spectrophotometrically and b) on agarose gels and were found to differ greatly. Spectrophotometrically the DNA estimate of P005 was approximately 500 ng/µl (not shown), however, on 1% agarose gels the concentration of P005 was estimated at 50-100 ng/µl (Fig. 2.4) (20 µl of λ marker II (*Hin*dIII digested) was loaded in the marker lane which was equivalent to 2500 µg DNA, with the upper band having a known concentration of 1250 µg DNA (Roche)). Good quality DNA was also obtained from host controls *Brassica oleracea*, *B. napus*, *B. rapa* and *B. juncea* hosts.

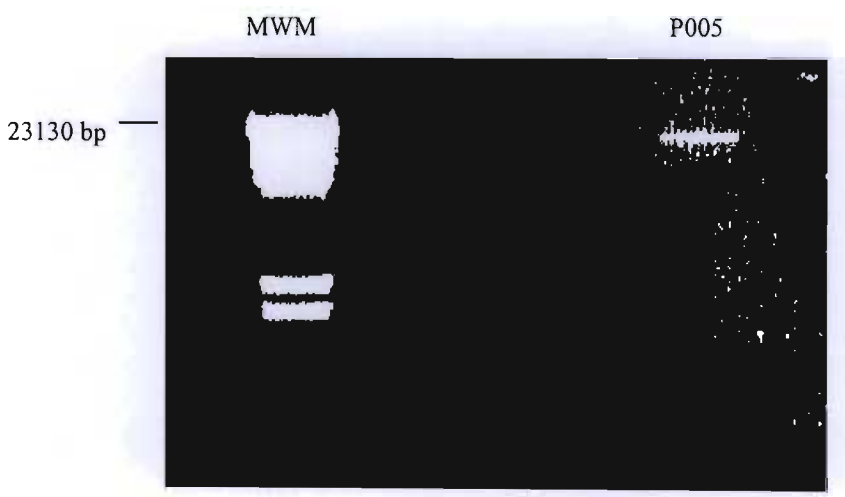

Fig. 2.4: Total genomic DNA purified from isolate P005 of *Peronospora parasitica*. MWM II (Roche, UK) - λ DNA, Hind III digested. 20 µl was loaded (2500 µg), therefore the 23130 bp band was estimated to be approximately 1250 µg and P005 was estimated to have a concentration of 50 ng/µl

Table 2.1: Isolates of *Peronospora parasitica* from *Brassica* species

Isolate Code	Location	Date Collected	Collector	Source/ Geographic Origin	Species of Origin	Cultivar of Origin	Maintenance Cultivar	Notes
IP05B	IACR – Rothamsted, UK	04/11/95	Wendy Heran	IACR – Rothamsted UK	*B. juncea*	Krishna	PPBJ1	Single Spore Isolate
IP09	IACR – Rothamsted, UK	04/01/94	Nash Nashaat	Pantnagar, India	*B. campestris (rapa)*	YST-151	RESR 263 or Sumo	Field Isolate from staghead
IP13	IACR – Rothamsted, UK	11/11/94	Nash Nashaat	Pantnagar, India	*B. campestris (rapa)*	PT303 Toria	RESR 263 or Sumo	Field Isolate from cotyledon
P003	IACR – Rothamsted, UK	U	John Lucas	Nottingham University, UK	*B. napus*	Jet Neuf	Capricorn or Karoo	Sexual progeny from homothallic field isolate
R1	IACR – Rothamsted UK	Nov-88	Nash Nashaat	IACR – Rothamsted UK	*B.napus*	Cobra	Capricorn or Karoo	Single Spore Isolate
Br1F	BBV, France & UD-W, SA	U	Drissa Silué	BBV - France	*B. oleracea*	U	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
Br5A	BBV, France & UD-W, SA	U	Drissa Silué	BBV - France	*B. oleracea*	U	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate

Pp9	BBV, France & UD-W, SA	U	Drissa Silué	BBV - France	*B. oleracea*	U	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
P501	BBV, France & UD-W, SA	U	Drissa Silué	Portugal	*B. oleracea*	U	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
SSP6	IACR-Long Ashton, UK	U	Brita D. Jensen	Mozambique	*B. oleracea*	U	Flowers of Spring	Single spore isolate
P005	UD-W, IACR, Long Ashton, UK	1979	John Lucas	Lincolnshire, UK	*B. oleracea* (*cabbage*)	Flowers of Spring	Hercules F1 Hybrid	Single Spore Isolate
ACAT	UD-W, SA	1999	P.N. Achar	Transkei, Eastern Cape, SA	*B. oleracea*	Conquestador F1 Hybrid	Hercules F1 Hybrid	Field Isolate
ACAT2B	UD-W, SA	1999	Maneshree Jugmohan	Transkei, Eastern Cape, SA	*B. oleracea* (broccoli)	N/A	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
ACAT2C	UD-W, SA	1999	Maneshree Jugmohan	Transkei, Eastern Cape, SA	*B. oleracea* (broccoli)	N/A	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
ACAT2D	UD-W, SA	1999	Maneshree Jugmohan	Transkei, Eastern Cape, SA	*B. oleracea* (broccoli)	N/A	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
ACAT2E	UD-W, SA	1999	Maneshree Jugmohan	Transkei, Eastern Cape, SA	*B. oleracea* (broccoli)	N/A	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
SSH	UD-W, SA	15/5/99	Maneshree Jugmohan	Sunshine Seedling Services, Pietermaritzburg, KZN, SA	*B. oleracea* (cabbage)	Hercules F1 Hybrid	Glory of Enkhuizen (open-pollinated)	Field Isolate

SSH1	UD-W, SA	1999	Maneshree Jugmohan	Sunshine Seedling Services, Pietermaritzburg, KZN, SA	*B. oleracea* (cabbage)	Hercules F1 Hybrid	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
SSH2	UD-W, SA	1999	Maneshree Jugmohan	Sunshine Seedling Services, Pietermaritzburg, KZN, SA	*B. oleracea* (cabbage)	Hercules F1 Hybrid	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
SSH3	UD-W	1999	Maneshree Jugmohan	Sunshine seedling Services, Pietermaritzburg, KZN, SA	*B. oleracea* (cabbage)	Hercules F1 Hybrid	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
SSH4	UD-W, SA	1999	Maneshree Jugmohan	Sunshine Seedling Services, Pietermaritzburg, KZN, SA	*B. oleracea* (cabbage)	Hercules F1 Hybrid	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
SSH5	UD-W	1999	Maneshree Jugmohan	Sunshine Seedling Services, Pietermaritzburg, KZN, SA	*B. oleracea* (cabbage)	Hercules F1 Hybrid	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
SSH6	UD-W, SA	1999	Maneshree Jugmohan	Sunshine Seedling Services, Pietermaritzburg, KZN, SA	*B. oleracea* (cabbage)	Hercules F1 Hybrid	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate

PPSAM	UD-W, SA	25/5/99	Maneshree Jugmohan	MacDonald Seeds, Pietermaritzburg, KZN, SA	*B. oleracea* (cabbage)	N/A	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
PPSAM1	UD-W, SA	1999	Maneshree Jugmohan	MacDonald Seeds, Pietermaritzburg, KZN, SA	*B. oleracea* (cabbage)	N/A	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
PPSAR	UD-W, SA	5/5/99	Maneshree Jugmohan	Roma Seedlings, Durban, KZN, SA	*B. oleracea (cabbage)*	Green Coronet F1 hybrid		Field Isolate
PPSAR2	UD-W, SA	1999	Maneshree Jugmohan	Roma Seedlings, Durban, KZN, SA	*B. oleracea* (cabbage)	N/A	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
PPSAR4	UD-W, SA	1999	Maneshree Jugmohan	Roma Seedlings, Durban, KZN, SA	*B. oleracea* (cabbage)	N/A	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
PPSAR5	UD-W, SA	1999	Maneshree Jugmohan	Roma Seedlings, Durban, KZN, SA	*B. oleracea* (cabbage)	N/A	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
PPSAR6	UD-W, SA	1999	Maneshree Jugmohan	Roma Seedlings, Durban, KZN, SA	*B. oleracea* (cabbage)	N/A	Glory of Enkhuizen (open-pollinated)	Single Spore Isolate
TC	UD-W, SA	15/5/99	Maneshree Jugmohan	Top Crop Nurseries, Pietermaritzburg, KZN, SA	*B. oleracea* (cabbage)	Green Coronet F1 Hybrid	Green Coronet F1 Hybrid	Field Isolate
TC1	UD-W, SA	1999	Maneshree Jugmohan	Top Crop Nurseries, Pietermaritzburg, KZN, SA	*B. oleracea* (cabbage)	Green Coronet F1 Hybrid	Green Coronet F1 Hybrid	Single Spore Isolate

TC2	UD-W, SA	1999	Maneshree Jugmohan	Top Crop Nurseries, Pietermaritzburg, KZN, SA	*B. oleracea* (cabbage)	Green Coronet F1 Hybrid	Green Coronet F1 Hybrid	Single Spore Isolate
Bremia	UD-W, SA	1999	Maneshree Jugmohan	Roma Seedlings, Durban, KZN, SA	*Bremia lactucae*	Cobham Green	N/A	Field Isolate
Noks1	IACR-Long Ashton & Horticulture Research International (HRI), UK	N/A	U	Norwich, Norfolk, UK	*Arabidopsis thaliana*	Niederenz (Nd-0)	Columbria (Col-*g11*)	Oospore of *Noco2* (*Noco2* - Norwich-maintained on Columbia)

BBV - Bretagné Biotechnologie Végetalé

IACR - Institute of Arable Crops Research

KZN - KwaZulu-Natal

SA - South Africa

UD-W - University of Durban-Westville

UK - United Kingdom

U - unknown

2.4 DISCUSSION

Much of the early work on *Peronospora parasitica* focused on methods to culture this pathogen in the laboratory with varied success (Kröber 1969, Kröber 1970, Ingram 1969, McMeekin 1981). Callus culture appeared to be advantageous since the pathogen initially survived for longer periods and there was reduced cross-contamination. However this method was not widely used since calluses could not be transferred directly (Ingram 1969) and the cell wall structure in callus tissue was believed to differ from whole plant cells thus affecting the colonisation and growth of the pathogen. Furthermore, callus culture could not be used for studies on resistance since the pathogen response to the pathogen did not necessarily reflect that of the whole plant.

In the last 10-15 years, culture of *P. parasitica* on host seedlings has become increasingly popular. Detailed investigations of the epidemiology of several *Peronosporas* have yielded valuable information on optimal conditions that promote infection and sporulation of *P. parasitica* (Hill 1966, Davison 1968b, Pinckard 1942). In general, low temperatures, high humidity and a dark-light cycle favour maintenance of cultures *in vitro* maintenance. These observations, together with the ability to freeze the inoculum on host plants for long periods makes this a simple procedure for providing a ready source of inoculum when required. In the current study, the optimum conditions for maintenance of *P. parasitica* was in the Schnidjers climatic chamber set at a dark-light cycle: 9 hrs dark and 15 hrs light (40% light) at a constant temperature of 16°C and a light intensity of 100 $\mu Es^{-1}m^{-2}$. At these conditions optimal sporulation was observed after approximately six to seven days after inoculation.

The maintenance conditions described above were also ideal for the derivation of single spore isolates. Hydration, a necessary ingredient for infection was provided by the agar block. The additional droplet of water resulted in higher success rate of the single spore technique. Hydration removes water-soluble inhibitors thus enhancing infection (Tommerup 1981). A single cotyledon, showing profuse sporulation was sufficient to bulk the new single spore isolate on a fresh pot of seedlings.

One of the drawbacks of culturing the pathogen in this way is loss of isolates during climatic chamber or freezer malfunction. Therefore it was necessary to keep backup cultures in different freezers (-20°C or -80°C) and in different locations. For import/export of isolates from diverse locations, special precautions had to be exercised during transportation. Some success was achieved by transporting inoculating seedlings 24 hrs after inoculation on moistened filter paper. 100% viability was retained by transporting frozen cultures in thermos flasks in dry ice over 2 days.

In the present study, isolates were subcultured onto host species of the original isolate and where possible on the original cultivar itself. Sub-culturing in this manner would be unlikely to have phenotypic consequences or for example induction of SAR or HR since the cultivars used were susceptible. Thus, selection was negligible and uniformity of the isolate was retained.

DNA was isolated from conidia from subcultured isolates which thus reflects a number of generations of the same line. Asexual reproduction of *P. parasitica* involves mitosis and ideally single spore isolates or monoconidial lines collected, as described in the present study for DNA isolation, should be alike. Therefore repeated subculturing should not have any significance with respect to variation within a particular isolate. The possibility of heterokaryosis in *P. parasitica* has not been completely ruled out since each conidium contains up to 30 nuclei (Davison 1968a). The implications of the above would have a greater impact on a field isolate than on a single spore isolate. Homothallism has been demonstrated to occur in field and in single spore isolates. Homothallism in single spore isolates may also influence variation within these lines.

The isolates collected in this study are representative of five pathotypes of *P. parasitica*. Within the *B. oleracea* pathotype, various single spore isolates from the same field population, as well as isolates from various geographical regions are represented. This provides an appropriate isolate set for sampling the diversity within *P. parasitica* as described in the subsequent chapters which form the basis of this study. The DNA extracted was of a very high quality and PCR testing using ITS primers for the absence of host contamination was therefore possible.

CHAPTER THREE

MOLECULAR PHYLOGENY OF PATHOTYPES OF *Peronospora parasitica*

3.1 INTRODUCTION

Taxonomic studies involve developing systems of classification, making expert identifications, describing nomenclaturally significant taxa, and preparing monographic studies. Classical fungal taxonomy relies heavily on using the size and shape of fruiting structures, spore morphology and release mechanisms, colouration and habitat to define taxa, and while this adequately describes the grouping its does not explain its origins. To do this a molecular phylogenetic approach must be taken (Mitchell et al. 1995).

Peronospora parasitica (Pers. ex. Fr.) Fr. belongs to the order Peronosporales, in the class Oomycetes. The Oomycetes were originally classified within the Mastigomycotina in the Kingdom Fungi (Agrios 1997). In the family Peronosporaceae, generic delimitation has traditionally been based on morphological characters such as sporangiophore and spore type as well as different modes of germ tube or zoospore. Gaumann (1918), on the basis of conidial measurements and cross-inoculations recognised 52 species of *Peronospora* on crucifer hosts. Later Yerkes and Shaw (1959) concluded that there were no morphological criteria for distinguishing *Peronospora* isolates from different host species and all collections of downy mildew from the Cruciferae have been grouped into a single aggregate species. Work of Dickinson and Greenhalgh (1977) concluded that the lack of consistent and unique morphological differences between isolates from different crucifers, coupled with the doubt surrounding their host range must preclude any subdivision of *Peronospora* on crucifers until such time as more information is gathered concerning the host range of isolates of this pathogen.

While *P. parasitica* is very specific in its host range it also has the ability to colonise heterologous host species to different extents i.e. hosts different to that from

which it was derived. Several studies investigating the differential response of isolates of *Peronospora parasitica* to *Brassica* accessions (Silué et al. 1996, Nashaat and Awasthi 1995, Jugmohan 1997) support the idea that host adaptation exists at the species level. It is possible that co-evolution with plant hosts over long periods is likely to have led to divergent forms of the pathogen adapted to different host taxa (Lucas et al. 1994). This dependence of downy mildews on their hosts might suggest that forms of the pathogen found on closely related plants share some common phylogeny.

Great advances in the systematics of fungi in general and in the Oomycetes, in particular, have occurred recently following the development and application of modern methods of systematic analysis (Hall 1996). Recent trends in systematics incorporate molecular, morphological and ecological data to reconstruct phylogenetic trees which will improve the understanding of the evolution and behaviour of downy mildews and are necessary to produce classifications which accurately reflect the species as they occur in nature (Bruns et al. 1991). Some of these methods have shown that the Oomycetes possess morphological, physiological, biochemical and molecular characters that are completely different from those found in fungi (the Mycota), which has lead to a fundamental reappraisal of their taxonomic position outside the Mycota (Hall 1996). Phylogenetic analysis of the Oomycete *Achyla bisexualis* revealed close relationships between the Oomycetes and Chrysophytes (Gunderson et al. 1987). Other methods have allowed the unravelling of the complex genetic behaviour of the Oomycetes, whilst still others have given new insights into the relationships between genera and species.

Molecular genetic techniques now allow mycologists to pursue lines of research that were previously inaccessible, mainly because of a paucity of effective characters (Kohn 1992). Molecular studies focus on DNA analysis including DNA-DNA hybridisation, RFLP analysis and sequence data are being used to investigate evolutionary relationships within and among fungal species (Bruns et al. 1991). In such instances, evidence for genetic similarity (or non-similarity, as may be the case) provides the main criterion for recognising members of a common group (Mitchell et al. 1995).

Sequences with useful phylogenetic information are the ribosomal RNA (rRNA) genes from the nuclear and mitochondrial genomes (a genome consists of all the autonomously replicating chromosomes in an organelle or individual cell), cytochrome oxidase genes and certain ribosomal protein elongation factors. These genes have the same function in all taxa, evolve at approximately the same the same rate, and are present only once in a genome or behave like a single copy region (Mitchell et al. 1995). Phylogenetic analyses are possible because mutations (changes) occur in the rRNA gene sequence, which can be detected and used to estimate divergence from common ancestors. Approximate estimations are usually made, as the common ancestor is not known, by comparing the sequences of modern species (Mitchell et al. 1995).

The advantages of using rRNA genes is that they are well characterised, easily accessible and are a mosaic of conserved and variable regions which can be informative at many taxonomic levels (Hibbett 1992). Genes of the ribosomal cluster are usually present in large numbers as tandem repeats along the chromosome but they evolve as a single unit (Mitchell et al. 1995). This comprises three structural regions coding for the 5.8S, 18S and 28S ribosomal RNA genes (Mitchell et al. 1995) (Fig. 3.1). These are transcribed into RNA molecules that form part of the ribosome. Interspersed between the genes are spacer regions that are also transcribed. Between the 18S and 5.8S, and the 5.8S and 28S genes are the internally transcribed spacers (ITS), while the region that separates the cluster of three genes along the chromosome is called the non-transcribed spacer (NTS). Just prior to where the 18S gene is transcribed there is another small spacer region called the externally transcribed spacer or ETS. Together, the ETS and NTS regions comprise the intergenic spacer region or IGS.

The structural genes, transcribed spacers and non-transcribed spacers of the rDNA operon, evolve at different rates, and their sequences can be used to discriminate between taxa at different levels. The structural genes are the most conserved - so conserved in fact that sequences present in these genes can be found in all organisms - and these can be used to delineate species to their order. The ITS and IGS regions are much more variable and can be used to separate taxa from classes to species (Mitchell et al. 1995).

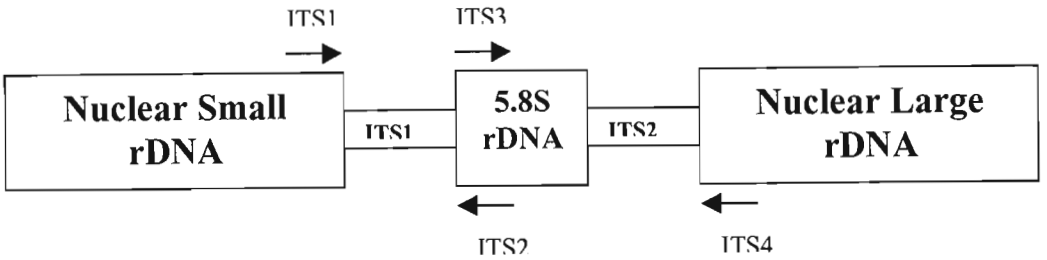

Fig. 3.1: Organisation of the ribosomal RNA operon showing the location of PCR primers for internal transcribed spacers of the nuclear rDNA map. The *arrowheads* represent the 3' end of each primer. The sequence for each primer is written in the 5' - 3' direction.: ITS1, TCCGTAGGTGAACCTGCGG; ITS2, GCTGCGTTCTTCATCGATGC; ITS3, GCATCGATGAAGAACGCAGC; ITS4, TCCTCCGCTTATTGATATGC (Yao et al. 1992).

No one particular gene sequence can determine all the taxonomic or evolutionary relationships of any organism as the analysis of different genomic regions may yield different results (Bunyard et al. 1994). Delineating organisms below the genus level, in particular, using rRNA gene sequences can be difficult and it is often necessary to use different gene sequences to determine close relationships. Sequences commonly used are the rRNA and cytochrome genes from the mitochondrial genomes as well as the nuclear 5.8 and 28s ribosomal genes.

PCR fingerprinting provides an extremely rapid view of genetic variability within a particular study group (Mitchell et al. 1995). PCR analysis of the ribosomal RNA operon has been widely used to study the phylogeny of various fungi. Based on 5.8S and ITS1-ITS2 rDNA sequences the clamped nematode trapping fungi were clearly distinguished from the clampless nematode trapping fungi (Liou and Tzean 1997). The wild and edible mushrooms *Cantharellus* and *Craterellus* were previously distinguished by morphological characteristics such as clamp connections, type of basidiome and configuration of the hymenophore but these proved unreliable as different characters produced different phylogenies. Sequence analysis of the ITS1-LR5 region indicated that *Cantharellus* and *Craterellus* should be treated as distinct genera (Feibelman et al. 1997).

Sequence analysis of the ITS1-ITS4 regions could distinguish important wheat pathogenic fungi such as *Stagnospora nodorum* and *Septoria tritici* (Beck and Lignon 1995). DNA amplification and direct sequencing of PCR products from parts of the LSU rDNA, SSU rDNA as well as the 5.8S rRNA was used to define relationships in the family Sclerotiniaceae (Holst-Jensen et al. 1997). The inferred rDNA phylogeny supports and refines the hypothesis that there are two distinct lineages within the Sclerotiniaceae viz. species including inoperative asci from stipitate apothecia that arise from i) an indeterminate substratal stoma or ii) a determinate sclerotial stroma (Holst-Jensen et al. 1997).

PCR-RFLPs of the ribosomal RNA operon have been widely used to compare different species of a fungus (Bunyard et al. 1994, Personn et al. 1993, Viljoen et al. 1993, Beck and Lignon 1995, Buscot et al. 1996). In the Ascomycetes, species discrimination of most morels including *Gyromitra, Discoitis, Mitrophera* and two morphological groups of *Morchella* was possible using ITS and IGS PCR (Bunyard et al. 1994, Buscot et al. 1996). Furthermore, species specific PCR primers were designed allowing specific detection of these pathogens in infected tissues. PCR-RFLPs of nuclear rDNA are also useful in distinguishing sub-specific groups of *Sclerotium* (Athelia) *rolfsii* (Harlton et al. 1995).

The systematics of obligate parasitic downy mildew has not benefited from the application of molecular techniques as much as other fungi due to the dependence of many of these techniques on DNA quantity. Ribosomal DNA analysis utilising the polymerase chain reaction (PCR) makes this possible.

Traditional taxonomy of the Peronosporomycetes (oomycota) distinguishes four orders Lagenidiales, Leptomitales, Peronosporales and Saprolegniales. The taxonomy of the Peronosporomycetes has however been the subject of much controversy and has been re-organised several times. Phylogenetic relationships between the Chlorophytes (*Chlamydomonas reinhartii*), Chrysophytes (*Oochromonas danica*) and the Oomycetes (*Achyla bisexualis*) were recently explored by sequence analysis of the SSU ribosomal

coding regions. Phylogenetic trees thus constructed reveal a close similarity between Chrysophytes (golden-brown algae) and the Oomycetes (Gunderson et al. 1987). A similar study by Förster and co-workers (1990a) expanded on this knowledge by including the Oomycetes (*Lagenidium gigantum* and *Phytophthora megasperma* f. sp. *glycinea*) and the Chytridiomycete (*Blastocladiella emmersoni*) and comparing them to the published sequences of *Achyla bisexualis*, *Saccharomyces cerevisiae* and *Neurospora crassa* and those of other fungi. Analysing SSU rRNA Forster et al. (1990a) confirmed the finding that Oomycetes were monophyletic and derived from a heterokont photosynthetic alga. Furthermore, the diatom *Skeletonema costatum* was found on the same branch as the Oomycetes and chrysophytes which supported the theory of Cavalier-Smith (1986) that Oomycetes, chrysophytes and diatoms are members of the plant Kingdom Chromista derived from a common ancestor.

Most recently, on the basis of partial sequences of the large ribosomal subunit (LSU rDNA), the Peronosporomycetes were predicted to have evolved as two major lineages (Peterson and Rosendahl, 2000). One lineage includes the members of the Rhipidiales, Leptomitales and Saprolegniales and the other includes members of the Pythiales and Peronosporales. The obligate parasite *Albugo* was placed on the most basal branch within the Peronosporomycetidae. *Phytophthora* showed a closer relationship to *Peronospora* and *Peronophythora* than to *Pythium* suggesting that this genus should be removed from Pythiales to Peronosporales. Similar observations were made by Cooke et al. (2000) analysing the ITS6-I -TS4 region of *Phytophthora* and other Oomycetes. *Phytophthora* was grouped with *Pythium*, *Peronospora* and *Halophythora* distant from the Saprolegniales, however, Albugo was intermediate between these two major groups. A close relationship was again suggested between *Phytophthora megakarya* and *Phytophthora palmivora* (Cooke et al. 2000). *Peronospora* was further suggested to be derived from a *Phytophthora* that has lost the ability to produce zoospores and has become an obligate biotroph (Cooke et al. 2000).

Within the Oomycetes, species delimitation using ITS-PCR has been possible for a number of organisms including *Pythium* (Klassen et al. 1996), *Peronospora sparsa*

(Lindqvist et al. 1998) as well as *Peronosclerospora*. Primers complementary to conserved regions were used to amplify the ITS-2 region of the rDNA repeat units of *Peronosclerospora* isolates *(P. sorghi, P. maydis, P. sacchari and P. zeae*). Length heterogeneity was evident and each species had a unique banding pattern (Yao et al. 1992). Sequences of the ITS region of isolates of *Peronospora sparsa* infecting the cultivated arctic bramble (*Rubus arcticus*) were determined and primers were designed for its detection (Lindqvist et al. 1998); It was further observed that arctic bramble host DNA was not amplified. Work on *Pythium* species (Klassen et al. 1996), focused on the amplification of 5S RNA and its subsequent use as species-specific probes to identify different species. The 5S spacer sequence was sufficiently homogenous within a species to allow recognition of that species by one probe (Klassen et al. 1996). Furthermore certain species of *Pythium* have tandem repeats of 5S genes unlinked to the ribosomal DNA (rDNA) repeat unit. The 5S rRNA gene spacer region was amplified with primers complementary to conserved sequences at the ends of tandemly repeated 5S rRNA genes. These amplimers were used as probes against 90 species of *Pythium*. All probes were species specific and this demonstrated that the 5S rRNA gene spacer sequence might be useful in defining species boundaries in the genus *Pythium*.

Polymerase chain reaction analysis of ITS sequences has recently shown that isolates of *Peronospora parasitica* from different hosts could be differentiated. PCR analysis of the ITS1 region of the rDNA operon indicate isolates of *Peronospora parasitica* from *Brassica* species to be clearly differentiated from isolates from *Arabidopsis thaliana* (Rehmany et al. 2000). A PCR product of 150-220 bp was amplified using the ITS2 and ITS5 primers and sequences. AFLP analysis was also performed which confirmed the groupings obtained with the ITS sequences. European *P. parasitica* (*At*) isolates and *P. parasitica* (*Bo*) isolates show absolute conservation of the ITS1 region when collected from the same host species but share only 85% identity between hosts. BLASTN homology searches using the ITS1 sequences of *P. parasitica* (*At*), *P. parasitica* (*Bo*) isolates, and *Bremia lactucae* isolates detected homology with *P. sparsa* and numerous *Phytophthora* species. Additional low abundance PCR products were identified as *Fusarium, Alternaria,* and *Cladosporium* species and suggested they

were amplified from contaminating DNA. Rehmany and co-workers (2000) concluded significant divergence between *P. parasitica* isolates from *A. thaliana* and *B. oleracea* and therefore question Yerkes and Shaw (1959) amalgamation of crucifer downy mildews as the species *P. parasitica*. However, it was suggested that any division of the *P. parasitica* species should follow a wider study that examines *P. parasitica* isolates from a variety of brassica hosts.

The aim of this study was to determine the phylogenetic relationships between isolates and/ pathotypes of *P. parasitica* in relation to other downy mildews. The region selected for analysis was the ITS1-ITS4 region of the ribosomal RNA operon which is generally universally conserved.

3.2 MATERIALS AND METHODS

3.2.1 Isolation and purification of genomic DNA

ITS1, 5.8S and ITS2 sequence analyses of the rDNA operon of 29 isolates of *P. parasitica* (Table 2.1) from diverse localities including the UK, India, France, Portugal and South Africa were performed (Table 2.1). The majority (22) of these were single spore isolates. Isolates of five pathotypes of *P. parasitica* were included (Table 2.1), viz. the *Brassica oleracea*, *B. napus*, *B. rapa*, *juncea* and *Arabidopsis thaliana pathotypes*. Isolate IP05B was a single spore isolate of the *Brassica juncea* pathotype from the UK. Isolates IP09 and IP13 were field isolates of the *B. campestris* pathotype from India; due to low infection levels derivation of single spore isolates from these field isolates was not possible. Isolate P003 and R1 were isolates of the *B. napus* pathotype from the UK. The majority (26) of the isolates were of the *B oleracea* pathotype and included isolates from Transkei, South Africa, Mozambique, France, Portugal, India and UK. Isolates BR1F, Br5A and Pp9 were from France whereas isolate P501 was from Portugal. Isolate P005 was from the UK and isolates ACAT2B, ACAT2C, ACAT2D, ACAT2E from a single field population in Eastern Cape, South Africa (ACAT), the bulk of the isolates were from Kwa-Zulu Natal, South Africa and included isolates, SSH1, SSH2, SSH3, SSH4, SSH5, SSH6 from a single field population (SSH) and PPSAM1 from PPSAM. Isolates

PPSAR2, PPSAR4, PPSAR5, PPSAR6 and TC1, TC2 were from the PPSA and TC field groups respectively. All single spore isolates from South Africa were derived as described in 2.2.2. Noks1 was an *Arabidopsis thaliana* isolate. Additional ITS1 sequences for the *Brassica oleracea* isolates Preston (AF241764), P005 (AF241754), Bb (AF241753), P006 (AF241755), P204 (AF241756), P211 (AF241757), P216 (AF241759), P218 (AF241761), P502 (AF241763), P501 (AF241762), P217 (AF241760), P214 (AF241758) were selected from the NCBI database for and included in the phylogenetic analyses (Cooke et. al. 2000). NCBI database sequences of the ITS1, 5.8S and ITS2 regions of the ribosomal DNA operon for *Peronospora sparsa* (AF266783), *P. manshurica* (AB021711), *P. destructor* (AB021712), *Phytophthora phaseoli* (AF266778), *Ph. Infestans* (AF228084) and *Pythium longandrum* (AY039713) were also included (Casimiro et al. 2000, Saito 1999a,b, Hong et al. 2000).

Brassica oleracea seeds were surface sterilised in 3% sodium hypochlorite and grown in autoclaved pots (1 kPa, 121°C for 15 min.) at 25°C in the greenhouse. For each isolate of *P. parasitica*, the maintenance host (described in Table 2.1) was inoculated with a conidial suspension (5 x 10^5 conidia/ml) and incubated in a Schnidjers Climatic Chamber. The climatic chamber was programmed with a light dark cycle; 9 hr dark and 15 hr light (100 $\mu Es^{-1}m^{-2}$) at a constant temperature of 16°C. Approximately 24 hrs after inoculation cotyledons were excised and axenic cultures were prepared as described in 2.2.3. Axenic culture involved surface sterilisation of excised cotyledons in 70 % ethanol and 1% sodium hypochlorite. Cotyledons were then dried briefly on sterile filter paper and placed on Murashige and Skoog (1962) medium. Seven days after inoculation, conidia were collected by tapping spores onto sterile water and were concentrated by centrifugation.

Good quality genomic DNA was extracted from conidia of isolates of *P. parasitica* (Tham et al. 1994) and from control host DNA (Edwards et al. 1991) as described in 2.2. Conidia, collected as described, were centrifuged and the conidial pellet washed once in sterile distilled water. Clean conidia were then vortexed for 1 min. with a mixture of 1- and 6 mm diameter glass beads (Sigma) in 600 µl lysis buffer (100 mM tris-

HCl (pH 7.20), 100 mM EDTA, 10 % (w/v) SDS and 2 % (v/v) 2-mercaptoethanol). DNA was recovered from the suspension of broken conidia using the protocol described by Lee and Taylor (1990). The suspension was incubated at 65 ^{O}C for 1.5 hr and vortexed every 20 min. for 30 sec. To remove the protein and cell debris, 600 µl chloroform:phenol (1:1) was added, vortexed briefly, and microcentrifuged at 10,000 x gr for 15 min. at room temperature or until the aqueous (top) phase is clear. Approximately 300 to 500 µl of the aqueous phase containing the DNA was transferred to a new tube. Precipitation of DNA was achieved using 10 µl 3 M sodium acetate and 0.54 volumes isopropanol and centrifugation at 10 000 x gr for 2 min. DNA pellets were washed in 70 % alcohol and resuspended in 30 µl of TE (10 mM Tris-HCl, 0.1 mM EDTA) buffer. Following resuspension, the concentration and the purity of the DNA was determined by gel electrophoresis with concentration standards and ethidium bromide staining. DNA was stored at -20 ^{0}C.

3.2.2 PCR amplification of the ITS1→ITS4 region of the rRNA operon

A part of the ribosomal DNA operon was analysed with ITS1 and ITS4 primers which extend over the ITS1, 5.8S and ITS2 regions of the rDNA operon (Fig. 3.1) using the modified method of Viljoen et al. (1993). Genomic DNA of 28 isolates of *P. parasitica* (27 *B. oleracea* and 1 *A. thaliana* pathotype) and 1 isolate of *B. lactucae* were amplified using these primers. Genomic DNA of *P. parasitica* hosts were also amplified in control reactions.

Polymerase chain reaction mixtures were set up having 2.5 µl of 10 X PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 250 µM dNTPs, 4 mM $MgCl_2$ (Life Technologies, GibcoBRL), 5 ng ITS1, 5 ng ITS4 (DNA Agency), 100 ng genomic DNA and 1 U T*aq* DNA polymerase (GibcoBRL) in a total volume of 25 µl Alternatively, Ampli*taq* DNA polymerase and Ampli*taq* PCR Buffer (Perkin Elmer Applied Biosystems, UK) was utilised in ITS_PCR reactions; the concentration of all other reaction components were as described above. Reactions were carried out either in a Gene Amp 9700 or Progene PCR machine under the following conditions: 5 min. @ 96 ^{O}C, followed by 35 cycles of 30 sec @ 55 ^{O}C,

1 min. @ 72 ^{O}C and 1 min. @ 92 ^{O}C. A final elongation of 5 min. @ 72 ^{O}C completed the reaction. PCR products (12 µl) were analysed on a 2 % agarose gel following staining with ethidium bromide, and Marker XIV (Roche, SA) used as a standard DNA marker.

3.2.3 Sequencing of the ITS1→ITS4 PCR Products

The ITS1, 5.8S and ITS2 regions of 20 isolates of *P. parasitica* were sequenced. Sequencing was attempted in one of three ways: a) Direct cycle sequencing using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, USA) after PCR product cleanup with ExoSAP-IT™ (USB corporation, USA) b) cloning of PCR products using the PCR Script™ Amp Cloning kit (Stratagene, USA) followed by cycle sequencing and c) Purification of PCR product using the Qiagen gel extraction kit (Qiagen Ltd., UK) followed by cycle sequencing.

3.2.3.1 *Cloning PCR products into the pPCR-Script CAM SK(+) cloning vector*

Polymerase chain reaction products for twelve isolates of *P.parasitica* were cloned using the PCR-Script™ Amp Cloning kit (Stratagene, USA). 100 µl PCR reactions were performed as described above (3.2.2). Cloning of PCR products involved purification of PCR products by re-precipitation (Maniatis et al. 1982), followed by polishing of purified PCR products, ligation to the pPCR Script CAM SK (+) cloning vector and transformation of Epicurian Coli XL-10-Blue MRF' supercompetent cells using the protocol described in the PCR-Script™ Amp Cloning kit (Stratagene, USA).

3.2.3.1.1 Purification of PCR products

The PCR products were purified by re-precipitation (Maniatis et al. 1982); 16 µl 3 M ammonium acetate (pH 5.2) and 2 µl sterile distilled water (SDW) were added followed by 2.5 volumes 100% ethanol at RT. Samples were then centrifuged in a benchtop centrifuge (10000 x gr) for 20 min. The supernatant was discarded and the pellet washed with 200 µl 80% ethanol followed by centrifugation for 10 min. at RT. The ethanol was

removed with a pipette and the pellet was air dried in a laminar flow unit for two hrs. then resuspended in 20 µl TE (pH 8). 5 µl purified PCR product was analysed on a 2% agarose gel.

3.2.3.1.2 Polishing of purified PCR products

Purified products were then polished to ensure blunt ends. To 10 µl of the products 1 µl 2.5 mM dNTP mix, 1.3 µl polishing buffer and 1 µl (0.5 U) cloned *pfu* DNA polymerase were added mixed gently and overlayed with 20 µl of mineral oil. The polishing reaction was incubated for 30 min. at 72°C. An aliquot of the polished PCR product was added directly to the ligation reaction or stored temporarily at 4 °C.

3.2.3.1.3 Ligation to the pPCR-Script CAM SK(+) cloning vector

Polished (blunt ended PCR products) were ligated to the pPCR-Script CAM SK(+) cloning vector (10 ng/µl). This vector consisted of 2961 bp (Genbank® Accession #U46017; Stratagene, USA). The insert to vector molar ratio was adjusted to an optimum range of 40:1 to 100:1 using the following formula:

$$\text{Xng of PCR product} = \frac{(Number\,of\,bp\,of\,PCR\,product)(10\,ng\,of\,pPCR\,cloning\,vector)}{2961\,bp\,of\,pPCR\,Cloning\,Vector}$$

The ligation reaction consisted of the following: 1 µl pPCR-Script Amp SK (+) cloning vector (10 ng/µl), 1 µl PCR-Script 10 X reaction buffer, 0.5 µl 10 mM γATP, 2 µl blunt end PCR product/4 µl control PCR insert, 1 µl *Srf*I restriction enzyme (5 U/µl), 1 µl T4 DNA ligase and 3.5 µl of SDW to a final volume of 10 µl. The ligation was incubated at RT for 1 hr, heated to 65 °C for 10 min. then stored at –20°C until needed.

3.2.3.1.4 Transformation

Epicurian Coli XL-10-Blue MRF' kan supercompetent cells (stored at –80 °C) were thawed on ice; cells were gently mixed by hand before being aliquoted (40 µl) into chilled microcentrifuge tubes. 1.6 µl XL10-GOLD β-mercaptoethanol was added to each tube, swirled gently to mix and incubated on ice (10 min.) with gentle swirling every two minutes. Ligated insert (cloning reaction, 3.2.3.1.3) was added to the tube, mixed and incubated on ice for 30 min. (Two controls, a test insert and a PUC18 control plasmid were used). NZY^+ broth (10 g/l casein hydrolysate, 5g/l yeast extract, 5 g/l NaCl with supplements (12.5 mM MgCl2, 12, 5 mM MgSO4, 20 mM, 20 mM glucose, filter sterilised)) was preheated at 42°C. At the end of the 30 min. incubation, tubes were heat pulsed at 42°C precisely for two min., then incubated for two min. on ice. NZY^+ broth (450 µl) was added to each tube and incubated at 37°C for one hr with shaking at 250 rpm. Transformants (100 µl) was spread onto LB agar plates (10 g/l NaCl, 5 g/l yeast extract, 10 g/l trypsin, 20 g/l agar) containing 100 mg/ml ampicillin. Prior to plating, 100 µl 2% 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) and 100 µl 10 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) were spread onto the LB agar plates containing the ampicillin and allowed to air dry for approximately 45 min. White colonies containing insert DNA were selected for plasmid preparation.

3.2.3.1.5 Preparation of plasmid DNA

NZY^+ broth + Ampicillin (3 ml) was inoculated with a single colony and incubated at 37°C on a shaker (250 rpm) overnight for 15 hrs. Plasmids were isolated using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega, USA) using the protocol described in the kit.

Bacterial cultures (3 ml) were pelleted by centrifugation for 5 min. at 10 000 x gr in a tabletop centrifuge. The supernatant was poured off. 250 µl of Wizard® *Plus* SV Cell Resuspension solution was added and the cell pellet resuspended by vortexing well. The cells were then lysed by the addition of 250 µl Wizard® *Plus* SV Cell Lysis Solution

and mixed by inverting the tube 4 times. The suspension was observed for clearing to ensure lysis was complete.

Protein was then precipitated by the addition of 10 µl Alkaline Protease solution. The solution was mixed by inverting the tube four times and then incubated for 5 min. at RT. 350 µl of Wizard® *Plus* SV Minipreps neutralization solution was added and the tubes inverted 4 times to mix. Bacterial lysate was centrifuged at 14 000 x gr in a microcentrifuge for 10 min. at RT.

Approximately 850 µl of the cleared lysate was transferred by decanting into the Wizard® *Plus* SV minipreps spin column inserted into a 2 ml collection tube. The cleared lysate was centrifuged at 14 000 x gr for 1 min. at RT. The Wizard® *Plus* SV minpreps spin column was then removed from the tube and flow through discarded from the collection tube.

The lysate was washed first by the addition of 750 µl of Wizard® *Plus* SV column wash solution to the spin column. The tube was centrifuged at 14,000 x gr for 1 min. at RT. The Wizard® *Plus* SV spin column was removed and the flow through discarded from the collection tube. The wash was repeated with 250 µl column wash solution and centrifuged at 14 000 x gr for 2 min. at RT.

The Wizard® *Plus* SV spin column was transferred to a clean, sterile 1.5 ml microcentrifuge tube. The plasmid was eluted by the addition of 100 µl Nuclease free water to the spin column and centrifugation at 14 000 x gr for 1 min. at RT in a microcentrifuge. An aliquot of the isolated plasmid (5 µl) was run on 1% agarose gel to determine success of the procedure. To confirm whether an insert of the expected size was present, an aliquot was digested with restriction enzymes *Eco*RI and *Not*I (Promega, USA).

3.2.3.2 *Purification of PCR products for direct sequencing*

3.2.3.2.1 Purification of PCR products with ExoSAP-IT™

Polymerase chain reaction products were prepared for direct sequencing by removing the excess primers and nucleotides using the ExoSAP-IT™ kit (USB Corporation, USA). ExoSAP-IT prepares PCR products for numerous applications such as radioactive and fluorescent detection methods. Once PCR is complete, any excess primers and nucleotides remaining may interfere with these applications. ExoSAP-IT utilises two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase, to remove unwanted dNTPs and primers. The Exonuclease I degraded residual single-stranded primers and any extraneous single-stranded DNA produced by the PCR. The Shrimp Alkaline Phosphatase hydrolysed the remaining dNTPs from the PCR mixture which would interfere with the sequencing reaction. 1 µl of ExoSAP-IT™ was added to 4 µl of PCR product and incubated at 37 °C for 15 min. (usually in a PCR machine) followed by inactivation of the enzyme at 80 °C for 15 min. The enzymes in ExoSAP-IT™ were active in the buffer used for PCR, hence no buffer exchange was required. The sequencing mix described below in 3.2.3.3 was added directly to the above enzyme-treated samples.

3.2.3.2.2 Qiagen purification

Polymerase chain reactions (100 µl) were set up as described previously. Samples were then separated on 2% agarose gels, the bands excised and purified using the Qiaex II gel extraction kit (Qiagen Ltd., UK). The amplified band was excised from the gel and excess agarose removed. A 1.5 ml eppendorf tube was used for processing up to 250 mg agarose. The weight of the gel slice was determined and 300 µl of Buffer QX1 was added per 100 mg of gel. The Qiaex II solution was resuspended by vortexing for 30 sec; 10 µl Qiaex II was added to the sample. The sample was then incubated at 50°C for 10 min. to solubilize the agarose and allow the Qiaex II silica-gel particles to bind the DNA. During the incubation the sample was vortexed every 2 min. to keep Qiaex II in

suspension. At the end of the incubation samples were checked to ensure that the solution was yellow. The adsorption of DNA to Qiaex II was only efficient at pH ≤ 7.5; Buffer QX1 contains a pH indicator which is yellow at pH ≤ 7.5. The sample was then centrifuged for 30 sec and the supernatant removed with a pipette. The pellet was then washed with 500 µl Buffer QX1, followed by two washes in 500 µl Buffer PE. The pellet was air dried for 10-15 min. DNA was eluted by the addition of 20 µl SDW, incubation at RT for 5 min., followed by centrifugation for 30 sec. The supernatant containing the purified DNA was transferred to a new tube. Purified products were sequenced using the direct sequencing method.

3.2.3.3 Direct sequencing using the ABI Prism cycle sequencing kit

Cloned samples, samples purified from agarose gels and samples purified using the ExoSAP-IT™ kit were prepared for cycle sequencing using the Big Dye™ Sequencing kit (Perkin-Elmer). Sequencing reaction was performed in 10 µl volume. 4 µl of Big Dye™ Terminator sequencing mix was added to 1 µl of primer and 5 µl of plasmid DNA (or DNA excised from the gel) and amplified in a Gene Amp 9700 PCR machine (Perkin Elmer). The cycling conditions were 25 cycles of 30 sec @ 96°C, 15 sec @ 50°C and extension for 3 min. @ 60°C, and finally a hold temp of 25°C for 10 min. PCR products were precipitated using four volumes of 75% isopropanol and incubated at RT for 15 min. Thereafter samples were spun at 13000 rpm for 20 min. The supernatant was carefully removed and the pellet was washed with 100 µl 75% isopropanol, centrifuged for 10 min, the supernatant was removed and the pellet was dried under low vacuum. Pellets were resuspended in 2 µl sterile distilled water prior to analysis on the ABI 377 Automated Sequencer (Perkin Elmer). Both forward and reverse sequences were performed; for the cloned PCR products the T3 and T7 primers were used and for direct sequencing ITS1 was used as the forward primer and ITS4 primer as the reverse primer.

3.2.3 Sequence analysis

For each isolate, forward and reverse sequences were assembled and edited using the programme Sequencher 2.0. The sequences ITS1→ ITS4 were aligned and edited with Bioedit Sequence Alignment Editor (Version 5.0.9, Hall 1999, 2001). Forward and reverse alignments were checked manually and ambiguities manually examined and edited. Sequences were then compared to the National Center for Biotechnology Information (NCBI) database for BLASTN analysis. The Bioedit programme was then used to align all sequences of the different isolates and species. Phylogenetic trees were drawn using the Phylogeny Inference Package (Phylip) (version 3.6 (alpha 2), Felsentein 2001).

3.3 RESULTS

3.3.1 PCR Amplification of the ITS1→ITS4 rDNA operon of *Peronospora parasitica*

PCR analysis of the ribosomal RNA operon revealed a single 970 bp fragment present in all isolates of *P. parasitica* (Fig. 3.2). Furthermore, this band was consistent among all pathotypes of *P. parasitica* viz. the *Brassica napus*, *Brassica oleracea*, *Brassica rapa*, *Brassica juncea* and including the *Arabidopsis thaliana (At)* pathotypes (Fig. 3.2). The use of Amplitaq DNA polymerase (Perkin Elmer, UK) produced bands that were clear and unambiguous (Fig. 3.2) compared to Gibco BRL Taq DNA polymerase (Fig 3.3 and Fig 3.4). The lettuce downy mildew isolate *Bremia lactucae* amplified a faint band, similar in size to that of *P. parasitica* (Fig 3.2 & 3.3).

Amplification of pure DNA of the *B. oleracea* host Glory of Enkhuizen (Fig. 3.4. lane 11) produced a band of 670 bp. The occurrence of host DNA contamination indicated by the co-amplification of the 670 and 970 bp band (not shown) was used as the criterion to exclude contaminated *P. parasitica* DNA for sequencing and subsequent analysis in later chapters. As an additional control for *Bremia lactucae*, amplification of the *Lactucae sativa* host revealed a band of approximately 770 bp (Fig 3.3).

Attempts to purify PCR products from the gel using the Qiagen kit resulted in reduced yield of the product (Fig. 3.3). Alternatively, purification of the product followed by cloning suggested inserts of the correct size (970 bp) in 7 of 9 isolates (Fig. 3.5); the remaining 2 isolates viz. lanes 3 and 10 contained inserts of a different size (Fig. 3.6). The pPCR Script SK (+) plasmid is a 2961 bp plasmid designed by incorporating an *Srf*I site into the pBluescript SK (+) phagemid, and cloned fragments were expected to be 3931 bp. Overall, the cloning of PCR products showed 50 % efficiency with only 5 of the 10 isolates (Fig. 3.6) demonstrating inserts of the desired size after digestion with *Xho* I. The expected *Xho*I digest pattern is an insert (970 bp) and the pPCR-Script SK(+) plasmid. Some undigested product (3931 bp) was also observed. One isolate (lane 5) showed an insert of a different size (Fig. 3.6).

3.3.2 Direct sequencing of ITS-PCR products

Direct cycle sequencing of the ITS-PCR products using the BigDye™ cycle sequencing kit was successful in producing good quality DNA sequences for 12 isolates of *P. parasitica*. Post-PCR purification of PCR products (Fig. 3.2) with ExoSap-It produced excellent sequences with chromatograms showing strong amplification signals (fluorescence) when analysed on the ABI Prism Sequencer. Furthermore, utility of ExoSAP-IT™ ensured sequences with longer sequence reads (compared to samples where this purification step was excluded) when sequenced with the ITS1 forward and ITS4 reverse primers. Sequences for 11 isolates IP09, IP13, IP05B, P003, R1, SSP6, PPSAR4, Pp9, P501, PPSAR6 and SSH1 obtained using this method were of sufficient length and therefore overlapped when forward and reverse sequences were aligned using the program Sequencher 2.0. For the isolate Noks1 good quality forward sequence alone was obtained.

Sequencing of PCR products without ExoSAP-IT™ resulted in low sequencing efficiency. Fig. 3.4 illustrates ITS- PCR products following purification by re-precipitation. Following cloning of these fragments into pPCR-Script CAM SK(+) cloning vector and subsequent plasmid preparation (Fig. 3.5), plasmids of the appropriate size were produced. Digestion of plasmid DNA with *Xho*I revealed insert sizes of

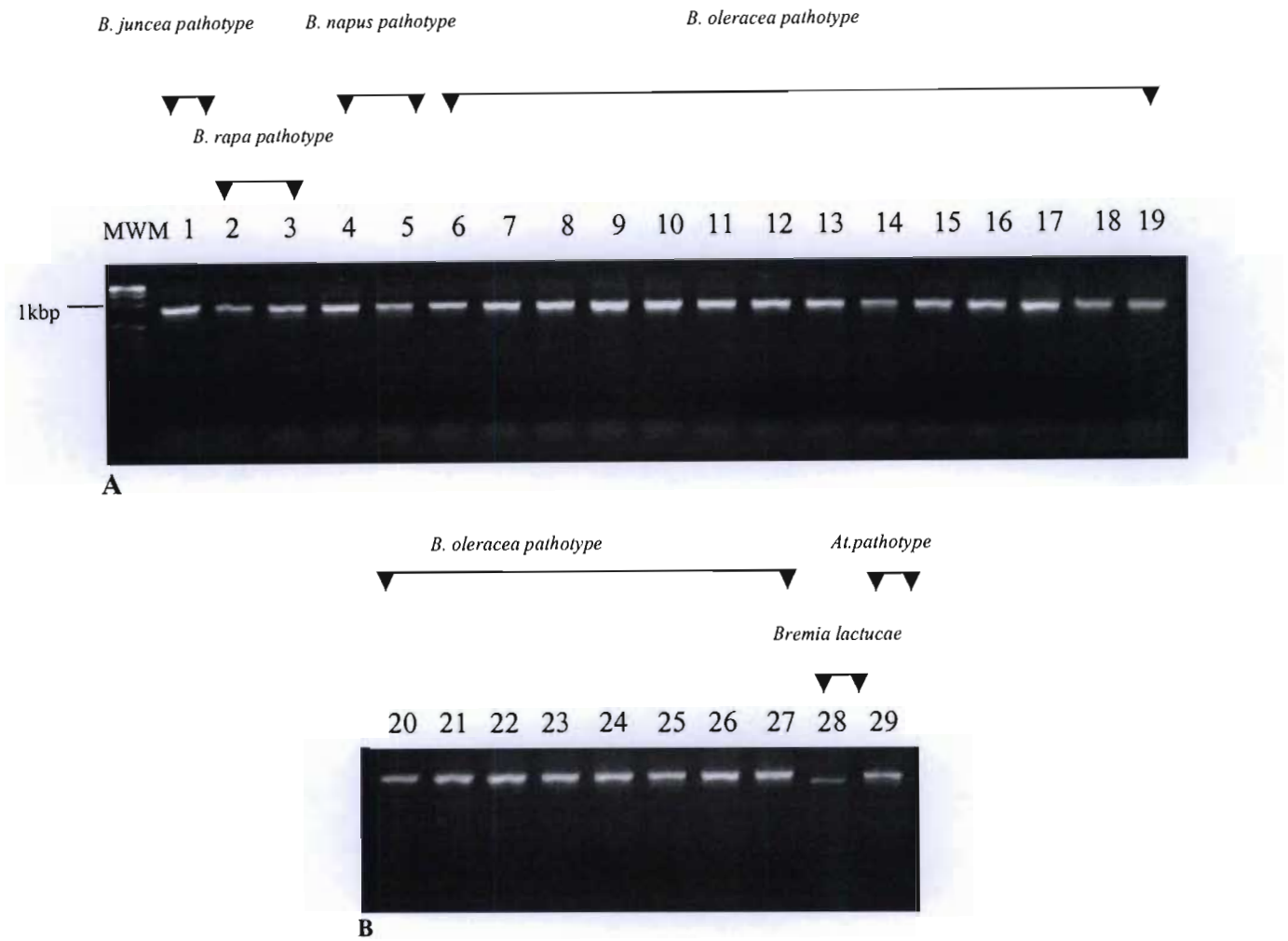

Fig. 3.2 A-B: Polymerase chain reaction amplimers of ITS 1→ITS 4 (including ITS 1, 5.8 s r, ITS 2 rDNA) region of the rDNA operon of pathotypes of *Peronospora parasitica* analysed on 2% agarose gels. MWM - 1 kbp ladder (Promega), Isolates 1-IP05B, 2-IP13, 3 - IP09, 4 - R1, 5 - P003, 6 - P005, 7 - Br1F, 8 - Br5A, 9 - P501, 10 - Pp9, 11 - P216, 12 - SSH1, 13 - SSH2, 14 - SSH3, 15 - SSH4, 16 - SSH5, 17 - SSH6, 18 - TC1, 19 - PPSAR2, 20 - PPSAR4, 21 - PPSAR5, 22 - PPSAR6, 23 - ACAT2B, 24 - ACAT2C, 25 - ACAT2D, 26 - ACAT2C, 27 - PPSAM1, 28 - *Bremia lactucae*, 29 - Noks1. *At* - *Arabidopsis thaliana* pathotype.

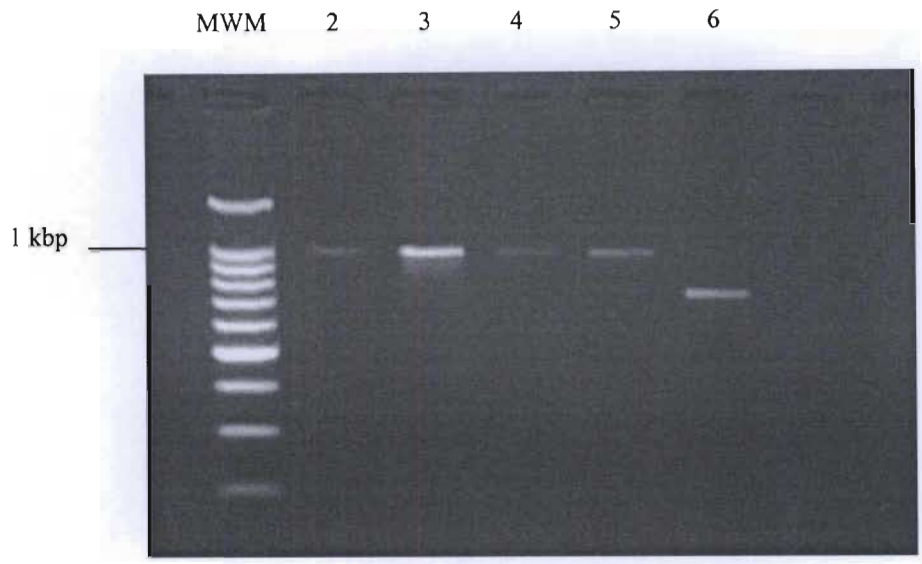

Fig. 3.3: ITS-PCR products of *Peronospora parasitica* visualised on 2% agarose gels following purification with Qiagen kit.
Lane 1 - MWM 100 bp ladder (Promega), 2 - P005, 3 - SSP6, 4 - SSH1, 5 - *Bremia lactucae*, 6 - *B. lactucae* and its host *Lactucae sativa*.

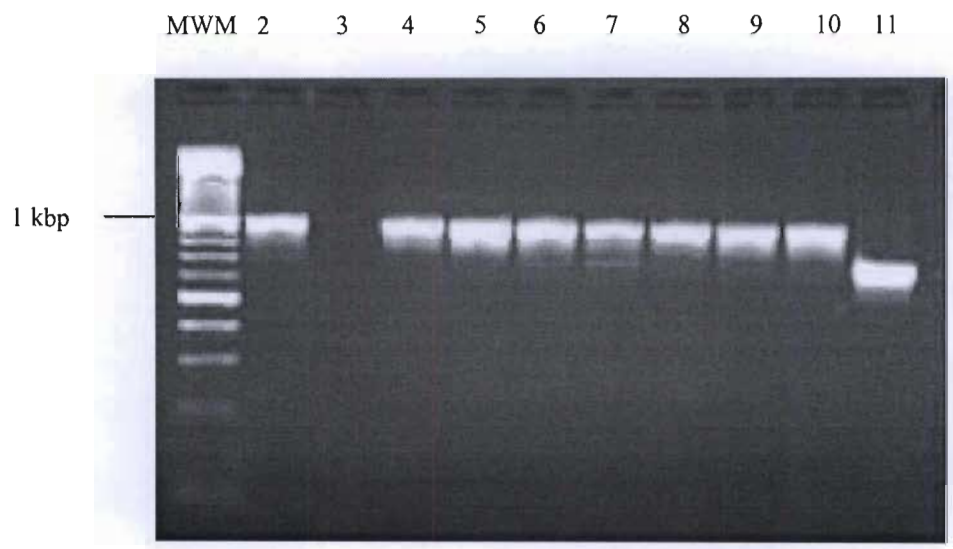

Fig. 3.4: ITS-PCR products of *Peronospora parasitica* purified by re-precipitation and separated on 2% agarose gels.
Lane 1 MWM XIV (Roche, SA), 2 - IP05B, 3 - P003, 4 - ACAT2B, 5 - ACAT2E, 6 - SSH6, 7 - PPSAM1, 8 - Br1F, 9 - Br5A, 10 - P501, 11 - GE (host DNA).

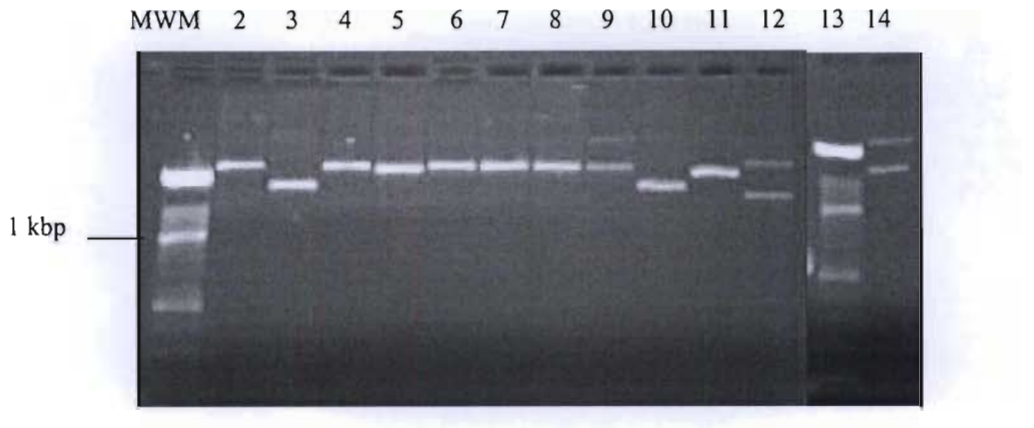

Fig. 3.5: ITS-PCR fragments (970 bp) of *Peronospora parasitica* cloned within pPCR-Script CAM SK(+) Cloning Vector (2961 bp). Expected Cloned fragments are 3961 bp. Lanes 3 and 10 contain inserts of a different size.
Lane 1 MWM XIV (Roche, SA), 2 - IP05B, 3 - P003, 4 - ACAT2B, 5 - ACAT2E, 6 - SSH6, 7 - PPSAM1, 8 - Br1F, 9 - Br5A, 10 - P501, 11 - GE, 12 - PUC18 Control DNA, 13 - MWM XIV, 14 - Test insert.

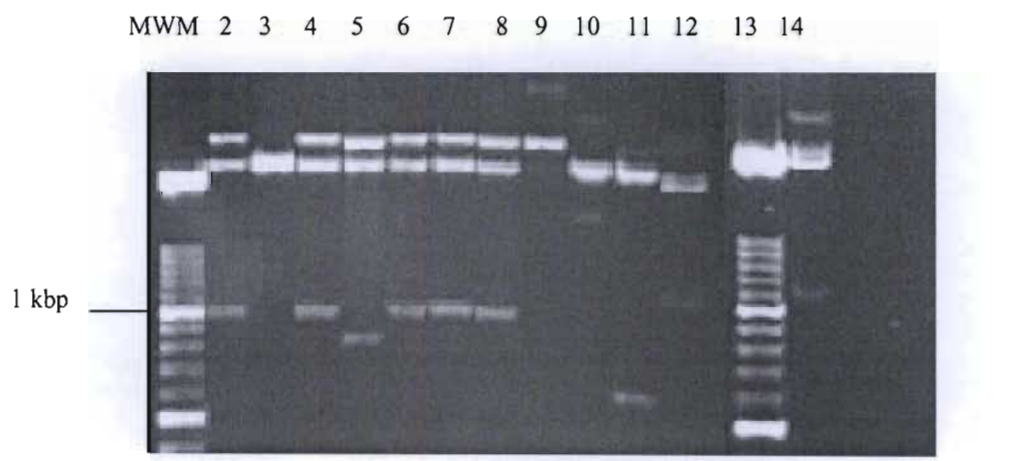

Fig. 3.6: ITS-PCR fragments of *Peronospora parasitica* cloned within pPCR-Script CAM SK(+) cloning vector and digested with *Xho* I.
Digested products are 970 bp for isolates in lanes 2,4,6-8 and 670 bp for the host in lane 11). Some undigested product (3961 bp) is observed.
Lane 1 MWM XIV (Roche, SA), 2 - IP05B, 3 - P003, 4 - ACAT2B, 5 - ACAT2E, 6 - SSH6, 7 - PPSAM1, 8 - Br1F, 9 - Br5A, 10 - P501, 11 - GE, 12 - PUC18 Control DNA, 13 - MWM XIV, 14 - Test insert.

approximately 970 bp (Fig. 3.6). However, sequence of cloned PCR fragments yielded very poor sequences (not shown) and these were excluded. Purification of PCR products by excising bands from agarose gels and Qiagen purification indicated distinct bands (Fig. 3.3), direct cycle sequencing of these purified products using the BigDye cycle sequencing kit however revealed good quality sequence for only the Mozambique isolate SSP6 (see alignment Table 3.1).

3.3.3 Alignment of ITS1→ITS4 sequences of the rRNA operon of *Peronospora parasitica* and related Oomycetes

Following alignment of forward and reverse sequences of the ribosomal DNA operon with Sequencher 2.0, sequences were edited manually and ambiguities clarified by comparing with original chromatograms. BlastN searches revealed significant homologies to ITS1, 5.8S and ITS2 sequences of isolates of *Peronospora parasitica* (Rehmany et al. 2000, Casimiro et al. 2000). In addition BLASTN searches also identified varying homologies to other species of *Peronospora* including *P. sparsa* (Cooke et al. 2000), *P. manshurica* and *P. destructor* (Saito 1999a,b) as well as other Oomycetes including *Phytophthora* (Cooke et al. 2000) and *Pythium* (Paul 2001).

Complete sequences of the ITS1, 5.8S rDNA and ITS2 regions of the rRNA operon of 12 isolates of *P. parasitica* viz. IP09, IP13, IP05B, Mozambique SSP6, P003, R1, P501, PPSAR4, PPSAR6, Pp9, P501, Noks1, were aligned using Bioedit. Sequences were selected from those obtained from BlastN searches and added to the alignment. These included all *P. parasitica* sequences present on the database as well as a few isolates of the same genus and others in the class Oomycetes. This final alignment is represented in Table 3.1 and 3.2. While amplification of the *Arabidopsis thaliana* pathotype Noks1 indicated a single band (Fig. 3.2) only partial sequence of the ITS1 region was possible. Attempts to sequence the isolate *B. lactucae* were unsuccessful thus *B. lactucae* was excluded from further analyses.

Table 3.1: Alignment of ITS1 sequences of isolates of *Peronospora parasitica* and other Oomycetes

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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         5         15         25         35         45         55         65         75         85
[a]IP09             AAAACCTTCC ACGTGAACCG TATTAACAAT TATAAT-TGG GGGTGAT--- -CTTAATGGA GGC-TACTAG T-CATTTTG- GCTGGA---G
[b]IP05B            AAAACTTTCC ACGTGAACCG TATTAACAAT TATAAT-TGG GGGTGAT--- -CTTAATGGA GGC-TACTAG T-CATTTTG- GCTGGA---G
[c]SSP6             ---------- ---------- -ACCAACAAT TATAAT-TGG GGGTGAT--- -CTTAATGGA GGC-TACTAG T-CATTTTG- GCTGGA---G
[cf]PPSAR4          AAAACTTTCC -CGTGAACCG GATTAACAAT TATTAT-TGG GGGTGAT--- -CTTAATGGA GGC-TACTAG T-CATTTTG- GCTGGA---G
[c]P501             AAAACTTTCC ACGTGAACCG TATTAACAGT TATAAT-TGG GGGTGAT--- -CTTAATGGA GGC-TACTAG T-CATTTTG- GCTGGA---G
[a]IP13             AAAACTTTCC -CGTGAACCG TATTAACAAT TATAAT-TGG GGGTGAT--- -CTTAATGGA GGC-TACTAG T-CATTTTG- GCTGGA---G
[d]P003             AAAACTTTCC -CGTGAACCG TATTAACAAT TATAAT-TGG GGGTGAT--- -CTTAATGGA GGC-TACTAG T-CATTTTG- GCTGGA---G
[c]Pp9              AAAACTTTCC -CGTGAACCG TATTAACAAT TATAAT-TGG GGGTGAT--- -CTTAATGGA GGC-TACTTG T-CATTTTG- GCTGGA---G
[c]PPSAR6           AAAACTTTCC ACGTGAACCG TATTAACAAT TATAAT-TGG GGGTGAT--- -CTTAATGGA GGC-TACTAG T-CATTTTG- GCTGGA---G
[ce]Preston         AAAACTTTCC ACGTGAACCG TATTAACAAT TATAAT-TGG GGGTGAT--- -CTTAATGGA GGC-TACTAG T-CATTTTG- GCTGGA---G
[g]Noks1            AAAACTTTCC ACGTGAACCG TATCAACAAC TTTAAT-TGG GGGTGAT--- -CTTGACGGT AGC-TACTGG T-CATTTTG- ACTGGC---G
P.destructor        AAAACTTTCC ACGTGAACCG TATCAACCCA ATTAAT-TGG GGGTTTA--- -CTTGGCGGC TGC-TGGTGG CATGTTTTT- GCTGGC--TG
Ph.phaseoli         AAAACTTTCC ACGTGAACCG TTTCAACC-C AATAGT-TGG GGGTCTT--- ACTTGGCGGC GGC-TGCTGG C--TTTATT- GCTGGC---G
Ph.infestans        AAAACTTTCC ACGTGAACCG TTTCAACC-C AATAGT-TGG GGGTCTT--- ACTTGGCGGC GGC-TGCTGG C--TTTATT- GCTGGC---G
P.sparsa            AAAACTTTCC ACGTGAACCG TATCAACCAA CATAAT-TGG GGGTTTA--- -TTTGGCGGC GGC-TGCTGG C--ATTTTGT GCTGGC--TG
P.manshurica        AAAACTTTCC ACGTGAACCG TATCAAACCA ATTCAT-TGG GGGTTTG--- -TTTGGCGGC GGC-TGCTGG CATCTTTTT- GCTGGC---T
Py.longandrum       AAAACTCTCC ACGTGAACTG TTTGTATCAG ATTAGCGCCG AGATTTTCGT GCGTGTTTGT GGTATCGCGA TGTATTCGTA CGTGGCGTTA

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        95        105        115        125        135        145        155        165        175
[a]IP09             GCTACTATCG AGTGAACCTT ATC--AT--G GCGGAAA-CT TAACTTT-TG TTGGGTGAAG TAG--TGAAA -TTTTAACCC --TTACTTAA
[b]IP05B            GCTACTATCG AGTGAACCTT ATC--AT--G GCGAAAA-CT AGACTTT-TG TTTGGTGAAG TAG--TGAAA ATTTTAACCC --TTACTTAA
[c]SSP6             GCTACTATCG AGTGAACCTT ATC--AT--G GCGAAAA-CT AGACTTT-TG TTTGGTGAAG TAG--TGAAA ATTTTAAACC --TTACTTAA
[cf]PPSAR4          GCTACTATCG AGGGAACCTT ATC--AT--G GCGAAAA-CT AGACTTT-TG TTTGGTGAAG TAG--TGAAA ATTTTAAACC --TTACTTAA
[c]P501             GCTACTATCG AGGGAACCTT ATC--AT--G GCGAAAA-CT AGACTTT-TG TTTGGTGAAG TAG--TGAAA ATTTTAAACC --TTACTTAA
[a]IP13             GCTACTATCG AGGGAACCTT ATC--AT--G GCGAAAA-CT AGACTTT-TG TTTGGTGAAG TAG--TGAAA ATTTTAAACC --TTACTTAA
[d]P003             GCTACTATCG AGTGAACCTT ATC--AT--G GCGAAAA-CT AGACTTT-TG TTTGGTGAAG TAG--TGAAA ATTTTAAACC --TTACTTAA
[c]Pp9              GCTACTATCG AGTGAACCTT ATC--AT--G GCGAAAA-CT AGACTTT-TG TTTGGTGAAG TAG--TGAAA ATTTTAAACC --TTACTTAA
[c]PPSAR6           GCTACTATCG AGTGAACCTT ATC--AT--G GCGAAAA-CT AGACTTT-TG TTTGGTGAAG TAG--TGAAA ATTTTAAACC --TTACTTAA
[ce]Preston         GCTACTATCG AGTGAACCTT ATC--AT--G GCGAAAAACT AGACTTT-TG TTTGGTGAAG TAG--TGAAA ATTTTAAACC --TTACTTAA
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[g]Noks1          GTAACCATTG AGTGAACCTC ATC--AT--G ACGACCG-TT GGGCTTT-TG CCTGACGTAG TAG--AATAA ATTTTAAACC --TTACTTAA
P.destructor  GCTACTGCTG AGCGAACCCT ATC--AT--G GCGAGTGTTC TGACCTC-GG TTGGAGCTAG TAG--CTTAA ATTGTAAACC CATT-CTTAA
Ph.phaseoli   GCTACTGCTG GGCGAGCCCT ATC--AAAAG GCGAGCGTTT GGGCTTC-GG TCTGAGCTAG TAGCTTTTTT ATTTTAAACC CTTTACTTAA
Ph.infestans GCTACTGCTG GGCGAGCCCT ATC--AAAAG GCGAGCGTTT GGACTTC-GG TCTGAGCTAG TAGCTTTTTT ATTTTAAACC CTTTACTTAA
P.sparsa      GCTGCTACTG GGCAAGCCCT ATC------- GCGAGCGTTT GGGCCTT-GG CCTGAGCTAG TAG--CTTTT ATTTTAAACC -ATT-CTTAA
P.manshurica GCTGCTGCTG AGCGAGCCCT ATC--AT--G GCAAGCGCTC GGACCTC-GG TCGGAGCTAG TAG--CATTC ATTTTAAACC CATT-CCTAA
Py.longandrumGCAAGCATTG TATGGAGCTT GGCTGATCGA AGGTCGGTGC GCACCTTGTG TGTGTGTTGG CTGATTAA-C CTTTCAAACC CTTTAAATTA

                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....
                     185        195        205        215        225        235        245        255
[a]IP09           ATACTGATTA TACTGTGGG- ACGAAAGTCT CTACTTTTAT TCTAGATACC AACTTTCACC AGTGGATGTC TAGGCTCGC
[b]IP05B          ATACTGATTA TACTGTGGGG ACGAAAGTCT CTACTTTTAT TCTAGATACC ACCTTTCAGC AGTGGATGTC TAGGCTCGC
[c]SSP6           ATACTGATTA TACTGTGGGG ACGAAAGTCT CTACTTTTAT TCTAGATAAC AACTTTCAGC AGAGGATGTC TAGGCTCGC
[c]P501           ATACTGATTA TACTGTGGGG ACGAAAGTCT CTACTTTTAT TCTAGATAAC AACTTTCAGC AGTGGATGTC TAGGCTCGC
[cf]PPSAR4        ATACTGATTA TACTGTGGGG ACGAAAGTCT CTACTTTTAT TCTAGATAAC AACTTTCAGC AGTGGATGTC TAGGCTCGC
[a]IP13           ATACTGATTA TACTGTGGGG ACGAAAGTCT CTACTTTTAT TCTAGATAAC AACTTTCAGC AGTGGATGTC TAGGCTCGC
[d]P003           ATACTGATTA TACTGTGGGG ACGAAAGTCT CTACTTTTAT TCTAGATAAC AACTTTCAGC AGTGGATGTC TAGGCTCGC
[c]Pp9            ATACTGATTA TACTGTGGGG ACGAAAGTCT CTACTTTTAT TCTAGATAAC AACTTTCAGC AGTGGATGTC TAGGCTCGC
[c]PPSAR6         ATACTGATTA TACTGTGGGG ACGAAAGTCT CTACTTTTAT TCTAGATAAC AACTTTCAGC AGTGGATGTC TAGGCTCGC
[ce]Preston       ATACTGATTA TACTGTGGGG ACGAAAGTCT CTACTTTTAT TCTAGATAAC AACTTTCAGC AGTGGATGTC TAGGCTCGC
[g]Noks1          TTA-TGATTA TACTGTGGGG ACGAAAGTCT CTGCTTTTAT TCTAGATAAC AACTTTCAGC AGTGGATGTC TAGGCTCGC
P.destructor  ATACTGATTA TACTGTGGGG ACGAAAGTCT CTGCTTTTAA -CTAGATAGC AACTTTCAGC AGTGGATGTC TAGGCTCGC
Ph.phaseoli   -TACTGATTA TACTGTGGGG ACGAAAGTCT CTGCTTTTAA -CTAGATAGC AACTTTCAGC AGTGGATGTC TAGGCTCGC
Ph.infestans -TACTGATTA TACTGTGGGG ACGAAAGTCT CTGCTTTTAA -CTAGATAGC AACTTTCAGC AGTGGATGTC TAGGCTCGC
P.sparsa      TTACTGATTA TACTGTGGGG ACGAAAGTCT CTGCTTTTAA -CTAGATAGC AACTTTCAGC AGTGGATGTC TAGGCTCGC
P.manshurica TGACTGAATG TACTGTGGGG ACGAAAGTCT CTGCTTTTAA -CTAGATAGC AACTTTCAGC AGTGGATGTC TAGGCTCGC
Py.longandrumATACTGATTA TACTGTGAGG ACGAAAGTCT TTGCTTTTA- TCTAGATAAC AACTTTCAGC AGTGGATGTC TAGGCTCGC
```

[a] *Brassica rapa* pathotype
[b] *Brassica juncea* pathotype
[c] *Brassica oleracea* pathotype
[d] *Brassica napus* pathotype
[e] Preston = sequence identical to *Brassica oleracea* isolates P005, Bb, P006, P204, P211, P216, P218, P502, P501, P217, P214 (ncbi)
[f] PPSAR4 = sequence identical to *Brassica oleracea* isolate SSH1
[g] *Arabidopsis thaliana* pathotype

Table 3.2: Sequence alignment of the ITS1, 5.8S and ITS2 regions of the rDNA operon of isolates of *Peronospora parasitica*

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ...|....|
                      5         15         25         35         45         55         65         75         85
[c]PPSAR4        ATTAACAATT ATTATTGGGG GTGAT-CTTA ATGGAGGCTA CTAGTCATTT T-GGCTGGA- GGCTACTATC GAGGGAACCT TATCAT--GG
[c]SSH1          ATTAACAATT ATTATTGGGG GTGAT-CTTA ATGGAGGCTA CTAGTCATTT T-GGCTGGA- GGCTACTATC GAGGGAACCT TATCAT--GG
[d]R1            ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
[c]SSP6          ACCAACAATT ATAATTGGGG GTGAT-CTTA ATGGAGGCTA CTAGTCATTT T-GGCTGGA- GGCTACTATC GAGTGAACCT TATCAT--GG
[d]P003          ATTAACAATT ATAATTGGGG GTGAT-CTTA ATGGAGGCTA CTAGTCATTT T-GGCTGGA- GGCTACTATC GAGTGAACCT TATCAT--GG
[a]IP13          ATTAACAATT ATAATTGGGG GTGAT-CTTA ATGGAGGCTA CTAGTCATTT T-GGCTGGA- GGCTACTATC GAGGGAACCT TATCAT--GG
[c]PPSAR6        ATTAACAATT ATAATTGGGG GTGAT-CTTA ATGGAGGCTA CTAGTCATTT T-GGCTGGA- GGCTACTATC GAGTGAACCT TATCAT--GG
[c]Pp9           ATTAACAATT ATAATTGGGG GTGAT-CTTA ATGGAGGCTA CTTGTCATTT T-GGCTGGA- GGCTACTATC GAGTGAACCT TATCAT--GG
[c]P501          ATTAACAGTT ATAATTGGGG GTGAT-CTTA ATGGAGGCTA CTAGTCATTT T-GGCTGGA- GGCTACTATC GAGGGAACCT TATCAT--GG
[b]IP05B         ATTAACAATT ATAATTGGGG GTGAT-CTTA ATGGAGGCTA CTAGTCATTT T-GGCTGGA- GGCTACTATC GAGTGAACCT TATCAT--GG
[a]IP09          ATTAACAATT ATAATTGGGG GTGAT-CTTA ATGGAGGCTA CTAGTCATTT T-GGCTGGA- GGCTACTATC GAGTGAACCT TATCAT--GG
Ph.phaseoli      TTCAACCCA- ATAGTTGGGG GTCTTACTTG GCGGCGGCTG CTGGCTTTAT --TGCTGGC- GGCTACTGCT GGGCGAGCCC TATCAAAAGG
Ph.infestans     TTCAACCCA- ATAGTTGGGG GTCTTACTTG GCGGCGGCTG CTGGCTTTAT --TGCTGGC- GGCTACTGCT GGGCGAGCCC TATCAAAAGG
P.sparsa         ATCAACCAAC ATAATTGGGG GT-TTATTTG GCGGCGGCTG CTGGCATTTT G-TGCTGGCT GGCTGCTACT GGGCAAGCCC TATC-----G
P.manshuri       ATCAAACCAA TTCATTGGGG GT-TTGTTTG GCGGCGGCTG CTGGCATCTT TTTGCTGGC- TGCTGCTGCT GAGCGAGCCC TATCAT--GG
P.destructor     ATCAACCCAA TTAATTGGGG GT-TTACTTG GCGGCTGCTG GTGGCATGTT TTTGCTGGCT GGCTACTGCT GAGCGAACCC TATCAT--GG

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                     95        105        115        125        135        145        155        165        175
[c]PPSAR4        CGAAAACT-A GACTTTTGTT TGGTGAAGTA GTGAA--AAT TTTAAA-CCT TACTTAAATA CTGATTATAC TGTGGGGACG AAAGTCTCTA
[c]SSH1          CGAAAACT-A GACTTTTGTT TGGTGAAGTA GTGAA--AAT TTTAAA-CCT TACTTAAATA CTGATTATAC TGTGGGGACG AAAGTCTCTA
[d]R1            ---------A GACTTTTGTT GGGTGAAGTA GTGAA--AAT TTTAAA-CCT TACTTAAATA CTGATTATAC TGTGGGGACG AAAGTCTCTA
[c]SSP6          CGAAAACT-A GACTTTTGTT TGGTGAAGTA GTGAA--AAT TTTAAA-CCT TACTTAAATA CTGATTATAC TGTGGGGACG AAAGTCTCTA
[d]P003          CGAAAACT-A GACTTTTGTT TGGTGAAGTA GTGAA--AAT TTTAAA-CCT TACTTAAATA CTGATTATAC TGTGGGGACG AAAGTCTCTA
[a]IP13          CGAAAACT-A GACTTTTGTT TGGTGAAGTA GTGAA--AAT TTTAAA-CCT TACTTAAATA CTGATTATAC TGTGGGGACG AAAGTCTCTA
[c]PPSAR6        CGAAAACT-A GACTTTTGTT TGGTGAAGTA GTGAA--AAT TTTAAA-CCT TACTTAAATA CTGATTATAC TGTGGGGACG AAAGTCTCTA
[c]Pp9           CGAAAACT-A GACTTTTGTT TGGTGAAGTA GTGAA--AAT TTTAAA-CCT TACTTAAATA CTGATTATAC TGTGGGGACG AAAGTCTCTA
[c]P501          CGAAAACT-A GACTTTTGTT TGGTGAAGTA GTGAA--AAT TTTAAA-CCT TACTTAAATA CTGATTATAC TGTGGGGACG AAAGTCTCTA
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[b]IP05B          CGAAAACT-A GACTTTTGTT TGGTGAAGTA GTGAA--AAT TTTAA-CCCT TACTTAAATA CTGATTATAC TGTGGGGACG AAAGTCTCTA
[a]IP09           CGGAAACT-T AACTTTTGTT GGGTGAAGTA GTGAA---AT TTTAA-CCCT TACTTAAATA CTGATTATAC TGTGGG-ACG AAAGTCTCTA
Ph.phaseoli       CGAGCGTTTG GGCTTCGGTC TGAGCTAGTA GCTTTTTTAT TTTAAACCCT TTACTTAATA CTGATTATAC TGTGGGGACG AAAGTCTCTG
Ph.infestans      CGAGCGTTTG GACTTCGGTC TGAGCTAGTA GCTTTTTTAT TTTAAACCCT TTACTTAATA CTGATTATAC TGTGGGGACG AAAGTCTCTG
P.sparsa          CGAGCGTTTG GGCCTTGGCC TGAGCTAGTA GCTTT--TAT TTTAAACCAT T-CTTAATTA CTGATTATAC TGTGGGGACG AAAGTCTCTG
P.manshurica      CAAGCGCTCG GACCTCGGTC GGAGCTAGTA GCATT--CAT TTTAAACCCA TTCCTAATGA CTGAATGTAC TGTGGGGACG AAAGTCTCTG
P.destructor      CGAGTGTTCT GACCTCGGTT GGAGCTAGTA GCTTA--AAT TGTAAACCCA TTCTTAAATA CTGATTATAC TGTGGGGACG AAAGTCTCTG

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                     185        195        205        215        225        235        245        255        265
[c]PPSAR4         CTTTTATTCT AGATAACAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAT TGCGATACGT AATGCGAATT
[c]SSH1           CTTTTATTCT AGATAACAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAT TGCGATACGT AATGCGAATT
[d]R1             CTTTTATTCT AGATAACAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAT TGCGATACGT AATGCGAATT
[c]SSP6           CTTTTATTCT AGATAACAAC TTTCAGCAGA GGATGTCTAG GCTCGCACAT CGATAAAAGA CGCTGCGAAT TGCGATACGT AATGCGAATT
[d]P003           CTTTTATTCT AGATAACAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAT TGCGATACGT AATGCGAATT
[a]IP13           CTTTTATTCT AGATAACAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAT TGCGATACGT AATGCGAATT
[c]PPSAR6         CTTTTATTCT AGATAACAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAT TGCGATACGT AATGCGAATT
[c]Pp9            CTTTTATTCT AGATAACAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAT TGCGATACGT AATGCGAATT
[c]P501           CTTTTATTCT AGATAACAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAT TGCGATACGT AATGCGAATT
[b]IP05B          CTTTTATTCT AGATACCACC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAT TGCGATACGT AATGCGAATT
[a]IP09           CTTTTATTCT AGATACCAAC TTTCACCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAT TGCGATACGT AATGCGAATT
Ph.phaseoli       CTTTTAA-CT AGATAGCAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAC TGCGATACGT AATGCGAATT
Ph.infestans      CTTTTAA-CT AGATAGCAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAC TGCGATACGT AATGCGAATT
P.sparsa          CTTTTAA-CT AGATAGCAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAC TGCGATACGT AATGCGAATT
P.manshurica      CTTTTAA-CT AGATAGCAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAC TGCGATACGT AATGCGAATT
P.destructor      CTTTTAA-CT AGATAGCAAC TTTCAGCAGT GGATGTCTAG GCTCGCACAT CGATGAAGAA CGCTGCGAAC TGCGATACGT AATGCGAATT

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                     275        285        295        305        315        325        335        345        355
[c]PPSAR4         GCTAAATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCTTGG GCTAGTCCTA CGAAGTATGC CTCTATCAGT GTCCACATAT
[c]SSH1           GCTAAATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCTTGG GCTAGTCCTA CGAAGTATGC CTCTATCAGT GTCCACATAT
[d]R1             GCTAAATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCTTGG GCTAGTCCTA CGAAGTATGC CTCTATCAGT GTCCACATAT
[c]SSP6           GCTAAATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCTTGG GCTAGTCCTA CGAAGTATGC CTCTATCAGT GTCCACATAT
[d]P003           GCTAAATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCTTGG GCTAGTCCTA CGAAGTATGC CTCTATCAGT GTCCACATAT
[a]IP13           GCTAAATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCTTGG GCTAGTCCTA CGAAGTATGC CTCTATCAGT GTCCACATAT
[c]PPSAR6         GCTAAATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCTTGG GCTAGTCCTA CGAAGTATGC CTCTATCAGT GTCCACATAT
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[c]Pp9          GCTAAATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCTTGG GCTAGTCCTA CGAAGTATGC CTCTATCAGT GTCCACATAT
[c]P501         GCTAAATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCTTGG GCTAGTCCTA CGAAGTATGC CTCTATCAGT GTCCACATAT
[b]IP05B        GCTAAATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCTTGG GCTAGTCCTA CGAAGTATGC CTCTATCAGT GTCCACATAT
[a]IP09         GCTAAATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCTTGG GCTAGTCCTA CGAAGTATGC CTCTATCAGT GTCCACATAT
Ph.phaseoli     GCAGGATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCC-GG GTTAGTCCTG -GAAGTATGC CTGTATCAGT GTCCGTACAA
Ph.infestans    GCAGGATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCC-GG GTTAGTCCTG -GAAGTATGC CTGTATCAGT GTCCGTACAA
P.sparsa        GCAGGATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCC-GG GTTAGTCCTG -GAAGTATGC CTGTATCAGT GTCCGTACAT
P.manshurica    GCAGGATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCC-GG GTTAGCCCTG -GGAGTATGC CTGTATCAGT GTCCGTACAT
P.destructor    GCAGGATTCA GTGAGTCATC GAAATTTTGA ACGCATATTG CACTTCC-GG GTTATCCCTG -GGAGTATGC CTGTATCAGT GTCCGTACAT

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                   365        375        385        395        405        415        425        435        445
[c]PPSAR4       CAAACTTGGT TTTCTTTTAT C-GTGTATTC GATAAAGGAT ATGCCAGATG TGAAGTGTCT TGCGGTTGAT TTTCGAATTG ACTACGAGTC
[c]SSH1         CAAACTTGGT TTTCTTTTAT C-GTGTATTC GATAAAGGAT ATGCCAGATG TGAAGTGTCT TGCGGTTGAT TTTCGAATTG ACTACGAGTC
[d]R1           CAAACTTGGT TTTCTTTTAT C-GTGTATTC GATAAAGGAT ATGCCAGATG TGAAGTGTCT TGCGGTTGAT TTTCGAATTG ACTACGAGTC
[c]SSP6         CAAACTTGGT TTTCTTTTAT C-GTGTATTC GATAAAGGAT ATGCCAGATG TGAAGTGTCT TGCGGTTGAT TTTCGAATTG ACTACGAGTC
[d]P003         CAAACTTGGT TTTCTTTTAT C-GGGTATTC GATAAAGGAT ATGCCAGATG TGAAGTGTCT TGCGGTTGAT TTTCGAATTG ACTACGAGTC
[a]IP13         CAAACTTGGT TTTCTTTTAT C-GTGTATTC GATAAAGGAT ATGCCAGATG TGAAGTGTCT TGCGGTTGAT TTTCGAATTG ACTACGAGTC
[c]PPSAR6       CAAACTTGGT TTTCTTTTAT C-GAGTATTC GATAAAGGAT ATGCCAGATG TGAAGTGTCT TGCGGTTGAT TTTCGAATTG ACTACGAGTC
[c]Pp9          CAAACTTGGT TTTCTTTTAT C-GTGTATTC GATAAAGGAT ATGCCAGATG TGAAGTGTCT TGCGGTTGAT TTTCGAATTG ACTACGAGTC
[c]P501         CAAACTTGGT TTTCTTTTAT C-GTGTATTC GATAAAGGAT ATGCCAGATG TGAAGTGTCT TGCGGTTGAT TTTCGAATTG ACTACGAGTC
[b]IP05B        CAAACTTGGT TTTCTTTTAT C-GTGTATTC GATAAAGGAT ATGCCAGATG TGAAGTGTCT TGCGGTTGAT TTTCGAATTG ACTACGAGTC
[a]IP09         CAAACTTGGT TTTCTTTTAT C-GTGTATTC GATAAAGGAT ATGCCAGATG TGAAGTGTCT TGCGGTTGAT TTTCGAATTG ACTACGAGTC
Ph.phaseoli     CAAACTTGGC TTTCTTCCTT CCGTGTAGTC GGTGGAGGAG ATGCCAGATG TGAAGTGTCT TGCGGTTGGT TTTCGGACCG ACTGCGAGTC
Ph.infestans    CAAACTTGGC TTTCTTCCTT CCGTGTAGTC GGTGGAGGAG ATGCCAGATG TGAAGTGTCT TGCGGTTGGT TTTCGGACCG ACTGCGAGTC
P.sparsa        CAAACTTGGT TTTCTTCTTT CCGTGTAGTC GGTGGAGGAT ATGCCAGATG TGAAGTGTCT TGCGGCTGAT TTTCGGATCG GCTGCGAGTC
P.manshurica    CAAACTTGGT TTTCTTCTTT CCGTGTAGTC GGTGGAGGAT ATGCCAGATG TGAAGTGTCT TGCGGCTGGT TTTTTGATCG GCTGTGAGTC
P.destructor    CAAACTTGGT TTTCTTCTTT CCGTGTAGTC GGTGGAGGAT ATGCCAGATG TGAAGTGTCT TGCGGCTGGT TTTCGGATCG GCTGTGAGTC

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                   455        465        475        485        495        505        515        525        535
[c]PPSAR4       CTTTTAAATA TAT--GACTG TACATCTCAT -GCTAGAAAA GCG-TGACGA TACTAATT-T GAAGGCTGTC CGTGCTGACC TGTCGGTGAC
[c]SSH1         CTTTTAAATA TAT--GACTG TACATCTCAT -GCTAGAAAA GCG-TGACGA TACTAATT-T GAAGGCTGTC CGTGCTGACC TGTCGGTGAC
[d]R1           CTTTCAAATA TAT--GACTG TACATCTCAT -GCTAGAAAA GCG-TGACGA TACTAATT-T GAAGGCTGTC CGTGCTGACC TGTCGGTGAC
[c]SSP6         CTTTTAAATA TAT--GACTG TACATCTCAT -GCTAGAAAA GCG-TGACGA TACTAATT-T GAAGGCTGTC CGTGCTGACC TGTCGGTGAC
[d]P003         CTTTCAAATA TAT--GACTG TACATCTCAT -GCTAGAAAA GCG-TGACGA TACTAATT-T GAAGGCTGTC CGTGCTGACC TGTCGGTGAC
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[a]IP13          CTTTTAAATA TAT--GACTG TACATCTCAT -GCTAGAAAA GCG-TGACGA TACTAATT-T GAAGGCTGTC CGTGCTGACC TGTCGGTGAC
[c]PPSAR6        CTTTTAAATA TAT--GACTG TACATCTCAT -GCTAGAAAA GCG-TGACGA TACTAATT-T GAAGGCTGTC CGTGCTGACC TGTCGGTGAC
[c]Pp9           CTTTTAAATA TAT--GACTG TACATCTCAT -GCTAGAAAA GCG-TGACGA TACTAATT-T GAAGGCTGTC CGTGCTGACC TGTCGGTGAC
[c]P501          CTTTTAAATA TAT--GACTG TACATCTCAT -GCTAGAAAA GCG-TGACGA TACTAATT-T GAAGGCTGTC CGTGCTGACC TGTCGGTGAC
[b]IP05B         CTTTTAAATA TAT--GACTG TACATCTCAT -GCTAGAAAA GCG-TGACGA TACTAATT-T GAAGGCTGTC CGTGCTGACC TGTCGGTGAC
[a]IP09          CTTTTAAATA TAT--GACTG TACATCTCAT -GCTAGAAAA GCG-TGACGA TACTAATT-T GAAGGCTGTC CGTGCTGACC TGTCGGTGAC
Ph.phaseoli      CTTTTAAATG TACTAAACTG TACTTCTCTT TGCTCCAAAA GTGGTGGCAT TGCTGGTTGT GGACGCTGCT ATTG-TAGCG AGTTGGCGAC
Ph.infestans     CTTTTAAATG TACTAAACTG TACTTCTCTT TGCTCCAAAA GTGGTGGCAT TGCTGGTTGT GGACGCTGCT ATTG-TAGCG AGTTGGCGAC
P.sparsa         CTTTGAAATG TACAGAACTG TACTTCTCTT TGCTCGAAAA GCA-TGGCGT TGCTGATTGT GGAGGCTGTC CGCG-TGGCC AGTCGGCGAT
P.manshurica     CTTTGAAATG TACAGAACTG TACTTCTCTT TGCTCGAAAA GCG-TGGCGT TGCTGGTTGT GGAGGCTGTG CGTG-TGACC AGTCGGCGAT
P.destructor     CTTTGAAATG TATGGAACTG -AC-TCTCTT TGTTCGAAAA GCG-TGGCGC TGCTGGTTGT GAAGGCTGTC AGTA-TGGCT AGTCGGCGAC

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                   545        555        565        575        585        595        605        615        625
[c]PPSAR4        TAGTTTGTTA ACTGTGACTA TATATATGAA GAAGTATTCG ATTCGCGGTA TGATTGGCCG TTCGGCTGGA CTACGTGTAT TGGATCTTTT
[c]SSH1          TAGTTTGTTA ACTGTGACTA TATATATGAA GAAGTATTCG ATTCGCGGTA TGATTGGCCG TTCGGCTGGA CTACGTGTAT TGGATCTTTT
[d]R1            TAGTTTGTTA ACTGTGACTA TATATATGAA GAAGTATTCG ATTCGCGGTA TGATTGGCCG TTCGGCTGGA CTACGTGTAT TGGATCTTTT
[c]SSP6          TAGTTTGTTA ACTGTGACTA TATATATGAA GAAGTATTCG ATTCGCGGTA TGATTGGCCG TTCGGCTGGA CTACGTGTAT TGGATCTTTT
[d]P003          TAGTTTGTTA ACTGTGACTA TATATATGAA GAAGTATTCG ATTCGCGGTA TGATTGGCCG TTCGGCTGGA CTACGTGTAT TGGATCTTTT
[a]IP13          TAGTTTGTTA ACTGTGACTA TATATATGAA GAAGTATTCG ATTCGCGGTA TGATTGGCCG TTCGGCTGGA CTACGTGTAT TGGATCTTTT
[c]PPSAR6        TAGTTTGTTA ACTGTGACTA TATATATGAA GAAGTATTCG ATTCGCGGTA TGATTGGCCG TTCGGCTGGA CTACGTGTAT TGGATCTTTT
[c]Pp9           TAGTTTGTTA ACTGTGACTA TATATATGAA GAAGTATTCG ATTCGCGGTA TGATTGGCCG TTCGGCTGGA CTACGTGTAT TGGATCTTTT
[c]P501          TAGTTTGTTA ACTGTGACTA TATATATGAA GAAGTATTCG ATTCGCGGTA TGATTGGCCG TTCGGCTGGA CTACGTGTAT TGGATCTTTT
[b]IP05B         TAGTTTGTTA ACTGTGACTA TATATATGAA GAAGTATTCG ATTCGCGGTA TGATTGGCCG TTCGGCTGGA CTACGTGTAT TGGATCTTTT
[a]IP09          TAGTTTGTTA ACTGTGACTA TATATATGAA GAAGTATTCG ATTCGCGGTA TGATTGGCCG TTCGGCTGGA CTACGTGTAT TGGATCTTTT
Ph.phaseoli      CGGTTTGTCT GCTGCGGCGT TA---ATGGA GAAATGCTCG ATTCGTGGTA TGGTTGGC-- TTCGGCTGAA C--------- ----------
Ph.infestans     CGGTTTGTCT GCTGCGGCGT TA---ATGGA GAAATGCTCG ATTCGTGGTA TGGTTGGC-- TTCGGCTGAA C--------- ----------
P.sparsa         CGGTTTGTCT GCTGTGGC-T TA---ATGGA GGAGTGTTTG ATTCGCGGTA TGATTGGC-- TTCGGCTGAA C--------- ----------
P.manshurica     CGGTTTGTCT GCTGTGGCGT TA---ATGGA GGAGTGTTTG ATTCGCGGTA TGGTTAGC-- TTCTGCTGAA C--------- ----------
P.destructor     CGGTTTGTCT GCTATGGTAT CA---ATGGA GGAGTGTTCG ATTCGCGGTA TGATTGGC-- TTCGGCTAAA C--------- ----------
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                  635        645        655        665        675        685        695        705        715
[c]PPSAR4       TTATGTATGA AGAAGTATTC GATTTGCGGT ATGATTGGCC GTTCGGCTGG ACTACGTTTA TTGGATCTTT TTCTGGTTGT GGTA-GATGA
[c]SSH1         TTATGTATGA AGAAGTATTC GATTTGCGGT ATGATTGGCC GTTCGGCTGG ACTACGTTTA TTGGATCTTT TTCTGGTTGT GGTA-GATGA
[d]R1           TTATGTATGA AGAAGTATTC GATTTGCGGT ATGATTGGCC GTTCGGCTGG ACTACGTTTA TTGGATCTTT TTCTGGTTGT GGTA-GATGA
[c]SSP6         TTATGTATGA AGAAGTATTC GATTTGCGGT ATGATTGGCC GTTCGGCTGG ACTACGTTTA TTGGATCTTT TTCTGGTTGT GGTA-GATGA
[d]P003         TTATGTATGA AGAAGTATTC GATTTGCGGT ATGATTGGCC GTTCGGCTGG ACTACGTTTA TTGGATCTTT TTCTGGTTGT GGTA-GATGA
[a]IP13         TTATGTATGA AGAAGTATTC GATTTGCGGT ATGATTGGCC GTTCGGCTGG ACTACGTTTA TTGGATCTTT TTCTGGTTGT GGTA-GATGA
[c]PPSAR6       TTATGTATGA AGAAGTATTC GATTTGCGGT ATGATTGGCC GTTCGGCTGG ACTACGTTTA TTGGATCTTT TTCTGGTTGT GGTA-GATGA
[c]Pp9          TTATGTATGA AGAAGTATTC GATTTGCGGT ATGATTGGCC GTTCGGCTGG ACTACGTTTA TTGGATCTTT TTCTGGTTGT GGTA-GATGA
[c]P501         TTATGTATGA AGAAGTATTC GATTTGCGGT ATGATTGGCC GTTCGGCTGG ACTACGTTTA TTGGATCTTT TTCTGGTTGT GGTA-GATGA
[c]IP05B        TTATGTATGA AGAAGTATTC GATTTGCGGT ATGATTGGCC GTTCGGCTGG ACTACGTTTA TTGGATCTTT TTCTGGTTGT GGTA-GATGA
[a]IP09         TTATGTATGA AGAAGTATTC GATTTGCGGT ATGATTGGCC GTTCGGCTGG ACTACGTTTA TTGGATCTTT TTCTGGTTGT GGTA-GATGA
Ph.phaseoli     ---------- ---------- ---------- ---------- ---------- AATGCGCTTA TTGGGTGATT TTCCTGCTGT GGCGTGATGG
Ph.infestans    ---------- ---------- ---------- ---------- ---------- AATGCGCTTA TTGGGTGATT TTCCTGCTGT GGCGTGATGG
P.sparsa        ---------- ---------- ---------- ---------- ---------- AG-GCGCTTA TTGGACGTTC TTTCTGCTAT GGCGGTATGG
P.manshurica    ---------- ---------- ---------- ---------- ---------- AG-ACGCTTA TTGGACATTC TTCCTGCCGT GGTGTTATGA
P.destructor    ---------- ---------- ---------- ---------- ---------- AG-GCGCTTA TTGAACGTTC TTCCTGCTAT GGCGGTACGA

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                  725        735        745        755        765        775        785        795        805
[c]PPSAR4       ATAGGTAAAC CGTAGTTGCA TGTGTGACTT GGCTTTTGTA CTTGTATTGT CAGTGCGAAG TAAACCGTCC CCTTCGGTTG TCGAGACGTC
[c]SSH1         ATAGGTAAAC CGTAGTTGCA TGTGTGACTT GGCTTTTGTA CTTGCATTGT CAGTGCGAAG TAAACCGTCC CCTTCGGTTG TCGAGACGTC
[d]R1           ATAGGTAAAC CGTAGTTGCA TGTGTGACTT GGCTTTTGTA CTTGTATTGT CAGTGCCAAG TAAAGCGTCA ACTTCGCTTG TCGAGACGTC
[c]SSP6         ATAGGTAAAC CGTAGTTGCA TGTGTGACTT GGCTTTTGTA CTTGTATTGT CAGTGCGAAG TAAAGCGTCA ACTTCGGTTG TCGAGACGTC
[d]P003         ATAGGTAAAC CGTAGTTGCA TGTGTGACTT GGCTTTTGTA CTTGTACCGT CAGTGCGAAG TAAAGCGTCA ACTTCGGTTG TCGAGACGTC
[a]IP13         ATAGGTAAAC CGTAGTTGCA TGTGTGACTT GGCTTTTGTA CTTGTATTGT CAGTGCGAAG TAAAGCGTCA ACTTCGGTTG TCGAGACGTC
[c]PPSAR6       ATAGGTAAAC CGTAGTTGCA TGTGTGACTT GGCTTTTGTA CTTGTATTGT CAGTGCGAAG TAAAGCGTCA ACTTCGGTTG TCGAGACGTC
[c]Pp9          ATAGGTAAAC CGTAGTTGCA TGTGTGACTT GGCTTTTGTA CTTGTATTGT CAGTGCGAAG TAAAGCGTCA ACTTCGGCTG TCGAGACGTC
[c]P501         ATAGGTAAAC CGTAGTTGCA TGTGTGACTT GGCTTTTGTA CTTGTATTGT CAGTGCGAAG TAAAGCGTCA CCTTCGGTTG TCGAGACGTC
[b]IP05B        ATAGGTAAAC CGTAGTTGCA TGTGTGACTT GGCTTTTGTA CTTGTATTGT CAGTGCGAAG TAAAGCGTCA ACTTCGGTTG TCGAGACGTC
[a]IP09         ATAGGTAAAC CGTAGTTGCA TGTGTGACTT GGCTTTTGTA CTTGTATTGT CAGTGCGAAG TAAAGCGTCA ACTTCGGTTG TCGAGACGTC
Ph.phaseoli     ACTGGTGAAC CATGGCTCT- ---TTAGCTT GGCATTTGAA TCGGCTTTGC TGTTGCGAAG TAGAGTGGCG GCTTCGGCTG CCGAGG-GTC
Ph.infestans    ACTGGTGAAC CATGGCTCT- ---TTAGCTT GGCATTTGAA TCGGCTTTGC TGTTGCGAAG TAGAGTGGCG GCTTCGGCTG CCGAGG-GTC
P.sparsa        ACTGATGAAC CGTAGCTATG CG-TTGACTT GGCTTTTGAA TTGGCTTTGC TGTTGCGAAG TAGAGTGGCA GTTTCAGCTG TCGAGG-GTC
P.manshurica    ACTGATGAAC CGTAGTCATG T--GTGACTT GGCTTTTGAA TCGGCTTTGC TGTTGCGAAG TAGAGTGACA GCTTTTGCTG TCGAGG-GTC
P.destructor    ACTGGTGAAC CGTAGTTCAT GC-ATGACTT GGCTTTTGAA TCGGCTTTGC TGTTGCGAAG TAGAGCGACA GTTTCGGCTG TCGAGT-GTC
```

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
                   815        825        835        845        855        865        875
[c]PPSAR4       GACAAAA--T GAAAAAT-GT GTTGTAAAGC TTCGGCTGCA CCCCACCTCC AATGGACCTG ATATCAGGCA AG
[c]SSH1         GACAAAA--T GAAAAAT-GT GTTGTAAAGC TTCGGCTGCA CCCCACCTCC AATGGACCTG ATATCAGGCA AG
[d]R1           GACCTTT--T GGAAAAT-GT GTTGTAAAGC TTCGGCTGCA CTTCACCTCC AATAGGCCTG ATATCAGGCA AG
[c]SSP6         GACCTTT--T GGGAAAT-GT GTTGTAAAGC TTCGGCTGCA CTTCACCTCA AATGGACCTG ATATCAGGCA AG
[d]P003         GACCTTT--T GAGAAAT-GT GTTGTAAAGC TTCGGCTGCA CTTCACCTCC AATGGACCTG ATATCAGGCA AG
[a]IP13         GACCTTT--T GGGAAAT-GT GTTGTAAAGC TTCGGCTGCA CTTCACCTCA AATGGACCTG ATATCAGGCA AG
[c]PPSAR6       GACCTTT--T GGAAAAT-GT GTTGTAAAGC TTCGGCTGCA CTTCACCTCC AATGGACCTG ATATCAGGCA AG
[c]Pp9          GACCTTT--T GGAAAAT-GT GTTGTAAAGC TTCGGCTGCA CTTCACCTCA AATGGACCTG ATATCAGGCA AG
[c]P501         GACCTTT--T GGGAAAT-GT GTTGTAAAGC TTCGGCTGCA CTTCACCTCC CATGGACCTG ATATCAGGCA AG
[b]IP05B        GACCTTT--T GGGAAAT-GT GTTGTAAAGC TTCGGCTGCA CTTCACCTCA AATGGACCTG ATATCAGGCA AG
[a]IP09         GACCTTT--T GGGAAAT-GT GTTGTAAAGC TTCGGCTGCA CTTCACCTCC CATGGACCTG ATATCAGGCA AG
Ph.phaseoli     GATCCAT-TT GGGAAAT--- GTTGTGTA-C TTCGGTAT-- --GCATCTCA A--------- ---------- --
Ph.infestans    GATCCAT-TT GGGAAAT--- GTTGTGTA-C TTCGGTAT-- --GCATCTCA ATTGGACCTG ATATCAGGCA AG
P.sparsa        GACCCATATT GGGAAATTGT GCTGTGCGGC TTCGGTCGTG TGGCATCTCA A--------- ---------- --
P.manshurica    GACCCAT-TT GGGAATTTGT GCGGTGCGGC TTCTGTCGCG CGGCATCTCA ATTGGACCTG ATATCAGGCA AG
P.destructor    GACCCAT-TT GGGAAATTGT GC-GTGCGAC TTCGGTCGGG TGGCATCTCA ATTGGACCTG ATATCAGGCA AG
```

[a] *Brassica rapa* pathotype
[b] *Brassica juncea* pathotype
[c] *Brassica oleracea* pathotype
[d] *Brassica napus* pathotype

3.3.4 Statistical Analysis of ITS1→ITS4 aligned sequences of the rDNA operon of isolates of *Peronospora parasitica* and related Oomycetes

A large number of sequences were available on the database for ITS1 therefore the final alignment data was analysed in two ways: firstly for the ITS1 region separately and secondly the combined ITS1, 5.8S and ITS2 regions. Using the program Mega 2.1 (Kumar et al. 2001), the frequency of the A,C, G and T residues for the ITS1 data set was found to be 0.26278, 0.18572, 0.23043 and 0.32107 respectively, and the transition/transversion ratio was 1.0588. For the combined ITS1, 5.8S and ITS2 data set the frequency of the A, C, G, T residues was 0.23872, 0.17835, 0.25368 and 0.32925 respectively with a transition/transversion ration of 1.617647. The program Phylip 3.6 was used to analyse each of the data sets. For each of the data sets Seqboot (Phylip 3.6) based on a 1000 bootstraps was used together with DNADIST (Phylip 3.6) to compute a distance matrix based on the F84 model (Kishino and Hasegawa, 1989; Felsentein and Churchill 1996) distance Algorithm; the distance for each pair of isolates/species estimates the total branch length between the two species. These distances were then used in the distance matrix program, Neighbour (Nei and Saitou 1987, cited by Felsentein 2001); Neighbor constructs a tree by successive clustering of lineages, setting branch lengths as the lineages join - in this study a square distance matrix was used. The program CONSENSE was then used to read the file of computer-readable trees and print out (and may also write out onto a file) a consensus tree. The consensus trees (Figs 3.7 and 3.8) are radial unrooted and the distances were transferred from the distance analysis to give a more accurate representation of the similarities between the isolates/species.

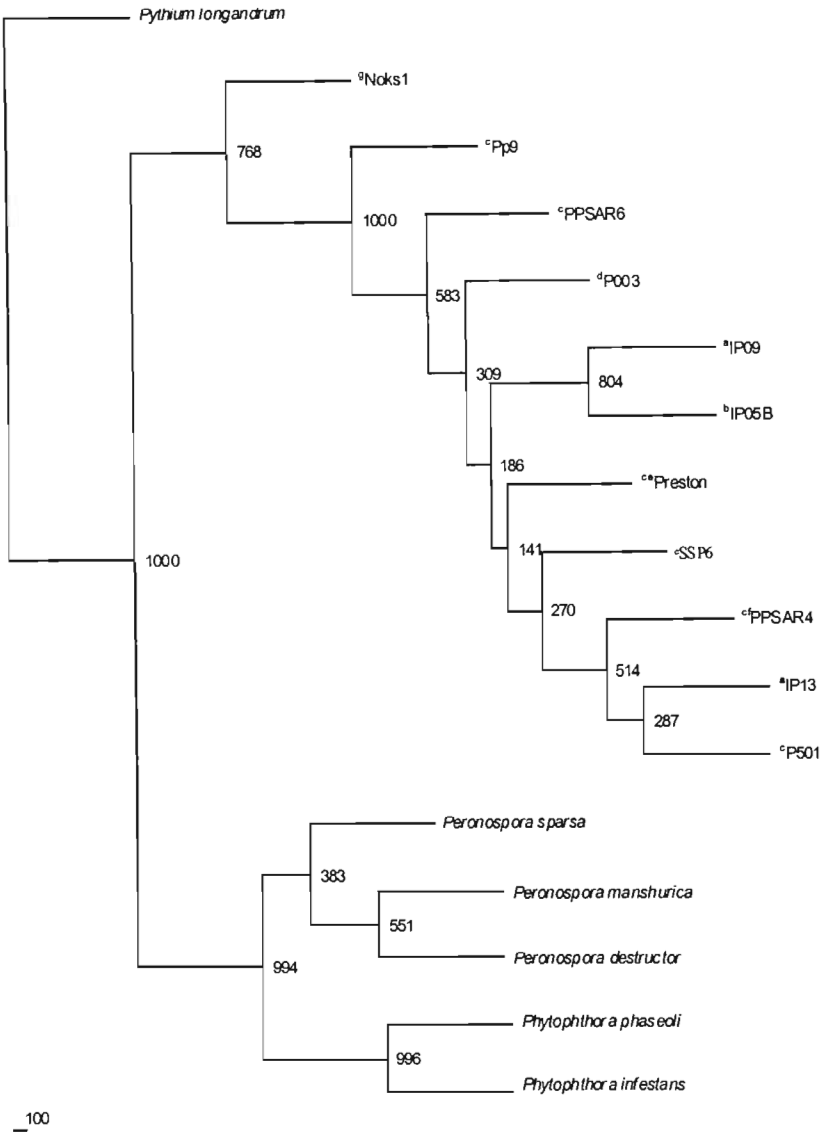

Fig. 3.7: ITS1 Cladogram of four *Peronospora* species (including 4 pathotypes of *P. parasitica*), two species of *Phytophthora*, and one Pythium species.
Peronospora parasitica isolates:
[a] *Brassica rapa* pathotype; [b] *Brassica juncea* pathotype
[c] *Brassica oleracea* pathotype [d] *Brassica napus* pathotype
[e] Preston = sequence identical to *Brassica oleracea* isolates P005, Bb, P006, P204, P211, P216, P218, P502, P501, P217, P214 (ncbi database)
[f] PPSAR4 = sequence identical to *Brassica oleracea* isolate SSH1
[g] *Arabidopsis thaliana* pathotype.

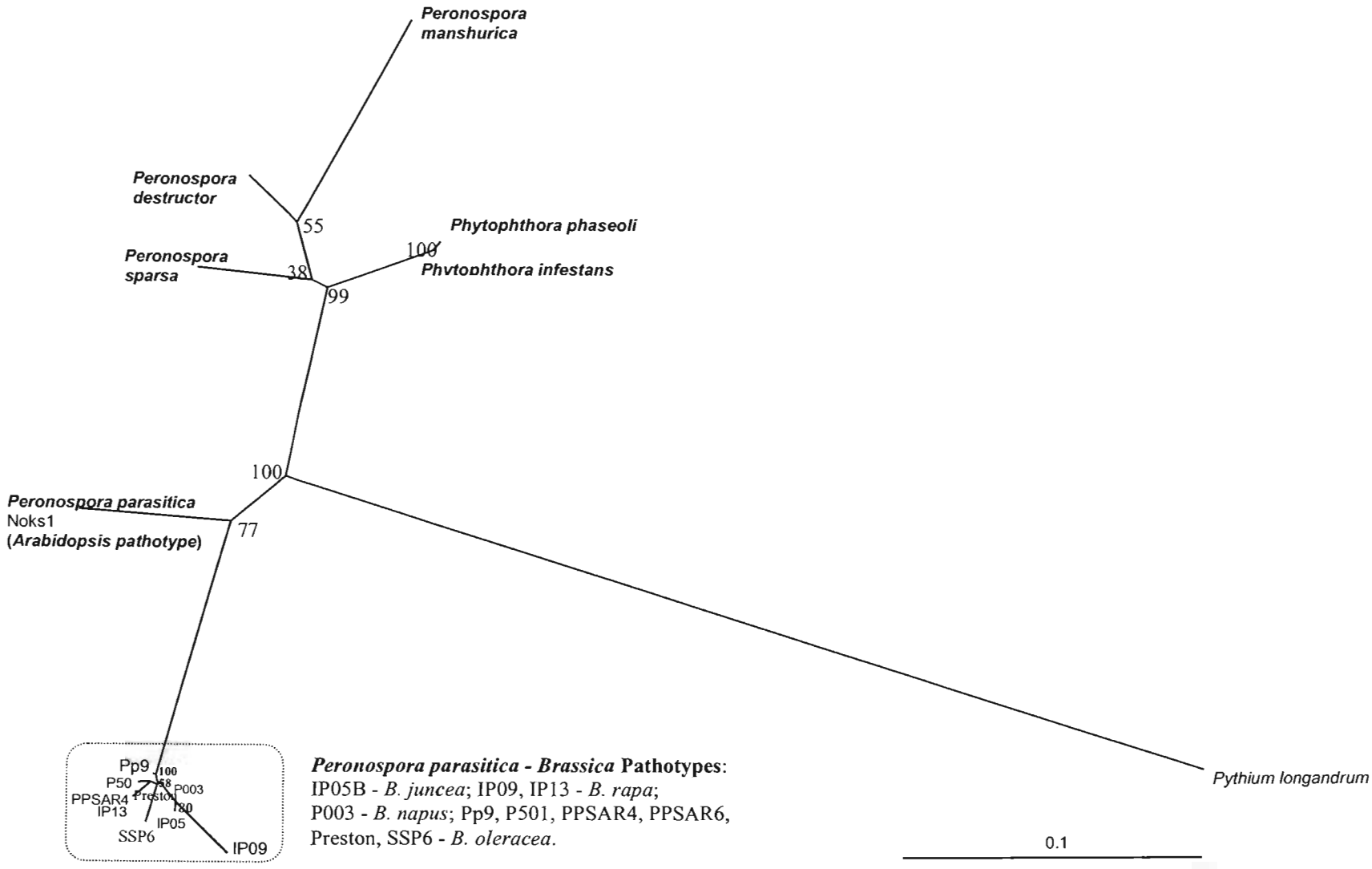

Fig. 3.8: Radial Tree of Fig 3.7 showing the evolutionary trends among 21 isolates of *Peronospora parasitica* in relation to members of the genus *Peronospora* and other Oomycetes, *Phytophthora* and *Pythium*, constructed after distance based analysis of the ITS1 region of the ribosomal RNA operon. The isolate Preston (ncbi) was identical to *Brassica oleracea* isolates P005, Bb, P006, P204, P211, P216, P218, P502, P501 (ncbi), P217, P214.

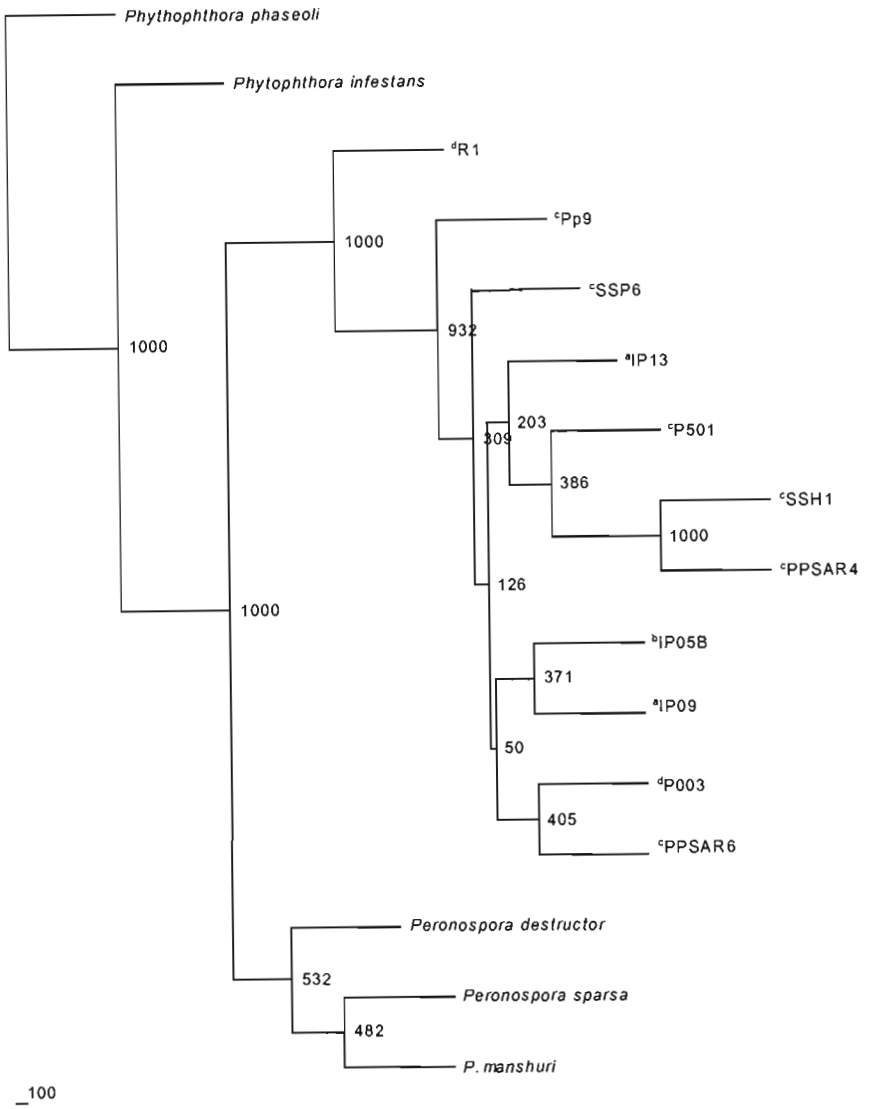

Fig. 3.9: ITS1, 5.8S and ITS2 Cladogram of 4 *Peronospora* species (including 4 pathotypes of *P. parasitica*) and 2 species of *Phytophthora*.

Peronospora parasitica isolates:

[a] *Brassica rapa* pathotype

[b] *Brassica juncea* pathotype

[c] *Brassica oleracea* pathotype

[d] *Brassica napus* pathotype.

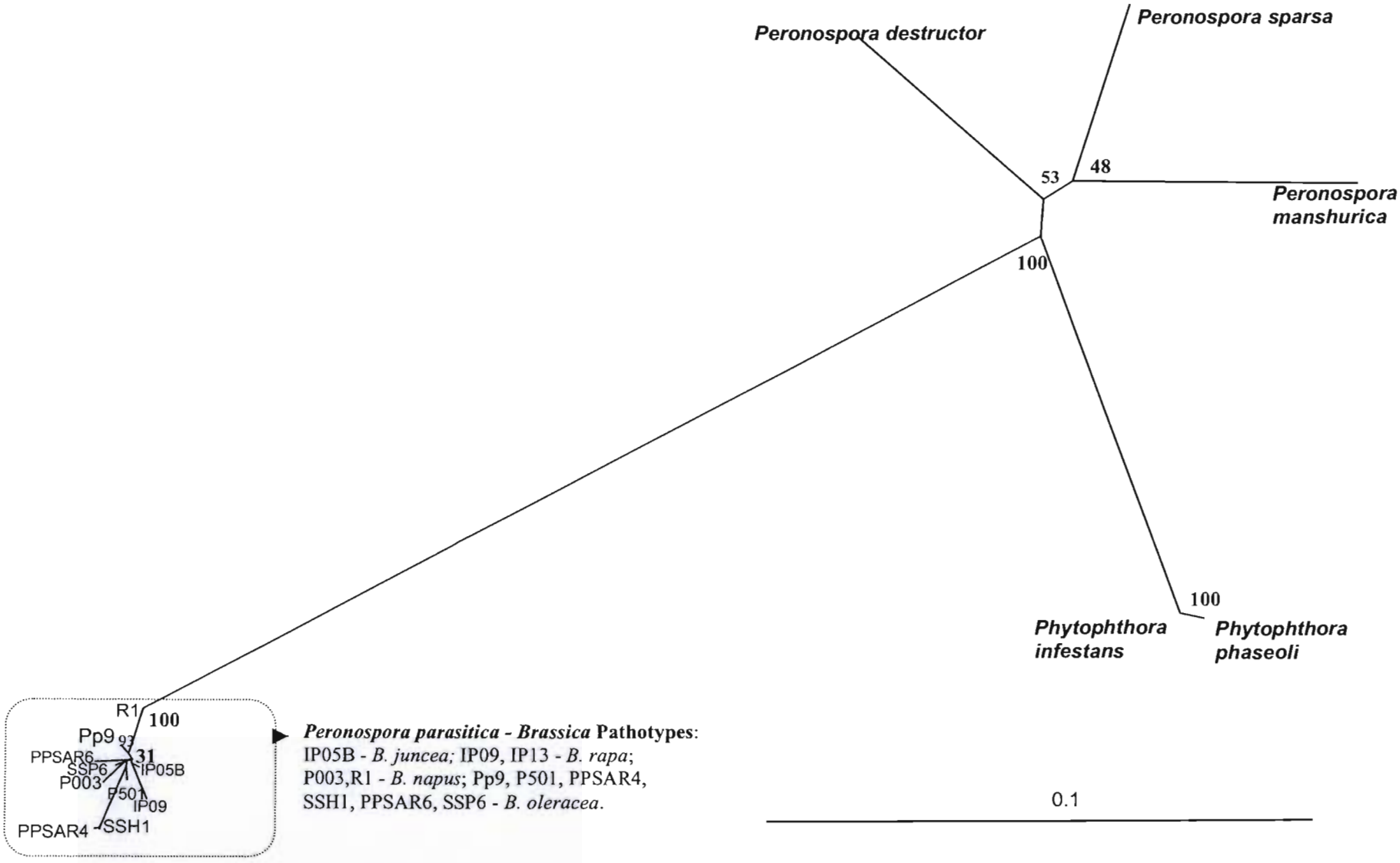

Fig. 3.10: Radial Tree of Fig. 3.9 showing the evolutionary trends among 11 isolates of *Peronospora parasitica* in relation to members of the genus *Peronospora* and the Oomycete, *Phytophthora*, constructed after distance based analysis of the ITS1, 5.8S and ITS2 regions of the ribosomal RNA operon.

3.4 DISCUSSION

The current study provides the first comprehensive ITS-based phylogeny of pathotypes of *Peronospora parasitica*. Early theories regarding host-adapted types of this diverse pathogen were tested using molecular evidence.

Phylogenetic analysis of eukaryotes based on the internally transcribed spacer (ITS) regions of rDNA has in the last decade challenged many of the traditional methods based on morphology. Evidence obtained by PCR and sequencing provides new insights into the evolutionary biology of pathogens. In the present study ITS-PCR analysis clearly separates *P. parasitica* isolates from other *Peronospora* species as well as Oomycetes such as *Pythium* and *Phytophthora* (Figs. 3.9 and 3.10). Sequence analysis of the combined ITS1, 5.8S and ITS2 regions of the ribosomal DNA operon (Fig. 3.10) revealed that three genera *Phytophthora*, *Pythium* and *Peronospora* are distinct entities. The radial distance trees generated for the ITS1 and the combined ITS1, 5.8S and ITS2 are very similar to each other. Based on ITS1 analysis *Pythium* is clearly an outlier group. Based on combined ITS sequence distance analysis, the *Phytophthora* species were more closely related to *Peronospora manshurica*, *Peronospora sparsa* and *Peronospora destructor* than *Peronospora parasitica*. This reiterates recent observations (Cooke et al. 2000) that support Gaümann's (1952) theory that biotrophic downy mildews evolved relatively recently from a hemibiotrophic or nectrotrophic *Phytophthora* ancestor. This is further supported by suggestions (Cooke et al. 2000) that *Peronospora* appears to be a biotrophic *Phytophthora* lineage that has lost the ability to produce zoospores.

The majority of the *P.parasitica* isolates formed a monophyletic group. Based on ITS1 analysis a significant difference was found between the *P. parasitica* isolates from *Arabidopsis thaliana* and *P. parasitica* isolates of *Brassica* pathotypes. This correlates with the findings of Rehmany et al. (2000) which indicate that isolates of the *Brassica oleracea* pathotype are divergent from those from *Arabidopsis thaliana*. This observation reinforces the scepticism of Rehmany et al (2000) concerning the

amalgamation of crucifer downy mildews as the single aggregate species *P. parasitica*. The results of the present study provide the first genetic evidence that isolates of the *Brassica* pathotypes are more evolutionary conserved. Isolates R1 of the *B. napus* pathotype and Pp9 of the *B. oleracea* pathotype were >70% similar as was evident by their genetic distances (Fig. 3.10).

Phylogenetic systematics attempts to understand evolutionary interrelationships of living things and interpret the way in which life has diversified and changed over time. Molecular analysis focussing on the genes which code the small subunit rRNA has in recent years elucidated many new theories of the mechanisms of evolution. Phylogenetic analysis based on rDNA has challenged traditional taxonomy of many organisms at various levels and represents the most comprehensive trees to date (Cavalier-Smith, 1993, Sogin and Silberman 1998). Eukaryotes have been subdivided into four kingdoms: animals, plants, fungi and protists. Appendix B provides an overview of the major lineages of eukaryotes. The plant, animal and fungal kingdoms are well-defined monophyletic groups, but the "Kingdom Protista" is not monophyletic; it contains organisms that are more closely related to other kingdoms than they are to other protists. The protists include Alreolates, Stramenopiles, Rhodophyta and other protists such as Cryptomonads (Appendix B.1). Recent studies of protist DNA and ultrastructure has shown that the protists are far more diverse than had been previously expected and should probably be classified in several kingdom-level taxa.

The Oomycetes were once classified as fungi, because of their filamentous growth, and because some feed on decaying matter like true fungi. The cell wall of Oomycetes, however, is not composed of chitin, as in the fungi, but is composed of cellulosic compounds and glycan. The nuclei within the hyphae are not haploid as in the fungi. The ultrastructure, biochemistry, and molecular sequences of these organisms indicate that they belong with the Kingdom Chromista. The Oomycetes incorporate approximately 500 species which include the water molds and downy mildews (Appendix B1 and B2). The Oomycetes are classified within the Stramenopiles and separate from the fungi (Patterson and Sogin 2002).

The most comprehensive trees are those based on 16S ribosomal RNA (Patterson and Sogin 2002) (Appendix A). Phylogenetic analyses at the kingdom, genus and species level was possible using this important discriminatory tool (Beck and Lignon 1995, Bunyard et al. 1994, Forster et al. 1990, Klassen et al. 1996, Mitchell et al. 1995). Furthermore molecular systematics based on rDNA sequences is continuously challenging and refining existing classification systems (Chen 1995, Paul 2001, Viljoen et al. 1993). For example phylogenetic analyses of rDNA sequences demonstrated that *Phytophthora* was more closely related to *Peronospora* than to *Pythium* and suggested that it be moved from the order Pythiales to Perononosporales (Peterson and Rosendahl 2000, Cooke et al. 2000). The results of the present study are concordant with this theory. Furthermore, analysis of *Pythium* revealed that *Pythium arrhenomanes* is more closely related to *Lagenidium giganteum* than to *Phytophthora megasperma*, which disagrees with the current classification system (Chen 1995). More recently, *Pythium longandrum* was found to be entirely different from all other species of *Pythium* (Paul 2001) based on analysis of the ITS region of rDNA.

The above observations demonstrated that ITS-PCR is a useful tool for discrimination of Oomycetes at the species level (Cooke et al. 2000, Rehmany et al. 2000). The resolution of *P. parasitica* based on both ITS1 as well as the combined ITS1, 5.8S and ITS2 analyses in the present study further reiterate this (Figs. 3.2-3.9). Furthermore the *Arabidopsis thaliana* pathotype could be discriminated from the *Brassica* pathotypes which is similar to the finding of Rehmany et al. (2000). However overall there was a lack of definition at the pathotype level as it did not allow differentiation of the *Brassica* pathotypes (Figs. 3.7-3.9).

Specificity of *P. parasitica* on its various hosts is a well documented phenomenon. Isolates of *P. parasitica* are morphologically similar (Yerkes and Shaw 1959, Jugmohan 1997) yet more virulent on their species of origin (Kluczewski and Lucas 1983, Nashaat and Rawlinson 1994, Nashaat and Awasthi 1995, Silué et al. 1996). Host-range experiments are insufficient for fully differentiating the host-adapted pathotypes of *P. parasitica* (Waterhouse 1973, Dickinson and Greenhalgh 1977).

Furthermore, the results of the present study indicate that ITS-PCR provides limited information on pathotypes of *P. parasitica*. This is as expected since analysis of intraspecies variation necessitates molecular markers with a higher degree of polymorphism. Molecular markers such as RAPDs and AFLPs have been found to be useful in assessing variation between *P. parasitica* isolates (Tham et al. 1994, Rehmany et al. 2000). Both RAPDs (Tham et al. 1994) and AFLPs together with ITS-PCR (Rehmany et al. 2000) were able to differentiate between isolates from *B. napus* and *B. oleracea*. Further investigations involving molecular tools with a higher discriminatory power such as AFLPs and microsatellites are required to further elucidate genetic relationships between isolates of the *B. oleracea*, *B. napus*, *B. juncea*, *B. rapa* and *Arabidopsis thaliana* pathotypes.

CHAPTER FOUR

ISOLATION OF MICROSATELLITES FROM *Peronospora parasitica*

4.1 INTRODUCTION

Microsatellites, also known as short tandem repeats (STRs) or simple sequence repeats (SSRs) occur as tandem repeats of di-, tri-, tetra- and penta-nucleotides. They may consist of 10-50 copies of motifs from 1-6 bp and are usually less then 100 bp long. Microsatellites are ubiquitous in eukaryotic genomes (Gross and Garrard 1986, Tautz and Renz 1984), however they can occur to lesser extent in prokaryotes or eubacteria (Tautz 1989). Surveys of Genbank have revealed long polymorphic microsatellites in simple organisms such as lower eukaryotes and prokaryotes, viz., the slime molds, fungi, protists, prokaryotes, viruses and plasmids (Field and Wills 1996).

The different types of microsatellites found in eukaryotes include dinucleotides $(AT)_n$, $(CA)_n$, $(CT)_n$, trinucleotides $(TCC)_n$ and tetranucleotides $(GACA)_n$, $(GA^C/_TA)_n$, $(GATA)_n$, $(CGAT)_n$, , where n is the number of repeating units within the microsatellite locus (Epplen 1988, Ostrander et al. 1992, Gross and Garrard 1986). Of the most studied repeats $(GT)_n$, $(GA)_n$, $(GATA)_n$ and $(GACA)_n$, the copy number of $(GT)_n$ per haploid genome varies from 100 copies in yeast to 100 000 copies in the mouse genome (Stallings et al. 1991). In the human genome there are 50,000-100,000 interspersed $(CA)_n$ (i.e. $(TG)_n$, $(GT)_n$, $(AC)_n$)* blocks, with the range of n being roughly 15-30 (Tautz and Renz 1984, Gross and Garrard 1986, Litt and Luty 1989). Computer database searches of microsatellites in the yeast chromosome III revealed the occurrence of AT dinucleotide repeats at a high frequency (Valle

* CA and AC are permutations of the same repeat. Similarly, TG and GT are permutations. Databases select reverse compliments automatically. Therefore, AC = CA = GT = TG, and TC = CT = GA = AG (Pentcheff 1999).

1993). In comparison Genbank surveys revealed a predominance of trinucleotide microsatellites in lower eukaryotes and prokaryotes (Field and Wills 1996).

Microsatellite repeat sequences are thought to arise by unequal crossovers or slippage-like events during DNA replication or DNA repair (Tautz 1990). Free ends are transiently created during the replication process. If such a free end lies within a simple sequence stretch, it may basepair out of register and when subsequent elongation occurs, the stretch may be longer or shorter than before (Tautz 1990). The analysis of slippage synthesis of simple sequence DNA *in vitro* (Schlötterer and Tautz 1992) sheds some light the question of how simple sequences arise *in vivo*. Schlötterer and Tautz (1992) demonstrated that it is possible to synthesise all types of repetitious di- and trinucleotide motifs starting from short primers and a polymerase *in vitro*. A basic PCR reaction was employed utilising the use of γ-^{32}P-ATP labelled primers such as CA_9. It was evident that the fragments grew continously with a constant rate until the components were exhausted. The length variability of the fragments generated in the reaction is very small at any given time point. It was only when the synthesis cycle was repeated on the original product that apparent secondary reaction products started to accumulate. Synthesis was found to be independent of the length of the primer fragments and depended therefore only on slippage occurring at the ends of these fragments. Simple sequences with a tri-nucleotide repeat unit behaved in a similar way as the dinucleotide repeats. It was however evident that the growth of the tri-nucleotide repeat products was slower, which suggested that the slippage rate was lower for these repeats. Overall the results supported the notion that *in vitro* synthesis of simple sequences proceeds via slippage reactions. These produce continously unpaired free ends which can be filled up by a DNA polymerase. Slippage synthesis also occurs on a fixed template where only one strand is free to move, a situation which resembles chromosome replication *in vivo*. It seems therefore likely that slippage during replication is the cause of the observed length polymorphism of simple stretches between individuals of a population (Schlötterer and Tautz 1992).

A number of models have been proposed for replication slippage (Walsh 1987, Tachida and Iizuka 1992, Valdes et al. 1993, Shriver et al. 1993). If the number of repeats increases, the rate of slippage is thought to increase because the probability of

mispairing increases. However, in a population genetic model developed by Tachida and Iizuka (1992), this was found to make little difference at equilibrium, especially for a large number of repeats, as long as a linear function was used for the slippage rate and assuming selective neutrality with regards to repeat number. However, sequences were also found which did not fit the model. In contrast to unequal crossing over between homologous chromosomes (Purandare and Patel 1997), replication slippage is a process which does not involve the homologous chromosome. Thus it is considered to be a type of mutation process and two models have been used to study the population genetics of VNTR loci viz. the infinite allele model (IAM) and the stepwise mutation model (SMM) (Valdes et al. 1993, Shriver et al. 1993). Valdes and co-workers (1993) surveyed frequency distribution of microsatellites at 108 loci and found this to be consistent with the stepwise mutation model. The model makes the assumption that mutations cause an increase or decrease in repeat number by one and under the condition that the product Nu, where N is the effective population size and u is the mutation rate, is larger then one. It was further shown that the variance of the distribution of allele sizes is a useful estimator of Nu and suggests that the mutation rate at these loci is independent of allele size (Valdes et al. 1993). However, taking into consideration a closer look at microsatellite frequency distributions, Shriver et al. (1993) found that not all measures (number of alleles, number of modes and range in allele size) followed the SMM strictly as each of the measures are subject to different mutational forces. Longer repeats (3-5 bp) were found to follow the SMM model more closely whereas 35 % of shorter microsatellites (1-2 bp) were different from the simulations for at least one of the three summary measures Shriver et al. (1993). This was predicted to be due to slipped strand mispairing.

More recently, Toth and co-workers (2000) reported that strand-slippage theories alone are insufficient to explain microsatellite evolution in the genome as a whole. Both slippage reactions and unequal crossovers would lead to a constant formation and deletion of simple sequences and one would expect to find them in all regions of the genome which do not undergo strong selection (Tautz and Renz 1984). Hence the occurrence of simple sequences in eukaryotes is not a matter of evolutionary conservation but instead depends on a number of factors including the (i) the frequency of accidental amplifications and deletions, (ii) the extent to which such

mechanisms spread the sequences between homologous chromosomes, (iii) the degree to which the sequences are tolerated in the genome and (iv) on the amount of possible formation sites for simple sequences, namely redundant DNA. The absence of large amounts of simple sequences in prokaryotes could be due to any one of these factors, singly or in combination (Tautz and Renz 1984).

Microsatellites share some of the properties of minisatellites. Minisatellite regions are composed of tandem repeats of a short (8-90) bp repeated sequence; allelic variation at these loci also derives from variation in the number of tandem, repeats, and hence in the length of the region (Armour and Jeffreys 1992). Since both minisatellite and microsatellite alleles vary in the number of constituently repeated elements a general designation for both is variable number of tandem repeat loci (VNTRs). Microsatellites have several advantages over minisatellites. Minisatellites are much larger and usually the repetitive elements within minisatellites may be as large as 200 bp with minisatellite allele sizes up to 50 kb, thus only a subset of the variation may be analysed by PCR. Being much smaller than 1 kb and varying over a narrow size range, microsatellites could be readily analysed by PCR and electrophoresis circumventing the need for large quantities of DNA and Southern blotting. In characterising allelic variability at microsatellite loci PCR products may be analysed on polyacrylamide gels which allows the resolution of alleles which differ by as little as 1 bp and several loci can be analysed simultaneously on a single gel (Armour and Jeffreys 1992, Ciofi et al. 1998).

Because of the great variability in repeat number at various loci, microsatellites are ideal markers for mapping, DNA forensic studies and population studies. In humans, especially, microsatellites have attracted much attention because certain simple sequences (viz. trinucleotide repeats) were shown to be associated with disease genes when they are abnormally expanded. Examples where such instability of repeats have been found include the fragile-X mental retardation syndrome, myotonic dystrophy, spino-bulbo-muscular dystrophy and Huntingtons disease (Brook et al. 1992, Valdes et al. 1993, Rubinsztein et al. 1995). An assessment of microsatellites in human and other vertebrates revealed inter-species and intra-species polymorphism at most loci (Rubinsztein et al. 1995). When allele lengths at different

loci were compared between humans and chimpanzees, 33 were longer in humans compared to seven that were longer in the chimpanzees. Similar significant excesses of longer human alleles were found with gorillas, oranguatans and macaques. Thus a directionality in the evolution of microsatellites was suggested, as opposed to there being an equal probability for microsatellite loci to increase or decrease in length over time. In addition, the consistent length difference observed also requires a difference in the rate of microsatellite evolution between human and other primates (Rubinsztein et al. 1995). Microsatellites have been found be widespread in eukaryotes (Hamada et al. 1982, Tautz and Renz 1984, Toth et al. 2000), particularly in plants (Beyermann et al. 1992) and fungi (Groppe et al. 1995, Field and Wills 1996). More recently microsatellites have been shown to be extremely useful in studies on fungal diversity (Morjane et al. 1994, Hantula et al. 1996, Longato and Bonfante 1997, Geistlinger et al. 1997a,b, Müller and Hantula 1998, Barve et al. 2001). An extensive review of the occurrence and utility of microsatellites in fungi is given in Chapter Six.

The mutation rate of microsatellites is estimated to be between 5×10^{-4} and 5×10^{-5}, which is low enough to permit their use in linkage studies, as probes for linkage disequilibrium with disease mutations in population studies, and in forensic applications (Dietrich 1992, cited by Hearne et al. 1992). In comparison, the mutation rate for minisatellites increases with variability in accord with the neutral mutation/random drift hypothesis and rises to 5% per gamete for the most unstable human minisatellite isolated (Jeffreys et al. 1988). In addition to occurring at many different loci, microsatellites may also be polyallelic (Barroso et al. 2000, Jones et al. 1997). Furthermore, analysis of a number of markers from families indicated co-dominant Mendelian inheritance for all families (Weber and May 1989, Litt and Luty 1989).

In general, simple repeat sequences do not have a defined function in the genome. They are included in those classes of DNA which do neither harm nor good and are often referred to as "junk" DNA (Tautz 1990). They may however reflect the occurrence of genetic "change" via the production or deletion of these sequences. Microsatellites may be flanked by unique sequences. In yeast AT repeats showed a close association with the core consensus of autonomously replicating sequences

(Valle 1993). Interestingly, 50% of microsatellites found in lower eukaryotes and prokaryotes were found in exons (Field and Wills 1996). More specifically, $(CA)_n$ repeats were occasionally located within protein coding regions, but very often were found within introns or between genes (Weber and May 1989). Various functional roles have been attributed to SSRs; for example, hot spots of recombination (Purandare and Patel 1997, Kabori et al. 1986; Bullock et al. 1986) and the regulation and expression of genes (Hamada et al. 1984, Murphy et al. 1989).

The ubiquitous presence of microsatellites in eukaryotes was first determined by DNA fingerprinting using microsatellite-specific oligonucleotides as hybridisation probes to characterise individual strains or genotypes (Beyermann et al. 1992, DeScenzo and Harrington 1994, Morjane et al. 1994). However, these methods primarily targeted a single microsatellite species which produced only a small number of clones thus resulting in markers which did not detect the level of polymorphism necessary for diversity studies (Edwards et al. 1996). Both yeast like and filamentous fungi have been investigated using microsatellite-primed PCR (also referred to as random amplified microsatellites (RAMS)) (Couto et al. 1996, Hantula et al. 1996, Hantula and Müller, 1997, Longato and Bonfante 1997, Müller and Hantula 1998, Geistlinger et al. 1997b). Only a few recent studies have been devoted to cloning of fungal microsatellites and/or the generation of STS markers (Groppe et al. 1995, Geistlinger et al. 1997b, Barroso et al. 2000, Burgess et al. 2001).

Generation of a high-density map of markers for an entire genome or a single chromosome requires the isolation and characterisation of hundreds of markers such as microsatellite repeats (Ostrander et al. 1992). Two simple yet tedious approaches have generally been used for this task. The first approach was to screen a large-insert library with an end-labelled $(CA)_n$ or $(TG)_n$ oligonucleotide (n >15). Clones that hybridized to the probe were purified and divided into subclones, which were then screened by hybridization for a fragment containing the repeat. The fragment was then sequenced, and a sequence tagged site (STS) was created by choosing unique primers that flanked the repeat and produced a fragment of convenient, discrete size upon amplification by PCR. The drawbacks of this method was the requirement for many blot hybridizations and the difficulty of sequencing the relatively large

subclones. An alternative approach was to construct a small insert (200-500 bp) genomic library constructed in a plasmid vector (Ostrander et al. 1992, Jones et al. 1997). The library was then screened with a number of microsatellite probes to identify inserts carrying SSRs. The inserts were then sequenced and primers chosen which match unique flanking sequences for particular loci. PCR amplification was used to generate DNA banding patterns on a gel and to reveal the polymorphism based on different numbers of repeats at the alleles of a locus. The expected frequency of $(CA)_n$ repeats in this small-insert library was low, about 1 per 100-400 colonies. Consequently, large numbers of plates needed to be screened at relatively low densities to obtain a significant pool of markers. To overcome the limitations of these approaches, many workers have developed efficient methods for genetic selection of libraries that are highly enriched for microsatellite sequences (Ostrander et al. 1992, Karagozov et al. 1993, Kijas et al. 1994, Edwards et al. 1996, Burgess et al. 2001, Mba et al. 2001).

Ostrander et al. (1992) constructed a genomic library with an average insert size of less than 500 bp in a phagemid vector. Amplification of this library in a *dut ung* strain of *Escherichia coli* allowed the recovery of the library as closed circular single-stranded DNA with uracil frequently incorporated in place in thymine. This DNA was used as a template for second-strand DNA synthesis, primed with $(CA)_n$ or $(TG)_n$ oligonucleotides, at elevated temperatures with a thermostable DNA polymerase. Transformation of this mixture into wild-type *E. coli* strains resulted in the recovery of primer-extended products as a consequence of the strong genetic selection against single-stranded uracil-containing molecules. In this manner, a library highly enriched for the targeted microsatellite-containing clones was obtained. Furthermore colony hybridisation with the radiolabeled $(CA)_{15}$ oligonucleotide resulted in a 50 fold enrichment of CA repeats.

An alternative enrichment method by Kijas and co-workers (1994) used synthetic oligonucleotide probes bound to magnetic beads in solution to hybridise complementary microsatellite core sequences. Following stringent washing the bound sequences were released, amplified by PCR, blotted on nylon membranes and hybridised with the biotinylated probe $(TAA)_8$. This resulted in a microsatellite

enriched sequence population suitable for cloning and screening in the conventional manner. Karagyyozov et al. (1993) developed an efficient method for constructing random small-insert genomic libraries enriched for $(CA)_n$ repeats. The method involved fragmentation of DNA by sonication and ligation of an adaptor to the fragments followed by PCR amplification and enrichment for $(CA)_n$ repeats by hybridization to $(GT)_{15}$ oligonucleotides bound to a nylon membrane. The degree of enrichment achieved was 50 fold. Success in isolation of (CA)*n* repeats was also achieved by Kölliker et al. (2001). Sequence analysis of 1123 clones from genomic library of *Trifolium repens* L. enriched for $(CA)_n$ repeats yielded 793 clones containing SSR loci. The majority of SSRs consisted of perfect dinucleotide repeats consisted, only 7% being trinucleotide repeats. After exclusion of redundant sequences and SSR loci with less than 25 bp of flanking sequence, 397 potentially useful SSRs remained. Primer pairs were designed for 117 SSR loci and PCR products in the expected size range were amplified from 101 loci. These markers were highly polymorphic, 88% detecting polymorphism across seven white clover genotypes with an average allele number of 4.8.

Of significant note is that the above workers (Karagyozov et al. 1993, Kijas et al. 1994, Ostrander et al. 1992, Kölliker et al. 2001) have achieved enrichment of a single type of microsatellite only. Since little is known about the level of polymorphism of individual microsatellite sequences Edwards et al. (1996) suggested that this approach resulted in markers that do not necessarily detect the level of polymorphism often required in diversity studies. Furthermore, targeting of a single microsatellite species produced only a small number of clones necessitating the construction of further libraries in the future.

Edwards and co-workers (1996) have modified these methods to yield clones that contain a variety of microsatellites. Microsatellite libraries of various plant genomes suggested that the clones produced by this procedure were sufficiently polymorphic to be used in population genetic and/or breeding studies. The enrichment procedure for such multiplex libraries employed hybridisation of restricted, ligated and pre-amplified DNA to a membrane containing the various bound oligonucleotides *viz.* $(GA)_{15}$, $(GT)_{15}$, $(AT)_{15}$, $(GC)_{15}$, $(CAA)_{10}$, $(CATA)_{10}$,

$(ATT)_{10}$, $(GATA)_{10}$, $(GCC)_{10}$, $(ATAG)_{10}$. Mba et al. (2001) have reported similar methods with much success. Mba et al. (2001) used two methods to enrich for microsatellites in the cassava (*Manihot esculenta* Crantz) genome. The first method yielded 35 SSR loci, for which primers could be designed, out of 148 putative DNA clones. A total of 137 primer pairs could be designed from 544 putative clones sequenced for the second enrichment. Overall, 172 new SSR markers for the cassava genome were developed. Most of the SSRs (95%) were di-nucleotide repeats, and 21% were compound repeats. The major disadvantages of SSR development using various enrichment methods were the redundancy (20% duplication) and in addition, often primers could not be designed for many SSR loci that were too close to the cloning site (45% of total) (Mba et al. 2001).

The use of sequence information by designing primers flanking microsatellite repeats reveals single base-pair polymorphism and has proven successful in distinguishing individuals of vertebrate species (Fries et al. 1990, Love et al. 1990, Weber 1990). Furthermore it has enabled host-specific differentiation in the anther smut fungus *Microbotryum violaceum* (Bucheli et al. 2000). An additional, potentially valuable characteristic of microsatellites (and one not shared by minisatellites) is that primers developed in one species could be used in related taxa. PCR primers based on microsatellites identified in one whale species have been successfully amplified in many other related species and some cattle primers are useful in sheep. Primers based on SSRs have been designed and used in SPARSs (single primer amplification reactions) to demonstrate polymorphism across a panel of evolutionary diverse genomes including grapes, lettuce, tomato, pine, maize, salmon and chicken (Bruford and Wayne 1993). If many microsatellites prove to have a wide taxonomic range less time and effort will be expended in the development and screening of genomic libraries.

Microsatellite sequences are homogeneously distributed markers, which were found to be easily distributed and, readily transferred between laboratories. Microsatellites have the advantage of being co-dominant. In addition they are simple, PCR-based and extremely polymorphic, and highly informative due to the number and frequency of alleles detected and to their ability to distinguish between closely related

individuals. These attributes qualify microsatellites as attractive tools for mapping, cultivar identification, protecting germplasm, determination of hybridity, analysis of genepool variation, and as diagnostic markers for traits of economic value (Jones et al. 1997).

The aim of the current chapter was prepare a genomic DNA library from *Peronospora parasitica* enriched for microsatellites.

4.2 MATERIALS AND METHODS

A genomic DNA library from *Peronospora parasitica* enriched for microsatellites was prepared using the modified method of Edwards et al. (1996). Fig. 4.1 provides an overview of the methods used for preparation of the library.

4.2.1 Axenic cultures of *Peronospora parasitica* and extraction of genomic DNA

An isolate of *Peronospora parasitica*, P005, of the *Brassica oleracea* pathotype was utilised in the preparation of the genomic DNA library enriched for microsatellites. Isolate P005 was a single spore of P1 mating type collected from Lincolnshire, UK (Table 2.1). P005 was maintained on cv. Hercules F1 hybrid as described in Chapter Two.

An axenic culture of P005 suitable for DNA isolation was prepared as described in 2.2.3. *B. oleracea* (cv. Hercules F1 hybrid) seeds were surface sterilised in 3% sodium hypochlorite and grown in autoclaved pots (1 kPa, 121°C for 15 min.) at 25°C in the greenhouse. Seven day old seedlings were inoculated with a conidial suspension (5 x 10^5 conidia/ml) of isolate P005 and incubated in a Schnidjers Climatic Chamber. The climatic chamber was programmed with a light dark cycle; 9 hr dark and 15 hr light (100 $\mu Es^{-1}m^{-2}$) at a constant temperature of 16°C. Approximately 24 hrs after inoculation cotyledons were excised and surface sterilised in 70 % ethanol and 1% sodium hypochlorite. Cotyledons were then dried briefly on sterile filter paper and placed on Murashige and Skoog (1962) medium. Seven days after inoculation, conidia were collected by tapping spores onto sterile water which were then concentrated by centrifugation.

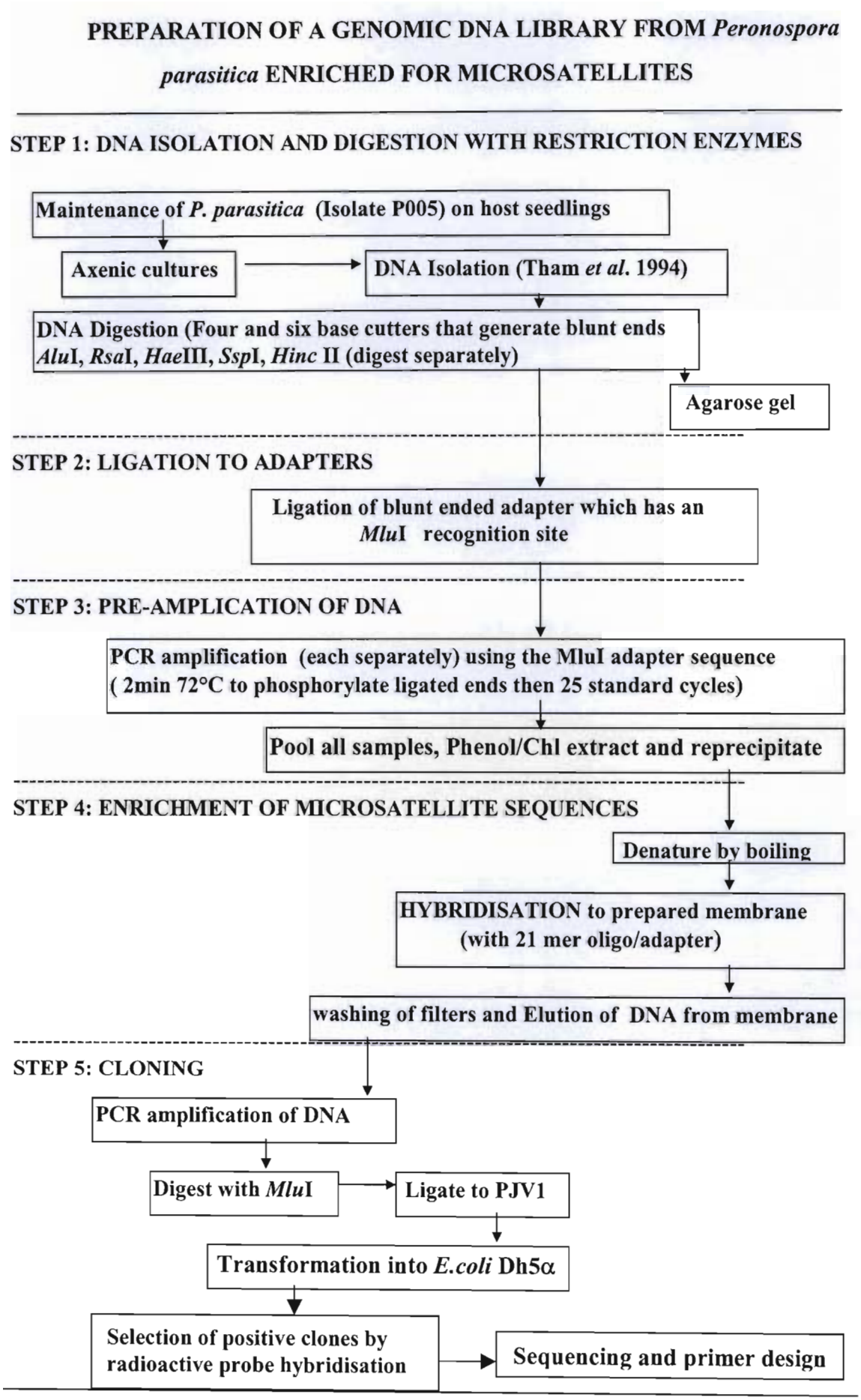

Fig. 4.1: Diagrammatic representation of the isolation of microsatellites from *Peronospora parasitica* (after Edwards et al. 1996).

Good quality genomic DNA was extracted from conidia of isolates of *P. parasitica* (Tham et al. 1994) and from control host DNA (Edwards et al. 1991) as described in 2.2.4. *P. parasitica* conidia, collected as described, were centrifuged and the conidial pellet washed once in sterile distilled water. Clean conidia were then vortexed for 1 min. with a mixture of 1- and 6 mm diameter glass beads (Sigma) in 600 µl lysis buffer (100 mM tris-HCl (pH 7.20), 100 mM EDTA, 10 % (w/v) SDS and 2 % (v/v) 2-mercaptoethanol). DNA was recovered from the suspension of broken conidia using the protocol described by Lee and Taylor (1990) (2.2.4). The suspension was incubated at 65 ^{O}C for 1.5 hr and vortexed every 20 min. for 30 sec. To remove the protein and cell debris, 600 µl chloroform:phenol (1:1) was added, vortexed briefly, and microcentrifuged at 10,000 x gr for 15 min. at room temperature or until the aqueous (top) phase is clear. Approximately 300 to 500 µl of the aqueous phase containing the DNA was transferred to a new tube. Precipitation of DNA was achieved using 10 µl 3 M sodium acetate and 0.54 volumes isopropanol and centrifugation at 10 000 x gr for 2 min. DNA pellets were washed in 70 % alcohol and resuspended in 30 µl of TE (10 mM Tris-HCl, 0.1 mM EDTA) buffer. Following resuspension, the concentration and the purity of the DNA was determined by gel electrophoresis of DNA samples on 1% agarose gels, together with Lamda (λ) marker II (i.e. *Hin*dIII digested λ DNA) and ethidium bromide staining (Fig. 2.1). DNA was stored at -20 ^{0}C.

A number of extractions were necessary to obtain sufficient DNA for library preparation. To ensure that the DNA was free from host contamination, every extraction of P005 was tested using ITS primers. A part of the ribosomal DNA operon was analysed with ITS1 and ITS4 primers which extend over the ITS1, 5.8S and ITS2 regions of the rDNA operon (Fig. 3.1). PCR reaction mixtures were set up as described in Chapter 3 and PCR products (12 µl) were analysed on a 2 % agarose gel following staining with ethidium bromide, and Marker XIV (Boehringer Mannheim) used as a standard DNA marker. Samples which amplified DNA of *P. parasitica* were selected and pooled for use in the library. Samples which showed amplification of host DNA were discarded.

4.2.2 Preparation of filters for microsatellite enrichment

Nylon membrane filters containing bound microsatellite oligonucleotide repeats were prepared for enrichment of the *P. parasitica* library. Ten µg of three oligonucleotide sets $(CT)_{15}$, $(GT)_{15}$, and a mixture of $(CAA)_{10}$, (ATT)10, $(GCC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$ were brought to 1 ml with 3 x standard saline citrate (SSC) (45 mM sodium citrate, pH 7, 450 mM NaCl). The above mixture (80 µl) was spotted onto a 0.5 cm^2 MSI nylon transfer membrane (Osmonics, UK), air dried for 1 hr and then baked in an oven for one hr at 65°C. Membranes were treated with a hand held UV transilluminator (260 nm) for 30 sec to crosslink the DNA to the membrane. Weakly bound oligonucleotides were washed off the membrane by washing twice in 10 ml hybridisation buffer (50% formamide, 3 x SSC, 35 mM Na-phosphate, pH 7 and 2.5% SDS) for two days each at 45°C followed by a final wash in 1 x SSC. Membranes were stored at -20°C until required.

4.2.3 Digestion of genomic DNA with restriction enzymes

Genomic DNA of isolate P005 (P1 mating type, UK) (Table 2.1) of *P. parasitica* was digested with restriction enzymes that recognise both four and six bases and generate blunt ends. 150 ng of high quality genomic DNA was digested separately with 3 µl of six restriction enzymes *Alu*I, *Rsa*I, *Hinc*II, *Hae*III, *Ssp*I (Roche, UK) for two hours at 37°C. Each of the separate restriction digests were then ligated to 1 µg adapter of an *Mlu*I adapter (consisting of a 21 mer: 5′CTCTTGCTTACGCGTGGACTA3′ and a phosphorylated 25 mer: 5′TAGTCCACGCGTAAGCAAGACACA3′) together with 2 µl 10 mM ATP along with 1U of T_4 DNA ligase (Amersham Pharmacia, USA). Ligation was then allowed to proceed for 3.5 hrs at 37°C. The adapter allowed blunt ended ligation to the restriction products and the 3′ overhang prevented concatemers that could occur as a result of a number of adapters linking together. Ligated DNA was stored at -20°C until required.

4.2.4 Pre-amplification of DNA

DNA fragments containing the adapter sequence were selected for by amplification using the 21 mer oligonucleotide of the adapter as the PCR primer. PCR reactions were set up in 50 µl reactions containing 2 µl of DNA, 1 x PCR buffer, 200 µM dNTPs, 1.5 mM $MgCl_2$ (Life Technologies, USA) and 0.5 µg of the 21 mer adapter primer. The amplification conditions were 72°C for 2 min followed by 25 cycles of 60 sec @ 94°C, 1 min. @ 60°C and 3 min. @ 72°C. In the initial 2 min. the *Taq* DNA polymerase bridges the gaps where the adapter has bound and seals it with a phosphate bond. The amplified DNA (5 µl) was separated on a 2% agarose gel to ensure that fragments of the correct size were amplified. At this juncture, all samples from the initial individual digests were pooled and subjected to further analysis.

4.2.5 Enrichment for microsatellites

Enrichment of microsatellite sequences was achieved by first denaturing the pre-amplified DNA (4.2.4) and then hybridising it to a Hybond N^+ filter containing bound oligonucleotide repeats. The three filters prepared for this purpose (4.2.2) contained the following a) $(CT)_{15}$, b) $(GT)_{15}$, and c) a cocktail of $(CAA)_{10}$, $(ATT)_{10}$, $(GCC)_{10}$, $(CTG)_{10}$, $(CAG)_{10}$ and $(CAT)_{10}$. The DNA was denatured in a boiling water bath for 3-5 minutes and immediately after denaturation, equal proportions of DNA were added to three separate eppendorf tubes containing 500 µl hybridisation buffer (50% formamide, 3 x SSC, 25 mM Na-phosphate pH 7.0 and 2.5% SDS), 1 µg of the 21 mer and the specific prepared Hybond N^+ filter and incubated overnight. The addition of 1 µg of the 21 mer oligonulceotide prevented concatemers in the reactions.

At the end of the incubation filters were subjected to post-hybridisation washing. The stringency of the post-hybridisation washing determined the level of enrichment of the bound material for microsatellites. Membrane filters were subjected to 5 washes of 5 min. each in 2 x SSC, 1% SDS, and three washes of 5 min. each in 0.5 x SSC, 1% SDS. DNA was eluted from the filters by submerging in a

boiling water bath for 5 min., transferred to new tubes and frozen at -20°C prior to cloning.

4.2.6 Cloning of Enriched DNA into PJV1

Peronospora parasitica DNA enriched for the dinucleotides CA, CT, and the cocktail of trinucleotides, were designated P-CA, P-CT and P-Tri's, respectively. Enriched DNA was first amplified by PCR. 5µl of DNA (in quadruplicate) was amplified in 50 µl reactions containing 0.3 µg of the 21 mer oligonucleotide, 1 x PCR Buffer, 200 µM dNTPs, 1.5 mM $MgCl_2$, and 1U *Taq* DNA polymerase (Life Technologies, USA). Amplification conditions consisted of 25 cycles of 30 sec @ 94°C, 60 sec @ 60°C and 180 sec @ 72°C. Following PCR, like samples were pooled, phenol extracted, chloroform extracted and then precipitated with 5µl 5M sodium chloride (i.e. 100 mM final concentration) and 100% ethanol. Samples were re-dissolved in sterile distilled water and 5µl was separated on a 2% agarose gel. The three samples were then digested with *Mlu*I, phenol extracted, chloroform extracted then processed through Pharmacia S-300 spin columns (2000 rpm for 2 min.), then precipitated with NaCl and 100% ethanol. At this stage the DNA was electrophoresed on agarose gels to confirm whether the products were not lost during the purification steps.

Enriched, digested DNA was cloned into a modified PUC19 vector, PJV1 (Edwards, unpublished results). The DNA was ligated to the *Bss*HII linearised plasmid in the presence of T4 DNA ligase, 1 mM ATP and 1µl *Mlu*I (New England Biolabs, UK). The addition of *Mlu*I to the mixture prevents the ends of digested DNA from re-annealing. Two µl of the ligation mix was transformed into BRL *E. coli* DH5α (Life Technologies) competent cells. These were then plated onto Luria agar plates (12.5 g/l NaCl, 6.25 g/l yeast extract, 12.5 g/l tryptone 25 g/l agar) containing 100 µg/ml ampicillin and 250 mg/ml X-gal. Following incubation overnight at 37°C positive colonies (indicated by a white colour) were transferred into microtitre plates incubated overnight; 50% glycerol was then added and the plates are frozen at -80°C for long term storage.

All colonies were transferred from the microtitre plates onto MSI nylon transfer membranes (Osmonics) using a Biomek® 2000 workstation (Beckman Instruments, USA) and probed with oligonulceotide probes to determine which of the clones contained microsatellites. The membranes were incubated overnight at 37°C, pre-hybridised, air dried for 20 min., crosslinked to the membrane for 30 sec using a UV crosslinker (260 nm), baked for an hour at 60°C, then stored at 4°C until required. Membranes were prehybridised in hybridisation buffer (6 x SSC, 0.25% Marvel skimmed milk, 0.5% SDS) at 65 °C for 1 hr before hybridisation to labelled probes. The probes contained the following: 50μC adenosine (γ - ^{33}P) triphosphate labelled PUC primer (which label the plasmid) and 10μC adenosine (α - ^{32}P) triphosphate labelled $(GT)_{15}$, $(CT)_{15}$ as well as some trinucleotide repeats and were allowed to hybridise for 5 hrs. Post-hybridisation washing involved washing in 1.25 l hybridisation buffer until most of the excess radioactivity was washed away. The membrane filter was washed twice (10 min. each) in hybridisation buffer at 50°C then allowed to dry for 20 min. before being exposed to an autoradiograph.

4.2.7 Plasmid preparation

Plasmids were prepared from positive clones grown overnight in Luria broth, using the Promega Wizard™ kit (Promega, USA) or the Qiagen kit. Alternatively clones were grown overnight in Terrific broth (12 g/l bactotryptone, 24 g/l Bacto yeast extract, 0.4% (v/v) glycerol, with 17 mM KH_2PO_4 and 72 mM K_2HPO_4 supplement) containing 100 μg/ml Ampicillin. Plasmids were prepared on the Biomek® 2000 Laboratory Automated Workstation (Beckman, USA). Plasmids were digested with *Xho*I to confirm the presence of an insert.

4.2.8 Sequencing of positive clones

Cloned fragments were sequenced using the Amersham Sequencing kit or the ABI Prism® BigDye™ Terminator Cycle Sequencing ready reaction kit (PE Applied Biosystems, UK) and the 5' to 3' universal forward primer. In some instances the microsatellites were also reverse sequenced using the universal reverse primer. The

ABI Prism® BigDye™ Terminator Cycle Sequencing ready reaction kit contains a set of dye terminators labelled with novel high sensitivity dyes. This dye contains a fluorescein donor dye, for e.g. 6-carboxyfluorescein (6-FAM) linked to a dichlorohodamine (dRhodamine) acceptor dye. The dye is optimized to absorb the excitation energy of the argon ion laser in the PE Applied Biosystems DNA sequencing instruments. The linker affords extremely efficient energy transfer (quantum efficiency nearly 1.0, i.e., 100%) between the donor and acceptor dyes. The BigDye™ terminators are 2-3 times brighter than the rhodamine dye terminators when incorporated into cycle sequencing products. The BigDye™ terminators also have narrower emission spectra than the rhodamine dye terminators, giving less spectral overlap and therefore less noise. The brighter signal and less noise provide an overall increase in sensitivity. Furthermore the nucleotide dinucleotide mixes have been optimized to give longer, more accurate reads above 700 bases, therefore long templates can be sequenced more readily. The ready reaction mix contains the dye terminators, deoxynucleoside triphosphates (dNTPs), Amplitaq DNA polymerase,FS, r*TtH* pyrophosphates (to eliminate problems associated with pyrophosphorolysis), magnesium chloride and buffer. The dNTP mix includes dUTP in place of dGTP to minimize band compressions. The dNTP mix also uses dUTP in place of dTTP. dUTP improves the incorporation of the T terminator and results in a better T pattern.

4.2.8 Pre-screening of the microsatellite library

In a pilot study, DNA of two isolates of *P. parasitica* was amplified using the primer pairs designed (Table 4.3). 50-100 ng genomic DNA, 1 X PCR buffer, 200 µM dNTPs, 1U *Taq* DNA polymerase (Gibco BRL, Life Technologies, USA) and 150 ng of each primer were combined in 30 µl reaction volumes and subjected to the following amplification conditions: 95°C for 2 min. followed by 36 cycles of 94°C for 40 sec, 55°C for 1 min. and 72°C for 1 min. and lastly a final elongation of 10 min. at 72°C. Samples were analysed on 2% agarose gels. Primer sets that showed good amplification of single bands were then analysed using radiolabelling and polyacrylamide gels, using the method described by Edwards et al. (1996), Ciofi et al. (1998). Eight primers pairs viz. M7, M37, M42F1/R2, M110, M6(2), M26, M55 and M34 were selected for pre-screening of microsatellites on polyacrylamide gels. This

was achieved by labelling one of the primers with adenosine K-^{33}P triphosphate (Dupont, UK) and T4 polynucleotide kinase (PNK) (Pharmacia, USA) at 37°C for 30 min. followed by 10 min. at 72°C to denature the enzyme. The labelled primer was then used in the standard 30 µl PCR reaction described above. 17 µl of formamide dye was added to each tube and the sample denatured for 5-10 min. at 96°C, cooled to 4°C and then loaded onto a 6% polyacrylamide gel. Samples were run for 3 hrs at 1.3 kV. The glass plates were separated and the gels blotted with 3 mm Whatman , covered with Saran wrap, then dried for 1-2 hrs in a gel dryer and exposed to an autoradiograph overnight.

4.3 RESULTS

4.3.1 Digestion of *P. parasitica* genomic DNA with restriction enzymes, ligation to adapters and pre-amplification

Good quality genomic DNA (50 ng/µl) was obtained from isolate P005 of *P. parasitica* using the method of Tham et al. (1994). The DNA was found to be free of host contamination as only a single band was obtained following amplification with ITS primers (Chapter Three). The digestion of genomic DNA with the four base cutters as well as six base cutters generated fragments in the size range of 500-3000 bp with the six base cutters *Hinc*II and *Ssp*I generating bands in the upper range after ligation to adapters and pre-amplification as expected (Fig. 4.2). The bands appeared smeared because they represent a whole range of sizes. Some of the individually separated bands at this stage suggest possible microsatellites, since they could not be mitochondrial DNA or plasmid DNA.

4.3.2 Amplification of enriched DNA sequences

Polymerase chain reaction amplification of enriched DNA fragments following hybridisation to bound oligonuceotides yielded fragments in the range of 500-1000 bp (Fig. 4.3). Furthermore the intensity of the bands in Fig. 4.3 compared to Fig. 4.2 was somewhat reduced. In addition, The CT products appeared more highly enriched than CA or the trinucleotides (Fig. 4.3). This was consistent with the observations that CT also had the highest percent transformation when cloned into PJV1 (Table 4.1).

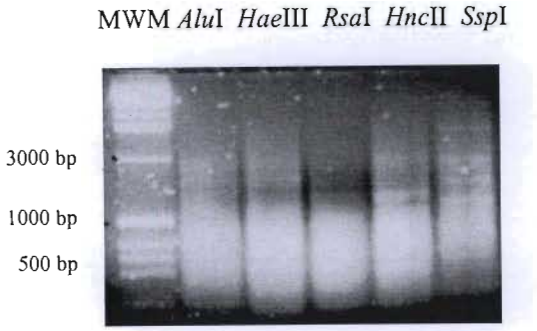

Fig. 4.2: Pre-amplification of DNA of isolate P005 of *Peronospora parasitica* following restriction digestion, with *Alu*I, *Hae*III, *Rsa*I, *Hnc*II, *Ssp*I, and ligation to *Mlu*I adaptor before Microsatellite enrichment. MWM - 1 kb ladder.

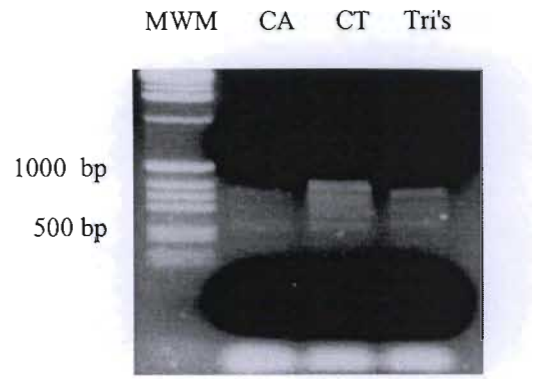

Fig. 4.3: PCR amplification of DNA of isolate P005 of *Peronospora parasitica* after enrichment by hybridisation to bound oligonucleotides CA, CT, and a cocktail of trinucleotides (Tri's). Microsatellites are bracketed by two loading dyes, xyelene cyanol FF and bromophenol blue MWM - 1 kbp ladder.

4.3.3 Cloning of enriched DNA into PJV1

Clones that contained inserts were identified as white colonies on L-agar following transformation in PJV1. Blue-white screening of 2863 clones revealed transformation percentages in the range of 49-70% (Table 4.1). The highest percentage transformation was achieved from CT repeats (70%).

Table 4.1: Transformation of CA, CT, and trinucleotide enriched sequences of *Peronospora parasitica*, into BRL DH5α competent cells

Ligation Mix	Total no. of clones, blue + white	No Transformed, white (%)
Ligation --CA	1094	557 (49%)
Ligation --CT	809	570 (70%)
Ligation --Tri's	960	587 (61%)

4.3.4 Selective hybridisation of positive transformed colonies

Following transformation into DH5α competent cells, 351 positive colonies were selected at random and transferred to MSI nylon membranes. Selective hybridisation indicated very strong hybridisation signals when hybridised with $(GT)_{15}$, $(CT)_{15}$ and tri-oligonucleotide probes (Fig. 4.4 and Fig. 4.5). Two autoradiographs were used and the universal primer sequence on the plasmid was labelled with ^{33}P which enabled the identification of the position of the colonies, while the ^{32}P indicated positive microsatellite hybridisation signals. The ^{32}P which usually generates a stronger signal penetrates the first autorad, strikes the second and bounces back onto the first in the presence of an enhancer in the X-ray cassette. Fig. 4.4 and Fig. 4.5 autoradiographs were superimposed to identify positive clones for plasmid preparation and sequencing. These were scored back to the original microtitre plates and the percent enrichment of CA, CT and Tri microsatellites was calculated (Table 4.2). Of the 351 clones transformed, 120 clones gave positive hybridisation signals and were selected for sequencing (Table 4.2 and Fig. 4.4 and 4.5).

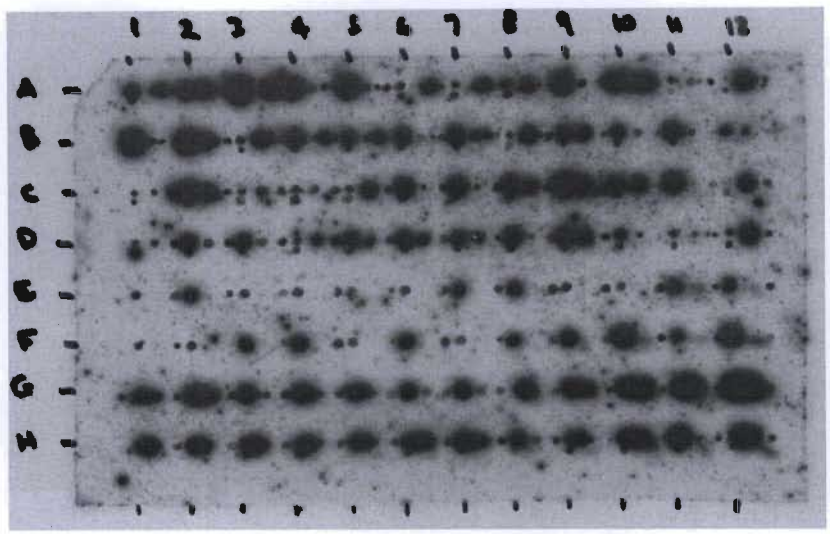

Fig. 4.4: Autoradiograph of hybridisation signals generated by ^{33}P labelled universal forward primers following background labelling of 351 PJV1 clones (containing possible microsatellite inserts of *Peronospora parasitica* fragments).

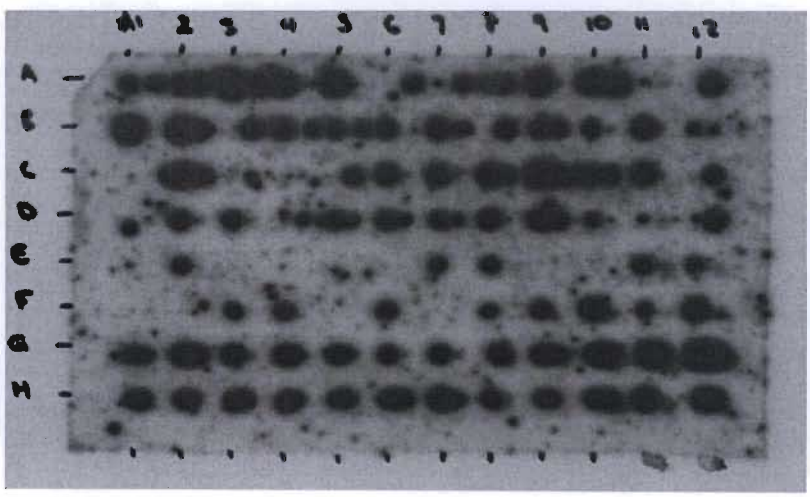

Fig. 4.5: Autoradiograph showing positive hybridisation signals generated by hybridisation of ^{32}P labelled microsatellite probes to insert sequences of PJV1 clones. Insert sequences were previous enriched sequences of *Peronospora parasitica*.

Table 4.2: Percent enrichment of positive transformed colonies after hybridisation with microsatellite probes

Positive Clones	Total positive clones screened	Enrichment after probe hybridisation (%)
P-CA	120	73%
P-CT	96	48%
P-Tri's	155	27%
Total	351	

4.3.5 Plasmid preparation and sequencing

Following selective hybridisation a total of 120 of the 351 clones screened showed positive hybridisation signals and were selected for plasmid DNA preparation and sequencing. Confirmation of the presence of an insert within plasmid DNA by cleaving the DNA with *Xho*I (Fig. 4.6) revealed insert sizes in the range of 100-500 bp. Typical sequences obtained are illustrated in Fig 4.7 and 4.8. 120 plasmids were sequenced and of the selected sequences 16 sequences were reverse sequenced to complete the sequence.

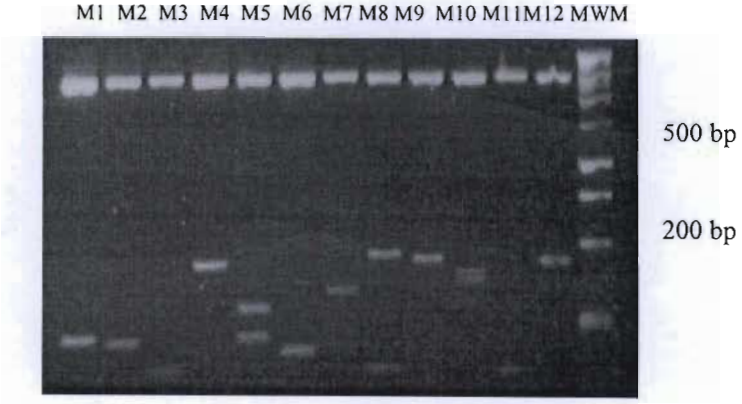

Fig. 4.6: *Peronospora parasitica* microsatellite insert sizes for 12 plasmid preparations of *E. coli*DH5α analysed on a 1.5% agarose gel. MWM - 100bp ladder (Promega).

4.3.6 Design of amplification primers

From sequence data 31 primer pairs were designed that mapped different loci of *P. parasitica*. For the design of primers it was necessary to first identify specific sites on PJV1 and then locate the insert.

The PJV1 vector had the following polylinker:

5'GAATTCCTCGAGGCGCGCCTCGAGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTT 3'

*Eco*RI *Xho*I *Bss*HII *Xho*I *Bam*HI *Xba*I *Sal*I *Pst*I *Sph*I *Hind*III

The polylinker was originally inserted into PUC19 to directly replace the original multiple cloning sites (Edwards et al. 1996). The adapter contained a *Mlu*I site which was ligated to the ends of the genomic DNA. The *Mlu*I site in the adapter/DNA was cut and the fragments cloned into the *Bss*HII site of the vector. Therefore, when plasmids were sequenced with either forward (*Eco*RI side) or reverse (*Hin*dIII side) universal primers, the polylinker sequence was identified prior to the start of the *Bss*HII site and until the start of the following sequence:

5'GGCGCGTGGACTAAC....insert.....

The final AC was part of the *Rsa*I (GTAC) site remaining on the insert. (Fig. 4.7 - 4.9).

Sequences and characteristics of primer sets (31) are indicated in Table 4.3. Primers were approximately 19 - 23 bp in length and were designed manually. Sequences were initially scanned to identify the polylinker sequences on both forward and reverse sequences. When designing primers it was necessary to ensure that a) forward and reverse primers (especially the end sequences) did not have any compatible bases that would result in primer dimers, b) a sufficient number of bases were available for primer design prior to the repeat, c) the reverse primer was read correctly (in reverse and by changing the base to its corresponding base pairing nucleotide unless reverse sequencing was done.

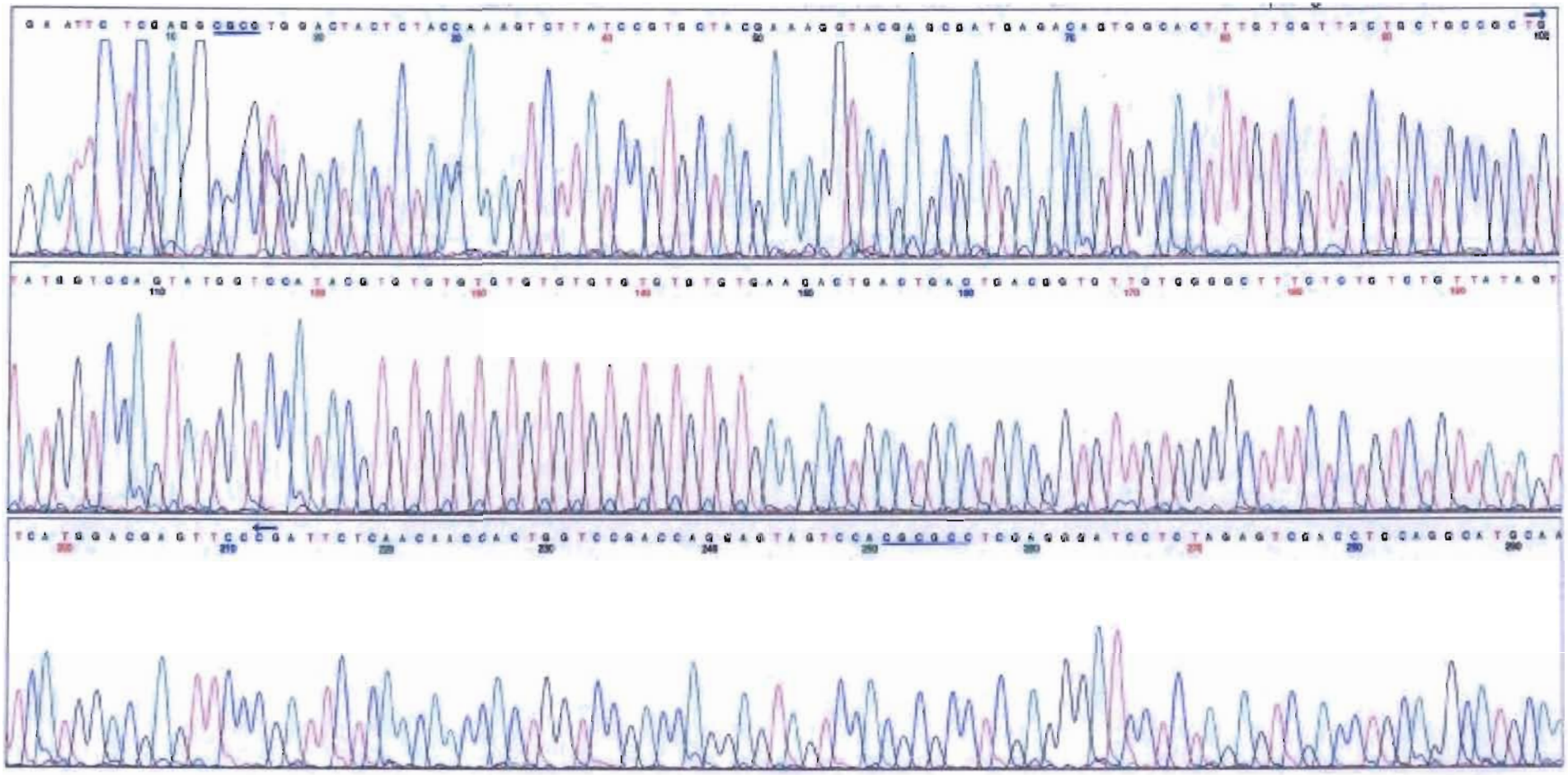

Fig. 4.7: Sequence of the M1 microsatellite locus of *Peronospora parasitica*. Microsatellite insert $(GT)_{12}\bullet(AC)_{12}$ cloned into the *Bss*HII site of PJV1 and sequenced with the universal primer M13. Inserts were identified by locating the polylinker sequence of PJV1 prior to the start of the *Bss*HII site and until the start of the following sequence: 5'GGCGCGTGGACTAAC……insert. Sites chosen for primer design of forward and reverse primers are indicated by arrows.

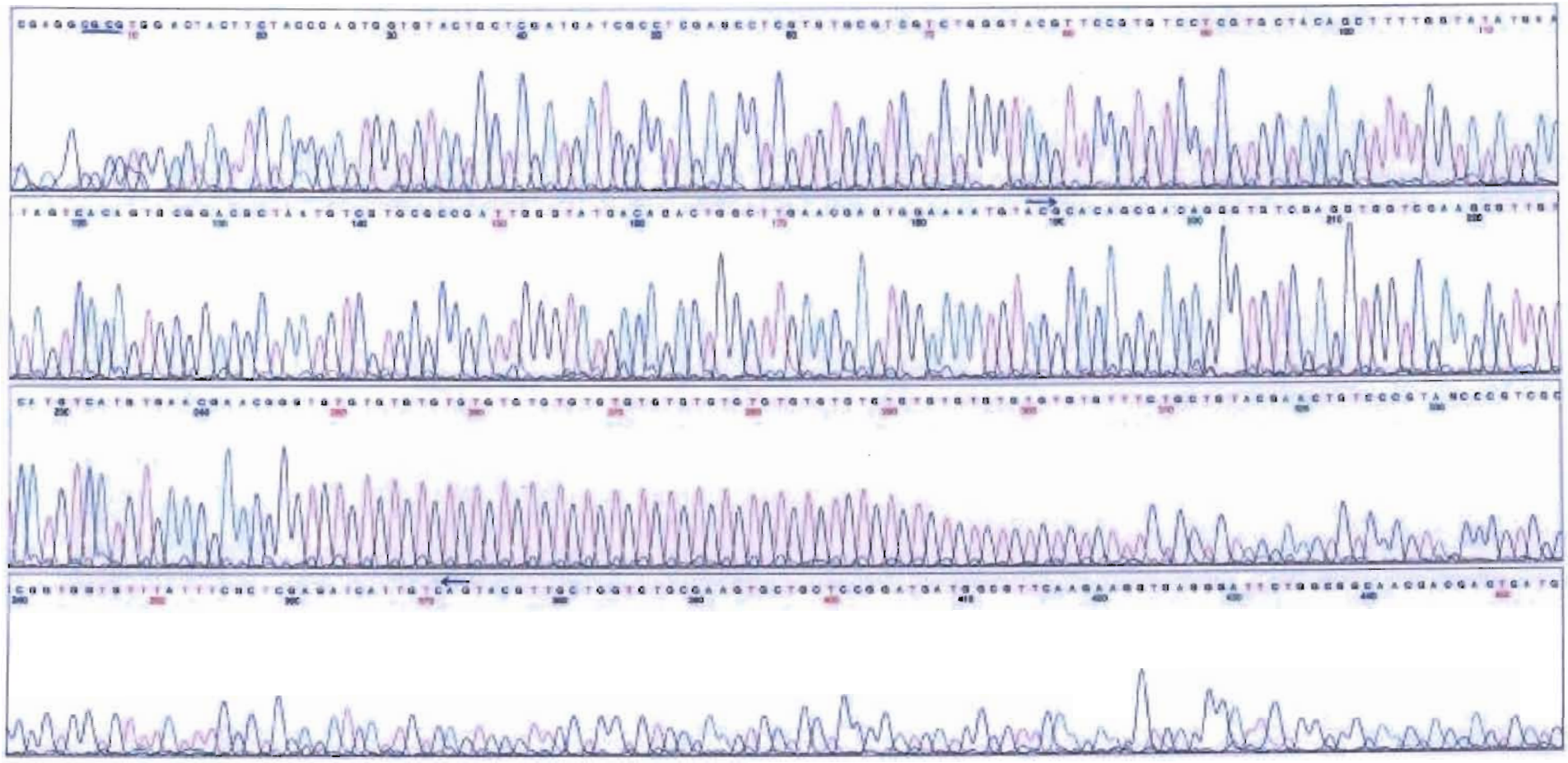

Fig. 4.8: Sequence of the M5 microsatellite locus of *Peronospora parasitica*. Microsatellite insert $(GT)_{30} \bullet (AC)_{30}$ cloned into the *Bss*HII site of PJV1 and sequenced with the universal primer M13. Inserts were identified by locating the polylinker sequence of PJV1 prior to the start of the *Bss*HII site and until the start of the following sequence: 5'GGCGCGTGGACTAAC.....insert. Sites chosen for primer design of M5(2) forward and reverse primers are indicated by arrows.

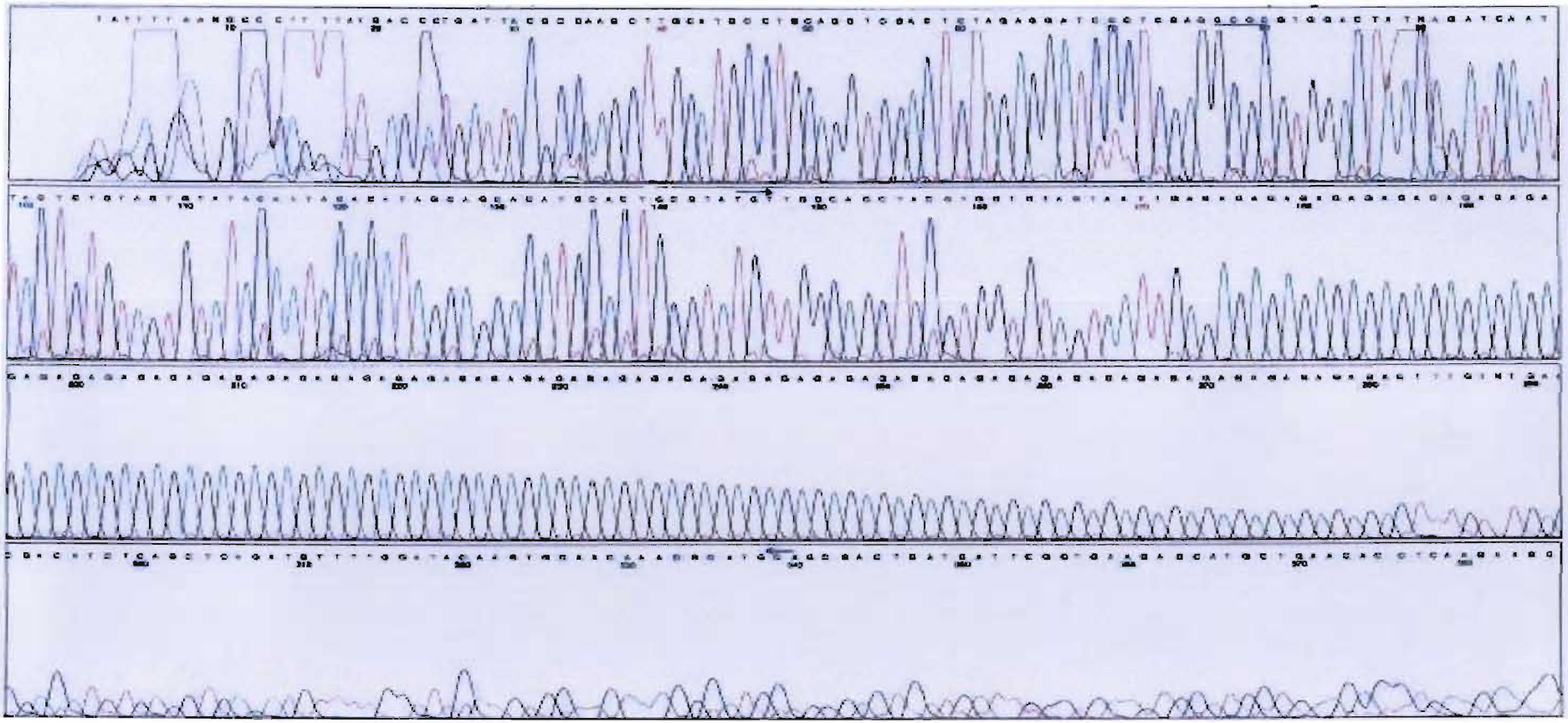

Fig. 4.9: Sequence of the M115 microsatellite locus of *Peronospora parasitica*. Microsatellite insert $(GA)_{55}$ •$(TC)_{55}$ cloned into the *Bss*HII site of PJV1 and sequenced with the universal primer M13. Inserts were identified by locating the polylinker sequence of PJV1 prior to the start of the *Bss*HII site and until the start of the following sequence: 5'GGCGCGTGGACTAAC.....insert. Sites chosen for primer design of forward and reverse primers are indicated by arrows. The reverse primer sequence was confirmed by reverse sequencing of PJV1 (not shown).

Table 4.3: Forward (F) and reverse (R) primer pairs designed from a genomic DNA library of *Peronospora parasitica* enriched for microsatellites

Name/ Locus	Primer sequence	T*m**	Repeat type**	Approx. Repeat length (bp)	Amplimer length (bp)
M1F	5'ACAGGGTGTCGAGGTGGTC3'	55.4	GT/CA	24	115
M1R	5'CTCGAGCGAAATAAACACC 3'	65.5			
M2F	5'AGCCGAGTGGGCGGCTGAG3'	74.4	GT/CA	62	141
M2R	5'GAGTCTGCTGTTCGCCCCA3'	69.0			
M4F	5'TCGAGAGGTTGCGACAATC3'	64.1	GT/CA	28	114
M4R	5'CAGCAGACCTGCAGCCACT3'	67.0			
M5F1	5'TGTATGGTCCAGTATGGTC3'	65.3	GT/CA	60	160
M5R1	5'CGGGAACTCGTCCATGAAC3'	59.8			
M5F2	5'ACGCACAGCGACAGGGTGTC3'	71.2	GT/CA	60	186
M5R2	5'CTGACAATGATCTCGAGCGA3'	64.2			
M6F1	5'CGGTAGGAGCAGCACCATA3'	63.8	CA/GT	44	163
M6R1	5'AGATTCAGTCGTTGCAGTC3'	57.8			
M6F2	5'GTAGGAGCAGCACCATACT3'	56.8	CA/GT	44	157
M6R2	5'TCAGTCGTTGCAGTCGATAAGG3'	66.3			
M7F	5'GGCTACTGGACAATGGCTT3'	61.7	GA/CT	~172	~225
M7R	5'TTGAACTGCACAGCCCGTG3'	68.6			
M8F	5'GTGAAAAGCCAGTCTGCCT3'	62.0	CT/GA	88	158
M8R	5'TCCCTTGATAGACGTGTGG3'	61.1			
M9F	5'CTCAACGACGGCGAGGGAC3'	70.5	CT/GA	114	~249
M9R	5'AGTAACGCGAACGTGTACC3'	60.2			
M10F	5'GTAACCATCATGTGCAATAGAC3'	58.4	CT/GA	88	~153
M10R	5'CAGAGACATGACCACTTGG3'	59.2			
M12F	5'CATTACTCTGAACGTCCAC3'	55.4	CT/GA	84	~332
M12R	5'GGTCAATTAAGACGGTGGATT3'	61.5			
M14F	5'GTCGAGGTGGTCGAAGCGT3'	67.8	GT/CA	72	166
M14R	5'GTCTGGTCGAACAGAGTCT3'	56.7			
M15F	5'TCCGTGTAGAGCGACCTCC3'	65.4	GT/CA	56	163
M15R	5'CCGTGCTTGATGTCAGTGT3'	62.5			
M21F	5'CATGTGGAAGAAACCAGCC3'	63.1	GT/CA	52	147
M21R	5'TGAGTCTGCTGTTGGCC3'	61.3			
M26F	5'AGTCGTTGCAGTCGATAAG3'	58.0	GT/CA	62	171
M26R	5'AGGAGCAGCACCATACTTG3'	60.4			
M27F	5'TGGTCACGCAGGCGGACGT3'	74.9	GT/CA	34	73
M27R	5'TACTTTGTGTGGTCC3'	45.5			
M31F	5'GGGACTTGCTGCAAAGACT3'	62.0	GT/CA	24	166
M31R	5'GACGAGATCGAGCGGGATT3'	66.6			
M34F	5'CCTCCGTGGTGCATCAACC3'	69.0	GT/CA	28	103
M34R	5'CAGACGAGACGCCAGTCGT3'	66.9			
M37F	5'TGTCGTCTGTACCATAGGG3'	58.5	ACAG	12	113
M37R	5'GAAACGCGACGGTGTAATT3'	62.4			
M42F1	5'TGTTTTTACAACGATACGCTACA3'	60.9	ACG? var	52	93
M42R1	5'GTCCGCCTGCGTGACCAG3'	70.8			
M42F2	5'CTGGTCACGCAGGCGGAC3'	70.8	GT/CA	34	92
M42R2	5'CAGAGCATGAGGTTTGGCT3'	63.0			
	NB. Use M42F and M42R02 → 93+92bp				185
M48F	5'TGGATCGAGTACGATCGCT3'	63.4	GT/CA	26	182
M48R	5'TCGAAGCAATCGTCGCCAC3'	69.8			
M54F	5'CACGAGCGCTCGCTGTCAC3'	71.1	$(CTTT)_3$ &	$(GT)_{13}$	>193
M54R	5'ACGCCAAGAGCAGTCGGAC3'	67.9			

M55F	5'GGCTGGTAGCGTCAAGACA3'	64.1	GT/CA	46	143
M55R	5'ACGCACGAGTGACCGTATG3'	64.9			
M108F	5'CTGAACGTCCACCTCTTTC3'	59.8	CT/GA	66	118
M108R	5'CGTTGCTGGATCGGCTGTT3'	68.6			
M110F	5'GTTAGTGTCAGTTGGCG3'	54.7	CA/GT	30	108
M110R	5'AACATGTCAAGCAGCGCGA3'	68.2			
M111F	5'CTGGTGCTCAATGAATGAC3'	59.2	GT/CA	44	100
M111R	5'GAGCAGCACCATACTCGTC3'	61.1			
M112F	5'CTTTGTAAGGCGTTTCGTGTGTC3'	66.5	GT/CA	64	187
M112R	5'TTGGGCATTATGACCAGTATGGG3'	68.3			
M115F	5'TGCATGCGTTTGTTGCACT3'	66.6	CT/GA	110	141
M115R	5'TGTTGGCAGCTACGTGGTG3'	66.1			
M119F	5'CACGAGCGAGTCGAGACGT3'	67.0	GA/CT	64	162
M119R	5'CCGACCATAGGACGCGC3'	68.0			

Total CAs (without double sets of primers)	→	18	
Total CTs	→	8	
Total variable i.e. not CA or CT only	→	4	(M37; M42F1 & M42R1; M42F1 & M42R2; M54)

*T*m* - melting temperature of primer

**Microsatellite repeat types are named as follows: CA and AC are permutations of the same repeat type; TG and GT are the reverse compliments. Databases such as Ephemeris 1.0 Readme (Pentcheff, 1999) requires only one unique repeat. Reverse compliments may be found automatically, i.e., AC = CA = GT = TG and, TC = CT = GA = AG. Hence, the convention CA/GT is a frequently used notation for these repeats (Groppe et al. 1995).

Of the 120 clones sequenced, 72 were CA clones, 36 were CT clones and 12 were trinculeotide clones. The predominant microsatellite repeats identified were dinucleotides with CT being present at a higher frequency; design of 18 primer sets for CA repeats compared to 8 for CT repeats was possible. A few (4) were variable for dinucleotide repeats. Furthermore, from the raw sequence data, and where possible, the approximate sizes of alleles were determined (Table 4.3). For two loci M5 and M6 primers were re-designed following poor amplification as indicated on polyacrylamide gels (data not shown). The M42 locus 1 and 2 were in close proximity to each other (Table 4.3). Repeat lengths varied from 12 bp to approximately 172 bp.

4.3.7 Pre-screening of the microsatellite library

The subset of primers screened confirm that the primer pairs of the *P. parasitica* library amplify microsatellite markers. Initial screening of 8 primer pairs of the

microsatellite library viz. M7, M37, M42F1/R2, M110, M6(2), M26, M55 and M34 and two isolates of *P. parasitica* viz. SSH6 and ACAT2E is represented in Fig. 4.7. Bands typical of microsatellites viz. a single major band associated with a minor lighter stutter band (usually 2 bp smaller than the major band) were observed (Fig. 4.7). Comparison to the estimated sizes (Table 4.3) revealed that allele sizes, with the exception of M7 were in the expected size range (Table 4.4). Of the 8 primer pairs, M7 and M37 appeared monomorphic across the two isolates of the *B. oleracea* pathotype while the other six primer pairs inferred different degrees of polymorphism.

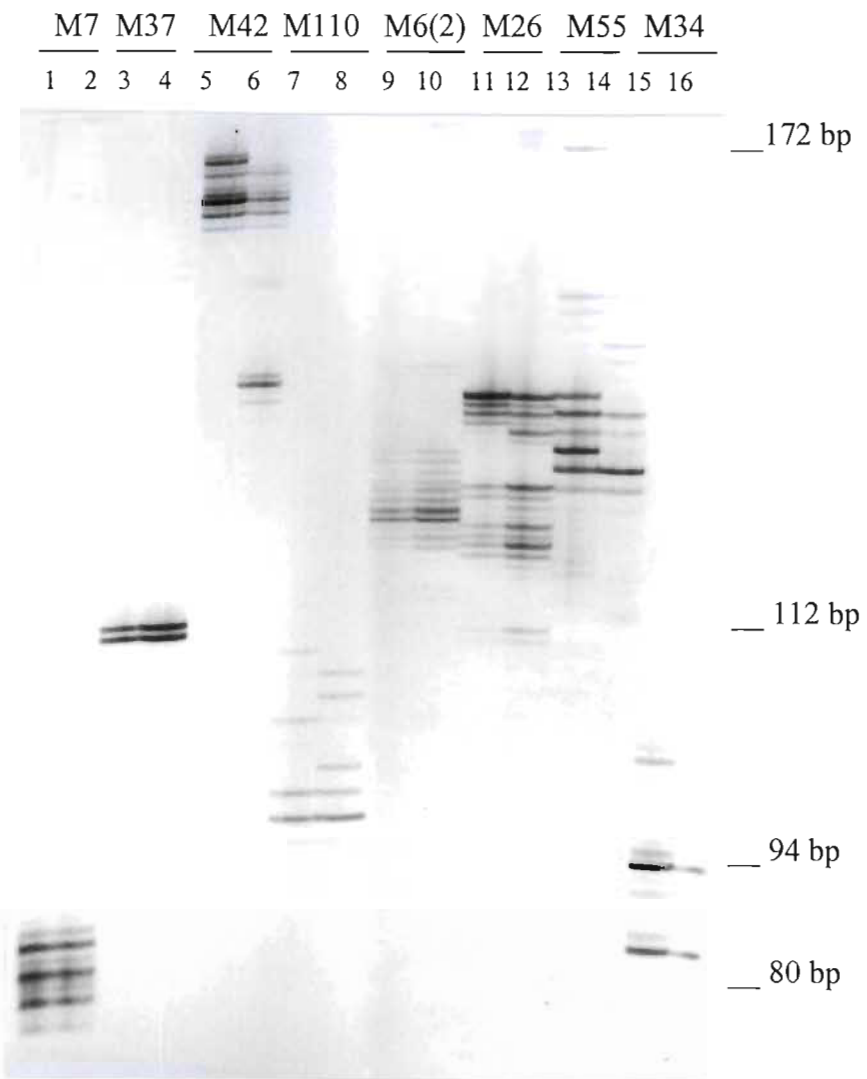

Fig. 4.10: Pre-screening of the M7, M37, M42F1/R2, M110, M6(2), M26, M55 and M34 loci of *Peronospora parasitica* on 6% polyacrylamide gels. Two isolates of *P. parasitica* viz. SSH6 (odd numbered lanes) and ACAT2E (even numbered lanes) were amplified utilising primers designed from a genomic DNA library enriched for microsatellites.

Table 4.4: Range of allele sizes at various microsatellite loci of *Peronospora parasitica* following pre-screening on 6 % polyacrylamide gels

Microsatellite locus	Range of allele sizes (Fig. 4.7) (bp)
M7	70 - 86
M37	112
M42F1/R2	136 - 170
M110	96 - 110
M6(2)	120 - 130
M26	112 - 136
M55	122 - 172
M34	84 - 110

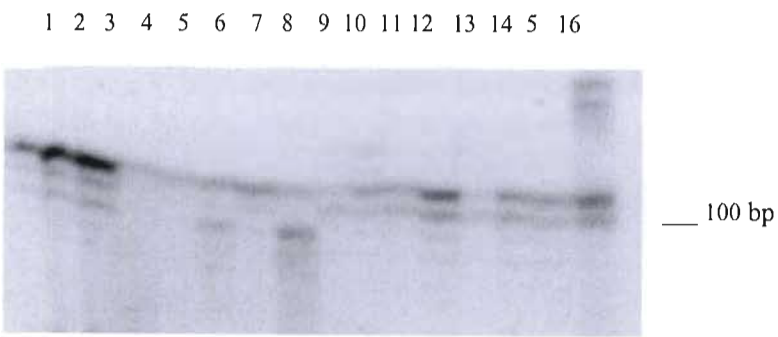

Fig. 4.11: Pre-screening of primers designed for the M1 microsatellite locus of *Peronospora parasitica*. Lanes 1-15: Amplimers generated by 15 isolates of *Peronospora parasitica*. Lane 16 - Amplimers of *Bremia lactucae*.

4.4 DISCUSSION

The sphere of molecular biology has in the last decade rapidly generated novel and intricate methods for cloning and manipulation of gene sequences. The selection of clones for sequencing by probe hybridisation and the versatility of PCR have encouraged plant pathologists to pursue lines of research that were previously inaccessible. This has resulted in the development of different molecular marker systems including highly discriminatory microsatellite libraries. The present study describes the development of a new tool for analysis of *Peronospora parasitica*. The first genomic DNA library enriched for microsatellites from an Oomycete pathogen, *P. parasitica*, has important applications in the understanding of host specificity of downy mildews in general and *P. parasitica* in particular.

Typically, successful detection of microsatellites from the genome of various eukaryotes was possible by the laborious task of screening genomic libraries with oligomeric probes (Jones et al. 1997, Kijas et al. 1994, Morjane et al. 1994). However, the screening of un-enriched genomic libraries was inefficient since large numbers of clones needed to be screened from small insert libraries for each positively hybridizing clone. Furthermore, it was expensive and time consuming. More recently, the construction and development of libraries highly enriched for microsatellites has enhanced the utility of microsatellites as a marker of choice (Karagyozov et al. 1993, Kijas et al. 1994, Ostrander et al. 1992, Kölliker et al. 2001, Edwards et al. 1996). In the present study, a genomic DNA library of *P. parasitica* enriched for several microsatellites was prepared using the protocol of Edwards et al. (1996). In comparison to other studies (Karagyozov et al. 1993, Kijas et al. 1994, Ostrander et al. 1992, Kölliker et al. 2001) which achieved enrichment of a single type of microsatellite only, the present study achieved enrichment of CA and CT repeats at high frequencies. While enrichment for trinucleotides was simultaneously attempted, trinucleotides were not detected. Similar to the findings of Edwards et al. (1996), the development of multiplex libraries resulted in a more representative sample of microsatellites in the genome of *P. parasitica*.

The sensitivity of enrichment for microsatellites was enhanced by the utilisation of two enrichment steps. The 1st enrichment step by hybridisation to bound oligonucleotides determined the variability of the sequences obtained and the 2nd enrichment step targeted good microsatellite inserts following cloning and therefore reduced the number of clones which needed to be sequenced. The stringency of the post-hybridisation washing in the 1st enrichment step determined how enriched the sequences were for various microsatellites. Over-enrichment by very stringent washing would result in only 1 type of microsatellite and can increase redundancy of characterised microsatellite loci. In the present study, the range of possible microsatellite sizes on agarose gels prior to cloning indicated high enrichment efficiency of this first step.

Following transformation a high percentage of the various microsatellites were recorded by blue-white screening, with CT microsatellites indicating the highest percentage transformation (68%). However, the percentage of these clones which demonstrated strong microsatellite probe hybridisation signals in the 2nd enrichment step reflected a different pattern. Only 73%, 48%, and 27% of each of CA, CT and Tri's, respectively, hybridised strongly to microsatellite probes. Using this novel method selection of 120 useful clones for sequencing, from a total of 351 positive transformed clones, was possible . The success of the 2nd enrichment step depends on the unique individual labelling of the plasmid (^{33}P) and the microsatellite (^{32}P) simultaneously in a single hybridisation step as well as the specific autoradiography manipulation employed here which enhanced detection of microsatelllites. The use of 2 enrichment steps in this study, one prior to cloning and then after cloning has resulted in the successful detection of good quality microsatellite sequences, for which primers could be assigned.

In the present study, the dinucleotide microsatellites were found to be abundant in the genome of *P. parasitica*. This was evident by the large number of clones that hybridised strongly to the ^{32}P microsatellite probes. Of the microsatellites obtained, CA repeats were abundant in comparison to CT and the trinucleotides $(CAA)_n$, $(ATT)_n$ and $(GCC)_n$, (CAG)n. Similarly, CA/GT repeats were abundant in vertebrates (Tautz and Renz 1984, Gross and Garrard 1986, Litt and Luty 1989).

However, they were present at lower frequencies in *Brassica spp* (Lagercrantz et al. 1993). CA/GT blocks were the most abundant repeat found in humans (Hamada et al. 1982). In the present study, the number of useful microsatellites for which primers could be designed was also the highest for CA and no useful trinucleotide microsatellites were obtained.

Whilst, microsatellites were reported to occur to a lesser extent in prokaryotes (Tautz 1989), recent studies indicate that microsatellites are widespread in simple organisms such as, the lower eukaryotes and prokaryotes, including fungi and protists (Groppe et al. 1995, Field and Wills 1996). Furthermore, surveys of Genbank revealed long polymorphic microsatellites in the simple organism (Field and Wills 1996). From the present study, sequence analysis revealed long repeats for those loci for which repeat lengths could be accurately determined (Table 4.3). Repeat lengths were in the range 12 - 110 bp (M7, M9, M10 and M12 excluded). Of significance was locus M115, having a repeat length of 110 bases, i.e., $(GA)_{55}$. The length of this repeat was confirmed by reverse sequencing.

The sequencing of 120 clones resulted in the identification of 29 microsatellite loci for which primers could be designed; the type of microsatellites identified were $(CA)_n$ and $(CT)_n$ repeats (Table 4.1, Figs. 4.7 - 4.9). Eight useful polymorphic loci were subsequently identified (Chapter five). The preparation of plasmid DNA from 120 positive clones was performed using both the Biomek™ 2000 robot as well as the Wizard Preparation Kit. Twenty nine clones yielded good sequence for primer design; at two loci (M5, M6) primers were redesigned to improve the amplimers generated. Reverse sequencing of PJV1 was often necessary since sequencing efficiency with the BigDye™ Sequencing kit was often reduced upon encountering a repeat. This was observed by the reduction in peak heights along or immediately after the repeat itself (Fig. 4.9). Of the remaining 91 clones (75%), primers could not be designed for various reasons. This included 1) microsatellites being too close to the polylinker site of the plasmid therefore leaving insufficient bases for primer design. 2) the presence of good forward sequence with long repeats together however, with poor reverse sequencing of the plasmid and reverse primers could not be designed and 3) poor quality sequence in some instances.

In the present study only 351 (21%) of the possible 1714 positive transformed clones were subjected to the 2nd hybridisation screening, thus indicating that only 21% of the total possible microsatellites were sampled. Of the 120 clones selected for sequencing, 29 primer sets could be designed (24%). This indicates that useful microsatellites where primers could be designed were found in 8% of the 351 positive transformed clones. This proportion is comparable to the lower percentages obtained in unenriched plant genomic libraries (LagerCrantz et al. 1993). LagerCrantz et al. (1993) obtained between 10-120 positive clones per 10^4 (i.e. between 0.1 - 1.2 %) clones screened.

The number of potential SSRs obtained (24%) with *P. parasitica* is very similar to recent reports of Mba et al. (2001) and Kölliker et al. (2001) who have reported high yields of microsatellite loci based on the enriched method of Edwards et al. (1996). In white clover (Kölliker et al. 2001), sequence analysis of 1123 clones yielded 793 clones containing SSR loci, however only 397 (35%) potentially useful SSRs after exclusion of redundancy and loci from which primers could be designed. In cassava, Mba et al. (2001) obtained 24 and 25% potentially useful SSRs using two different enrichment methods. There appeared to be no evidence of redundancy in the *P. parasitica* library.

The construction of microsatellite libraries is useful for diversity studies as the clones produced are sufficiently polymorphic to be used in the genetic analysis of populations and/or plant breeding. The added advantage is that they are co-dominant, homogenously distributed and readily transferred between laboratories. In the present study preliminary screening of the microsatellite library revealed the existence of both monomorphic and polymorphic microsatellite loci for two isolates of the *B. oleracea* pathotype (Fig. 4.10). Most of the alleles were in the expected size range (Table 4.4) and occasionally estimated sizes were longer than the actual sizes on polyacrylamide gels. Actual repeat sizes of a few sequences may vary from estimated sizes due to, reverse sequencing and the effect of the BigDye™ kit which loses efficacy along microsatellites during sequencing. Nevertheless, reverse sequencing usually enabled confirmation of reverse primers.

Microsatellites markers may be a good indication of diversity in a population and may possibly be useful in answering questions on the spatial distribution of isolates and the relationship between different pathotypes of *P. parasitica*. This was explored further and the number of useful microsatellites was determined by screening isolates of *P. parasitica* as described in Chapter five. Furthermore PCR amplification at the M1 microsatellite locus indicated unique alleles for the Oomycete pathogen *Bremia lactucae* compared to 15 isolates of *P. parasitica* of the *B. oleracea* pathotype. This illustrates the potential of microsatellites marker of *P. parasitica* for analysing diversity in other pathogens. These results demonstrate the well-documented advantage of microsatellites to detect high levels of polymorphism among closely related taxa, species and *forma specialis*.

CHAPTER FIVE

ASSESSMENT OF MICROSATELLITE DIVERSITY IN PATHOTYPES OF *Peronospora parasitica*

5.1 INTRODUCTION

Highly polymorphic and dispersed markers in a genome can be used to study the biological relatedness of organisms and facilitate the mapping and cloning of genes. In recent years a number of different molecular marker systems have been developed with microsatellite markers proving to be the most powerful (Kijas et al. 1994). The occurrence of simple sequence repeats in both coding and non-coding DNA has been referred to as "cryptic simplicity" (Tautz et al. 1986). Cryptically simple regions have also been regarded as biased in nucleotide composition and consist of scrambled rearrangements of repetitive motifs which differ within and between species.

Microsatellites, also known as short tandem repeats (STRs) or short sequence repeats (SSRs) have been described as an additional source of genetic markers. Microsatellites consist of around 10-50 copies of motifs from 1-6 bp that can occur in perfect tandem repetition, as imperfect (interrupted) repeats or together with another repeat type. An extensive review of microsatellite types, frequencies and their evolution is described in Chapter Five. Microsatellites display considerable polymorphism due to variation in the number of repeat units. This polymorphism is sufficiently stable for genetic analyses and is therefore ideal for constructing high resolution genetic maps (Hearne et al. 1992) in for studies on genetic diversity.

Microsatellites have been known to exist in eukaryote genomes since the 1970s (Bruford et al. 1998) however, the large numbers and widespread occurrence of these sequences were first demonstrated by Hamada and co-workers in 1982. By Southern blotting and hybridization analysis using ^{32}P-labeled poly (dT-dG)·poly (dC-dA) as a

probe, a huge number of stretches of dT-dG alternating sequence (i.e. CA repeats)* have been found in eukaryote genomes including human, calf, mouse, chicken, salmon, yeast, *Xenopus* and *Drosophila* (Table 5.1). This particular alternating sequence was found to adopt the Z-DNA conformation ("left-handed" double helix configuration) under certain conditions. The number of the sequence repeats ranged from about 100 in yeast to tens of thousands in higher eukaryotes. Furthermore, sequence analysis showed that different clones varied in the number of repeat units. A second alternating sequence dG·dC was found to be moderately repetitive in the human, mouse and salmon genomes (Hamada et al. 1982). Subsequently, Tautz and Renz (1984) hybridised different microsatellite sequences to genomic DNA from a variety of organisms (*viz.* man, *Drosophila*, Sea Urchin, *Stylonychia* and yeast) and found that many types of simple sequences (including CA, CT, AA, GG, CAG) were present in varying proportions.

Surveys of Genbank revealed long polymorphic microsatellites in simple organisms (Field and Wills 1996). Despite earlier reports of the lack of concentration of microsatellites in lower eukaryotes and prokaryotes, viz., the slime molds, fungi, protists, prokaryotes, viruses and plasmids, these organisms contributed 78 of the 375 examined sequences. These identified sequences were predominantly trinucleotides, 50% of which were in exons and mostly AT dinucleotide repeats. Computer database searches of microsatellites in the yeast chromosome III revealed the occurrence of only AT-repeats at a high frequency (Valle 1993). Furthermore some of these repeats showed a close association with the core consensus of autonomously replicating sequences

A study of microsatellite abundance in various *Brassica* species revealed that microsatellites are five times less abundant in the genomes of plants than in mammals (Lagercrantz et al. 1993). The most common plant repeat motif was AA/TT followed by AT/TA and CT/GA. This group comprised about 75% of all microsatellites with a length

* CA and AC are permutations of the same repeat. Similarly, TG and GT are permutations. Databases select reverse compliments automatically. Therefore, AC = CA = GT = TG, and TC = CT = GA = AG (Pentcheff 1999).

Table 5.1: Estimation of approximate copy number of the Z(T-G) element in various eukaryotic genomes (Hamada et al. 1982).

	Haploid Genome Size	Approximate copy number
Human	3×10^9	5×10^4
Calf	3×10^9	3×10^4
Mouse	3×10^9	10^5
Chicken	10^9	4×10^3
Xenopus	2×10^{10}	10^5
Salmon	6×10^9	2×10^5
Drosophila	2×10^8	2×10^3
Yeast	10^7	10^2

of more than six repeats. The GT/CA motif being the most abundant in vertebrates was present less frequently in plants. However, investigations of four *Brassica* species at five microsatellite loci indicated polymorphism across four of five loci.

A survey of all fungal DNA sequences currently deposited in the DNA sequence databases of EMBL and GenBank revealed that microsatellites of different repeating units are widespread in fungi (Groppe et al. 1995). The most abundant repeats in fungi were $(AT)_n$, $(AAT)_n$, $(AAC)_n$, $(AAG)_n$, $(ATC)_n$, $(AC)_n$, $(AG)_n$, $(AGC)_n$, $(CCA)_n$, $(C)_n$, $(CCT)_n$, $(ACG)_n$, $(ACT)_n$, and $(CCG)_n$; no $(CG)_n$ repeats were found (Table 5.2). A total of 626 microsatellite alleles were found in 31 fungal species. These included 501 microsatellite alleles found in *Saccharomyces cerevisiae*. Furthermore, an extensive computer search of the DNA sequence library of the European Molecular Biology Laboratory (EMBL) demonstrated that nearly all possible simple motifs occur 5-10 times more frequently than the equivalent sized random motifs (Tautz et al. 1986). The study incorporated scans for direct sequence homologies to 15-nucleotide long probes each consisting of all possible mono-, di-, and tri-nucleotide motifs. In order to detect accumulations of direct oligonucleotides which need not have a regular spacing (cryptic simplicity), a computer algorithm was devised to search for such regions.

Table 5.2: Abundance of microsatellites with sizes of 20 bp or more in fungal genomic sequences deposited in the GenBank and EMBL databases (Groppe et al. 1995)

Repeated nucleotide (s) (permutation[s]; Complementary sequence[s])	No. of microsatellites
AT (TA)	263
A (T)	160
AAT (ATA, TAA; TTA, TAT, ATT)	50
AAC (ACA, CAA; TTG, TGT, GTT)	22
AAG (AGA, GAA; TTC, TCT, CTT)	21
ATC (TCA, CAT; TAG, AGT, GTA)	19
AC (CA; TG, GT)	17
AG (GA; TC, CT)	15
AGC (GCA, CAG; TCG, CGT, GTC)	14
CCA (CAC, ACC; GGT, GTG, TGG)	11
C (G)	10
CCT (CTC, TCC; GGA, GAG, AGG)	9
ACG (CGA, GAC; TGC, GCT, CTG)	8
ACT (CTA, TAC; TGA, GAT, ATG)	5
CCG (CGC, GCC; GGC, GCG, CGG)	2
CG (GC)	0
Total:	626

The universal existence in DNA from monotonous arrays of single motif to variable permutations of relatively short-lived motifs suggests that ubiquitous slippage-like mechanisms are a major source of genetic variation in all regions of the genome, not predicted by the classical mutation process (Tautz et al. 1986). Replication slippage is believed to be a mechanism by which the number of short, tandemly repeated sequences increases or decreases when DNA is replicated (Schlötterer and Tautz 1992). A number of models have been proposed for replication slippage (Walsh 1987, Tachida and Iizuka 1992, Valdes et al. 1993, Shriver et al. 1993). A complete discussion of replication slippage, an explanation of how microsatellites arise as well as the various models is discussed in more detail in Chapter 4.

Primary demonstration of the utility of microsatellites as an important source of DNA polymorphism was first shown in humans by a number of researchers (Weber and May 1989, Litt and Luty 1989, Tautz 1989, Tautz 1990, Fu et al. 1991). In addition to

being associated with various disease genes (Brook et al. 1992, Valdes et al. 1993, Rubinszstein et al. 1995) these studies have provided insight into the way in which microsatellites evolve (Rubinsztein et al. 1995). Comparing allele length distributions Rubinsztein et al. 1995 concluded that consistent allele length difference was synonymous with a difference in the rate of microsatellite evolution between humans and other primates. In plants, research on the utility of microsatellites has advanced rapidly and a number of microsatellite enriched libraries have been prepared ((Beyermann et al. 1992, Udapa et al. 1999, Edwards et al. 1996, Burgess et al. 2001, Ribeiro et al. 2001). A recent study by Schmidt and Heslop-Harrison (1998) provides a model for the large-scale organisation of plant chromosomes; this model includes stretches of repetitive DNA which are recognised to have a characteristic genomic location within a genus and are important for evolutionary, genetic, taxonomic and applied studies.

Although SSR variation has been exploited increasingly in genetic studies involving mammalian and plant systems, fungal microsatellites have until recently, remain virtually unused. Early examples include the use of repetitive sequences for inferring phylogenetic relationships among crucifer infecting strains of *Fusarium oxysporum* (Kistler et al. 1991). A common ancestry was found for strains representing different races of *F. oxysporum* f. sp. *conglutinans*, but a distinct ancestry between these and strains of *F. oxysporum* f. sp. *raphani* and *F. oxysporum* f. sp. *matthioli*. The distribution of repetitive sequences supported the results obtained with plasmid typing, mitochondrial and RFLPs analysis that there is common ancestry for members of the same *forma specialis* and VCG. SSRs were useful in strain/race variation in *Saccharomyces cerevisiae* (Couto et al. 1996) and *Gremmeniella abietina* (Hantula and Müller 1997).

In general one of three popular approaches are utilised for the application of microsatellites as a discriminatory tool. These are a) the use of microsatellites probes as hybridisation probes for DNA fingerprinting, b) a technique combining the benefits of

RAPD and microsatellite analyses known as RAMS (random amplified microsatellites) or c) the preparation of microsatellite enriched libraries and subsequent analysis using PCR.

Fingerprinting probes specific for microsatellites have been useful in studies on the diversity of a number of fungi (Meyer et al. 1991, DeScenzo and Harrington 1994, Groppe et al. 1995). Species and strains of filamentous fungi, *Penicillium*, *Aspergillus* and *Trichoderma* spp., were differentiated using $(GATA)_4$ as a hybridisation probe; fingerprints of strains of the same species differed only slightly from each other while fingerprints of clones originating from one strain were identical (Meyer et al. 1991). The probe $(CAT)_5$ was used for delineating genotypes of *Ophiostoma piliferum* as well as identifying host-specialised variants of *Heterobasidiom annosum* (DeScenzo and Harrington 1994). Groppe and co-workers (1995) revealed the $(AAC)_8$ and $(AAG)_8$ repeats in fungal endophytes (*Epichloë* spp.) in grasses by sequencing size variants of a RAPD band which differed between isolates. The $(AGT)_5$, $(ATC)_5$, $(GATA)_4$ and $(GATA)_4$ hybridisation patterns were useful for distinguishing 4 races of *Fusarium oxysporum* f. sp. *ciceri*, the causal agent of chickpea wilt, from India (Barve et al. 2001). The distribution of microsatellite repeats in the genome further revealed races 1 and 4 to be closely related at a similarity index ratio of 76.6% as compared to race 2 at a similarity value of 67.3%; race 3 was very distinct at a similarity value of 26.7%. Discrimination of strains of *Saccharomyces cerevisiae* from spoiled wine and beer was possible using both RAPD markers as well as fingerprinting with microsatellite oligonucleotide primers $(GAC)_5$ and $(GTG)_5$ (Couto et al. 1996). Oligonucleotide fingerprinting of *Ascochyta rabiei* isolates using the probes $(CA)_8$, $(CAA)_5$, $(CAT)_5$ and $(GATA)_4$ revealed the occurrence of 12 different fungal haplotypes at various frequencies within a field of chickpeas (Morjane et al. 1994). Furthermore the haplotypes were unequally distributed throughout the sample locations. Of the four locations sampled, seven haplotypes were confined to one location only, four occurred at two, one at three and none at all locations. Furthermore, most of the genetic variability originated from diversity within rather than between locations. In some cases more than one haplotype was isolated from the same lesion on a single host plant. Subsequently Geistlinger et al. (1997b) utilised in-gel

hybridisation with repetitive probes targeting the *ArMS1* (*Ascochyta rabiei* microsatellite 1) locus revealed that two U.S. mating types share a considerable amount of genetic variability; a higher level of polymorphism was evident between these isolates and those from different geographical regions.

Random amplified microsatellites (RAMS), based on primers designed on microsatellite sequences, have been useful in characterising variation in fungi (Hantula et al. 1996, Longato and Bonfante 1997, Müller and Hantula 1998, Geistlinger et al. 1997a,b). This method was successful in detecting interspecific and intraspecific DNA polymorphisms in *Armillaria cepistipes*, *Gremmeniella abietina*, *Heterobasidion annosum*, *Phytophthora cactorum*, *Phlebiopsis gigantea*, and *Stereum sanguinolentum* (Hantula et al. 1996), mycorrhizal fungi (Longato and Bonfante 1997), *Tiarosporella parca* (Müller and Hantula 1998), and *Ascochyta rabiei* (Geistlinger et al. 1997a,b). Furthermore, RAMS analysis was able to separate 39 isolates of *Tiarosporella parca* into 35 different haplotypes (Müller and Hantula 1998). RAMS was effective in separating the races of *Gremmemiella abietina* from different geographical areas as well as measuring intraracial variation; in addition it provided more information as a result of the larger number of alleles present (Hantula and Müller, 1997).

A number of workers have explored the genetic differentiation of pathotypes (host-adapted types and races) of fungi (Levy et al. 1991, Sastry et al. 1995, Bucheli et al. 2000). Levy and co-workers (1991) employed a probe for a dispersed repeated DNA sequence called MGR to construct genotype specific, *EcoR*I restriction fragment length profiles (MGR-DNA fingerprints) from United States field isolates of the rice blast fungus *Magnaporthe grisea*. The absence of durable resistance in the field has been attributed to a high degree of polymorphism in virulence in pathogen populations. The accurate identification of pathotypes of isolates collected over a 30 yr period resolved the controversy regarding rice blast pathotype stability and has provided new insights to population and evolution dynamics on this fungus.

In a study of genetic variability of the pearl millet downy mildew pathogen *Sclerospora graminicola*, microsatellite were found to be more useful than minisatellites in discriminating pathotypes of this pathogen (Sastry et al.1995). The microsatellites $(GAA)_6$, $(GACA)_4$ and especially $(GATA)_4$ showed high levels of polymorphisms between the pathotypes and fingerprint profiles were predicted to be useful as diagnostic tools to formulate breeding strategies targeting resistance to local population and for monitoring the emergence of new virulent races.

Host-specificity in the anther smut fungus (*Microbotryum violaceum* = *Ustilago violacea*) subject to much controversy with all host specific lineages being grouped in *M. violacea* (Bucheli et al. 2000). Samples, from eight hosts of the Caryophyllaceae affected by this pathogen viz. *Silene*, *Saponaria*, *Dianthus* and *Gypsophila* species, were investigated. Microsatellite variation revealed an almost perfect differentiation among the anther smut fungi from different host species (Bucheli et al. 2000). This differentiation was supported by the non-random distribution of null alleles (caused by mutations in the priming site thereby inhibiting primer annealing) among samples from different host species and host genera.

In the last few years the advent of genomic libraries enriched for microsatellites has greatly enhanced the utility of microsatellites as a discriminatory tool. Recently, Barroso and co-workers (2000) isolated the first microsatellite from cultivated edible mushrooms. An *Agaricus bisporus* microsatellite with the tetranucleotide motif TATG tandemly repeated was isolated from an *A. bisporus* library enriched in repeated sequences. Direct amplification of the microsatellite-region DNA (termed DAMD-PCR) allowed the discrimination of four strains of *A. bisporus*. In addition TATG was present at numerous loci and scattered on different chromosomes. Closely related species of *Pleurotus* could also be differentiated. Olignucleotide probes and RAMs provide useful information on diversity however the methods employed target one or a few microsatellite loci. The preparation of enriched libraries described for other organisms was a more accurate representation of the microsatellite diversity (Edwards et al. 1996).

Since microsatellites are usually less than 100 bp long and are embedded in DNA with unique sequences, they can be amplified *in vitro* using the polymerase chain reaction (PCR) (Hearne et al. 1992). PCR has vast applications in the analysis of microsatellites. The repeated core sequences of microsatellites, usually two or three nucleotides in length, often vary in number and are flanked by conserved DNA sequences (Gupta et al. 1994). Using primers complementary to flanking regions, SSR sequences can be amplified via PCR and analyzed for variation in the number of repeats.

PCR amplification of microsatellites generally involves standard methods. Depending on which of the possible strategies for electrophoresis and subsequent scoring of alleles is used, PCR amplification employs either unlabelled primer pairs or primer pairs with one of the primers being radiolabelled or fluorolabelled. Electrophoresis of unlabelled PCR products followed by staining with immersions-staining with silver or ethidium bromide is possible which may be the most desirable method in low–budget and non-radioactive laboratories. The one disadvantage is that alleles differing by one or two base pairs are sometimes difficult to resolve. Polyacrylamide gel electrophoresis (PAGE) using large manual sequencing gel systems are ideal for electrophoresis of microsatellites.

Radioactive labelling with ^{32}P or ^{35}S may be used to label one end of a primer which may subsequently be incorporated into the product during amplification (Hearne et al. 1992). The use of ^{33}P to label primers has become increasingly popular due to reduced radiation properties, yet sensitive detection ability. Labelling DNA fragments at the 5′ end with polynucleotide kinase is one of the methods to end label DNA. The enzyme polynucleotide kinase is able to attach a labelled phosphate molecule to the 5′-OH group of the ("sticky end") DNA when the incubation is performed in the presence of the γ-^{33}P labelled ATP (Winnacker 1987).

Autoradiography of gels containing the ^{33}P radioactive label was used to detect and quantitate the radioactive label in a particular band or spot. Scoring microsatellite

gels or autoradiograms is usually a relatively simple process. This is because the electrophoresis systems used usually have very high resolution (to a single base-pair) and because alleles differ in a very predictable way (multiples of the microsatellite repeat unit, e.g. two base-pairs). The accurate sizing of alleles is achieved by running size markers, such as known DNA sequence, alongside the system and, in automated systems, internal size-markers using a unique fluorescent label results in the sizing of alleles in each individual of even greater accuracy (Ciofi et al. 1998).

Amplified alleles are visualised as bands on gels or were represented by peaks on electropherograms. PCR amplification of microsatellite loci typically produced a minor product band 4 bp shorter than the corresponding main allele band which was referred to as the stutter band (Walsh et al. 1996). Stutter bands were found to differ in size from the main allele by multiples of the repeat unit size, and has also been referred to as a shadow band (Schlötterer and Tautz 1992). In the case of dinucleotide repeats, the most prevalent stutter band was generally found to be two bases shorter than the main allele band, however additional stutter bands of four and six bases shorter were also visible (Murray et al. 1993, cited by Walsh et al. 1996). As a result of this multi-band pattern for each allele, interpretation of dinucleotide repeat loci was sometimes complicated, particularly for DNA samples that were mixtures from two or more individuals or when two alleles from a single individual were close in size (Litt et al. 1993, cited by Walsh et al. 1996).

The one difficulty with scoring microsatellite gels is that with mono- and dinucleotide repeat unit microsatellites, replication slippage during the amplification process can lead to the presence of sometimes confusing products on the gel. These slippage products are present as less intense bands of usually one to five repeat units smaller (and occasionally, greater) than the actual allele. The slippage bands become relatively less intense the more they deviate in size from the native allele, and are in practice usually easy to diagnose and ignore. However, where the second allele of a heterozygous individual overlays a slippage product from the first allele, confusion can occur, and here the difference of the relative intensity of the band (i.e. a fainter slippage

overlaid by a native allele results in a more intense band than even that of the first native allele) is usually diagnostic. The user can however, quickly become practised at visually scoring the more difficult systems.

More recently, fluorescence labelling of primers combined in standard PCR and automation on a 377 ABI sequencer has been successful as an efficient and economical method for large-scale screening of polymorphisms. Automated systems detect fluorolabelled PCR products using a laser and electrophoresis results are transmitted directly into a computer database where they are available for analysis using software such as Genescan™ and Genotyper® (Applied Biosystems/ABI). These analysis programs provide algorithms that separate native alleles automatically from slippage products (Ciofi et al. 1998). Furthermore the use of single-tube multiplex PCR reactions based on the above is being investigated as avenues for automated, economical and reduced output time analysis (Keith Edwards pers. commun.). Once established the use of this method for single nucleotide polymorphism (SNP) detection will be a valuable screening tool.

The aim of the current chapter was to assess microsatellite diversity in pathotypes of *P. parasitica* of diverse geographical origin by utilising the genomic library of *P. parasitica* prepared in Chapter Five. Pre-liminary screening of the microsatellite library of *Peronospora parasitica* revealed the both monomorphic and polymorphic microsatellite loci for two isolates of *P. parasitica* of the *B. oleracea* pathotype (section 5.3). All 31 primer pairs designed will be radioactively labelled followed by PCR analysis and polyacrylamide gel electrophoresis. Twenty nine isolates of *P. parasitica* including isolates from *Brassica oleracea* pathotypes from different geographic locations and isolates of different pathotypes will be analysed and the data accumulated for each primer pair or locus.

5.2 MATERIALS AND METHODS

5.2.1. DNA isolation from isolates of *Peronospora parasitica*

Good quality genomic DNA was isolated from 29 isolates of *P. parasitica* using the method described in Chapter Two. For convenience, isolates (Table 2.1) were labelled numbers 1 to 29 as indicated in Table 5.3. Control host DNA was extracted as described in Chapter 2.

Isolates of five pathotypes of *P. parasitica* were included (Table 2.1), viz. the *Brassica oleracea*, *B. napus*, *B. rapa*, *B juncea* and *Arabidopsis thaliana* pathotypes. Isolate IP05B was a single spore isolate of the *Brassica juncea* pathotype from the UK. Isolates IP09 and IP13 were field isolates of the *B. campestris* pathotype from India; due to low infection levels derivation of single spore isolates from these field isolates was not possible. Isolate P003 and R1 were isolates of the *B. napus* pathotype from the UK. The majority (26) of the isolates were of the *B oleracea* pathotype and included isolates from South Africa, Mozambique, France, Portugal, India and UK. Isolates BR1F, Br5A and Pp9 were from France whereas isolate P501 was from Portugal. Isolate P005 was from the UK and isolates ACAT2B, ACAT2C, ACAT2D, ACAT2E from a single field population in Eastern Cape, South Africa (ACAT); the bulk of the isolates were from KwaZulu Natal, South Africa and included isolates, SSH1, SSH2, SSH3, SSH4, SSH5, SSH6 from a single field population (SSH) and PPSAM1 from PPSAM. Isolates PPSAR2, PPSAR4, PPSAR5, PPSAR6 and TC1, TC2 were from the PPSA and TC field groups respectively. All single spore isolates from South Africa were derived as described in 2.2.2. Noks1 was an *Arabidopsis thaliana* isolate.

Brassica oleracea seeds were surface sterilised in 3% sodium hypochlorite and grown in autoclaved pots (1 kPa, 121°C for 15 min.) at 25°C in the greenhouse. For each isolate of *P. parasitica*, the maintenance host (described in Table 2.1) was inoculated

with a conidial suspension (5 x 10^5 conidia/ml) and incubated in a Schnidjers Climatic Chamber. The climatic chamber was programmed with a light dark cycle; 9 hr dark and 15 hr light (100 $\mu Es^{-1}m^{-2}$) at a constant temperature of 16°C. Approximately 24 hrs after inoculation cotyledons were excised and axenic cultures were prepared as described in 2.2.3. Axenic culture involved surface sterilisation of excised cotyledons in 70 % ethanol and 1% sodium hypochlorite. Cotyledons were then dried briefly on sterile filter paper and placed on Murashige and Skoog (1962) medium. Seven days after inoculation, conidia were collected by tapping spores onto sterile water which were then concentrated by centrifugation.

Good quality genomic DNA was extracted from conidia of isolates of *P. parasitica* (Tham et al. 1994) and from control host DNA (Edwards et al. 1991) as described in 2.2. Conidia, collected as described, were centrifuged and the conidial pellet washed once in sterile distilled water. Clean conidia were then vortexed for 1 min. with a mixture of 1- and 6 mm diameter glass beads (Sigma) in 600 µl lysis buffer (100 mM tris-HCl (pH 7.20), 100 mM EDTA, 10 % (w/v) SDS and 2 % (v/v) 2-mercaptoethanol). DNA was recovered from the suspension of broken conidia using the protocol described by Lee and Taylor (1990). The suspension was incubated at 65 ^{O}C for 1.5 hr and vortexed every 20 min. for 30 sec. To remove the protein and cell debris, 600 µl chloroform:phenol (1:1) was added, vortexed briefly, and microcentrifuged at 10,000 x gr for 15 min. at room temperature or until the aqueous (top) phase is clear. Approximately 300 to 500 µl of the aqueous phase containing the DNA was transferred to a new tube. Precipitation of DNA was achieved using 10 µl 3 M sodium acetate and 0.54 volumes isopropanol and centrifugation at 10 000 x gr for 2 min. DNA pellets were washed in 70 % alcohol and resuspended in 30 µl of TE (10 mM Tris-HCI, 0.1 mM EDTA) buffer. Following resuspension, the concentration and the purity of the DNA was determined by gel electrophoresis with concentration standards and ethidium bromide staining. DNA was stored at -20 ^{0}C.

5.2.2 Pre-screening of a genomic DNA library of *Peronospora parasitica* enriched for microsatellites by non-radioactive PCR

Initial screening of the *P. parasitica* library enriched for microsatellites involved the use of unlabelled primers (Table 4.3) in the amplification of the various loci and amplimers were analysed on agarose gels. To confirm non-amplification of host DNA, control host DNA was combined in PCR reactions at different primer annealing temperatures and analysed on agarose gels; an isolate representing each of the pathotypes, together with one deliberately contaminated with host DNA, and pure host DNA was amplified with different primer sets (viz. M1, M6). For the *B. oleracea* pathotype, ACAT2E and the host Glory of Enkhuizen were utilised. For the *B. napus* pathotype, isolate R1 and the corresponding host Capricorn were utilised. For the *B. juncea* pathotype, isolate IP05B and the host PPBJ-1 were used. For the *B. rapa* pathotypes, the isolate IP09 and the host Sumo were selected.

Polymerase chain reactions (30 µl) were set up containing the following: 1.5 µg of each primer, 10 x PCR Buffer (Gibco, BRL), 250 µM dNTPs (Promega), 50 ng genomic DNA and 1U *Taq* DNA polymerase (Gibco, BRL). Amplification was carried out in a GeneAmp 9700 PCR machine using the following cycling conditions; 95°C for 2 min. followed by 36 cycles of 94°C for 40 sec, 60°C for 1 min. and 72°C for 30 sec, and a final elongation at 72°C for 10 min. PCR products were electrophoresed on 2% agarose gels at 80 V for 2 hrs. Gel images were captured using a UVP gel documentation system.

5.2.3 Screening of a *Peronospora parasitica* pathotypes with γ-^{33}P labelled microsatellite primers

Genomic DNA of 29 isolates of *P. parasitica* (Table 5.3) was amplified using 31 primer sets (Table 4.3) designed in the microsatellite library of *P. parasitica* in Chapter Five. Genomic DNA of four hosts were included as controls. Screening involved PCR amplification with radiolabelled primers, separation on polyacrylamide gels and autoradiography.

Table 5.3: Numerical designations utilized for microsatellite marker screening of *Peronospora parasitica* and its hosts

Designated Number	*P.parasitica* isolate	Pathotype
1	IP05B	*Brassica juncea*
2	IP13	*Brassica rapa*
3	IP09	*Brassica rapa*
4	R1	*Brassica napus*
5	P003	*Brassica napus*
6	P005	*Brassica oleracea*
7	BR1F	*Brassica oleracea*
8	Br5A	*Brassica oleracea*
9	P501	*Brassica oleracea*
10	Pp9	*Brassica oleracea*
11	P216	*Brassica oleracea*
12	SSH1	*Brassica oleracea*
13	SSH2	*Brassica oleracea*
14	SSH3	*Brassica oleracea*
15	SSH4	*Brassica oleracea*
16	SSH5	*Brassica oleracea*
17	SSH6	*Brassica oleracea*
18	TC1	*Brassica oleracea*
19	PPSAR2	*Brassica oleracea*
20	PPSAR4	*Brassica oleracea*
21	PPSAR5	*Brassica oleracea*
22	PPSAR6	*Brassica oleracea*
23	ACAT2B	*Brassica oleracea*
24	ACAT2C	*Brassica oleracea*
25	ACAT2D	*Brassica oleracea*
26	ACAT2E	*Brassica oleracea*
27	PPSAM1	*Brassica oleracea*
29	Noks1	*Arabidopsis thaliana*
28	*Bremia lactucae*	*Lactucae sativa*
	Host control DNA	
	Cultivar	Species
30	Capricorn	*Brassica napus*
31	Sumo	*Brassica rapa*
32	PPBJ1	*Brassica juncea*
33	Glory of Enkhuizen	*Brassica oleracea*

5.2.3.1 Primer labelling with γ-^{33}P nucleotide triphosphate

All forward primers (Table 4.3) were end-labelled with γ-^{33}P nucleotide triphosphate. For each primer a labelling master mix was prepared. The primer labelling mix for a 25 µl amplification reaction consisted of the following: 25 ng or 0.25 µl (0.1 µg/µl) forward primer, 0.05 µl One-Phor-All Buffer (OPA; 100 mM Tris-acetate, 100 mM Magnesium

acetate, 500 mM potassium acetate, pH 7.5; Pharmacia, USA), 0.05 µl γ-^{33}P ATP (Amersham, UK), 0.008 µl T4 polynucleotide kinase (PNK) (Pharmacia, USA) and sterile distilled water (SDW) to make up final volume of 25 µl. Labelling mastermixes for 35 PCR reactions were prepared as described in Appendix C. The labelling mix was incubated at 37 °C for half an hour followed by 10 min. at 68-72°C to denature the PNK.

5.2.3.2 PCR Amplification of microsatellite loci with γ-^{33}P labelled primers

PCR mastermixes were prepared for each primer pair as described in Appendix C Mastermixes were prepared such that each PCR reaction (12.5 µl) contained the following: 1.25 µl 10 x PCR Buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, 0.01% gelatin, pH 8.5; Perkin Elmer), 0.1 µl dNTPS (25 mM), 0.25 µl Reverse Primer (1 µg/µl), 0.008 µl labelled Primer (6.2.1), 0.1 µl *Taq* DNA polymerase (5 U/µl) (Gibco BRL, Life Technologies) and SDW to 12.5 µl; the *Taq* DNA polymerase and labelled primer were added at the end. Amplification was conducted in a GeneAmp 9700 PCR machine (Perkin Elmer). The cycling conditions were as follows: an initial denaturation at 94 for 2 min. followed by 35 cycles of 1 min. @ 94°C, 1 min. @ 60°C and 1 min. @ 72°C, and a final extension for 10 min. @ 72°C. For some primers different annealing temperatures were attempted. Experiments were replicated thrice.

5.2.3.3 Resolution of microsatellite amplimers with polyacrylamide gel electrophoresis

Polymerase chain reaction products were electrophoresed on a manual sequencing system (Life Technologies, USA). Prior to commencement of the experiment, glass plates (including new plates) were soaked in 0.5 M NaOH overnight to strip them of all previous chemicals. Glass plates were washed thoroughly in liquid soap and warm water to remove all traces of previous gel debris. Plates were dried and cleaned with 100% ethanol. Each plate was carefully marked on the outside and Sigmacote (Sigma) was

added to the inside of one plate only. Gel spacers (0.3 mm) were used to prepare gel sandwiches and the plates were firmly clamped in place.

Six percent denaturing sequencing polyacrylamide gels were prepared for separation of microsatellite products. Urea (Separations) (29.4 g) was added to 7 ml 10 x TBE (54 g/l Tris, 27.5 g/l boric acid, 20 ml/l 0.5 M EDTA, pH 8), 10.5 ml Accugel (acrylamide: bisacrylamide; 19:1) and distilled water to a final volume of 70 ml. The urea was allowed to dissolve completely and 375 µl freshly prepared ammonium persulphate (10% stock) and 75 µl TEMED added prior to pouring. The gel was carefully yet quickly poured and allowed to polymerise for at least an hour; alternatively polyacrylamide gels were covered with cling film and used the next morning. Once the gel was set, the comb was removed and the wells washed thoroughly with 1 X TBE buffer using a syringe to remove all unpolymerised urea. The gel was pre-warmed at 70 watts for 20-30 min., the comb replaced and the samples prepared as described below and loaded into labelled wells.

5.2.3.4 Sample preparation for polyacrylamide gel electrophoresis (PAGE)

Polymerase chain reaction products were prepared for polyacrylamide gel electrophoresis (PAGE) by the addition of an equal volume of formamide loading buffer (80% formamide, 10 mM EDTA (pH 8), 1mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue; Maniatis et al. 1982). PCR products were then heat denatured at 94°C for 3-5 min. and immediately chilled on ice. 4 µl (for 48 lane gels) or 2 µl (for 96 lane gels) of each sample were loaded on PAGE gels. 1-2 µl of marker (Sequamark, Biomedical Inc. USA) was also loaded. Samples were electrophoresed in 1 x TBE at 55-60 W until the bromophenol blue dye reached the end of the gel (approximately 1 hr 15 min.). The plates were separated by carefully lifting the corner of the plate containing the Sigmacote repellent with a blade. The gel was transferred onto blotting paper (3 mm Whatman). When good quality plates were used (Life Technologies), Sigmacote (Sigma) was not necessary and plates were carefully separated by spreading ice for a few seconds on one

plate immediately after electrophoresis. The gels were then dried for approximately 2 hr at 80°C. The gel was then exposed to X-ray film (Kodak BioMax MR film, Kodak, USA) for 24-48 hrs, ensuring that it was placed correctly over the gel, with the notch in the top left hand corner. The emulsion coating of the film was placed in contact with the sample. *Kodak Biomax MR film provided maximum resolution, clarity and sensitivity for the detection of ^{33}P labelled samples.

5.2.3.5 Preparation of SequaMark – 10 bp ladder for size determination

A 10 bp ladder for size determination of microsatellites was prepared using the SequaMark protocol (Biomedical Inc. USA). Reagents of the kit viz. the template ssDNA was resuspended in 250 µl sterile distilled water and the SequaMark primer was resuspended in 100 µl sterile distilled water and stored at -20°C. Sequamark was prepared by mixing: 5 µl of template ssDNA, 2 µl SequaMark primer, 2.5 µl Perkin Elmer-Amplicycle 10 x cycling mix, 4 µl dTTP (PE-Amplicycle kit), 13.5 µl sterile distilled water and 1 µl of $\alpha^{33}P$ adenosine triphosphate (Amersham Pharmacia Biotech, UK). The labelling mix was subjected to an Amplicycle sequencing programme: An initial denaturation at 95° for 1 min., followed by 25 cycles of 30 sec @ 95°C, 30 sec @ 68°C and 1 min. @ 72°C, and finally 45 min at 4°C. The marker was stored at 4°C. When needed the required amount was transferred to a new PCR tube, an equal volume of formamide loading buffer was added, and denatured for 3 min. at 95°C. Depending on activity of the isotope, 1-2 µl was added for the large well combs and 0.5-1µl for the small well combs of PAGE gels. All experiments described were replicated thrice.

5.2.3.6 Autoradiography

X-rays were developed with an automatic X-ray film processor, Compact X-2 (X-OGRAPH Imaging Systems, LTD, UK). Different exposure times were utilised for visual optimisation.

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* Kodak Biomax MR film is coated with emulsion on one side of the film base only. When the emulsion identification is positioned in the upper right corner, the emulsion coating is facing up.

5.2.4 Post-PCR product purification

Radioactively labelled PCR products, which did not yield good resolution on polyacrylamide gels, were purified by solvent extraction using the modified method of J. Barker (personal communication) PCR products (with loading buffer) were transferred to 0.5 ml PCR tubes and volumes were made up to 50 µl with SDW. An equal volume of phenol (pH 8) was added, gently mixed and incubated at 37°C for 10 min. The mixture was spun at 13 000 rpm for 2 min. and the top layer transferred to a new tube. 50 µl phenol:chloroform (1:1) was added, mixed, centrifuged at 13 000 rpm for 2 min. and the top layer transferred to a new tube. 50 µl chloroform was added, mixed, centrifuged at 13 000 rpm for 2 min. and the top layer transferred to a new tube. PCR products were precipitated by the addition of 5 µl 3 M sodium acetate (Sigma, UK) and 125 µl 100% ethanol, mixed and incubated at RT for 10 min. The mixture was centrifuged at 13 000 rpm for 2 min., the supernatant discarded and 250 µl 75% ethanol added to wash the pellet. The mixture was spun, the supernatant discarded and the pellet dissolved in 7.5 µl sample buffer. The mixture was denatured and 2 µl loaded per sample as described previously.

5.2.5 Data analysis

Autoradiograms were viewed and alleles for each of the isolates were scored as presence (1) or absence (0) in a Microsoft Excel spreadsheet. A Jaccard similarity matrix was calculated for various sets of loci/alleles and a dendrogram constructed from a UPGMA cluster analysis of that matrix.

5.3 RESULTS

5.3.1 Pre-screening of *Peronspora parasitica* microsatellite library with non-radioactive primers

Preliminary screening with host DNA controls prior to screening the library (Chapter 4) for polymorphism across the isolate set (Table 5.3) revealed non-amplification of host DNA. The amplification of different pathotypes and their corresponding hosts at the M6 microsatellite locus revealed a band that was amplified from the *P. parasitica* pathotype, as well as from the *P. parasitica* pathotype with host contaminant, but not from the host DNA alone (Fig. 5.1). This was effective in reducing host contamination bias in the library.

Pre-screening of the M37 microsatellite locus on agarose gels across all pathotypes and isolates of *Peronospora parasitica* indicated a single monomorphic band of the expected size in all isolates (Fig. 5.2). The effect of different annealing temperatures was not significant. An absence of host DNA amplification was also noted.

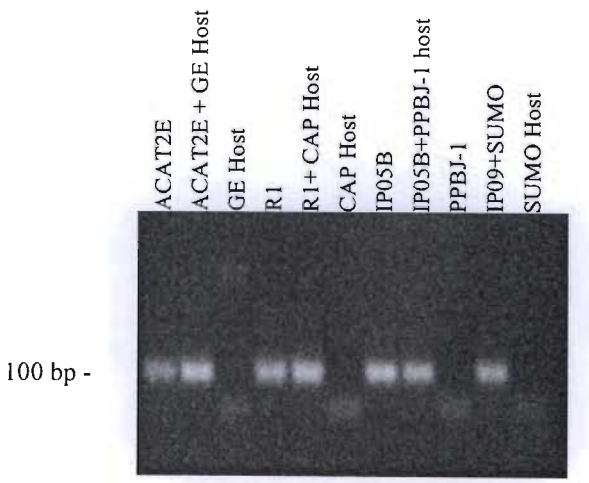

Fig. 5.1: Screening of four pathotypes of *Peronospora parasitica* together with host DNA controls at the M6 microsatellite locus on a 2% agarose gel.

Brassica oleracea pathotype - Isolate ACAT2E, host Glory of Enkhuizen (GE).
Brassica napus pathotype - Isolate R1, host Capricorn (CAP).
Brassica juncea pathotype - Isolate IP05B, host PPBJ-1.
Brassica rapa pathotype - Isolate IP09, host Sumo.

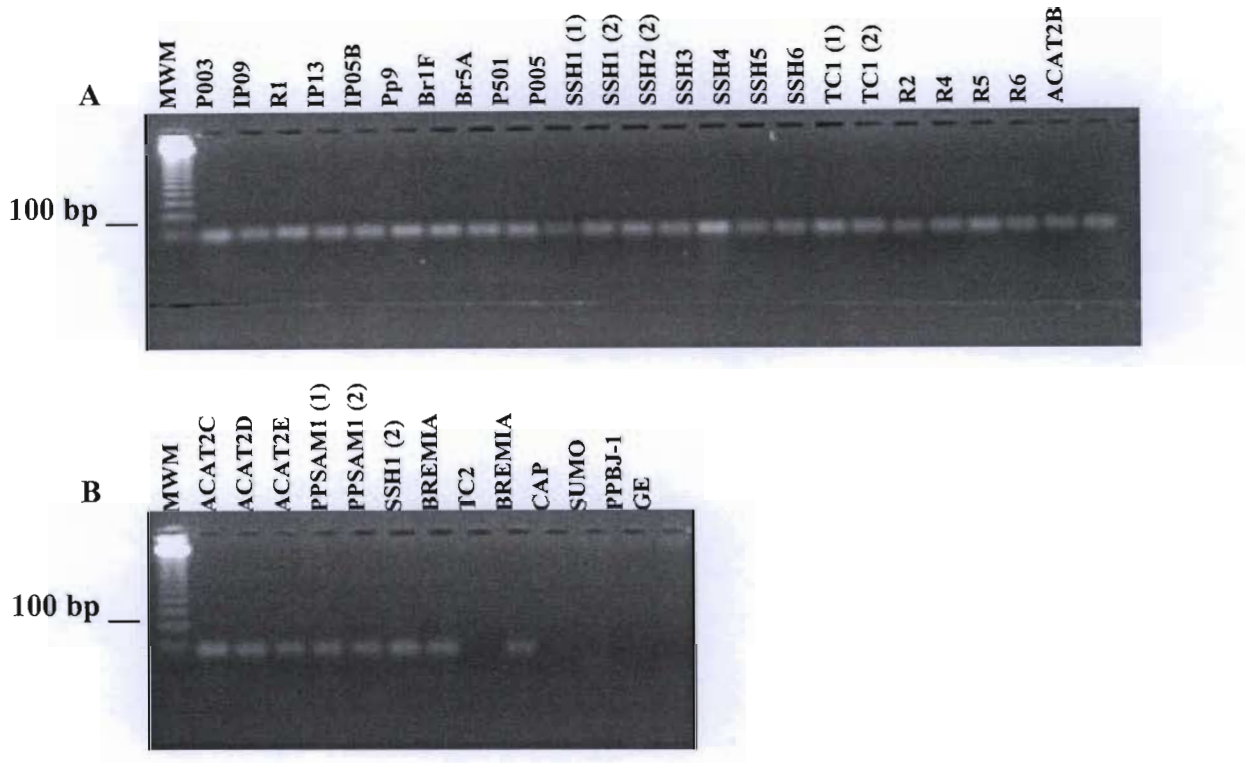

Fig. 5.2 A-B: PCR analysis of the M37 microsatellite locus of *Peronospora parasitica* on 2% agarose gels. MWM - 100 bp ladder.

P. parasitica isolates:

P003, R1 - *Brassica napus* pathotype. IP09, I

P13 - *Brassica rapa* pathotype.

IP05B - *Brassica juncea* pathotype.

Br1F, Br5A, P501, P005, SSH1 (1), SSH1 (2), SSH2 (2), SSH3, SSH3, SSH4, SSH4, SSH5, SSH6, TC1 (1), TC1 (2), R2, R4, R5, R6, ACAT2B, ACAT2C, ACAT2D, ACAT2E, PPSAM1 (1), PPSAM1 (2) - *Brassica oleracea* pathotype

Bremia lactucae: BREMIA

Host cultivars:

cv. Capricorn (CAP) - *Brassica napus*, cv. Sumo - *Brassica rapa*, cv. PPBJ-1 - *Brassica juncea*, cv. Glory of Enkhuizen (GE) - *Brassica oleracea*.

5.3.2 Screening of *Peronospora parasitica* pathotypes with γ-^{33}P labelled microsatellite primers

Screening of the microsatellite library using labelled primers was initiated once the following was established: 1) the library prepared in Chapter Five was indeed enriched for potential microsatellites (Section 4.3); 2) the DNA prepared from the isolate collection (Chapter Two and Table 5.3) was sufficiently pure for PCR analysis (Chapter Four); and 3) that the primers designed in Chapter Five were specific enough in that they did not amplify host DNA (Fig. 5.1).

Polymorphism across the isolate set (Table 5.3) was detected across 27 of the 29 loci analysed. A subset of this is represented by the M37, M26, M1, M5(2), M112, M21, M55, M48, M34 loci in Figs. 5.3 - 5.11 respectively. Microsatellites alleles were scored by recording the presence (1) or absence (0) of bands as shown in Tables 5.6 - 5.12. Usually an allele had its major band (darkest) and associated with this major band were minor stutter bands and shadow bands which differ from the main band by multiples of the repeat (in this case 2 since dinucleotides are considered). Occasionally what appeared as the stutter band for an allele in one sample was the same size as the allele for the other sample. Attempts were made to score these alleles as accurately as possible. Comparing the library isolate 6 (P005) to the Sequamark$^{\Psi}$ sequencing standard, the allele sizes are in the expected size range (Table 5.4). These sizes compared well with the estimated sizes in Table 4.3.

The simplest microsatellite locus was the M37 locus (Fig. 5.3). M37 was found to be monomorphic across all isolates since a single band was produced by all isolates. This correlated well with the image viewed on agarose gels. A single band was also amplified by the *Bremia lactucae* isolate, however host DNA was not amplified.

$^{\Psi}$ The 10 bp ladder, Sequamark was added to every gel and allele sizes were calculated from autoradiograhs. For some Figures the ladder may not be visible due to it being loaded away from the immediate lanes.

Table 5.4: Allele sizes of various microsatellite loci of *Peronospora parasitica*

Locus	Estimated Allele Size (Table 4.3) (bp)	True Allele Size range on PAA gels (bp)
M1	115	94-110
M5(2)	186	120-190
M112	187	110-160
M21	147	118-160
M55	143	130-170
M34	103	88-112
M48	182	140-160
M26	171	120-140
M37	113	112

The M26, M1, M5(2), M112, M21, M55, M48, M34 produced good polymorphic patterns (Figs. 5.4 - 5.1.1)[∞]. Each locus provided varying amounts of information. The M1 locus (Fig 5.4) shows some polymorphism and alleles were scored as shown in Table 5.5. For the M1 locus, each isolate contained only 1 or 2 alleles; the isolates 12-25, 27 were identical, however, isolates 1-3 were each unique. The M26 locus had a number of alleles common to most samples (Fig. 5.5 and Table 5.6). The middle obvious band was present in all samples and was therefore not scored. With the exception of the first few samples, the rest of the samples appear very similar. The number of alleles per sample varied from 2 in samples 4,9 to 6 in sample 6.

The M21 and M112 loci had very similar gel patterns (Figs. 5.6. and 5.7), but were slightly different sizes; the allele size range in M21 and M112 were 118-160 and 110-160 bp respectively. From Fig. 5.6 and 5.7, it was evident that the latter half of the isolates on both these gels were more alike. The isolates at the beginning were more unique and in the middle there was a "grey" area of shared alleles. From the allele distribution tables (Table 5.7 and 5.8 for M21 and M112 respectively) isolates 1-5 are more unique, isolates 6-11 shared at least one allele with isolates 12-27. It is important to

[∞] Banding patterns and allele labels approximate those seen on autoradiographs. Alleles were scored from autoradiographs. Furthermore, host DNA lanes may not be represented since no products were seen.

note that isolates 1-5 are of different pathotypes (*B. juncea*, *B. napus*, and *B. rapa*) whereas isolates 6-27 are of the *B. oleracea* pathotype. Furthermore, isolates 6-11 are of different geographical origin compared to 12-27 which were from South Africa. It appears that the South African isolates of the *B. oleracea* pathotype shared some alleles in common with isolates from the UK, France and other geographical areas.

The microsatellite locus M5(2) was most polymorphic as there were more unique genotypes (Fig. 5.8 and Table 5.9). While isolates of various pathotypes appeared unique there was also a wider degree of variation reflected within the *B. oleracea* pathotype. The number of alleles per genotype ranged from 1 to 5. Similarly the M55 locus (Fig. 5.10 and Table 5.11) appeared very polymorphic. However the alleles have more stutter. The number of alleles differs from 1-6 per sample. Phenol:chloroform purification did not improve resolution of the alleles or reduce overlapping (data not shown). The M34 locus (Fig. 5.11 and Table 5.12) was polymorphic with many alleles however stutter bands of noticeable longer length (and lighter appearance were associated with each allele). The M48 locus was moderately polymorphic; The allele distribution appeared simple/straightforward, however, there were more overlapping alleles present which sometimes made interpretation ambiguous (Fig. 5.9). Scoring of gels revealed many isolates each with a unique set of alleles (Table 5.10).

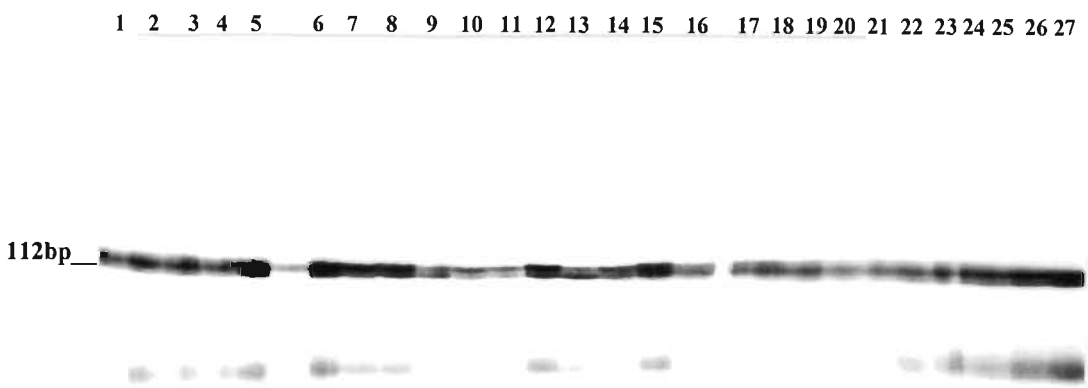

Fig. 5.3: Screening of the M37 microsatellite locus of *Peronospora parasitica* on a 6% polyacrylamide gel. *P. parasitica* isolates: Lane 1-IP05B, 2 - IP13, 3 - IP09, 4 - R1, 5 - P003, 6 - P005, 7 - BR1F, 8 - Br5A, 9 - P501, 10 - Pp9, 11 - P216, 12 - SSH1, 13 - SSH2, 14 - SSH3, 15 - SSH4, 16 - SSH5, 17 - SSH6, 18 - TC1, 19 - PPSAR2, 20 - PPSAR4,21 - PPSAR5, 22 - PPSAR6, 23 - ACAT2B, 24 - ACAT2C, 25 - ACAT2D, 26 - ACAT2E, 27 - PPSAM1. Isolates 1-27 were monomorphic at the M37 locus.

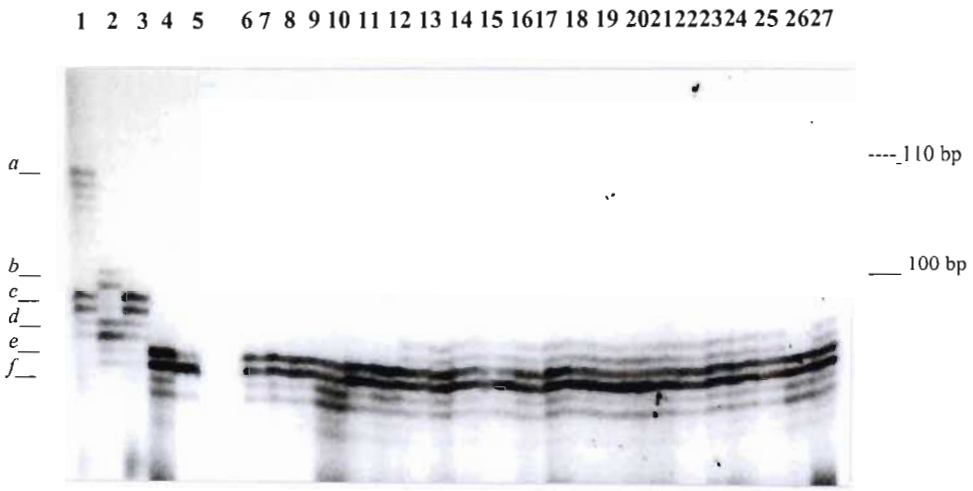

Fig. 5.4: Screening of the M1 microsatellite locus of *Peronospora parasitica* on a 6% polyacrylamide gel. *P. parasitica* isolates: Lane 1-IP05B, 2 - IP13, 3 - IP09, 4 - R1, 5 - P003, 6 - P005, 7 - BR1F, 8 - Br5A, 9 - P501, 10 - Pp9, 11 - P216, 12 - SSH1, 13 - SSH2, 14 - SSH3, 15 - SSH4, 16 - SSH5, 17 - SSH6, 18 - TC1, 19 - PPSAR2, 20 - PPSAR4,21 - PPSAR5, 22 - PPSAR6, 23 - ACAT2B, 24 - ACAT2C, 25 - ACAT2D, 26 - ACAT2E, 27 - PPSAM1. *a-f* indicate alleles shared by these isolates.

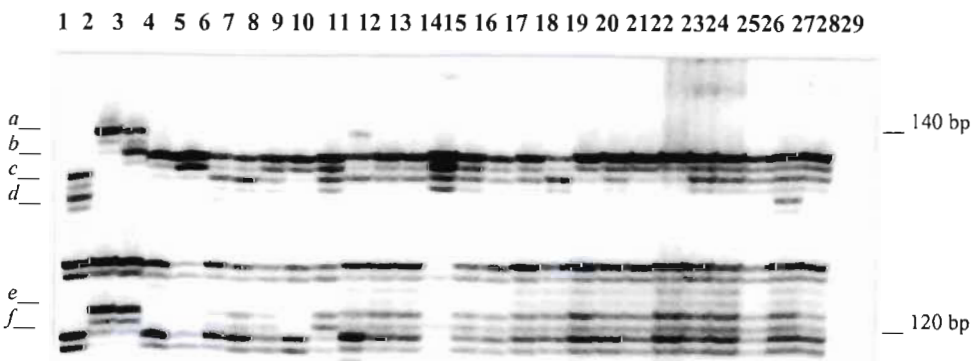

Fig. 5.5: Screening of the M26 microsatellite locus of *Peronospora parasitica* on a 6% polyacrylamide gel. *P. parasitica* isolates: Lane 1-IP05B, 2 - IP13, 3 - IP09, 4 - R1, 5 - P003, 6 - P005, 7 - BR1F, 8 - Br5A, 9 - P501, 10 - Pp9, 11 - P216, 12 - SSH1, 13 - SSH2, 14 - SSH3, 15 - SSH4, 16 - SSH5, 17 - SSH6, 18 - TC1, 19 - PPSAR2, 20 - PPSAR4,21 - PPSAR5, 22 - PPSAR6, 23 - ACAT2B, 24 - ACAT2C, 25 - ACAT2D, 26 - ACAT2E, 27 - PPSAM1, 29 - Noks1. *Bremia lactucae*: 28. *a-f* indicate alleles shared by these isolates.

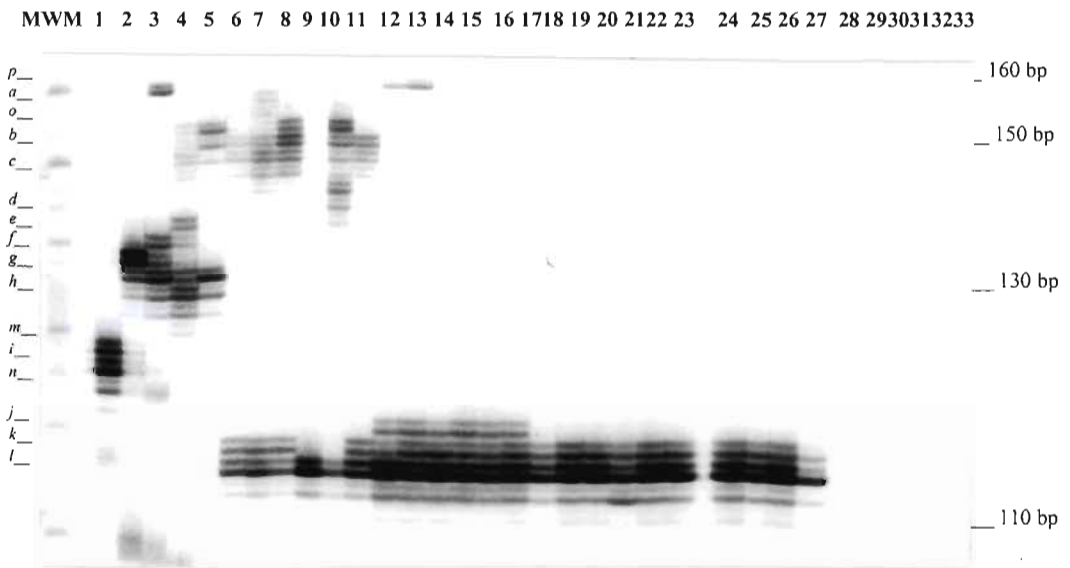

Fig. 5.6: Screening of the M21 microsatellite locus of *Peronospora parasitica* on a 6% polyacrylamide gel. *P. parasitica* isolates: Lane 1-IP05B, 2 - IP13, 3 - IP09, 4 - R1, 5 - P003, 6 - P005, 7 - BR1F, 8 - Br5A, 9 - P501, 10 - Pp9, 11 - P216, 12 - SSH1, 13 - SSH2, 14 - SSH3, 15 - SSH4, 16 - SSH5, 17 - SSH6,18 - TC1, 19 - PPSAR2, 20 - PPSAR4,21 - PPSAR5, 22 - PPSAR6, 23 - ACAT2B, 24 - ACAT2C, 25 - ACAT2D, 26 - ACAT2E, 27 - PPSAM1, 29 - Noks1. *Bremia lactucae*: 28. MWM: 10 bp ladder, Sequamark (Biomedical Inc. USA). Host DNA (controls): 30 - Capricorn, 31 - Sumo, 32 - PPBJ1, 33 - Glory of Enkhuizen. *a-p* indicate alleles shared by these isolates.

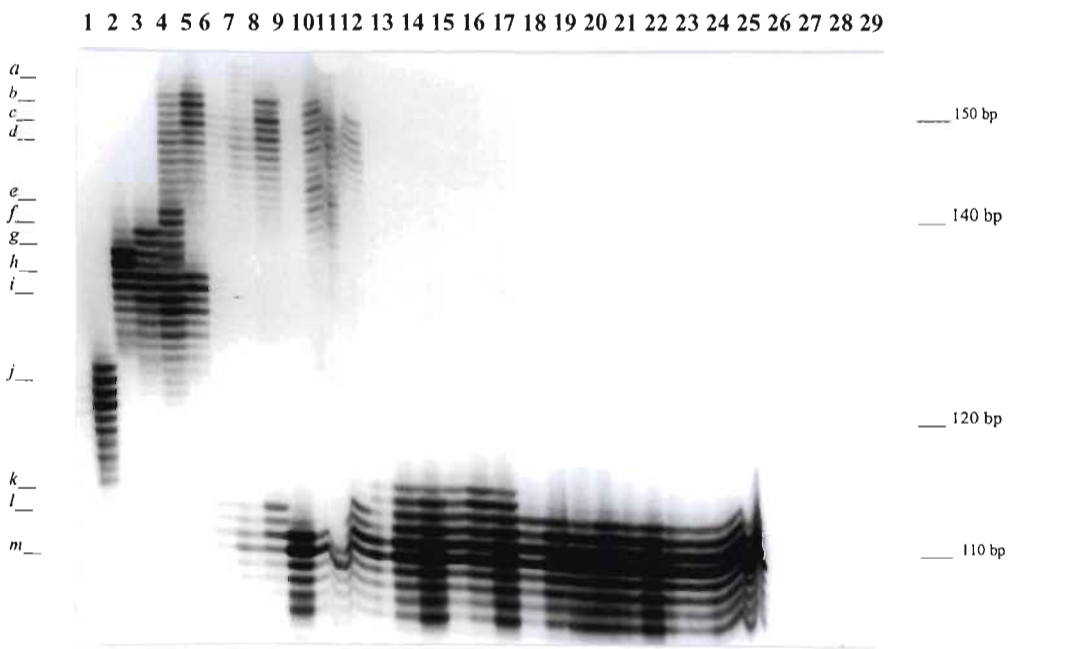

Fig. 5.7: Screening of the M112 microsatellite locus of *Peronospora parasitica* on a 6% polyacrylamide gel. *P. parasitica* isolates: Lane1-IP05B, 2 - IP13, 3 - IP09, 4 - R1, 5 - P003, 6 - P005, 7 - BR1F, 8 - Br5A, 9 - P501, 10 - Pp9, 11 - P216, 12 - SSH1, 13 - SSH2, 14 - SSH3, 15 - SSH4, 16 - SSH5, 17 - SSH6,18 - TC1, 19 - PPSAR2, 20 - PPSAR4,21 - PPSAR5, 22 - PPSAR6, 23 - ACAT2B, 24 - ACAT2C, 25 - ACAT2D, 26 - ACAT2E, 27 - PPSAM1, 29 - Noks1. *Bremia lactucae*: 28. *a-m* indicate alleles shared by these isolates.

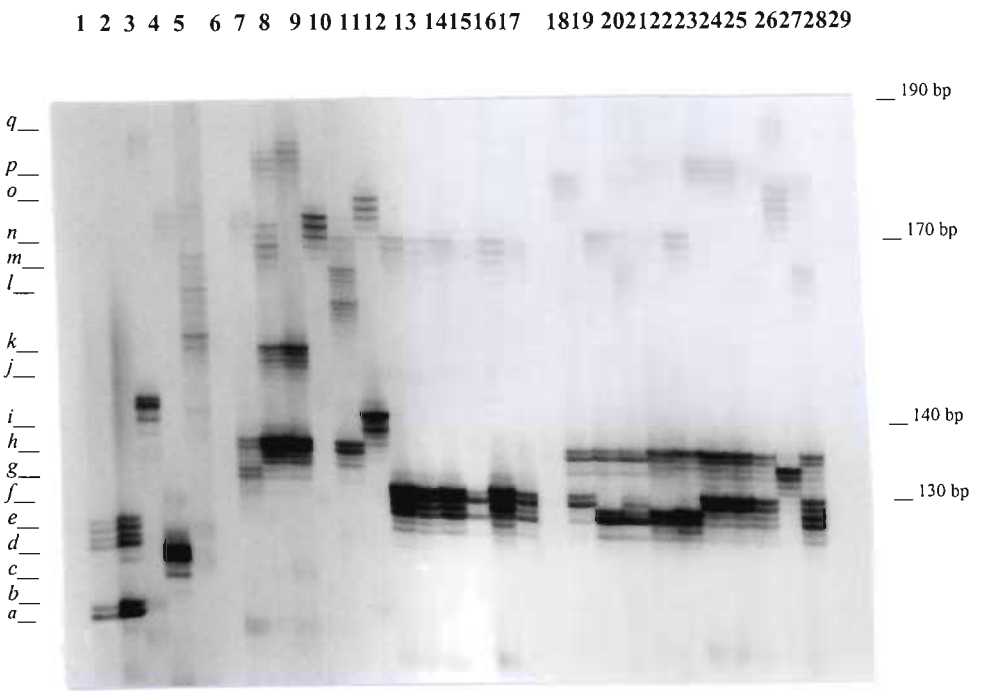

Fig. 5.8: Screening of the M5 (2) microsatellite locus of *Peronospora parasitica* on a 6% polyacrylamide gel. *P. parasitica* isolates: Lane 1-IP05B, 2 - IP13, 3 - IP09, 4 - R1, 5 - P003, 6 - P005, 7 - BR1F, 8 - Br5A, 9 - P501, 10 - Pp9, 11 - P216, 12 - SSH1, 13 - SSH2, 14 - SSH3, 15 - SSH4, 16 - SSH5, 17 - SSH6,18 - TC1, 19 - PPSAR2, 20 - PPSAR4,21 - PPSAR5, 22 - PPSAR6, 23 - ACAT2B, 24 - ACAT2C, 25 - ACAT2D, 26 - ACAT2E, 27 - PPSAM1, 29 - Noks1. *Bremia lactucae*: 28. *a-q* indicate alleles shared by these isolates.

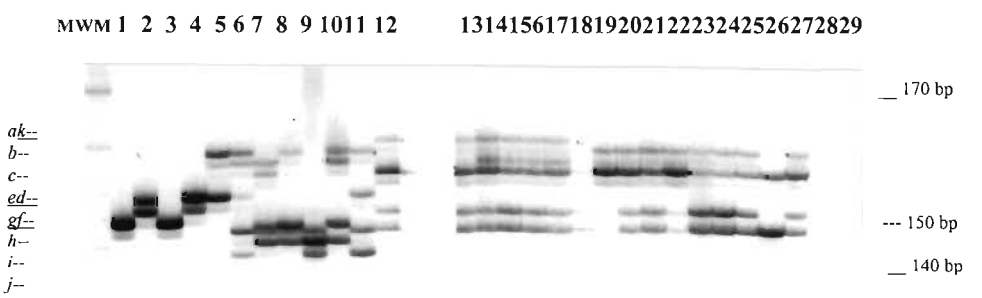

Fig. 5.9: Screening of the M48 microsatellite locus of *Peronospora parasitica* on a 6% polyacrylamide gel. *P. parasitica* isolates: Lane 1-IP05B, 2 - IP13, 3 - IP09, 4 - R1, 5 - P003, 6 - P005, 7 - BR1F, 8 - Br5A, 9 - P501, 10 - Pp9, 11 - P216, 12 - SSH1, 13 - SSH2, 14 - SSH3, 15 - SSH4, 16 - SSH5, 17 - SSH6, 18 - TC1,19 - PPSAR2, 20 - PPSAR4,21 - PPSAR5, 22 - PPSAR6, 23 - ACAT2B, 24 - ACAT2C, 25 - ACAT2D, 26 - ACAT2E, 27 - PPSAM1, 29 - Noks1. *Bremia lactucae*: 28. MWM: 10 bp ladder, Sequamark (Biomedical Inc. USA). *a-k* indicate alleles shared by these isolates.

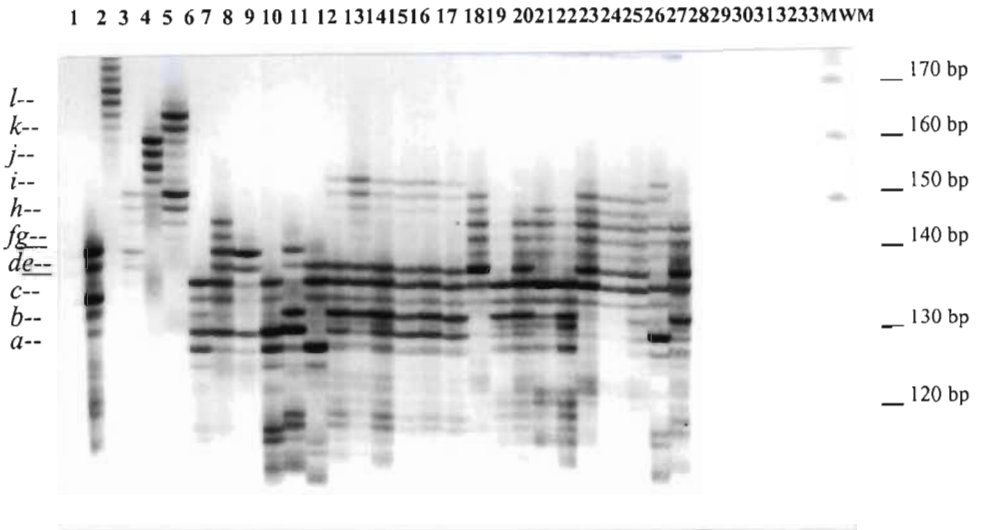

Fig. 5.10: Screening of the M55 microsatellite locus of *Peronospora parasitica* on a 6% polyacrylamide gel. *P. parasitica* isolates: Lane 1-IP05B, 2 - IP13, 3 - IP09, 4 - R1, 5 - P003, 6 - P005, 7 - BR1F, 8 - Br5A, 9 - P501, 10 - Pp9, 11 - P216, 12 - SSH1, 13 - SSH2, 14 - SSH3, 15 - SSH4, 16 - SSH5, 17 - SSH6,18 - TC1, 19 - PPSAR2, 20 - PPSAR4,21 - PPSAR5, 22 - PPSAR6, 23 - ACAT2B, 24 - ACAT2C, 25 - ACAT2D, 26 - ACAT2E, 27 - PPSAM1, 29 - Noks1. *Bremia lactucae*: 28. MWM: 10 bp ladder, Sequamark (Biomedical Inc. USA). Host DNA (controls): 30 - Capricorn, 31 - Sumo, 32 - PPBJ1, 33 - Glory of Enkhuizen. *a-l* indicate alleles shared by these isolates.

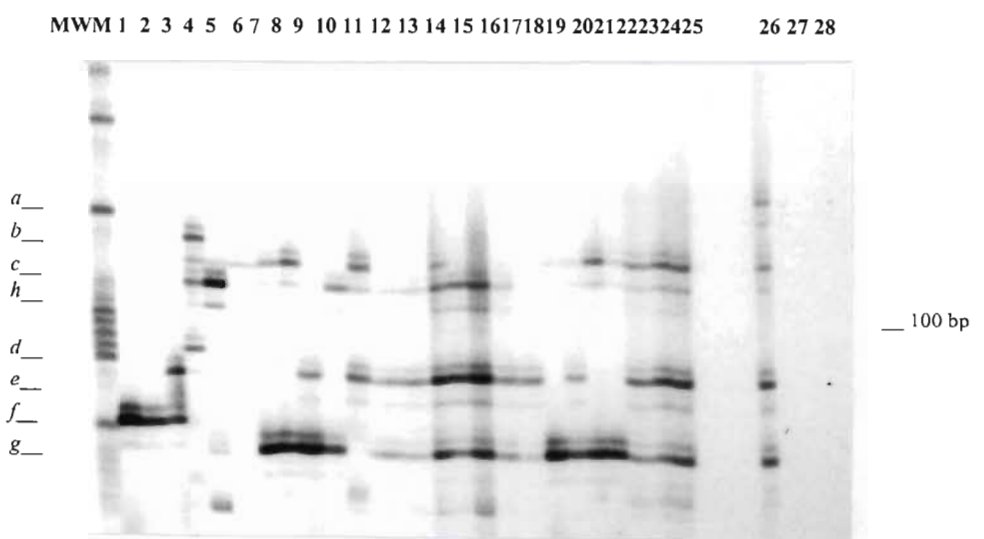

Fig. 5.11: Screening of the M34 microsatellite locus of *Peronospora parasitica* on a 6% polyacrylamide gel. *P. parasitica*isolates: Lane 1-IP05B, 2 - IP13, 3 - IP09, 4 - R1, 5 - P003, 6 - P005, 7 - BR1F, 8 - Br5A, 9 - P501, 10 - Pp9, 11 - P216, 12 - SSH1, 13 - SSH2, 14 - SSH3, 15 - SSH4, 16 - SSH5, 17 - SSH6,18 - TC1, 19 - PPSAR2, 20 - PPSAR4,21 - PPSAR5, 22 - PPSAR6, 23 - ACAT2B, 24 - ACAT2C, 25 - ACAT2D, 26 - ACAT2E, 27 - PPSAM1, *Bremia lactucae*: 28. MWM: 10 bp ladder, Sequamark (Biomedical Inc. USA). *a-h* indicate alleles shared by these isolates.

Table 5.5: Allele distribution among 29 isolates of *Peronospora parasitica* at the M1 microsatellite locus

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Allele																													
a	1																												
b		1																											
c	1		1																										
d		1	1									1	1	1	1	1	1	1	1	1	1	1	1	1	1		1		
e				1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
f				1					1																	1			

Table 5.6: Allele distribution among 29 isolates of *Peronospora parasitica* at the M26 microsatellite locus

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Allele																													
a		1	1			1					1																		
b			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
c	1				1	1	1	1		1	1	1	1	1	1	1	1	1		1			1	1	1	1	1		
d	1					1																				1			
e		1	1			1	1	1		1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1	1		
f	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		

Table 5.7: Allele distribution among 29 isolates of *Peronospora parasitica* at the M21 microsatellite locus

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Allele																													
a			1				1					1	1																
b				1	1			1		1																			
c				1		1	1	1			1																		
d										1																			
e				1																									
f			1																										
g		1																											
h			1	1	1				1																				
i	1																												
j												1	1	1	1	1	1												
k						1	1	1			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
l						1	1	1	1	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
m				1																									
n	1																												
o						1																							
p							1																						

Table 5.8: Allele distribution among 29 isolates of *Peronospora parasitica* at the M112 microsatellite locus

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Allele																													
a							1																						
b				1	1	1		1		1																			
c							1				1																		
d				1						1									1										
e			1																										
f		1																											
g		1	1	1	1																								
h				1																									
i	1																												
j												1	1	1	1	1	1												
k						1	1	1			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
l									1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	

Table 5.9: Allele distribution among 29 isolates of *Peronospora parasitica* at the M5 (2) microsatellite locus

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Allele																													
a	1																												
b			1																										
c	1																												
d												1	1	1	1	1	1		1	1	1	1					1		
e												1	1	1	1	1	1	1					1	1	1		1		
f					1																					1			
g						1	1	1		1							1	1	1	1	1	1	1	1	1		1		
h											1																		
i		1																											
j							1	1																					
k										1																			
l										1																	1		
m							1			1		1	1	1	1	1	1		1			1							
n											1						1									1			
o							1																1	1					
p								1																		1			
q				1																									
r				1																									

Table 5.10: Allele distribution among 29 isolates of *Peronospora parasitica* at the M48 microsatellite locus

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Allele																													
a					1	1		1		1	1								1	1	1	1	1	1	1		1		
b					1	1	1			1				1	1	1	1												
c							1					1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
d		1		1	1						1																		
e		1		1	1							1	1	1	1	1	1	1		1	1	1	1	1	1		1		
f	1		1				1	1		1																			
g	1		1			1	1	1	1		1	1	1	1	1	1	1	1											
h					1	1	1	1																					
i						1			1		1									1	1	1	1	1	1	1	1		
j					1																								
k												1	1	1	1	1	1												

Table 5.11: Allele distribution among 29 isolates of *Peronospora parasitica* at the M55 microsatellite locus

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Allele																													
a						1			1		1											1							
b						1	1	1	1	1		1	1	1	1	1	1					1				1			
c										1		1	1	1	1	1	1		1	1	1	1					1		
d	1																												
e						1	1		1		1	1	1	1	1	1	1		1		1	1	1	1	1	1	1		
f											1	1	1	1	1	1	1	1		1	1		1	1	1		1		
g	1		1				1	1		1								1		1	1		1	1	1		1		
h							1											1		1	1		1	1	1	1	1		
i			1		1													1			1		1	1	1				
j				1																									
k					1																								
l		1																											
m												1	1	1	1	1	1									1			

Table 5.12: Allele distribution among 29 isolates of *Peronospora parasitica* at the M34 microsatellite locus

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Allele																													
a																												1	
b				1																									
c				1		1	1	1			1			1					1	1	1	1	1	1	1			1	
d				1																									
e			1		1				1		1	1	1	1	1	1	1	1		1			1	1	1			1	
f	1	1	1																										
g					1	1	1	1		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			1	
h					1					1		1	1	1	1	1	1					1	1	1	1			1	

5.3.3 Data Analysis

Amplification at 27 of 29 loci revealed polymorphism across all isolates. However in total data were recorded for 8 loci for isolates 1-29. For isolates 28 and 29, very few alleles were recorded ("null" for most loci) so these were omitted from further consideration. At first glance the data appeared polyploid and therefore too complex to analyse. Loci M1, M26, M21, M112 and M5(2) appeared simpler and could be scored with accuracy. However for loci M48, M55 and M34, there were many overlapping alleles. The overlapping alleles and increased stutter created ambiguities which made analysis and interpretation difficult. In addition over all eight loci, the number of alleles at different loci for a particular sample varied widely. Phenol-chloroform extraction and replicate analysis of loci did not improve this resolution. At the outset, the optimisation of the PCR using different annealing temperatures did not result in any appreciable alteration in the products obtained. Therefore the data of all loci were subdivided into good and moderate based on the ability to score the alleles accurately. Loci M1, M26, M21, M112 and M5(2) were categorised as "good" and grouped together whereas M48, M55 and M34 were categorised as "moderate" and grouped. Locus M37 was excluded since it was monomorphic. The remainder of the loci were also excluded.

Due to the polyploid nature of the data, it was not possible to consider measures such as number of shared alleles at each locus to determine similarities, i.e., Polymorphism Information Content (PIC). A Jaccard similarity matrix was therefore calculated for various sets of loci/alleles and a dendrogram constructed from a UPGMA cluster analysis of that matrix. The two sets chosen for presentation are: (i) 5 'good' loci (58 alleles) and (ii) 3 'moderate' loci (31 alleles). In order to compare these results the ordering of the samples on these two dendrograms (Fig. 5.12 and Fig. 5.13) was done to match as closely as was possible, given the links on them (these can be visualised like mobiles, where ordering can be altered about the nodes, subject to the rotations being possible.

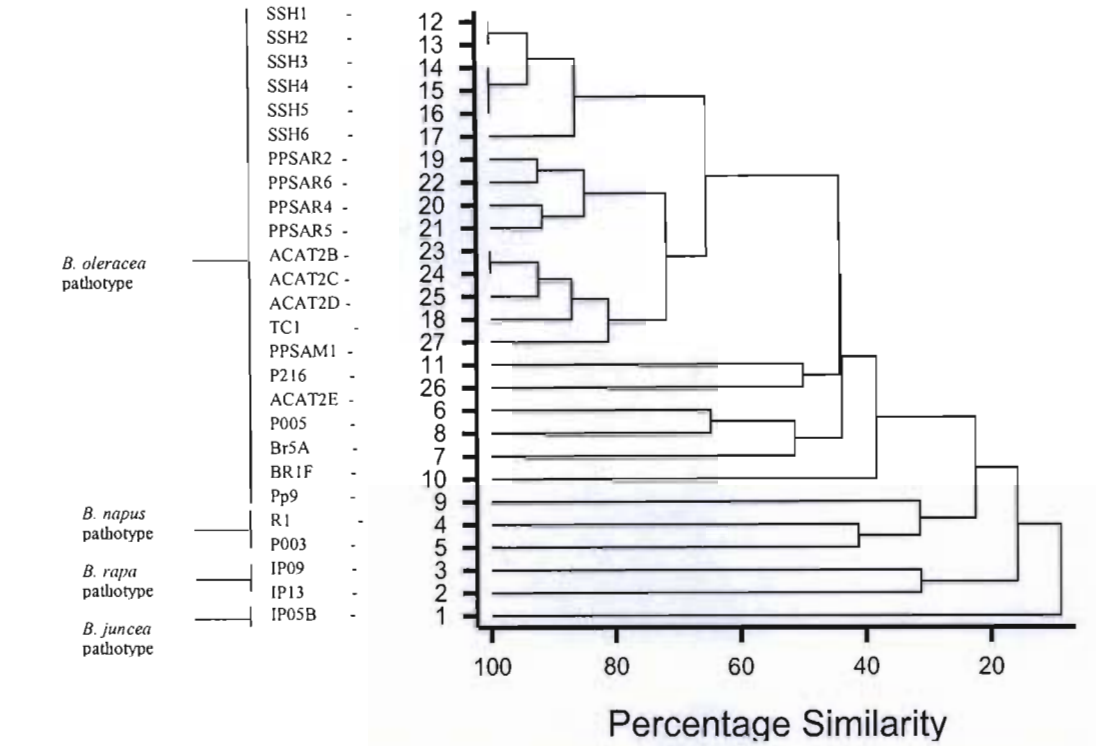

Fig. 5.12: Jaccard Similarity matrix constructed from UPGMA cluster analysis of 5 microsatellite loci, viz. M1, M21, M112, M26, and M5(2) ('good' loci, 58 alleles), of 27 isolates of *Peronospora parasitica*.

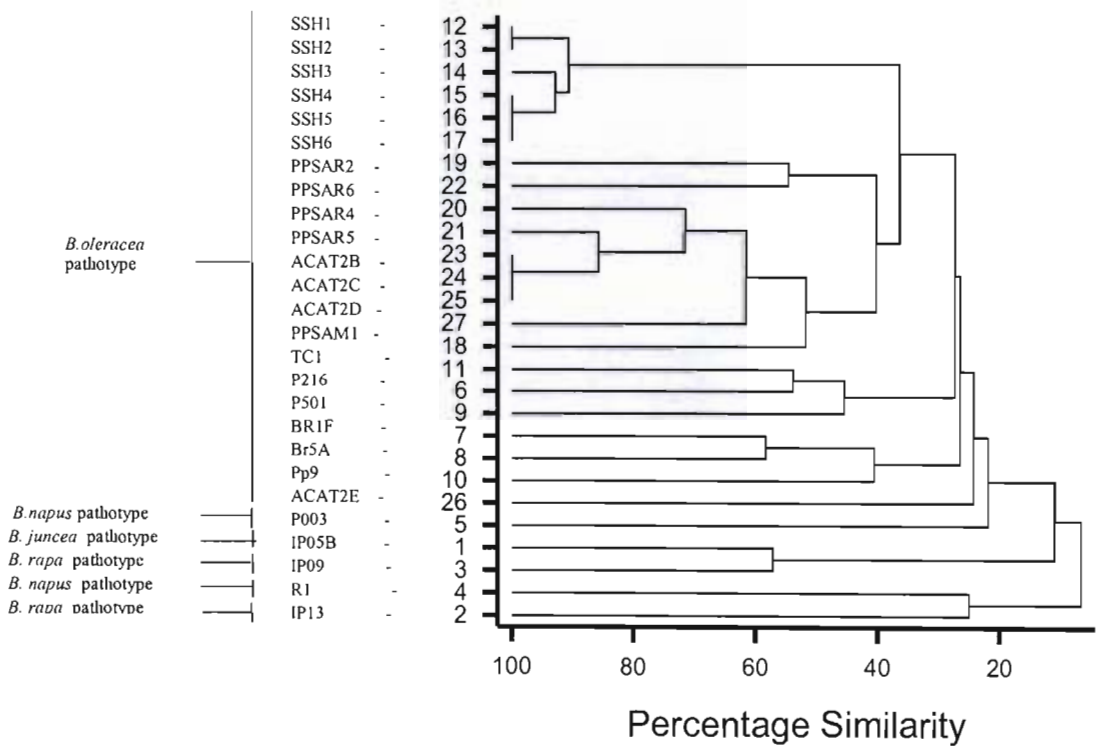

Fig. 5.13: Jaccard Similarity matrix constructed from UPGMA cluster analysis of 3 microsatellite loci, viz. M48, M55 and M34 ('moderate' loci, 31 alleles), of 27 isolates of *Peronospora parasitica*.

Viewing the dendrogram for the "good " and "moderate" loci, a pattern emerged (Fig. 5.12 and Fig. 5.13). Overall, the relationship between the different isolates in both dendrograms was approximately the same with the dendrogram of the "good" loci showing stronger clustering of isolates (Fig. 5.13). Within the *B. oleracea* pathotype, very high percentage similarities (≥ 80%) were found between single spore isolates arising from the same field isolate. This is evident in three groups of single spore isolates viz. isolates 12-17, isolates 19-22 and isolates 23-25. Furthermore within these groups single spore isolates 12 and 13 were identical and isolates 23 and 24 were identical.

A strong significant clustering was found between South African of the *B. oleracea* pathotype (isolates 12-25,27). Isolates 18-27 (excluding isolate 26) appeared to be reasonably closely related, although the strength of this does vary - strong for the 5 'good' loci (with two subgroups within this group, comprising 19-21 and 18, 23-25, 27) but average for the "moderate" loci.

Within the *B. oleracea* pathotype (isolates 12-27), isolates demonstrated geographical grouping; the South African isolates (KwaZulu Natal and Eastern Cape) (Southern hemisphere) are closely grouped together, however they share many common alleles with the isolates from France (isolates 7-10) and the UK (isolates 6,11) (Northern hemisphere) (Fig. 5.12). The dendrogram in Fig. 5.13 reflects similar relationships to Fig. 5.12 however overall isolates in Fig. 5.13 are more "loosely" clustered. Furthermore, the UK isolates (isolates 6 and 11) cluster together and the French isolates (isolates 7,8 and 10) cluster together in the "moderate" loci dendrogram (Fig. 5.13), however the percentage similarity was low.

Isolates 1-11 and 26 did not show any strong or consistent inter-relationships. Although the statistical significance was low, the dendrogram from the "good" loci grouped all isolates of the same pathotype together. Isolate IP05B (isolate 1), the only *B, juncea* pathotype was the most outlying group. Isolates 2 (IP13) and 3 (IP09) of the *B. rapa* pathotype were grouped together, while isolates of the *B. napus* pathotype R1

(isolate 4) and P003 (isolate 5) were grouped together. All other isolates (i.e. 6-27) of the *B. oleracea* pathotype were more closely related to each other than to the *B. juncea*, *B. rapa* and *B. napus* pathotypes.

5.4 DISCUSSION

The present study provides the first evidence of the occurrence and abundance of microsatellites in *Peronospora parasitica*. SSRs, specifically dinucleotide repeats, were powerful in dissecting variation between pathotypes of *P. parasitica*. In addition, it has provided greater insight into the genetics of this complex pathogen.

A genomic DNA library of *P. parasitica* enriched for microsatellites prepared in Chapter Four was screened for diversity among 29 isolates of *P. parasitica*. Confirmation that the library does in fact contain microsatellites was done in 4.3. For all loci screened, primers designed in the library amplified microsatellite alleles for 27 isolates representing four *Brassica* pathotypes of *P. parasitica* viz. *B. oleracea*, *B. juncea*, *B. rapa* and *B. napus*. The non-amplification (in some or most instances) of the *Arabidopsis thaliana* isolate Noks 1 and the genus *Bremia lactucae* was attributed to the poor quality DNA obtained in the absence of axenic culture methods utilised for all other isolates. However, it may also be due to a greater diversity in these pathotypes that means that eh primers are unsuitable. Pre-screening of host DNA by PCR amplification with the various library primer pairs (1-31) on agarose gels indicated that DNA of the different hosts used was not amplified thus reducing the bias of host contamination in the library and in the screening. Furthermore, screening of host control DNA, at various *Peronospora parasitica* microsatellite loci with labelled primers revealed no host amplification products on polyacrylamide gels and thus confirmed these findings.

Peronospora parasitica has a wide host range (Gaumann, 1952, Yerkes and Shaw, 1959). While host-range experiments employing differential response tests may be useful in clarifying host race or lineage specialisation, such studies in the greenhouse or

growth cabinet may not reflect what occurs in nature. Recently, microsatellite markers have demonstrated remarkable applicability to the differentiation of host-adapted types or species of pathogens (Bucheli et al. 2000, Levy et al. 1991, Sastry et al. 1995). In the present study, four pathotypes of *P. parasitica*, the *B. juncea*, *B. napus*, *B. rapa* and *B. oleracea* pathotypes could be distinguished using microsatellite markers. This is similar to the observation in other plant pathogens where different host adapted types of *Magnaporthe grisea* (Levy et al. 1991), *Microbotryum violaceum* (Bucheli et al. 2000), as well as the pearl millet downy mildew pathogen *Sclerospora graminicola* (Sastry et al. 1995) could be differentiated. It was also observed that the *B. juncea* pathotype was the most outlying group. Furthermore, the dendrogram (good loci) shows that the relationship between the various pathotypes can also be interpreted in terms of the genetic relationships between the host from which they are derived (Sheriff and Lucas 1987, Silué et al. 1996). The *B. oleracea* pathotype was found to be more similar to *B. napus* and *B. rapa* pathotypes than to the *B. juncea* isolate included. Genetic analysis provides a direct method of determining the extent of genetic exchange among isolates from different host species. Furthermore it allows one to determine whether the different host races exchange genes at all i.e. whether they should be assigned to a single species or to several species (Bucheli et al. 2000).

The occurrence of geographical and spatial diversity in isolates of *P. parasitica* has been previously documented. For example the predominant race of *P. parasitica* pathogenic to all types of *Brassica oleracea* grown in New York was designated race 1 (Natti et al. 1967). A physiological race of *P. parasitica* pathogenic to plants resistant to race 1 was designated race 2. The inheritance resistance to two races (1 and 2) of *P. parasitica* was found to be governed by separately inherited dominant genes (Lucas et al. 1988, Natti et al. 1967). In the present study isolates of the *Brassica oleracea* pathotype from KwaZulu-Natal, South Africa were found to be more closely related to the *Brassica oleracea* isolates from Eastern Cape (Transkei), South Africa than to the *Brassica oleracea* isolates from the UK. This demonstrated spatial distribution of similar alleles in the Southern Hemisphere as compared to the Northern hemisphere. Also, very high

percentage similarities (≥ 80%) were found between single spore isolates arising from the same field isolate. Analysis of F1 progeny of sexual crossings of these isolates would very likely yield more insight into the gene/s governing specificity.

The question of ploidy of *P. parasitica* was not easily resolved. While the Peronosporales have been suggested to be diploid there is insufficient evidence to support diploidy. Studies on genome size are useful in determining evolutionary relationships of plant pathogenic fungi as well as understanding pathogen cytology including the cell cycle and ploidy. Voglmayr and Greilhuber (1998) recently determined genome size in Peronosporales using Feulgen image analysis. Using this method, the genome size of a wide range of isolates was obtained. Within the 22 *Peronospora* species studied, genome size ranged from 46.31 to 138.86 megabases (Mb). A single *P. parasitica* isolate assessed had a genome size of 77.09 Mb. Data on the genome size of the Peronosporales indicated that it was difficult to identify polyploidy by genome size analysis alone without confirmation by chromosomal counts, as polyploidy seemed to be obscured by chromosomal rearrangements such as dysploidy and/or aneuploidy which gave a continuous distribution of genome size rather than discrete levels of ploidy (Voglmayr and Greilhuber 1998). Goodwin et al. (1992) found the first evidence of ploidy in *Phytophthora infestans*, the closest relative of *P. parasitica*; a five-banded allozyme phenotype was found to be attributable to three alleles on different chromosomes in the same individual. Later diploid, triploid and tetraploid strains as well as diploid-polyploid heterokaryons were identified in *Phytophthora infestans* by Feulgen absorbance photometry (Voglmayr and Greilhuber 1998). In the present study, the ploidy of *P. parasitica* could not be unequivocally determined; the number of alleles per genotype for each locus differed widely. The results of the present study suggest that *P. parasitica* is definitely not diploid or haploid but very likely polyploid. The exact level of ploidy could not be determined since the number of alleles per isolate and at different loci differed widely. Future studies involving the excision of the microsatellite bands from polyacrylamide gels and sequencing these products may assist in clarifying ploidy.

Microsatellites and other simple tandem repetitive loci generate PCR-amplifiable alleles that can in principle be sized with precision on DNA sequencing gels. However, restricted allelic variability and the frequent occurrence of PCR-artefact bands limit their usefulness (Tautz 1989, Jeffreys et al. 1991). In the present study, the use of the DNA marker Sequamark, together with correct interpretation of data was required for the analysis to be useful and accurate. Stutter bands may occur as a result of A nucleotide overhangs or loss of a repeat during the PCR amplification process. Although subject to much speculation the artefacts of a band are thought to be due to structural constraints within the fragments however, these can be clearly distinguished from contaminating bands since they appear to be equidistant from the major band for all alleles and thus form a pattern. PCR amplification of microsatellite loci typically produced stutter bands, a minor/lighter band which differed in size from the main allele by multiples of the repeat unit size (Walsh et al. 1996). This results in a multi-band pattern for each allele and interpretation of dinucleotide repeat loci is sometimes complicated particularly when two alleles from a single individual are close in size (Walsh et al. 1996). In the present study the analysis of the "good" loci yielded unambiguous stutter bands which were easily identified. Furthermore, these "good" loci yielded very consistent inter-relationships among the different isolates since all isolates of the same pathotype were grouped together. The "moderate " loci reflected a pattern very similar to the "good" loci however, the isolates were more "loosely" associated.

In the present study, the allele sizes for the "moderate loci" are very close in size. This was noticeable at the M48 locus. What appeared as an allele for one individual (dark prominent band) was of the same size as the stutter (lighter band) for another allele but in a different isolate. Consistent DNA quantity was utilised for each isolate and it seems unlikely that varying DNA concentration would result in these effects. Although good quality glass plates were used for gels (Life Technologies), slight changes in gel thickness or heat distribution across the plate may produce minor changes in band size and thus create ambiguities between alleles and their associated stutter bands. However, since repeated analysis on different gels of the same PCR products yielded the same

banding pattern, these effects were not considered significant. Furthermore the estimated length of the repeat (Table 4.3) did not influence the extent of stutter bands. Walsh et al. (1996) reported that stutter band formation is elevated in samples that have long stretches of core repeat sequences. With the exception of the monomorphic locus M37 which had the smallest repeat length of 12 bp and a single faint stutter band, no other consistent relationships between core repeat length and stutter formation was evident. M37 was a tetranucleotide, $(ACAG)_3$, and the reduced stutter is consistent with other observations with tetranucelotides (Walsh et al. 1996). Among the dinucleotides, M1, M21 and M26 had similar repeat sizes and showed no significant differences in amount of stutter. Furthermore, similar size repeats M5(2) and M112 showed marked differences in the degree of stutter. Of significance was that all the above loci were CA repeats; in general all CT repeats were much longer (Table 4.3) and these did not produce good patterns for use in diversity analysis. Further studies incorporating sequencing of more CT clones would enable conclusions about CT microsatellites.

Speculations regarding the nature of the alleles produced by the various isolates being attributed to the nature of *P. parasitica* itself were considered. With the exception of the Bremia and Noks1 isolates, all isolates considered in this study were single spore isolates (Chapter Two), therefore the possibility of mixed cultures (Walsh et al. 1996) as the reason for the multi-band appearance of alleles was ruled out. Of note however is that the isolates represent more than one generation of the same asexually producing isolate due to repeated sub-culturing of same single spore isolate, a widely accepted method of maintaining isolates of *P. parasitica* (Chapter Two). It seems unlikely that this would introduce new variation, except by somatic means.

The influence of other factors such as homothallism in *P. parasitica* or heterokaryosis may be worth investigating. Homothallism is a common phenomenon in isolates of *P. parasitica* (Sheriff and Lucas 1989a,b, Jugmohan 1997). Cytogenetic evidence revealed that homothallism is due to trisomy of the mating type alleles on the fifth chromosome (Sheriff and Lucas 1989a). Homothallism may introduce variation by

sexual recombination irrespective of whether it is a monoconidial line or not. This is likely to have a greater effect on a field population and on the stage of the life cycle. This would have a minimal effect on the isolates used here simply because isolates were regularly subcultured every seven days and while asexual and sexual reproduction may take place simultaneously on the same leaf, it is mainly the late cycles or periods of unfavourable conditions that favour sexual reproduction and the production of oospores (McMeekin 1960). The asexual cycle involves mitosis therefore heterokaryosis is not likely to influence the results irrespective of the fact that each conidium of *P. parasitica* contains approximately 30 nuclei (Davison 1968a).

CHAPTER SIX

CONCLUDING REMARKS

6.1 THE RESEARCH IN PERSPECTIVE

Host specificity of numerous plant pathogens has been a topic of considerable debate for the last century. A greater understanding of host-specific differentiation of pathogens is essential for studying the ecology and evolution of plant-pathogen interaction (Bucheli et al. 2000). Whether the differences in prevalence and incidence on the specific hosts are simply ecological phenomena or whether they are affected by adaptation of a host-specific pathogen strain depends on the amount of gene flow among pathogen strains on different host species, i.e. the presence of host-specific genetic variation (Bucheli et al. 2000). Knowledge of phylogenetic relationships between host-adapted strains of the pathogen provides insights into the evolution of host-adaptation of pathogens to plant populations and species.

Being obligate biotrophs downy mildews are host-dependent pathogens. *P. parasitica* is very specific in its host range, however, it also has the ability to colonise heterologous host species to different extents i.e. hosts different to those from which it was derived. Several studies based on the differential response test have shown that isolates are more virulent on their species of origin (Nashaat and Awasthi 1995, Silué et al. 1996, Jugmohan 1997) and the varying abilities to infect other hosts was estimated using a disease index (DI). The overall results support the idea that host adaptation exists at the species level. Co-evolution with plant hosts over long periods was suggested to have led to divergent forms of the pathogen adapted to different host taxa (Lucas et al. 1994). This dependence of downy mildews on their hosts further suggested that forms of the pathogen found on closely related plants share some common phylogeny and classification schemes could mirror hierarchical schemes used to delimit plant taxa.

Traditional fungal taxonomy was based on morphology. In the family Peronosporaceae generic delimitation was based on morphological characteristics such as based as sporangiophore structure or spore type. On this basis all collections of the downy mildew found on crucifers (Yerkes and Shaw 1959) have been grouped into a single

aggregate species, *Peronospora parasitica*. Host range experiments have been suggested to be an unreliable indicator of the evolutionary history of downy mildews (Lucas et al. 1994).

The tools of molecular biology promise to clarify many of the unresolved questions concerning host specificity. Genetic analysis provides a straightforward way of determining how much genetic exchange is occurring among fungal strains from different host species and whether they should be assigned to a single species or to several species (Bucheli et al. 2000). Furthermore to develop breeding strategies for cultivars with stable and durable host resistance to downy mildew molecular characterisation of pathogen population diversity is necessary (Sastry et al. 1995). Molecular markers such as RFLPs, RAPDs, AFLPs are now well developed and have been applied to the study of numerous plant pathogens including downy mildews (Drenth et al. 1993, Tham et al. 1994, Cooke et al. 2000).

Much controversy has surrounded the taxonomy of Oomycetes in general, which has resulted in the reappraisal of their taxonomic position outside the Mycota (Gunderson et al. 1987, Hall 1996). In recent years the analysis of rDNA has clarified the phylogenies of various Oomycetes including *Phytophthora*, *Pythium* and *Peronospora* (Cooke et al. 2000, Rehmany et al. 2000). ITS-PCR has proved to be a powerful tool for delineating plant pathogens at the genus and species level. The current study provides the first comprehensive ITS-based phylogeny of pathotypes of *P. parasitica*. Based on ITS1, 5.8S and ITS2 sequences all pathotypes of *P. parasitica* from *Brassica* species (viz. *Brassica oleracea*, *Brassica napus*, *Brassica juncea* and *Brassica rapa*) were monophyletic. Based on ITS1 sequences, the pathotype of *P. parasitica* from the wild host, *Arabidopsis thaliana,* was found to be significantly different from the *Brassica* pathotypes (i.e. cultivated host pathotypes). This correlated with findings of Rehmany and co-workers (2000) who showed that European *P. parasitica* (*Arabidopsis thaliana*) and *P. parasitica* (*Brassica oleracea*) isolates show absolute conservation when collected from the same host species but share only 85% identity between hosts. Furthermore, Cooke et al. (2000) proposed that the downy mildews have derived from a *Phytophthora* ancestor. The results of the present study indicates that the genetic distance between *Peronospora* species and *Phytophthora* is closer than to other taxa such as *Pythium*; ITS1 sequences clearly show the genus *Pythium* to be an outlier group. This is the first study that incorporates a range of different pathotypes of *P. parasitica*. The ITS sequences indicate that host adaptation

must exist at the species level. ITS sequences indicate differentiation of the more distinct pathotypes like *Arabidopsis thaliana* and the *Brassica* pathotypes. However this level of discrimination is insufficient to explain differences between *Brassica* pathotypes

Molecular markers such as AFLPs and microsatellites have been established as powerful discriminatory tools for intraspecies variation due to the polymorphic and co-dominant nature of the alleles generated (Hantula et al. 1996, Longato and Bonfante 1997, Rehmany et al. 2000, Van der Lee et al. 1997). Microsatellite libraries are important due to their ubiquitous distribution (Groppe et al. 1995), high copy number (Stallings et al. 1991) and varied location within protein coding regions and within introns or between genes (Weber and May 1989). In addition, if extensive allele sequence data is available, the mapping of characterised single nucleotide polymorphisms (SNPs) is an efficient approach to mapping of genes.

Microsatellite markers are readily analysed by PCR and have succeeded in clarifying some of the contrasting views of the diversity of pathotypes that exist for example, in the rice blast fungus *Magnaporthe grisea* (Levy et al. 1991), the anther smut fungus *Micobotryum violaceum* (Bucheli et al. 2000) as well as the pearl millet downy mildew pathogen *Sclerospora graminicola* (Sastry et al. 1995). In the present study, screening of the microsatellite library of *P. parasitica* resulted in clear differentiation of four *Brassica* pathotypes of *P. parasitica*, viz. *B. oleracae*, *B. rapa*, *B. napus* and *B. juncea* pathotypes. All isolates within a particular pathotype were grouped together and in addition geographical diversity within the *Brassica oleracea* pathotype was evident.

In the present study, due to the polymorphic nature of the alleles of isolates of *P. parasitica* it was not possible to look at measures such as polymorphic information content (PIC). The ploidy of Peronosporales was suggested to be diploid (Crute 1981) however subsequently the ploidy of Oomycetes was found to differ widely. In the *Phytophthora*, variations in ploidy between isolates from different geographical regions have been observed (Tooley et al. 1991). These observations were further supported by observations of Voglmayr and Greilhuber (1998) who found diploid, triploid, tetraploid and diploid-polyploid heterokaryons in *Phytophthora* by Feulgen absorbance photometry. Similarly in the downy mildew pathogen, *Bremia lactucae*, diploid and polyploid isolates have been reported (Hulbert et al. 1988). In the present study the number of alleles per genotype for each locus differed widely. In addition, the alleles showed pathotype specific

as well as geographical distribution within a particular pathotype. In view of the above and observations in other closely related downy mildews, the results of the present study suggest that *P. parasitica* is polyploid. Sequencing of the PCR products will enable confirmation of this inferred result.

6.2 POTENTIAL FOR FUTURE DEVELOPMENT OF THE WORK

Inadequate species definitions present a serious problem to the pathologist working in plant hygiene or quarantine which demands urgent attention (Hall 1996). It is imperative that more information regarding the breeding systems, gene flow, ecology, phylogeny and distribution be obtained before informed decisions about the delimitation of most species can be made. Significant advances in the analysis of diversity of obligate pathogens such as downy mildews have occurred in the last decade. The application of molecular markers such as RAPDs, AFLPs and microsatellites has succeeded in distinguishing between species and pathotypes of plant pathogens (Tham et al. 1994, Barve et al. 2001, Cooke et al. 2000, Sastry et al. 1995, Rehmany et al. 2000). These studies are essential because until patterns of genetic diversity are established a number of modern phylogenetic species concepts provide only an interim solution to the problem of species definition in the downy mildews (Hall 1996).

Several aspects meriting further investigation have emerged during the course of this study. The high level of polymorphism indicated in the microsatellite library produced in this study showed that microsatellite markers are a powerful tool for discriminating pathotypes of *P. parasitica*. The large number of alleles produced suggest that *P. parasitica* is polyploid and much more complex than previously imagined. Future work involving sequencing of the amplimer at microsatellite loci would provide more insight into the exact ploidy. The availability of such allele sequence data would then allow the mapping of characterised single nucleotide polymorphisms (SNPs) which may be useful in specific identification of pathotypes of *P. parasitica* which in turn would provide a more efficient method of clarifying the phylogeny of isolates, species and pathotypes of *P. parasitica*. In addition primers developed for analysis of *P. parasitica* has been shown to amplify other downy mildews such as *Bremia lactucae* and may therefore enable analogous studies in other Oomycetes.

The nature of the alleles of *P. parasitica* i.e. the high level of stutter together with the close size of the alleles of the single spore isolates themselves do not reflect a simple fingerprint pattern. The above observations possibly reflect the kinds of genetic "exchange" that may be operating in the field. The additional influence of homothallism or heterothallism or even heterokaryosis (which has not been ruled out) would further complicate this picture. The observation of spatial and geographical diversity within the *Brassica oleracea* pathotype indicates that the environment would also play a role in the diversity present. These reflect the ability of the pathogen to undergo continuous change and are essential when studying the sustained inheritance of resistance to *P. parasitica*.

Microsatellites may provide the key to unravelling many of the unanswered questions concerning downy mildews. Microsatellite libraries are expensive to develop because a) microsatellites are very often too close to the polylinker site of the plasmid therefore leaving insufficient bases for primer design b) only a proportion of the primers amplify, the reason being that there may be chimaeric clones present (where one priming site is possibly outside the flanking region) or the primers are designed in repetitive DNA resulting in smears c) only a proportion of the primers are polymorphic or they amplify several bands d) redundancy further reduces the number of markers obtainable. However once established they may be used in multiplexed PCR to simultaneously analyse various loci in a genome of a particular organism in a single PCR reaction, or enable the characterisation of Single nucleotide polymorphisms (SNPs). One disadvantage of microsatellites which must be cautioned against is the presence of null alleles. A deletion in the DNA flanking the microsatellite coincident with the priming site may result in heterozygotes being mistyped as homozygotes.

6.3 SPECULATIONS

Plant pathogen interactions of biotrophs are mediated by specific interactions between pathogen *avr* (avirulence) gene loci and an allele of the corresponding disease resistance (*R*) loci. When these are present both in the host and the pathogen, the result is disease resistance. If either is inactive or absent the result is susceptibility to disease. *R* gene products recognise *avr*-dependent signals and trigger the chain of signal transduction events leading to halt of pathogen proliferation (Ellis et al. 2000). The co-existence of host plants and their pathogens side by side in nature indicates that the two have been evolving together. Changes in the virulence of the pathogens must be continuously

balanced by changes in the resistance of the host so that a dynamic equilibrium of resistance and virulence is maintained and both the host and the pathogen survive (Agrios 1997). A complete understanding of the genetics of host specificity requires molecular analyses in both the host and the pathogen.

Knowledge on the host- pathogen interaction of plant pathogens is essential for the development of sustainable control measures for disease. Current control methods for downy mildew of brassicas include the use of fungicides (Brophy and Laing 1992). It has been established that *P. parasitica* has developed resistance to metalaxyl-based fungicides. This coupled with the possible environmental impacts of chemicals indicates that alternative control methods should be developed. Biological control has shown promise in this respect (Wilson 1997), however, very few commercial products have been developed. More recently, methods available for identifying molecular markers linked to resistance genes and thus the mapping and cloning of these genes have become available. This should speed up identification of novel sources of resistance and allow rational diversification of R genes in cultivar mixtures.

Plant *R*-genes involved in gene-for-gene interactions with pathogens are expected to undergo coevolutionary arms races in which plant specificity and pathogen virulence continually adapt in response to each other (Bergelson et al. 2001). A classic arms race is one that entails a series of selective sweeps as novel R-gene alleles which can recognise *Avr* factors that previously avoided detection in a plant population, spread to high frequency. A mutation in the pathogen *Avr* gene and prevents pathogen recognition and defeats the effectiveness of an R-gene specificity in the host. This in turn imposes selection pressure on the host for new resistance specificities, which may arise at the 'defeated' locus or elsewhere in the genome (Ellis et al. 2000). *Avr* genes (having a selective advantage) and the 'defeated' (yet functional) R-gene may be maintained in the host and pathogen populations balancing selection (Ellis et al. 2000). Adaptive divergence has been investigated in tomato, rice and *Arabidopsis*.

A wild host of *P. parasitica*, *Arabidopsis thaliana* has provided a model system for the identification of many resistance genes (*RPP* genes) to *P. parasitica* (Crute et al. 1994a,b, Holub et al.1994, Kock and Slusarenko 1990, Parker et al. 1993). Once these genes are cloned, the search for homologs in related crops can begin (Lucas et al. 1994). The majority of R genes cloned thus far encode proteins with a nucleotide binding site (NBS) and a leucine-rich repeat (LRR) region (Ellis et al. 2000, Jones and Jones 1997).

The solvent exposed amino acid residues of leucine-rich repeats, the domain of R-genes involved in recognising pathogens, often evolve at unusually fast rates (Bergelson et al. 2001). The numerous R-genes to *P. parasitica* from *Arabidopsis* encoding NBS and LRR regions were found to be highly polymorphic (Aarts et al. 1998a). In particular, three specificities have been identified at the *RPP13* locus which appears to be a single gene with highly variable multiple alleles that are subject to diversifying selection in the LRR region (Bittner-Eddy et al. 2000).

Similarity in the patterns of evolved differences have been observed among alleles at individual loci and between genes belonging to evolutionary clusters. R-gene alleles representing a wide range of evolutionary ages were found to undergo rapid adaptive evolution. Furthermore the rates of adaptive evolution appeared greatest between closely related *R-genes*, suggesting that genetic exchange has contributed to the production of new adaptive alleles (Bergelson et al. 2001).

Population genetic analysis of complex multi-allelic R genes in plant-pathogen ecosystems will shed light on the nature of the selection processes acting on these loci. In *Arabidopsis* R-genes show a wide range of ages, with some loci harbouring old alleles which may be the result of balancing selection (*RPP1 and RPP13*) and other loci showing modest levels of divergence. Furthermore *RPP8* segregates for a large number of alleles. While selection was suggested to play a profound role in *R*-gene dynamics, the classic arms race concept was considered a poor model since alleles were not young and loci were not monomorphic, as predicted by this model (Bergelson et al. 2001). The polymorphism data suggests that a simple arms race model involving repeated selective sweeps may apply to, at most, a small complement of *R*-loci (Bittner-Eddy et al. 2000). Further models exploring the age of alleles under different scenarios of adaptive evolution were needed to better understand short-term disease dynamics.

In the last two decades, molecular methods have resulted in the reappraisal of the Oomycetes outside the Mycota. Similar rearrangements in taxonomic position are occurring in the Plant Kingdom. In the last few years there has been considerable interest in the evolution and diversity of the Kingdom of Plants (Embryophyta). Most of the 470-million year history of plants on land belongs to bryophytes, pteridophytes and gymnosperms which eventually led to the ecological dominance of angiosperms 90 Myr ago (Pryer et al. 2001, Graham et al. 2000). Knowledge of angiosperm phylogeny has

recently been increased by the concurrence of multigene sequence analyses (Pryer et al. 2001).

Phylogenetic analyses of DNA sequences of mitochondrial, plastid and nuclear genes have been utilised to trace the earliest angiosperms and clarify the uncertainty regarding the sister group of angiosperms among extant and fossil gymnosperms (Qui et al. 1999). Phylogeny based on DNA sequences of the plastid genes rbcL and atpB and nuclear 18S rDNA for 560 species of angiosperms and 7 non-flowering seed plants have provided a well resolved tree for use in comparative biology (Soltis et al. 1999). The progress in reconstruction of angiosperm phylogeny using multigene sequence analysis has been reviewed by Kuzoff and Gasser (2000). Sophisticated multigene analyses are more reliable, statistically significant and superior to earlier studies which focussed on tentative inferences from relatively small data matrices (Kuzoff and Gasser 2000). This approach has succeeded in identifying the earliest extant lineages of angiosperms, confirming Amborella as a sister group of all other angiosperms, confirming some previously proposed lineages and redefining other groups consistent with their phylogeny.

The coevolution of downy mildews is likely to have led to the adapted forms of the pathogen found on different host taxa (Lucas et al. 1994). Furthermore, the differences in the pathotypes of *P. parasitica* exemplified by host specificity and genotypic differences are a reflection of the adaptation of this pathogen to its *Arabidopsis*, Brassica and other crucifer hosts. Current theories of adaptive evolution together with the observed polymorphism of R-gene loci in *Arabidopsis* add substance to the long speculation that host adaptation is a multigenic trait. Until now, there have been few genetic studies on the large scale host movement of Oomycetes from fungi to Chromista. This occurrence together with the new redefined classifications in angiosperms suggest convergent evolution of *P. parasitica* with its hosts. Further studies are needed to determine the extent of evolution and whether the host and pathogen are evolving at the same rate.

With systematic sequencing efforts in humans and other organisms in full swing there is an immense amount of sequence data being generated (Baxevanis 2000). The entire sequencing of the first plant genome, *Arabidopsis thaliana* provides an unparalleled resource for understanding the evolution of crop plants (Walbot 2000). In addition to storage of the data (bases and amino acids), online databases make logical connections to other information such as phenotypic or expression data facilitating biological discovery

(Baxevanis 2000). The linking of specialised database collections ensures data quality and fosters the ability to access and analyse sequence data. The Molecular Biology Database collection is an online resource, available at http://www.oup.co.uk/nar/Volume_28/Issue-_01/html/gkd115_gm.html and serves as an searchable, up-to-date, centralised jumping access point to individual web sites (Baxevanis 2000). It allows individual investigators to easily find and use specialised databases that are appropriate to their scientific needs. It facilitates online access to a vast number of specialised databases including Genbank (Gene Sequence Data Bank), EMBL (European Molecular Biology Laboratory), the various RNA sequence databases (De Rijk et al. 2000, Van der Peer et al. 2000, Szymanski et al. 2000), the NCBI taxonomy browser (Wheeler et al. 2000), Mendel Database (plant ESTs ad STS), Tree of Life Database, the *Arabidopsis* database (AtDB) and the *Phytophthora* Genome Initiative Database (Oomycete database) (Waugh et al. 2000).

Information on the genome and gene structure of species such as *Arabidopsis thaliana* and rice, together with hundreds of thousands of expressed sequenced tags (ESTs) from many plant species has drastically changed the strategy of plant geneticists and reduced discrimination among the plant materials studied (Tabata and Caboche 2001). EST databases are now becoming the basis for genomic approaches to drug discovery, plant and animal genetic improvement and the study of human genetic diseases. These databases are also a potential valuable source of genetic markers and provide an opportunity to construct syntenic genome linkage maps of expressed genes among related species (Cato et al. 2001). EST markers are more popular than RAPDs, AFLPs, and SSRs since they are derived from coding DNA and genetic mapping with ESTs would enable a more rapid transfer of linkage information between species. However, discriminatory microsatellite markers are important since ultimately, comparison across whole regions of the genome will be necessary. Since microsatellites are highly polymorphic and evolve directionally and at different rates in different species (Rubinsztein et al. 1995), the rate of evolution of *P. parasitica* in relation to its hosts may provide insight into the phenomenon of convergent evolution.

The *Phytophthora* Genome Initiative is a distributed consortium to study the genome and evolution of this destructive Oomycete and ultimately understanding the mechanisms of infection and disease (Waugh et al. 2000). The data derive from *Phytophthora infestans* and *Phytophthora sojae* ESTs (expressed sequence tags) and

Phytophthora sojae BAC (Bacterial Artificial Chromosome) libraries (Nuss et al. 1999, Randall and Judelson et al. 1999, Torto et al. 2000, Waugh et al. 2000). An overview of the collaborative EST project of *Phytophthora infestans* is presented in Appendix D). Of particular interest was the identification of several novel members of the elicitin family of proteins, a family of structurally related proteins that condition the hypersensitive response and avirulence of *P. infestans* on *Nicotiania* plants (Kamoun et al. 1999). The number of identified ESTs is growing at a rapid rate and many novel ESTs corresponding to *Phytophthora* extracellular protein genes have been identified (Torto et al. 2000). The sequencing initiative to understand and control *Phytophthora* is part of vision to integrate data from host species, which is necessary to understand a product of a co-evolutionary process (Hraber and Sobral 1999, Nuss et al. 1999).

The PGI Database consortium together with other databases including the *Arabidopsis* Database are key to the development in functional genomics of Oomycetes and allows the benefit of studying genome wide expression of important pathogenesis-related-transcripts early during pathogenesis. Furthermore, the information on the structure and function of genes in one plant species can easily be transferred to another. Many approaches to identifying the function of genes are now possible. These include the characterisation of knockout lines, generation of insertion lines using retrotransposons, gene silencing and microarrary technology (Tabata and Caboche 2001). Functional analysis of WRKY proteins and EDS1 in the signal transduction pathway have already been identified. Investigations of an isolate of *P. parasitica* and the *Arabidopsis thaliana* host are focussing on the identification and expression of WRKY genes (a superfamily of transcription factors which may be involved in the regulation of pathogen defense responses) which show an altered transcription level (induced or repressed expression) after challenge with the avirulent *P. parasitica* (Lippok 2002). The *EDS1* component of the signaling pathway is required for resistance conferred by *RPP1*, *RPP2*, *RPP4* and *RPP5* genes controlling resistance to *P. parasitica* (Aarts et al. 1998b).

The tools of molecular biology have set the platform for some exciting functional genomic studies of Oomycetes and other organisms since ultimately, a complete understanding of the evolutionary biology of any organism will involve comparison of whole regions of the genome. These developments have important applications in the improved classification and diagnosis of downy mildew diseases. Analysis of the ITS region of the rDNA has allows discrimination of the Brassica pathotypes and information

on discriminatory microsatellite markers will enable further subdivisions of *P. parasitica* and other downy mildews.

In South Africa, Brassica seed is imported and is not bred for resistance to *P. parasitica* or many of the other downy and powdery mildews. Discrimination of isolates and/pathotypes using molecular markers such as microsatellites are important tools for the development of sustainable resistance. The strategies used to engineer broader spectrum and more durable disease resistance include identification of pathogen traits that contribute to resistance and then the cloning or design of R-genes that confer recognition based on those traits (Bent 1996).

The functional characterisation of R genes would enable the development of an R-gene inventory and the possibility of R gene multilines (near isogenic lines differing in specific R genes configured to confound pathogen evolution/variation). In the present study, microsatellite loci were found to be highly polymorphic and resulted in the discrimination of pathotypes of *Peronospora parasitica*. In humans microsatellites were found to be associated with disease when they were abnormally expanded (Brook et al. 1992, Valdes et al. 1993, Rubinsztein et al. 1995). Since microsatellite markers may also be associated with regulation and expression of genes further investigations linking microsatellite loci to ESTs may establish correlations with existing heterologous R-gene products in *Arabidopsis*.

As newly designed R-genes are released into the field, it may be necessary to utilise old plant breeding methods to ensure durable resistance, for example, release varieties with different R-gene specificities in alternate growing seasons or planting of mixed resistant and susceptible seed so that avirulent genotypes remain predominant over rare virulent strains in the pathogen population (Bent 1996).

In addition the large number of alleles identified may be a reflection of the multiple *Avr* genes segregating in the pathogen population. The correlation of the observed genetic differences from microsatellites with other influences in the field such as homothallism and heterothallism may be important in the evaluation of factors that lead to emergence of new races and to predict future disease development. Identification of the exact ploidy of *P. parasitica* would allow the calculation of measure such as polymorphic information content (PIC) from microsatellites and genotype proportions may be determined. However, one must caution against the presence of null alleles which would

result in genotype proportions deviating from Hardy-Weinberg expectations (Bruford and Wayne 1993).

The identification of new genetic markers differentiating isolates of *P. parasitica*, together with the concurrent studies on characterisation of R-genes in its hosts will enable the development of sustainable resistance to this pathogen. Ultimately, it will enable the effective management of downy mildew in *Brassica* species.

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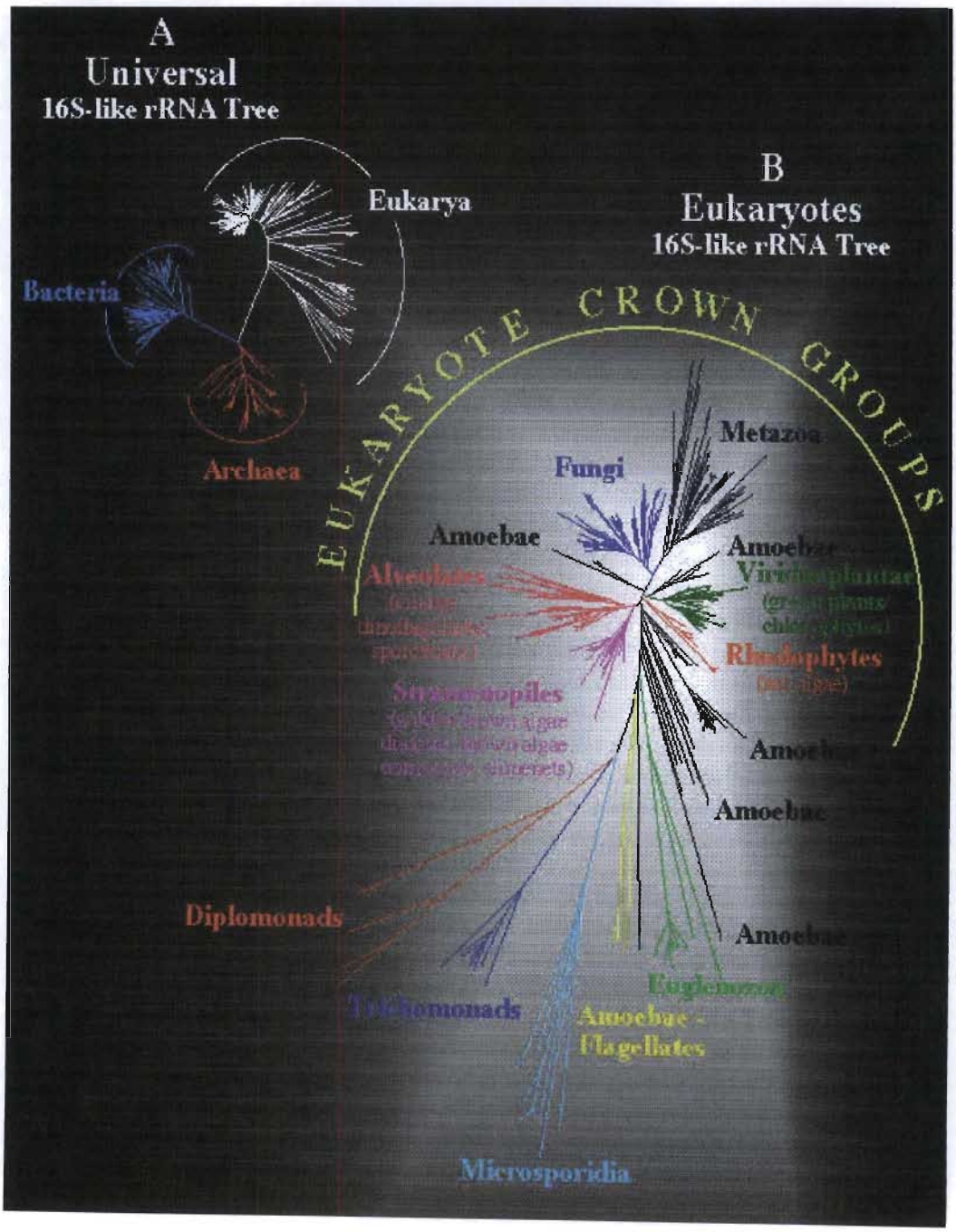
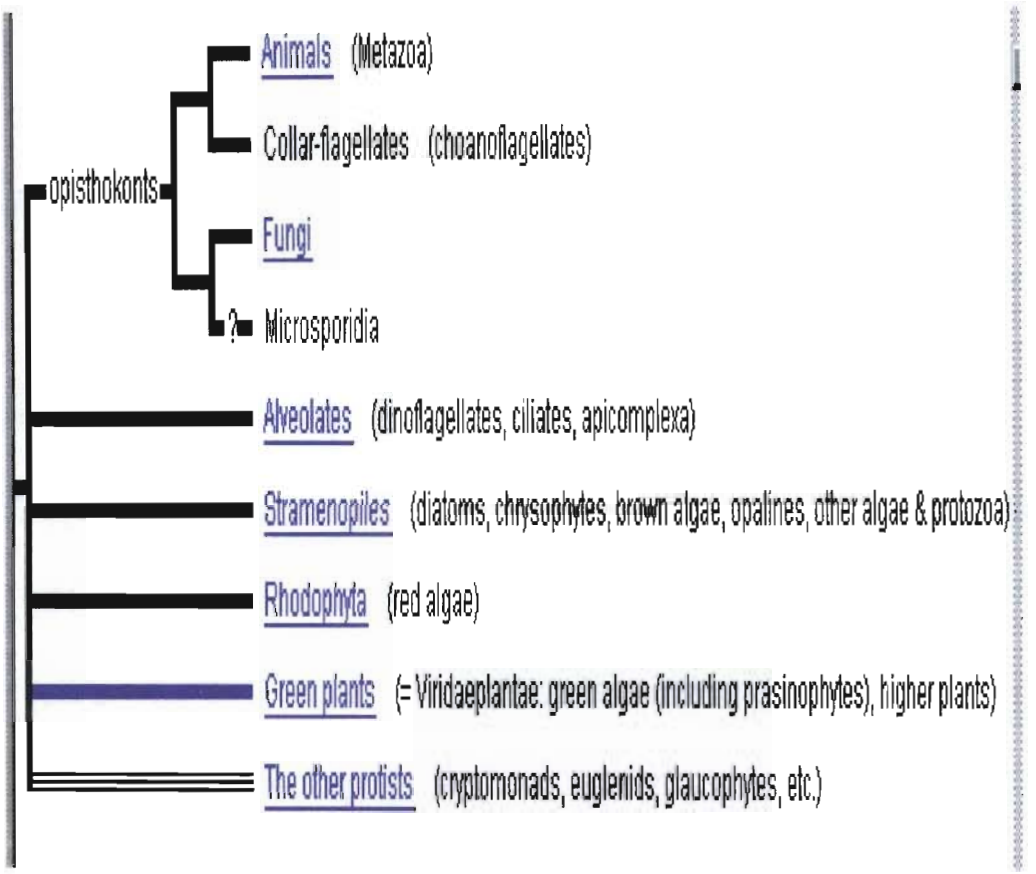
APPENDICES

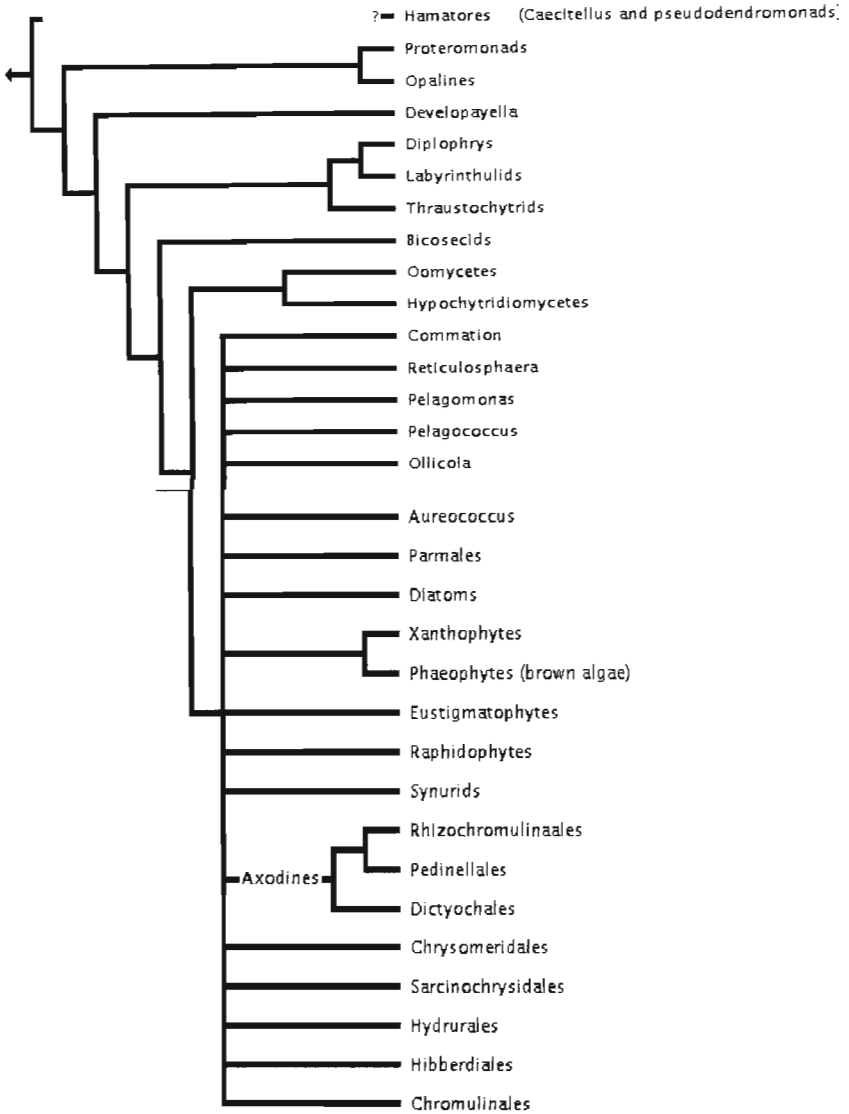
Appendix A: Representation of the 16s rRNA tree showing the relatedness among different eukaryotes (Patterson and Sogin 2002).



Appendix B: Overview of the classification of *Peronospora parasitica* within the Protists group of Eukaryotes (based on the Tree of Life Web Project, Patterson and Sogin 2002)



Appendix B.1: Overview of the major lineages of Eukaryotes (based on Patterson and Sogin 2002)



Appendix B.2: Subdivision within the Protist group, Stramenopiles, based on Patterson 1989).

Appendix C: Detailed protocol: PCR Analysis of microsatellite loci of *Peronospora parasitica* with γ-^{33}P Labelled nucleotide triphosphate

Appendix C.1: End labelling of primers with γ-^{33}P nucleotide triphosphate

All forward primers (Table 4.3) were end labelled with γ-^{33}P nucleotide triphosphate. For each primer a labelling master mix was prepared The primer labelling mix for a 12.5 µl amplification reaction consisted of the following: 25 ng or 0.25 µl (0.1 µg/µl) forward primer, 0.05 µl One-Phor-All Buffer (OPA; 100 mM Tris-acetate, 100 mM magnesium acetate, 500 mM potassium acetate, pH 7.5; Pharmacia), 0.05 µl γ-^{33}P (Amersham Pharmacia, UK), 0.008 µl T4 polynucleotide kinase (PNK) (Pharmacia, UK) and sterile distilled water (SDW) to make up final volume.

A typical labelling mix for 35 PCR reactions was prepared as follows:

10 x OPA Buffer	0.05 µl x 35	→ 1.75 µl
Forward Primer	0.25 µl x 35	→ 8.75 µl
γ-^{33}P ATP	0.05 µl x 35	→ 1.75 µl
T4 PNK	0.008 µl x 35	→ 0.28 µl
SDW	0.142 µl x 35	→ 4.97 µl
		17.5 µl

The labelling mix was incubated at 37 °C for half an hour followed by 10 min. at 68-72°C to denature the PNK.

Appendix C.2 PCR Amplification

A PCR master mix for 35 PCR reactions was prepared for each primer pair containing the following:

10 x PCR Buffer (Gibco, Lifetech, USA)	1.25 µl x 35 →	43.75 µl
dNTPS (25 mM)	0.1 µl x 35 →	3.5 µl
Reverse Primer	0.25 µl x 35 →	8.75 µl
Labelled Primer (C.1)	0.5 µl x 35 →	17.5 µl
Taq DNA polymerase (5 U/µl)	0.1 µl x 35 →	3.5 µl
SDW	9.8 µl x 35 →	343.0 µl
	12 µl	420.0 µl

Labelled primer were added last, mixed, aliquoted to PCR tubes followed by the addition of DNA. Amplification was conducted in a GeneAmp9700 PCR machine (Perkin Elmer Applied Biosystems, UK). The PCR cycling conditions were as follows: an initial denaturation at 94 for 2 min. followed by 35 cycles of 1 min. @ 94°C, 1 min. @ 60°C and 1 min. @ 72°C and a final extension for 10 min. @ 72°C. For some primers different annealing were attempted.

Appendix D: *Phytophthora infestans* EST Sequencing Project, an International resource accessible online as part of the Phytophthora Genome Initiative database (Nuss et al. 1999).