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Review on Molecular Epidemiology in Relation to Devastating Late Blight Pathogen, *P. infestans*, de Bary

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A B S T R A C T

Molecular epidemiology is a science that focuses on the contribution of potential genetic and environmental risk factors, identified at the molecular level, to the etiology, distribution and prevention of disease Molecular epidemiology provides the 'tools' (both laboratory and analytical) that have predictive significance and that epidemiologists can use to better define the etiology of specific diseases, and work towards their control. Application of these molecular techniques has increased the understanding of the epidemiology of the most important infectious agents, *Phytophthora infestans.* Recent progress in *P. infestans* genomics is providing the raw data for such methods and new bio molecular markers are currently being developed which have tremendous potential in the study of *P. infestans*. Closer collaborations between specialists in the fields of plant pathology, epidemiology, population genetics / molecular ecology, *P. infestans* molecular biology and plant breeding are advocated to enable such progress. Molecular techniques help to stratify and to refine data by providing more sensitive and specific measurements which facilitate epidemiologic activities including disease surveillance, outbreak investigations, identifying transmission patterns and risk factors among apparently disparate cases characterizing host pathogen interactions and providing better understanding of disease pathogenesis at the molecular level..

Introduction

Phytophthora infestans causes late blight on a range of solanaceous plant species and can devastate potato and tomato crops in most cool-temperate environments worldwide. Crop losses and costs of late-blight control constitute a significant financial burden on the potato industry. In many potato-growing areas, frequent fungicide applications are the main method of disease control. These applications commence when a local inoculums source is identified and/or environmental conditions are suitable for disease development. The potentially serious consequences of a late-blight infection result in many growers spraying their crops as a matter of routine from the time the plants meet in the rows through until harvest. There is a clear environmental and economic need for more sustainable late-blight control, through better management of primary inoculum, improved chemicals or more efficient application schedules and the use of 'engineered' or natural host resistance. Research has demonstrated that natural host

resistance has the potential to replace at least some of the chemical inputs (Gans, 2003; Kessel *et al.,* 2003). When released in the UK, the potato cultivars Pentland Dell and Maris Peer were highly resistant to late blight. Their resistance was, however, based on simple combinations of R genes and was overcome as the frequency of matching virulence genes in the *P. infestans* population increased (Malcolmson, 1969). This increase was as a direct result of the selection pressure imposed upon the pathogen population by the cultivation of these cultivars (Shattock *et al.*, 1977) and illustrates the potential problems of relying on host resistance for disease control without due consideration of how the pathogen population may respond to its deployment. Similarly, the widespread use of the phenylamide class of systemic blight fungicides soon after their release drastically increased the frequency of resistant isolates (e.g. Dowley and O'Sullivan, 1985) resulting in failures in disease control (Bradshaw and Vaughan, 1996). Predicting the sustainability of disease-management strategies is clearly dependent on an understanding of the pathogen and its population dynamics. This is especially true of potato late blight, as *P. infestans* has been classified as 'high risk' based on its evolutionary potential (McDonald and Linde, 2002). *Phytophothora infestans* is thus a moving target and the bodies (e.g. advisors, forecasters, agrochemical companies, researchers, regulatory bodies, breeders, etc.) responsible for practical long- and short term advice to the potato industry need data on contemporary pathogen populations. Fungi and oomycetes are the causal agents of many of the world's most serious plant diseases and are unique among the microbial pathogens in being able to breach the intact surfaces of host plants. Recently, there have been a number of studies published describing the genome sequences of a diverse set of fungi and oomycetes including one published in this issue of The Plant Cell (Hane *et al.,* 2007), and this provides an opportunity to review what we have learned so far from sequencing the genomes of pathogenic and free-living fungi and also to look forward to the mass of genome sequence information that is likely to be generated in the next few years. The deployment of low-cost, high-throughput DNA sequencing technologies and large-scale functional genomics to eukaryotic plant pathogens will provide new insight into their biology and into the evolution of pathogenicity. *Phytophthora* literally means plant destroyer, a name coined by Anton de Bary in 1861 when he proved that a microorganism, designated as a fungus, was the causal agent of a plant disease known as late blight of potato and was responsible for the Irish potato famine (Large 1940). The genus *Phytophthora* belongs to the oomycetes, a diverse group that includes both saprophytes and pathogens of plants, insects, fish, vertebrates and microbes. More than 150 years ago, the late blight pathogen *Phytophthora infestans* struck the Irish potato crop. Virtually the entire potato crop was wiped out in a single warm, wet week in the summer of 1846. In its aftermath over 1 million people died and another 2 million emigrated from Ireland.

Among the plant pathogenic oomycetes are more than 65 *Phytophthora* species, a hundred or more *Pythium* species, and a variety of obligate biotrophs, including downy mildews and white rusts (Agrios, 2005; Erwin and Ribeiro, 1996). They cause devastating diseases on numerous crops and have an enormous impact on agriculture. Fungal and oomycete plant pathogens occupy similar ecological niches. Yet the distinct evolutionary history of the two groups implies that their pathogenic behavior evolved independently and that convergent evolution has shaped the genomes of these two major groups of plant pathogen Only in recent years have genomes of eukaryotic plant pathogens been sequenced. The first one was

Magnaporthe grisea, the rice blast fungus (Dean *et al.*, 2005), and to date, a handful of draft genome sequences of fungal plant pathogens are available (Xu *et al.*, 2006). Overall, the genome sizes of fungi do not exceed 40 Mb and they are mostly haploid. In contrast, the genomes of oomycetes studied so far are all larger than 45 Mb and often double that size or more and they are diploid (Judelson and Blanco, 2005; Kamoun, 2003). It is, therefore, not surprising that it took some time before oomycete genome projects got off the ground. Advances in software and sequencing technologies have resulted in a decrease in costs and a sharp increase in the number of ongoing eukaryotic genome sequencing projects, and fortunately, oomycete sequencing projects are also on the rise. One incentive for funding a *Phytophthora* genome sequencing project was the emergence of a mysterious disease threatening California oak trees. *Phytophthora ramorum*, the causal agent of Sudden oak death, was described as a species in 2001 (Werres et al. 2001), and only four years later,a draft sequence of its genome was available. Emotion and scientific rationale clashed. The Californians cried because their magnificent oak trees were dying and they wanted immediate action to solve the problem. But the scientists raised doubts about the value of sequencing the genome of a relatively unknown species that had no history of research and that few people studied. The compromise was to include a second species, *Phytophthora sojae* that, next to 'the Irish potato famine fungus' *Phytophthora infestans*, has the status of being a model for molecular genetic research on oomycetes. *P.sojae* was first described in the 1950s as the causal agent of root and stem rot on soybean (Hildebrand 1959; Kaufmann and Gerdemann 1958). Thus *P. infestans* and *P. sojae* each attack major food and feed crops and are devastating pathogens worldwide. *Phytophthora infestans*, (Mont.) de Bary is the causative agent of the late blight disease of tomato and potato and is by far the most devastating disease of potato worldwide (Fry and Goodwin, 1997b). *P.infestans*, which has caused the Irish potato famine in the mid nineteenth century (de Bary, 1876), continues to cause multi-billion dollar losses annually in potato and tomato production (Fry and Goodwin, 1997a; Fry and Goodwin, 1997b). The havoc that *P. infestans* wreaks on potato and tomato is yet to be effectively controlled, and the problem worsened with the recent emergence of highly aggressive and fungicide in sensitive strains (Fry and Goodwin, 1997a; Fry and Goodwin, 1997b). In fact, recent reports warned that potato blight might cause catastrophic losses, and possibly famine, in Eastern Europe, and recent epidemics in that region resulted in as much as 70% losses in yield (Schiermeier, 2001; Garelik, 2002). *P. infestans* belongs to a unique taxonomic group of organisms called the oomycetes. This group includes various plant and animal pathogens as well as saprophytic species (Margulis and Schwartz, 2000). Historically, based on their fungal-like morphology and physiology, the oomycetes have been referred to as fungi. Increasing biochemical (Bartnicki-Garcia and Wang, 1983; Pfyffer *et al.*, 1990) and molecular (Paquin *et al.*, 1997; Sogin and Silberman, 1998) evidence has shown that oomycetes are not fungi, but are more related to heterokont algae. Their unique phylogenetic position suggests that molecular mechanisms underlying host infection and interaction could be unique. Invariably, fungal pathogens, for which molecular studies are more advanced, cannot serve as models to study oomycetes. Also, in light of the different evolutionary history of the fungi, the unique biochemical features of oomycetes render them insensitive to many of the fungicides available (Griffith *et al.*, 1992; Kirk *et al.*, 1999). Effective management of diseases caused by the oomycetes, will come from a thorough understanding of the mechanisms underlying

pathogenicity and plant responses to the pathogen and the development of specific fungicides. In this review, I discuss the life cycle of *P. infestans*, pathogenicity, elicitors and host/nonhost resistance, and finally I discuss recent genomic resources and functional genomic systems available for *P. infestans.*

Phytophthora infestans infection cycle

The *P. infestans* infection cycle is well known (Pristou and Gallegly, 1954: Coffey and Wilson, 1983; Agrios, 1988; Erwin and Ribeiro, 1996). Infection is initiated when sporangia come into contact with a moist leaf surface. The sporangia will either germinate directly at temperatures above 15ºC or release biflagellate zoospores at temperatures below 15ºC. The motile biflagellated zoospores then germinate after encystment on the surface of the plant. Following appressorium formation, infection tubes emerge and penetrate epidermal cells. In susceptible plants (compatible interactions), hyphae spread into the mesophyll layer, occasionally forming haustorium-like feeding structures. After colonization, sporangiophores are formed at the tip of emerging hyphae from the stomata. These become inocula for subsequent aerial spread of the pathogen (Fig. 1 and 2). Infected foliage becomes yellow, water soaked and ultimately turns black. In resistant plants (incompatible interactions), a form of programmed cell death known as the hypersensitive response (HR) is induced. Cytological studies demonstrated that the hypersensitive response is associated with all forms of resistance to *P. infestans*, albeit at different rates of induction (Vleeshouwers *etal*., 2000). In race specific resistant hostplants, induction of the HR is limited to one or a few cells and results in the arrest of pathogen growth in the early stages of infection (Kamoun *et al.,* 1999c; Vleeshouwers *et al.,* 2000). Other types of resistance, such as partial or rate-limiting resistance, also involve the HR, which can occur as a trailing type of necrosis (*et al.,* 1999c; Vleeshouwers *et al.,* 2000) and in nonhost.

During the growing season, infections usually start from primary infected potato plants with sporangiophores carrying sporangia. These sporangia are wind dispersed and can start new infections in two ways. Under wet conditions and temperatures below 12 oC, sporangia develop into zoosporangia that release a number of zoospores, each carrying two flagella. After a mobile period, which can last for over ten hours, these zoospores stop moving and a thick cell wall is formed creating a cyst. Alternatively, at higher temperatures sporangia act as sporangiospores that can germinate directly. Both cysts and sporangiospores germinate and at germtube tip an appressorium is formed a specialized structure from which a penetration peg emerges that pierces the cuticle and penetrates the epidermal cell. In the epidermal cell an infection vesicle is formed from which the colonization of the underlaying cell layers starts. *P. infestans* grows in between the mesophyl cells where feeding structures (haustoria) are formed.

After three to four days with conditions favorable to the pathogen, hyphae emerge through the stomata and sporangiophores with sporangia are formed which can start a new cycle of infection. At this time the leaf can still look healthy, without clear symptoms, but more often part of the leaf becomes necrotic and may be surrounded by a white fluffy area where the plant tissue is covered by sporangiophores. *P. infestans* can infect leaves, stems, berries and tubers. While infected tubers are the most common source of inoculum at the beginning of the season (Zwankhuizen *et al.,* 1998), infections can also start from oospores

that result from the sexual cycle and can survive several years in the soil (FLIER et al. 2001b). The sexual cycle starts when vegetative hyphae of two opposite mating types (A1 and A2) meet. This induces the formation of oogonia and antheridia. The oogonium grows through the antheridium and after meiosis a fertilization tube grows from the antheridium through the oogonial cell wall and delivers the haploid antheridial nucleus into the oogonium. Subsequently, a thick cell wall is formed making oospores persistent structures. Germinating oospores can form a sporangium, which can start infection of tubers, stems and leaves.

Molecular epidemiology: Focus on infection

Molecular biology techniques have become increasingly integrated into the practice of infectious disease epidemiology. The term "molecular epidemiology" routinely appears in the titles of articles that use molecular strain-typing ("fingerprinting") techniques—regardless of whether there is any epidemiologic application. What distinguishes molecular epidemiology is both the "molecular," the use of the techniques of molecular biology, and the "epidemiology," the study of the distribution and determinants of disease occurrence in plant populations. This reviews various definitions of molecular epidemiology and comment on the range of molecular techniques available and present some examples of the benefits and challenges of applying these techniques to infectious agents and their affected host using tuberculosis and urinary tract infection as examples.They close with some thoughts about training future epidemiologists to best take advantage of the new opportunities that arise from integrating epidemiologic methods with modern molecular biology. *Am J Epidemiol* 2001;153: 1135–41. Molecular epidemiology provides the 'tools' (both laboratory and analytical) that have predictive significance and that epidemiologists can use to better define the etiology of specific diseases, and work towards their control (Andrew Thompson Molecular epidemiology of infectious diseases 2000. 326p). It is a science that focuses on the contribution of potential genetic and environmental risk factors, identified at the molecular level, to the etiology, distribution and prevention of disease. Over the past two decades, there has been a proliferation of subspecialties among epidemiologists. Perhaps none of these subspecialties has been received with more controversy than "molecular epidemiology," as the term "molecular" describes neither a disease category nor a substantive area (1) but in jargonese refers to characteristics based on nucleic acid- or amino acid-based content. The issue is further confused by the independent emergence of the term molecular epidemiology during the 1970s and early 1980s in three separate substantive areas: cancer epidemiology, environmental epidemiology, and infectious disease epidemiology. In many epidemiologic textbooks, molecular epidemiology has been defined almost exclusively in terms of biomarkers (2), ignoring the many applications in both genetic and infectious disease epidemiology

What exactly is molecular epidemiology?

Many different definitions of molecular epidemiology have been published all mention the use of molecular tools, but not all explicitly mention epidemiology. This is unfortunate, as molecular epidemiology is not just molecular taxonomy, phylogeny, or population genetics but the application of these techniques to epidemiologic problems. Molecular taxonomy, phylogeny, population genetics, and molecular epidemiology may use the same laboratory techniques, but each follows distinct principles. In phylogeny/ taxonomy, the data are generated to describe

properties and characteristics of organisms. Population genetics often intersects with epidemiology: both use population approaches to describe the distribution of characteristics of interest and analyze data to identify the determinants of that distribution. Epidemiology attempts to identify factors that determine disease distribution in time and place, as well as factors that determine disease transmission, manifestation, and progression. Further, epidemiology is always motivated by an opportunity or possibility for intervention and prevention. What distinguishes molecular epidemiology is both the "molecular," the use of the techniques of molecular biology to characterize nucleic acid- or amino acid-based content, and the "epidemiology," the study of the distribution and determinants of disease occurrence in human populations.

Various definitions of molecular epidemiology

"The application of sophisticated techniques to the epidemiologic study of biological material"

"Molecular epidemiology is the use of biologic markers or biologic measurements in epidemiologic research"

"The application of molecular biology to the study of infectious disease epidemiology"

"Using molecular biomarkers in epidemiology"

"Molecular epidemiologic research involves the identification of relations between previous exposure to some putative causative agent and subsequent biological effects in a cluster of individuals in populations"

"The analysis of nucleic acids and proteins in the study of health and disease determinants in human populations"

"Molecular epidemiology uses molecular techniques to define disease and its pre-clinical states, to quantify exposure and its early biological effect, and to identify the presence of susceptibility genes"

"The practical goals of molecular epidemiology are to identify the microparasites responsible for infectious diseases and determine their physical sources, their biological relationships, and their route of transmission and those of the genes responsible for their virulence, vaccine relevant antigens and drug resistance"

"A science that focuses on the contribution of potential genetic and environmental risk factors, identified at the molecular level, to the etiology, distribution and prevention of disease".

Molecular techniques

Molecular techniques do not substitute for conventional methods. They address epidemiologic problems that cannot be approached or would be more labor intensive, expensive, and/or time consuming to address by conventional techniques. Today's molecular technique can become tomorrow's conventional diagnostic tool or even consigned to the wastebasket. For example, plasmid profile analysis was a mainstay of molecular fingerprinting just a short while ago and now has been almost entirely replaced by other techniques. Acknowledging that any list of molecular techniques will be outdated from the time it is published, and the techniques that have been applied in epidemiologic studies of infectious disease. They fall into two large categories: identification and fingerprinting (strain typing). Rather than describe the techniques themselves in detail, we describe how the application of some of these techniques has increased our

understanding of the epidemiology of two important infectious agents: *Mycobacterium tuberculosis*, which causes tuberculosis, and uropathogenic *Escherichia coli*, which causes urinary tract infection. Tuberculosis is the most common infectious cause of deaths in adults worldwide (3), and urinary tract infection is one of the most common bacterial infections, affecting half of all women (4) and one seventh of all men at least once during their lifetime (5). We will use these pathogens to illustrate the distinct approaches and principles that must be considered when conducting epidemiologic investigations using molecular technics. Molecular techniques are used to study and solve the epidemiological problems that the traditional epidemiological methods can not.

Basic molecular markers used in molecular epidemiology

Individual (Molecular diagnosis):

1. Hybridization,
2. PCR-based:
   a. classical PCR,
   b. nested PCR,
   c. real-time PCR.

Population

1. PCR-based assay: RAPD, ISSR (internal simple sequence repeat, MP-PCR), AFLP.
2. Hybridization RFLP.
3. DNA sequence: ITS, IGS, Protein genes- β tubulia, EF1α, Elongation factor.

Evolution is an important factor in predicting the effectiveness and durability of new management practices. A range of phenotypic and genotypic tests has been applied to achieve this goal, but each has limitations and new methods are sought. Recent progress in *P. infestans* genomics is providing the raw data for such methods and new high-throughput codominant biomolecular markers are currently being developed that have tremendous potential in the study of *P. infestans* population biology, epidemiology, ecology, genetics and evolution. This reviews some key applications, recommends some changes in approach and reports on the status and potential of new and existing methods for probing *P. infestans* genetic diversity of information familiar to plant pathologists concerning the aetiology and epidemiology of the disease; for example, understanding the origins of disease outbreaks on both local (e.g. individual seed tubers, dumps, soilborne oospores) and international (e.g. global seed trade or large-scale weather systems) scales. However, a greater understanding of the biology of *P. infestans* infection, genetics, genomics and evolutionary processes is also important. There must be a greater emphasis on *P. infestans*. Understanding the relative contributions and rates of mutation, recombination, natural selection, gene flow, random genetic drift and migration (Burdon and Silk, 1997) to the generation and maintenance of variation in populations is important, yet such factors remain little studied (McDermott and McDonald, 1993) and poorly understood. Similarly, the paucity of information on the below-ground and soilborne phases of the disease, the absence of a widely adopted and objective means of estimating *P. infestans* population diversity and a lack of understanding of the impact of selection pressure are also hampering scientific progress. Recent advances in *P. infestans* physical (Randall and Judelson, 1999; Whisson *et al.*, 2001) and genetic (van der Lee *et al.*, 1997) mapping, genomics (Kamoun *et al.*, 1999; Birch and Whisson, 2001; Birch *et al.*, 2003; Bos *et al.*, 2003), and the functional analysis of genes involved in growth, development and plant infection (Birch and Kamoun, 2000; Avrova *et al.*, 2003; Torto *et al.*, 2003) are revolutionizing

the field of *Phytophthora* research. They also form a crucial resource from which valuable DNA-based markers can be generated and this, coupled with advances in fingerprinting technology and laboratory automation, is facilitating affordable, high-throughput analysis of multiple DNA-based markers. It is therefore timely to review the types and likely contributions of such biomolecular markers in advancing *P. infestans* research in key fields such as population biology, epidemiology, genetics and the mapping and functional analysis of novel genes. In light of the threats from changing *P. infestans* populations in many regions worldwide (Fry and Goodwin, 1997), particular emphasis will be placed on the utility of existing phenotypic and genotypic markers and the potential of new methodology for examining *P. infestans* populations. It is suggested that new methods and approaches are needed to stimulate advances in this field.

The applications of marker technology

It is clear that no single marker system (Milbourne *et al.*, 1997) will be adequate for all aspects of *P. infestans* research. This review firstly considers the principal applications of new marker technology, examining the requirements of each type of study. Some key considerations in selecting an appropriate marker are depth of taxonomic resolution, run-in time and resources available, throughput required, running costs and proposed adaptation by other research groups.

Population diversity and population genetics

Probably the most common objective in the study of *P. infestans* populations is to ensure that management practices, prediction tools and potato breeding strategies are appropriate for the contemporary pathogen population. The monitoring of A1 and A2 mating-type ratios is important to aid predictions of the extent of sexual recombination and thus the risk of long-lived oospores serving as primary inoculum sources. In addition to its epidemiological impact, sexual recombination is likely to increase the rate of pathogen adaptation (Barton and Charlesworth, 1998), thus reducing the predictability of disease management practices. Understanding the population biology of *P. infestans* and closely related taxa (e.g. *P. phaseoli*, *P. ipomoeae* and *P. mirabilis*) in 'natural' ecosystems and comparing it with populations on cultivated crops are

Characteristics of an ideal marker system for the genetic analysis of *Phytophthora infestans*

High throughput uses the most widespread and affordable technology available (e.g. PCR), capable of being multiplexed (i.e. several traits can be analysed simultaneously within a single isolate), *robust*, optimized protocols for running and objective scoring of the assays to encourage widespread adoption of a standard marker system, *flexible*, can be applied to both pure *P. infestans* DNA samples and infected leaf material or spore washings, can be modified to the resolution appropriate to the study, e.g. from the study of closely related species to intrapopulation diversity, *suitable for rigorous genetic analysis.* Markers unlinked, simply inherited and, ideally, mapped to each linkage group codominant (both alleles at a locus revealed). A combination of nuclear and mitochondrial targets, *broadly applied*, widely disseminated protocols resulting in its universal adoption, *safe*, does not involve hazardous procedures or chemicals. It is important to distinguish between studies of population diversity and population genetics; the former yield the raw data, to which the latter can be applied to answer questions on the fundamental

mechanisms and processes of genetic change in populations (reviewed in Milgroom and Fry, 1997). Surveys are conducted by collecting isolates that represent a 'snapshot' of the overall population in time and space.

Temporal and geographic variations in phenotypic and/or genotypic diversity are then examined and interpreted in relation to the scientific goals of the study. There are many examples of this type of study in which the sophistication of the analysis has advanced from phenotypic (Malcolmson, 1969; Shattock *et al.,* 1977) to genotypic methods, such as analysis of isozymes (Shattock *et al.,* 1986; Tooley *et al.,* 1985), mtDNA and RG57 restriction fragment length polymorphism (RFLP) patterns (Goodwin *et al.,* 1994), amplified fragment length polymorphisms (AFLPs) (Cooke *et al.,* 2003; Flier *et al.,* 2003) and, more recently, simple sequence repeats (SSRs) (Knapova and Gisi, 2002). With the exception of the already diverse populations at its centre of origin (Goodwin *et al.,* 1992a), an overall trend of increasing diversity in *P. infestans* has been observed in many potato-growing regions of the world. Early studies described populations that were clonal or dominated by a few discrete lineages (Drenth *et al.,* 1994; Goodwin *et al.,* 1998; Cohen, 2002), whereas more recent analysis highlights the appearance of many new genotypes via migration and sexual recombination (e.g. Sujkowski *et al.,* 1994; Goodwin *et al.,* 1995a, 1998; Punja *et al.,* 1998; Hermansen *et al.,* 2000; Cooke *et al.,* 2003). Evaluating the evolutionary forces driving such population change and the practical significance to disease control remains difficult (Goodwin, 1997). Comparing regional studies to build up an international perspective of *P. infestans* population dynamics would be beneficial, but unfortunately has not proved possible. In part, the problem stems from the logistical difficulties of comparing data collected in different laboratories, but a more serious problem is the nature of the raw data. Mating type, RG57 loci and isozyme data have been central in elucidating the movement and displacement of major lineages (Goodwinn *et al.,* 1994) and data from more than 1500 isolates have yielded a valuable baseline description of the dominant lineages in many countries (Forbes *et al.,* 1998). However, the data are not appropriate for the type of powerful population genetic analysis needed to critically examine *P. infestans* populations on this scale. There is a clear need for both new markers and a new approach to interpreting fluxes in *P. infestans* populations.

The practical criteria that will encourage the uptake of any new marker and those necessary to ensure the data are appropriate for population genetic analysis. In terms of practicality, the methods should use commonly available technology, and be based on cost effective, high-throughput, robust and freely available detailed protocols to ensure their widespread adoption. Population genetic analysis is typically based upon five to 15 unlinked, simply inherited and codominant markers (Harper *et al.,* 2003; Maggioni *et al.,* 2003; Chauvet *et al.,* 2004).

Codominance, meaning both alleles at a locus can be unambiguously resolved, is particularly important as it allows a more robust and powerful population genetic analysis. It is critical that new markers are appropriate for comparison of isolates both within and between populations on local and intercontinental scales and can accommodate the problem of convergence while adequately describing the ever-expanding genotypic diversity. Convergence (or homoplasy) occurs when isolates of different genetic backgrounds share an identical fingerprint. Such apparent 'identity' occurs by chance alone, rather than common descent, and will confound genetic analysis.

AFLP (amplified fragment length polymorphisms

1. Genomic DNA is digested with both a restriction enzyme that cuts frequently (*Mse*I, 4 bp recognition sequence) and one that cuts less frequently (*Eco*RI, 6 bp recognition sequence).
2. The resulting fragments are ligated to end-specific adaptor molecules.
3. A preselective PCR amplification is done using primers complementary to each of the two adaptor sequences, except for the presence of one additional base at the 3' end. Which base is chosen by the user. Amplification of only 1/16th of *Eco*RI-*Mse*I fragments occurs.

AFLP fingerprinting, for example, discriminates isolates considered identical based on RG57 fingerprint (Purvis *et al.,* 2001) and two SSR markers (Knapova and Gisi, 2002). The converse, where a high proportion of isolates within a population have unique genetic fingerprints (e.g. Brurberg *et al.,* 1999; Zwankhuizen *et al.,* 2000; Cooke *et al.,* 2003), results in an endlessly expanding list of defined genotypes. The currently adopted system of designating genotypes (Goodwin *et al.,* 1994; Forbes *et al.,* 1998) is based on a country code followed by a unique number for each new genotype, with subcategories for isolates presumed to have emerged within a genotype. As a growing feature of *P. infestans* populations is a 'blurring' of the boundaries of genetically distinct subpopulations, the number of genotypes that need to be described in this way is likely to increase exponentially and, in the longer term, this may not be a helpful approach. There are now many variants of the US1 lineage (e.g. Forbes *et al.,* 1998; Reis *et al.,* 2003) and at least 19 'US' genotypes, some probably generated as recombinants of existing lineages (e.g. Gavino *et al.,* 2000; Wangsomboondee *et al.,* 2002). An accepted naming system is clearly needed for dominant subgroups of the population (i.e. asexual lineages), but it needs to be able to accommodate this increasing diversity. A possible solution is a population approach in which the genotype of each new isolate is examined in the context of allele types, combinations and frequencies in series of populations hierarchically sampled at geographic scales ranging from a single leaf to a continent and, ideally, duplicated over time. Analysis using *F* -statistics (Hartl and Clark, 1997) and genetic distances (Goldstein and Pollock, 1997) yields detailed objective descriptions of the population structure and the relatedness of different subgroups. Other methods are applied to estimate effective population size, demographic history and the magnitude and direction of gene flow between populations (Hartl and Clark, 1997). Such accurate partitioning of genetic diversity will, for example, allow a critical examination of whether any new genotype is a subset of the local population (i.e. is derived from sexual recombination within the population) or is the result of migration, a novel mutation or recombination between populations. An international database of isolates genotyped using similar protocols. *Markers for examining Phytophthora infestans* is crucial to this approach. Linking existing and new population-based systems of nomenclature will be a major challenge, but will answer many key questions on the historical and contemporary patterns of migration of *P. infestans*; for example, what is the relationship between the US lineages and the populations currently dominant in Europe? *Phytophthora infestans* populations are characterized by patchiness and high rates of extinction and recolonization from one season to the next (Fry *et al.,* 1992). Such a metapopulation structure means that small-scale sampling in a single season is unlikely to yield a true picture of the population structure. More extensive

sampling over time and space is needed and sample throughput is therefore important for any new marker system. The direct testing of sporangia from sporulating lesions without lengthy isolation procedures is an obvious way to increase throughput, particularly if key phenotypic tests can be converted into reliable molecular assays (see below). Another crucial means of achieving this scaled-up approach is the coordination of research groups involved in the study of *P. infestans*. The recent EU-funded Concerted Action project EUCABLIGHT (http://www. eucablight.org) aims to develop, harmonize and disseminate protocols and data on *P. infestans* populations within Europe and, in the longer term, worldwide. As stated, the most powerful analysis tools rely on codominant data in which allele frequencies and distributions can be monitored over time.

RFLP (restriction fragment length polymorphisms

- Use the restriction endonucleases to recognize the specific DNA sequences.
- Hybridize to probe DNA or amplify by PCR.
- Analyze the variation of amplified bands.

Real time PCR

(1) Forward and reverse primers are extended with *Taq* polymerase as in a traditional PCR reaction. A probe with two fluorescent dyes attached anneals to the gene sequence between the two primers.

(2) As the polymerase extends the primer, the probe is displaced.

(3) An inherent nuclease activity in the polymerase cleaves the reporter dye from the probe.

(4) After release of the reporter dye from the quencher, a fluorescent signal is generated.

SSR-PCR (Simple sequence repeats)

Bands are generated by a primer of simple sequence repeats. SSRs offer the greatest combination of required attributes for population analysis and their potential should be explored more fully. The increasing use of such biomolecular markers has great potential, but a move away from simply cataloguing *P. infestans* variation and towards experiments with sampling strategies designed to test specific hypotheses, using such markers within a theoretical framework of population genetics, is needed. In the coming years, the tracking of allele frequencies and distributions over time will advance the understanding of the spatial and temporal dynamics of *P. infestans* populations, as well as helping to estimate gene flow and investigate the balance between the forces of natural selection and chance effects of genetic drift and migration. From these data, the processes driving population change and how it may best be managed to the benefit of long-term disease control can be considered. For this to be realized, a coordinated approach is needed, in which the strengths of the disciplines of plant pathology, population genetics, molecular ecology and epidemiology are combined.

Tracking isolates in epidemiological studies

A major goal of the population analyses detailed above is to infer the processes driving population change. The resultant hypotheses based on such 'observational' survey data will, however, require rigorous testing. Such testing is not easy; even the suggestion that 'new' genotypes have replaced 'old' types in the UK because of increased aggressiveness has proved surprisingly difficult to test experimentally (Day and Shattock, 1997). Empirical data are needed from which the relative fitness of different strains can be compared directly. High-throughput markers

will facilitate rapid isolate discrimination and thus direct comparisons of the frequency of recovery of two or more preselected isolates during the course of field epidemics. A single genetic marker that discriminates the test strains would be sufficient, offering a higher throughput than equivalent studies based on allozymes (Legard *et al.,* 1995; Lebreton *et al.,* 1999). Direct fingerprinting of *Plasmopara Viticola* lesions has been demonstrated (Gobbin *et al.,* 2003) and work at SCRI showed that sporangia harvested from a single lesion or even single sporangia grown for a few days in a small volume of pea broth in a 96-well microplate yielded sufficient DNA for rapid PCR fingerprinting (Hussain, 2003). Fingerprinting using a more comprehensive range of markers also has potential for larger-scale tracking of isolates with specific traits. For example, understanding the origin and spread of strains that have overcome novel host resistance, or developed resistance to an important fungicide, is fundamental to managing the risk that such strains pose. Such isolate tracking can also be used effectively to determine sources of primary inoculum (Zwankhuizen *et al.,* 2000). The association between seedborne infection and subsequent field outbreaks, for example, is important to the understanding of infection pathways and control methods, as well as having commercial and regulatory implications. Similar approaches have been used to identify source populations in the surveillance of human pathogens (Fisher *et al.,* 2002). Tracking of inoculum using powerful genetic markers will also add detail to the fascinating palaeogeographical reconstruction of the spread of *P. infestans* across the world (Ristaino *et al.,* 2001) and may influence international quarantine issues in the context of contemporary pathogen movement. SSRs offer the greatest potential for studies of comparative fitness, as multiple combinations of alleles are possible at each specific locus, thus increasing the likelihood of identifying unique test isolates for any given experiment. For tracking particular strains, or monitoring inoculums movement on a larger scale, SSRs again have the greatest potential to uniquely discriminate each strain. However, further work is needed to investigate whether the resolution offered by SSRs will be sufficient in populations with limited genetic diversity. If the specific mutation responsible for the change in phenotype is known, as in the case of QoI resistance in *P. viticola* (Gisi *et al.,* 2002), the combined tracking of both selectable and neutral markers will yield the most useful data.

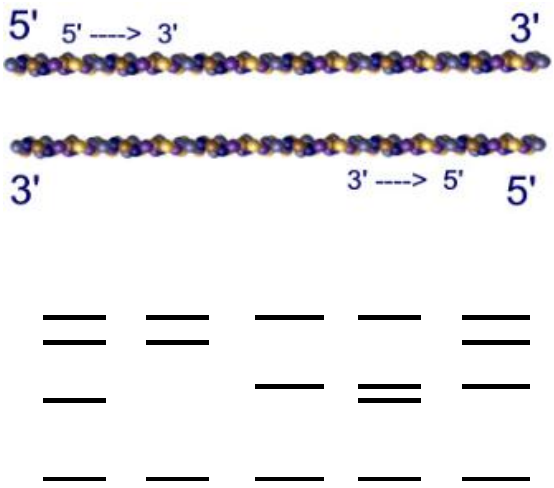
Genetic mechanisms

Phytophthora has a tremendous range of mechanisms for creating and maintaining genetic diversity (Brasier, 1992). However, the contribution of each mechanism to its adaptability under natural conditions remains poorly understood (Goodwin, 1997; Judelson, 1997b).

In addition to conventional genetic recombination of A1 and A2 mating types, self-fertility (Smart *et al.,* 1998), segregation of heterokaryons (Pipe *et al.,* 2000), zoospore-mediated hyphal fusion (Judelson and Yang, 1998), mitotic recombination (Goodwin, 1997), polyploidy (Tooley and Therrien, 1991) and aneuploidy (Carter *et al.,* 1999) have all been reported in *P. infestans.* Phenotypic variation during clonal reproduction (Caten and Jinks, 1968; Judelson, 1997a; Abu-El Samen *et al.,* 2003) also remains poorly understood. Many phenotypic or genotypic markers have been used in the analysis of the above mechanisms, but a collection of well-characterized, PCR-based, codominant and, ideally, mapped markers such as single nucleotide polymorphisms (SNPs) or SSRs would be of great benefit in resolving such processes and their relative importance.

Mapping and functional analysis of genes

The isolation of genes responsible for key traits, such as avirulence, pathogenicity, fungicide resistance or mating type, is an important target in *P. infestans* research (Judelson, 1997b; Birch *et al.*, 2003; Kamoun, 2003). Positional, or map-based, cloning approaches rely on a high density of mapped markers in a segregating population and, in the absence of genomic resources, randomly generated AFLPs and RAPDs proved the most appropriate markers (Judelson *et al.*, 1995; van der Lee *et al.*, 1997, 2001).RAPD (Randomly amplified polymorphic DNA)**)** Bands are generated by a 10-bp Operon primer.



Three of the bands in the diagram are RAPD bands, and there are 5 polymorphic phenotypes

There is an urgent need for a genome-wide set of high-density markers in *P. infestans* to aid gene discovery and allow approaches such as 'natural selection mapping' to be applied. Unique patterns of linkage disequilibrium were recently confirmed around the region responsible for warfarin resistance in natural rat populations under a strong selection pressure (Kohn *et al.*, 2000). Such an approach could be used in *P. infestans* to identify key fitness-related genes. Whether the candidate gene is identified by the above methods or comparative genomics (Bos *et al.*, 2003), a first step towards confirming its function requires genetic markers either tightly linked to or within the gene. Association genetics can then be used to examine the correspondence of the phenotypic trait and the linked marker in multiple isolates from natural populations or progeny from test crosses. Clearly, marker position is critical for such analysis and SNPs are likely to be the most valuable markers as they occur at a high frequency (Brumfield *et al.*, 2003) and can precisely target the specific nucleotide responsible for the amino acid change (e.g. Bos *et al.*, 2003).

The importance and potential of phenotypic markers

Like most *Phytophthora* species, there are relatively few reliable morphological characters by which to discriminate *P. infestans* isolates (Shaw, 1991; Shaw and Shattock, 1991). The most studied of these phenotypic traits, and those that remain most informative, are mating type (Gallegly and Galindo, 1957), virulence (Malcolmson and Black, 1966) and fungicide resistance (Dowley and O'Sullivan, 1981).

Mating type

Studying the spatial and temporal distribution of the A1 and A2 strains of *P. infestans* is fundamental to understanding the significance of mating type to both the generation and maintenance of genetic diversity and to disease aetiology. Considerable efforts have therefore been made to estimate mating-type frequencies in *P. infestans* populations worldwide (Hermansen *et al.*, 2000; Zwankhuizen *et al.*, 2000; Cooke *et al.*, 2003). Apart from the complication of self-fertility (Judelson, 1997a), the mating-type assay,

based on the pairing of an unknown isolate with known tester A1 and A2 strains and screening for oospore production, is robust and reliable. However, an axenic culture of each isolate is required, which can be a bottleneck in the screening process. A reliable molecular assay for mating type would be valuable, but the genetic bases of mating-type determination are not yet fully understood and non-Mendelian segregation and frequent rearrangement in the region encoding the mating type loci (Judelson, 1997a) will make the design of the assay challenging.

Virulence

Genetic analysis of the resistance introgressed into *Solanum tuberosum* from wild *Solanum* species demonstrated a gene-for-gene interaction with single R genes in the host and corresponding virulence genes in the pathogen (Malcolmson *and* Black, 1966). An isolate's 'race', or virulence phenotype, is determined by inoculating a series of 11 genetically defined 'differential' potato genotypes, each carrying a specific R gene, then scoring the resultant compatible or incompatible reactions. The breakdown of Rgene- based resistance in cvs Pentland Dell (R1, R2, R3) and Maris Peer (R1, R2) prompted studies on how R-gene deployment may drive changes in the frequency of specific virulences in the pathogen population (Malcolmson, 1969; Shattock *et al.,* 1977). Virulence has been monitored ever since and alongside an overall increase in virulence complexity with increasing sexual recombination (Drenth *et al.,* 1994; Cohen, 2002), marked temporal and spatial variation in virulence has been reported (Lebreton *and* Andrivon, 1998; Peters *et al.,* 1998; Hermansen *et al.,* 2000). The emergence of virulence against all 11 R genes in a clonal lineage (Goodwin *et al.,* 1995b) and the variation in virulence types in single-zoospore progeny of a single isolate (Abu-El Samen *et al.,* 2003) indicate that there is still much to learn about the generation and inheritance of virulence. It must also be acknowledged that additional R genes exist (Trognitz, 1998) and differential sets should be continually updated to accommodate them. Potential inconsistencies in virulence scores arise from variation in the differential sets used in different laboratories and the sensitivity of such assays to environmental conditions or changes in protocol (Stewart, 1990). The reduced use of R-gene-based resistance and the paucity of information on the R genes present in commonly grown cultivars make the interpretation of the evolutionary forces driving changes in specific virulence difficult. Furthermore, different genetic mutations may result in identical virulence phenotypes. The isolation of the specific avirulence effector genes (*Avr* genes) from *P. infestans* is, however, a major goal of many research programmes (van der Lee *et al.,* 2001; Bos *et al.,* 2003) and once the polymorphisms have been identified, specific DNA-based assays will be available. The combination of markers for such functional genes and neutral markers will be a powerful means of testing contemporary theories in host pathogen specificity. The 'guard hypothesis' (Dangl and Jones, 2001) proposes that a complex of the pathogen *Avr* gene product with a plant virulence target is recognized by an R gene product. Implicit in this is that the *Avr* gene products themselves play a role in pathogenicity. Mutation to a statethat avoids host recognition (i.e. virulence) will, in the absence of that R gene, impose a 'fitness cost' on the pathogen. It is proposed that the opposing forces of fitness costs for resistance in the plant and virulence in the pathogen result in frequency-dependent balancing selection that maintains the alleles in both host and pathogen populations (Van der Hoorn *et al.,* 2002). A cost of plant resistance has been demonstrated (Tian *et al.,* 2003), and the specific tracking of different *Avr* allele frequencies in natural or experimental

populations will be critical in determining whether a corresponding 'fitness cost' to virulence exists. This hypothesis needs to be tested to predict the longevity of engineered resistance based on the pyramiding of R genes.

Fungicide resistance

Fungicide resistance testing has, with the exception of routine testing within the agrochemical industry, predominantly targeted the well-documented resistance to phenylamides (reviewed in Gisi and Cohen, 1996). Agar-based (Shattock, 1988) or *in vivo* testing of many isolates (e.g. Dowley and Sullivan, 1985; Dowley *et al.,* 2002) has indicated clear fluctuations in the frequency of resistant strains according to the fungicide deployment strategy (Davidse *et al.,* 1981). It is unclear whether the reduced frequency of resistant strains, in response to reduced phenylamide use, is the result of random genetic drift to a low but stable level of resistance (Gisi and Cohen, 1996) or a fitness cost to metalaxyl resistance (e.g. Day and Shattock, 1997; Dowley *et al.,* 2002). Again, the development of DNA-based assays, either within or linked to the genes conferring resistance, would be beneficial. However, the genetic basis of resistance is not fully understood (Shattock, 1988; Shaw, 1991; Lee *et al.,* 1999) as it is likely that multiple loci are involved (Judelson and Roberts, 1999) and no reliable DNA-based assay for fungicide resistance is available. Limited study of the sensitivity of *P. infestans* to protectant fungicides revealed no marked variation (Kato *et al.,* 1997). Resistance to the recently released QoI (quinine outside inhibitors) group of fungicides has been reported in cereal fungal pathogens and the oomycetes *P. viticola* and *Pseudoperonospora cubensis* (Ishii *et al.,* 1999; Gisi *et al.,* 2002). With the release of QoI fungicides for late blight control, active resistance monitoring in the commercial sector is ongoing. The mode of action and specific mutation to resistance has been located in the mitochondrial cytochrome *b* gene (Gisi *et al.,* 2002) and monitoring of this specific allele in *P. infestans* may be of interest.

Other phenotypic characters

Variation in other phenotypic characters has been tested on a limited scale. Differences in aggressiveness have been cited as an explanation for population displacements (Day and Shattock, 1997; Kato *et al.,* 1997). Aggressiveness is a multicomponent trait and since many factors may affect infection efficiency, lesion size, incubation period, latent period and sporulation capacity (Spielman *et al.,* 1992), it is a difficult character to measure objectively. Ploidy levels (Tooley and Therrien, 1991) and antibiotic resistance (Shattock and Shaw, 1975) have also been examined. Temperature response, which has important implications for decision support systems, has also been shown to vary amongst different populations (Mizubuti and Fry, 1998), but none of these characteristics has been systematically tested.

The importance and potential of genotypicmarkers

Whilst phenotypic traits are important for understanding the selection pressures on *P. infestans* populations, in isolation they do not fulfil many of the criteria in many different genotypic markers have been used to study *P. infestans* and here the status and future applications of each are considered.

Isozymes

Before the development of DNA-based molecular methods, isozyme variation was used extensively (Tooley *et al.,* 1985). Isozyme data continues to provide valuable

insights into the genetics (Shattock *et al.,* 1986) and population diversity of *P. infestans* (Sujkowski *et al.,* 1994) and was integral to the international naming system (Forbes *et al.,* 1998). Isozymes are based on affordable technology and are codominant, yielding data amenable to population genetic analysis (Goodwin, 1997). However, of the many isozymes tested, only glucosephosphate isomerase and peptidase have proved suitable for widespread use (Spielman *et al.,* 1990; Fry *et al.,* 1992). Furthermore, despite improvements introduced with the cellulose-acetate method (Goodwin *et al.,* 1995c), isozymes fulfil few of the requirements of an ideal marker system. For example, migration distance is expressed in relative terms and can be difficult to interpret, a different stain is required for each enzyme, the precise nature of the genetic change that alters migration distances is unknown and the assays are time-consuming.

RFLPs

The moderately repetitive RFLP probe RG57 (Goodwin *et al.,* 1992b) yields a genetic fingerprint of 25–29 bands (Forbes *et al.,* 1998) and has proved a valuable tool in monitoring *P. infestans* genetic diversity. Many thousands of isolates worldwide have been fingerprinted and an international database of the results constructed (Forbes *et al.,* 1998). The dataset has been important in defining and monitoring (Goodwin and Drenth, 1997) lineages of *P. infestans* and tracking inoculum sources (Zwankhuizen *et al.,* 2000). The method does have disadvantages, however; large amounts of pure DNA are required, it is timeconsuming, the banding patterns can be difficult to interpret and the resultant data are dominant. Furthermore, very little is known about the individual loci that make up the fingerprint, so assessing the likelihood of homoplasy is difficult.

mtDNA haplotype analysis

The *P. infestans* mitochondrial genome has been sequenced (Paquin *et al.,* 1997) and its RFLP diversity studied in some detail (Carter *et al.,* 1990; Goodwin, 1991; Gavino and Fry, 2002). Uniparentally inherited (Whittaker *et al.,* 1994) mitochondrial DNA markers enable the tracking of specific lineages, providing a useful comparison to markers in the nuclear genome. Although it is a powerful tool for the phylogeographic analysis of many organisms, *P. infestans* mtDNA diversity is relatively limited, with the vast majority of tested isolates falling into two [Ia(A) and IIa(B)] of the six defined haplotypes (Griffith and Shaw, 1998; Gavino and Fry, 2002). Marked regional variation in mtDNA haplotype frequency (Forbes *et al.,* 1998; Griffith *and* Shaw, 1998) and associations between haplotype and nuclear DNA fingerprint have been observed (Purvis *et al.,* 2001), but neither the cause nor the functional significance (if any) is known. There is no known mechanism of selection acting on the mtDNA (Gavino and Fry, 2002), but the emergence of mtDNA-based resistance to QoI fungicides in other oomycetes (Gisi *et al.,* 2002) indicates a potential selection pressure to consider in future monitoring. The principal method for characterizing *P. infestans* mtDNA type is a PCR-RFLP method (Griffith and Shaw, 1998), but recent sequencing of the IGS has identified additional SNP variation (Wattier *et al.,* 2003) within these groups. Further screening and the design of new protocols suited to high-throughput methods are therefore required.

AFLPs

Amplified fragment length polymorphisms (Vos *et al.,* 1995) have proved very powerful markers, since they yield many loci per primer combination (Milbourne *et al.,* 1997). They have been central to the genetic mapping of *P.*

infestans (van der Lee *et al.,* 1997) and resolve at a level appropriate for examining intrapopulation diversity (Knapova and Gisi, 2002; Cooke *et al.,* 2003; Flier *et al.,* 2003). Fingerprinting by AFLPs discriminated almost every isolate (Flier *et al.,* 2003) or every second *P. infestans* isolate (Knapova and Gisi, 2002; Cooke *et al.,* 2003) in studies in Mexico and Europe, respectively. The data are dominant, however, which increases the number of markers required to estimate population parameters (Jorde *et al.,* 1999)Since the method traditionally relies on acrylamide gel electrophoresis and radioactive labelling, the gel-to-gel normalization of the resultant fingerprints represents a challenge, even within a single laboratory. The method is also sensitive to changes in DNA quality and comparisons between laboratories may only be possible when common protocols are adopted and a combination of fluorescent labelling and capillary electrophoresis yields accurately sized digital output under standardized running conditions. The method is also time-consuming and requires very pure *P. infestans* DNA, which means it cannot be applied to infected plant material. In addition, conversion of AFLP bands to locus-specific markers is not straightforward. Comparisons of AFLPs and the methods described below are needed to assess the relative merits of each. Their suitability for examining fine-scale diversity in local populations and high-throughput population genomics (Luikart *et al.,* 2003) is likely to result in their continued use in specific applications.

SSRs

Simple sequence repeat markers, or microsatellites, have many of the attributes detailed in. With their high variability and dense distribution throughout the genome they have revolutionized the fields of molecular ecology and phylogeography (e.g. Goldstein and Pollock, 1997; Goldstein *et al.,* 1999) as well as proving to be powerful tools for genetic analysis (e.g. Kohn *et al.,* 2000). However, they have not, to date, been exploited widely by plant pathologists, with only a few recent examples of their use in *P. infestans* (Knapova *et al.,* 2001; Knapova and Gisi, 2002), *Plasmopara* (Gobbin *et al.,* 2003) and *Magnaporthe* (Kaye *et al.,* 2003). Microsatellites are short fragments of DNA in which motifs of 1–6 bases occur in tandem repeats. Slippage during DNA replication (Goldstein and Pollock, 1997; Li *et al.,* 2002) results in periodic alteration of the repeat length, which is scored by accurate sizing of the PCR-amplified repeat and its immediate flanking sequence. They offer a taxonomic resolution suitable for the analysis of individual isolates within a population and phylogenetic relationships between closely related taxa. Unlike multilocus marker systems such as AFLPs, SSR analysis tends to focus on relatively few markers, but the precise nature of each locus and its length variation are unambiguously defined. This objective 'locus-specific' approach facilitates interisolate and interlaboratory comparisons, which are of great benefit in the analysis of global populations of important taxa such as *P. infestans*. Both alleles at a locus are amplified and discriminated simultaneously, yielding codominant data appropriate for detailed population genetic analysis. Genetic distance, calculated on the basis of allele sharing and size divergence (Goldstein and Pollock, 1997), is also suited to intraspecific and interspecific phylogenetic analysis. Individual loci can be positioned onto a physical map by PCR against multidimensional pools of bacterial artificial chromosome (BAC) clones (Whisson *et al.,* 2001) or onto a genetic map by scoring the alleles in existing mapping populations (van der Lee *et al.,* 1997). The assay is PCR-based and only tiny amounts of relatively 'crude' DNA are required. Thus, DNA extracted from spores washed from a lesion or even a section

of the infected leaf itself (Gobbin *et al.,* 2003) is adequate, obviating the need for pathogen isolation. A disadvantage of SSRs is the relatively long lead-in time. Fortunately, the lengthy process of enriching a small insert DNA library and sequencing to yield candidate markers for further optimization (Knapova *et al.,* 2001) can now be avoided thanks to the increasing availability of pathogen sequence data. A large amount of such *P. infestans* expressed sequence tag (EST) and noncoding sequence data is now available through public [*Phytophthora* genome consortium (Waugh *et al.,* 2000)] and private [Syngenta *Phytophthora* consortium (Lam, 2001)] consortia. At SCRI, *P. infestans* sequences were screened for the presence of SSR motifs. Subsequent testing of the candidate PCR primer sets against a panel of *P. infestans* isolates showed that approximately 10% of the putative SSRs were suitable markers (A. K. Lees, SCRI, Dundee, personal communication). Once the discovery and optimization phase is complete, throughput may be increased by amplifying more than one locus per PCR, termed multiplexing. Such approaches are well developed in human forensic testing (Wallin *et al.,* 2002) and may be further refined by the generation of genome-wide frameworks of multiplexed markers (e.g. Tang *et al.,* 2003). Such a system would be a tremendous resource for *P. infestans* research. The first reports on the use of SSRs in the study of *P. infestans* (Knapova *et al.,* 2001; Knapova and Gisi, 2002) demonstrate both the difficulties and the great potential of these markers. Of six SSR loci screened, only three were polymorphic amongst European populations (Knapova and Gisi, 2002). Two of these three loci were tested against 176 isolates from Switzerland and France and revealed four and six allele sizes, respectively, in 21 different combinations, indicating that they have a resolution appropriate for population analysis. No strong associations between SSR genotype, AFLP pattern, mating type or metalaxyl resistance were revealed, suggesting independent segregation of these traits via sexual recombination. Null alleles were recorded with one SSR locus (Knapova and Gisi,2002) and some of the loci recently developed at SCRI (A. K. Lees, SCRI, personal communication) show limited allele diversity. This demonstrates the need for detailed studies to optimize and, if needed, extend the marker set to ensure an appropriate range of SSR loci are available for different research objectives.

SNPs

Single base-pair differences in DNA, which occur as a result of point mutations (substitutions or insertions/ deletions), are termed single nucleotide polymorphisms. They represent the main source of genetic variation in the genome, comprising, for example, approximately 90% of the variation in the human genome (Collins *et al.,* 1998). SNP-based markers share many of the advantages of SSRs) and they are thus powerful tools for genetic analysis, as well as for the estimation of population parameters such as genetic distances, divergence times and gene flow (reviewed in Brumfield *et al.,* 2003). Their potential is being investigated because the estimation of such parameters from sequences of single loci (gene trees) is less powerful than from a suite of unlinked markers representing a genome-wide picture of population history (Brumfield *et al.,* 2003). Because of the difficulty of estimating mutation rates at hypervariable SSR loci, it is argued that SNPs are a more powerful tool. However, approximately three times more SNPs than SSRs are required and the concepts and tools for interpreting SNP diversity on the basis of coalescent theory are still under development (Brumfield *et al.,* 2003). SNP discovery is a lengthy process and assay development more technologically

challenging than for SSRs. These factors suggest SSRs will remain the marker of choice for *P. infestans* population analysis for the immediate future. SNP markers do, however, have specific advantages for particular applications. Unlike SSRs they are not constrained to tandem repeat regions and assays may be based on the specific SNP responsible for an amino-acid replacement in a functional protein. They will thus be vital in the direct monitoring of the frequencies of functional alleles (e.g. virulence loci) in natural and experimental populations. In combination with neutral markers, such data will be critical in the estimation of selection pressures on a range of key functional traits. Technical advances in SNP discovery and scoring and increasing *P. infestans* genome sequence data are likely to increase the use of SNPs. A small number of SNPs have already been identified and much-needed comparisons of population parameters on the basis of SNPs, SSRs and AFLP markers are underway (D. Cooke and S. Hussain, SCRI, personal communication).

Sequence analysis

Recognized as a powerful method for reconstructing phylogenetic relationships between species (Cooke *et al.,* 2000), sequence analysis is increasingly being applied to the reconstruction of gene genealogies or phylogeography within fungal species (Koufopanou *et al.,* 1997; Carbone and Kohn, 2001a; Banke *et al.,* 2004). Improved sequencing chemistry and reduced costs, combined with new concepts (Carbone and Kohn, 2001b; Templeton, 2004) and analysis tools (Clement *et al.,* 2000; Stephens *et al.,* 2001), are making the sequencing of several alleles from many individual isolates from a population a viable option. It is increasingly recognized that traditional phylogenetic methods are based on assumptions that do not apply at a population level (Clement *et al.,* 2000), and more sophisticated methods, in which haplotype data are analysed by coalescent-based methods to create a population genetic framework, are being developed (e.g. Posada *et al.,* 2000; Templeton, 2004). From this framework, the probability of obtaining a given genealogical structure under different population genetic models is calculated. A haplotype is a set of polymorphisms in a defined length of DNA sequence that can be assigned unambiguously to one chromosome (Brumfield *et al.,* 2003) and is the raw data for much phylogeographic analysis. A complication with diploid organisms such as *P. infestans* is that, for each allele, the two haplotypes must first be 'extracted' from the pooled PCR-derived diplotype sequence data. To obviate the need to derive haplotypes by expensive cloning and sequencing, accurate theoretical approaches have been developed (Clark, 1990; Stephens *et al.,* 2001). Sequencing, and subsequent haplotype analysis, is perhaps most appropriate for investigating the broader issues of *P. infestans* diversity, e.g. in phylogeographic analyses to confirm the centre of diversity and patterns of longrange migration or to examine the interface between populations and species to unravel the origins of *P. infestans* and its sister taxa on wild *Solanum* species (Gomez *et al.,* 2003). As well as their use as neutral markers in population analysis, sequence data are valuable in estimating the type and extent of selection pressure on functional genes. The sequencing of a putative virulence gene from many isolates and analysis of the ratio of the mutations in the DNA sequence that result in a change (replacement or nonsynonomous) or no change (synonomous or silent) to the resultant amino acid yields the Ka/Ks ratio (Hurst, 2002). From this ratio, inferences can be made about whether the gene is under stabilizing or diversifying selection, and, from this, predictions on the functional role of the gene can be made (Bos *et al.,* 2003).

Main areas of molecular epidemiology

A. Pathogen Identification and diagnosis

B. Quantification of Initial inoculums and infection

C.Pathogen temporal development

D. Pathogen spatial distribution.

E. Pathogen long distance dispersal and migration
F. Dynamics of pathogen population structures

G. Interactions between host resistance and pathogen virulence and pathogenicity

A. Pathogen Identification and diagnosis

It is required for the pathogens because it is difficult to distinguish from other species from morphologically and also for the pathogens to be Identified from soil, seed, plant tissue, water, and other bio or abio environments

Basic steps are-

- Design and create the species-specific primer(s),
- Test the primer's specificity using a broad range of species,
- Test the sensitivity of the primer(s).

The design of species-specific primers is based on:

Species-specific sequences of internal transcribed spacer (ITS)

Species-specific sequences of randomly amplified polymorphic DNA region (RAPD) and Species-specific repetitive sequences.

B. Quantification of initial inoculum and infection

Initial inoculum:
- Propagule density in soil, water or plant debris,
- Spore density in the air,
- Latent infections in plant tissues,
- Infected seeds of seed-borne diseases,
- Disease intensity in the original source of the long-distance dispersal pathogens.

Molecular techniques can be used to quantify the initial inoculum of some diseases which is not at all possible in traditional epidemiological methods.

C. Pathogen temporal development

This studies focus on pathogen population dynamics over time and the isolates should be collected from different time in same or different fields.

Also proper molecular makers should be used to produce polymorphic DNA haplotypes or genotypes.

The analysis will include:

1. Genetic distances among sampling populations at different times.

2. Determination of genetic identity and distance among sampling populations.

3. Determination of changes in genetic structure among populations.

Percentage of polymorphonic loci and genetic diversity for each sampling group

orchard	Sampling group	Percentage of polymorphic loci	Genetic diversity	Shannon's info index
potato	Gt	78.05	0.25(0.20)	0.37(0.28)
	Gf	75.61	0.25(0.20)	0.38(0.29)
	Gff	63.41	0.21(0.19)	0.31(0.28)
	Gh	60.98	0.21(0.19)	0.32(0.28)
tomato	AS	56.1	0.17(0.19)	0.26(0.27)
	FL	58.54	0.20(0.21)	0.31(0.29)
	FR		0.22(0.20)	0.33(0.26)

D. Pathogen spatial distribution

Pathogen isolates should be collected from different geographical locations and proper molecular makers should be used for the spatial distribution and these markers should show the polymorphism among isolates. Also a PCR with the special primers or probes should be performed, and the polymorphic DNA patterns or a specific DNA fingerprint can be used in data analysis.

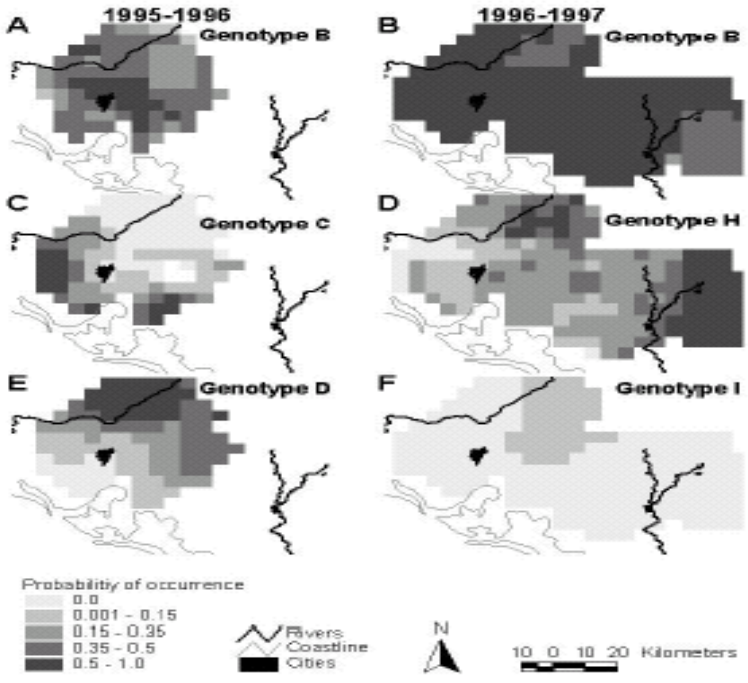
The data analyses may include:

1. geographical populations,
2. Clustering analysis to determine the existence of isolation or gene flow among geographic populations.
3. Using UPGMA and genetic identity to determine the relationship between genetic distance and geographic distances among populations.

GIS is an useful tool to quantify the above relationships

Each isolate was characterized by

1) According to their mating types, allozyme at the *glucose-6-phosphate isomerase (Gpi)* and *peptidase (Pep)* loci,
2) restriction fragment length polymorphizm (RFLP) with probe RG57 (different races), metalaxyl sensitivity and aggressiveness



- There are two small areas where the three genotypes had a similar probability of occurrence.
- Six different RFLP genotypes were represented.
- In 1996-1997, three RFLP banding pattern genotypes were found. Genotype "B" had the highest probability of occurrence in most areas
- The genotype "I" had a low probability of occurrence, with the probability above 0.1 only in a small area.

E. Pathogen long-distance dispersal and migration

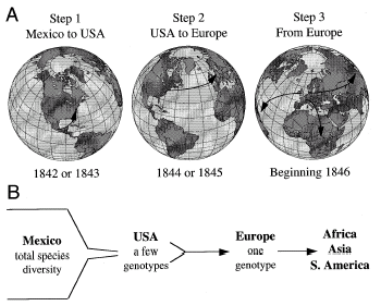
Here the studies focus on origin or long-distance dispersal of the plant pathogens. The pathogen isolates should be collected from different ecological or geographical regions or even different continents. Also different PCR processes could be used by using some specific molecular makers including species-specific makers, race-specific makers, and so forth. Analysis of Population Structures and Dynamics of *Phytopthora infestans* in Mexico by Using Microsatellite Primed-PCR. Here the isolates were collected from potato and tomato orchards in 2001.

The actual time of collection in the plum orchard, apothecia, fruit is at mid-season and fruit at harvest. And the time of collection in the prune orchard: mummies in early spring, blossoms, fruit at mid-season, and fruit before harvest.

F. Dynamics of pathogen population structures

The information on pathogen population structure is important to understand pathogen evolution, population diversity and related disease development. And the special molecular makers are needed to determine the variation of genetic structures of different populations and their changes over time and space. Specific analyses are needed to determine how disease development is related to changes in pathogen population structures. This information is useful to determine disease management strategies.

Example of *Phytothora infestans*



From Goodwin, (Phytopathology 87:462-473).

G. Interactions between host resistance and pathogen virulence

This studies emphasize on determination of pathogen pathogenicity or virulence by using fast and accurate molecular methods. The important areas may include:

1. Fast identification of pathogen races,
2. Determination of geographic distribution of races and different pathogenicities,

3. Evaluation of pathogen evolution over time and space.
4. Providing information on resistance deployment and decision support for resistance applications.
5. Prediction of disease development and dynamics of pathogen races.

Various diseases caused by *P.infestans*

Disease cycle of the fungus *P.infestans*

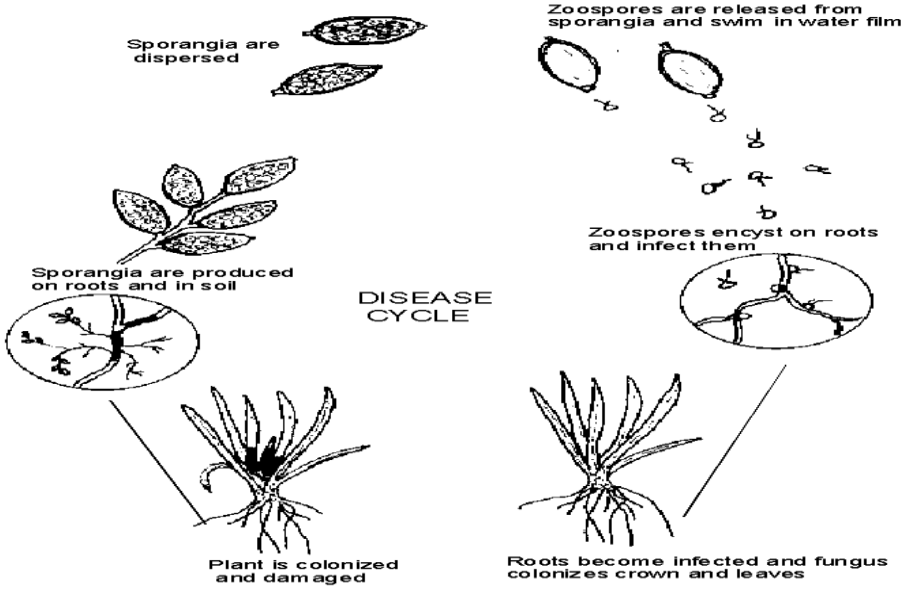
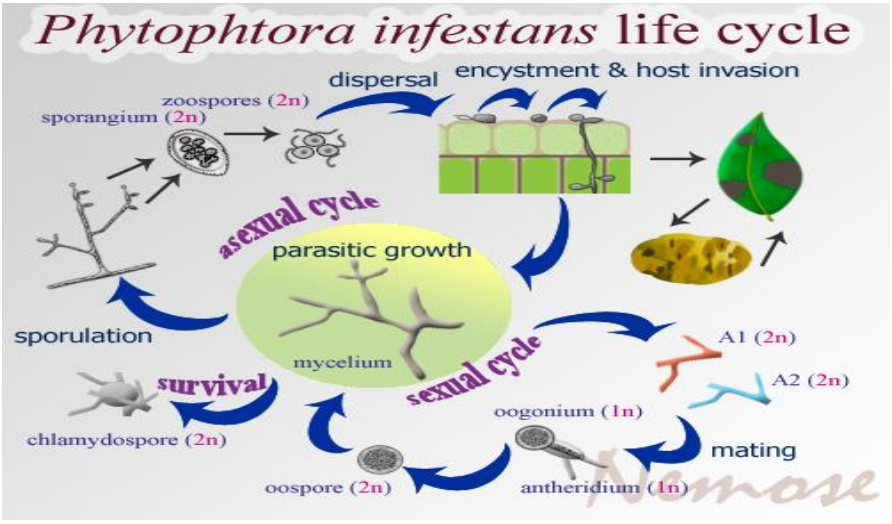


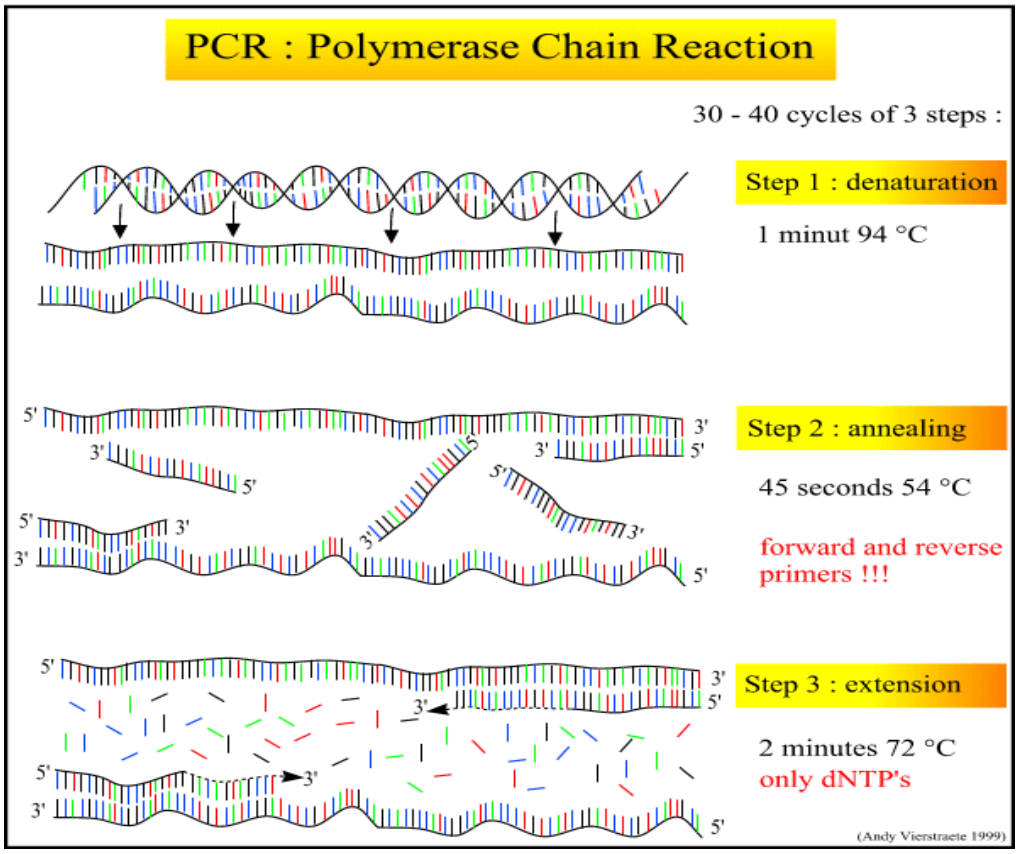
Fig.1 The fungus is dispersed by wind-borne sporangia, which are produced on branched hyphae (sporangiophores) that emerge from the stomata of infected leaves in humid conditions

Fig.2 sporangia germinate either by releasing zoospores or by producing a hyphal outgrowth

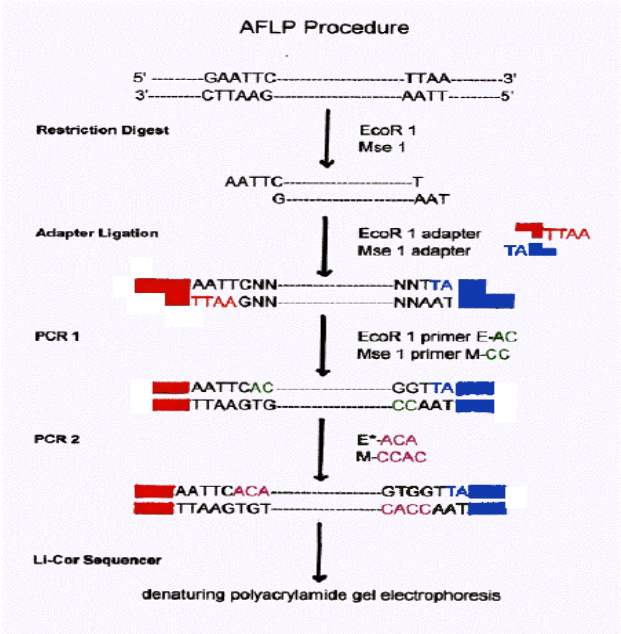
Life cycle of the fungus *P.infestans*



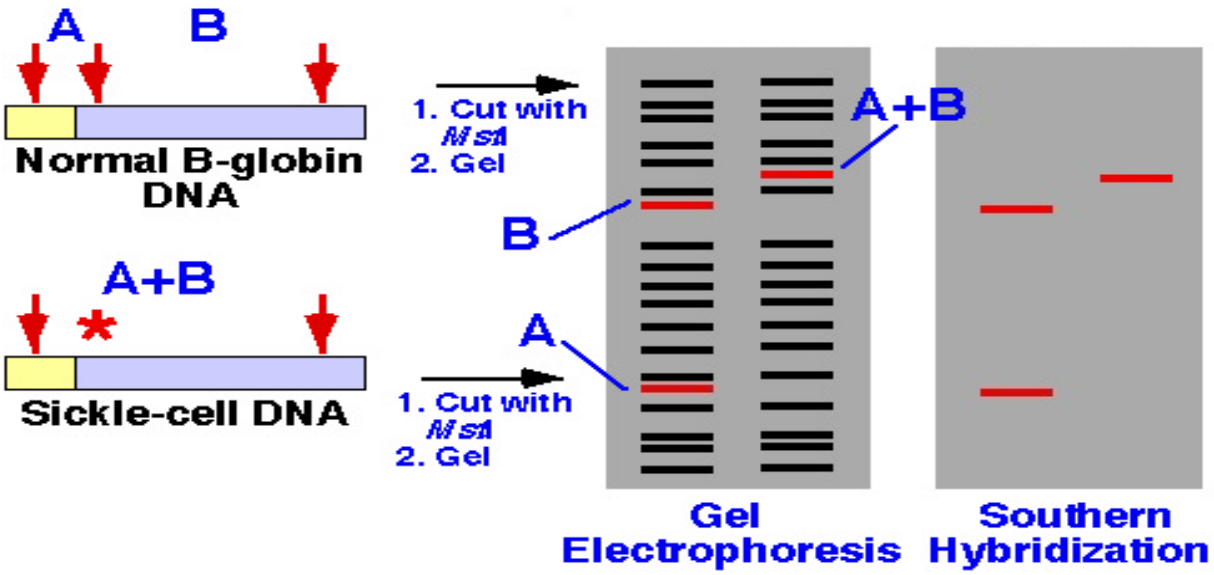
Basic steps of PCR



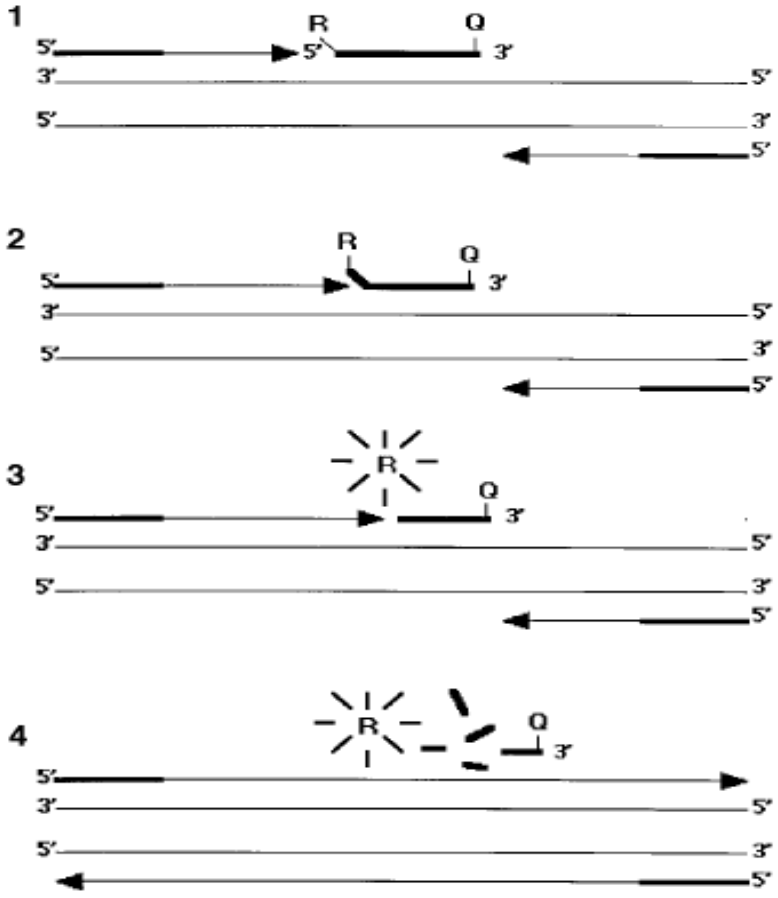
AFLP procedure



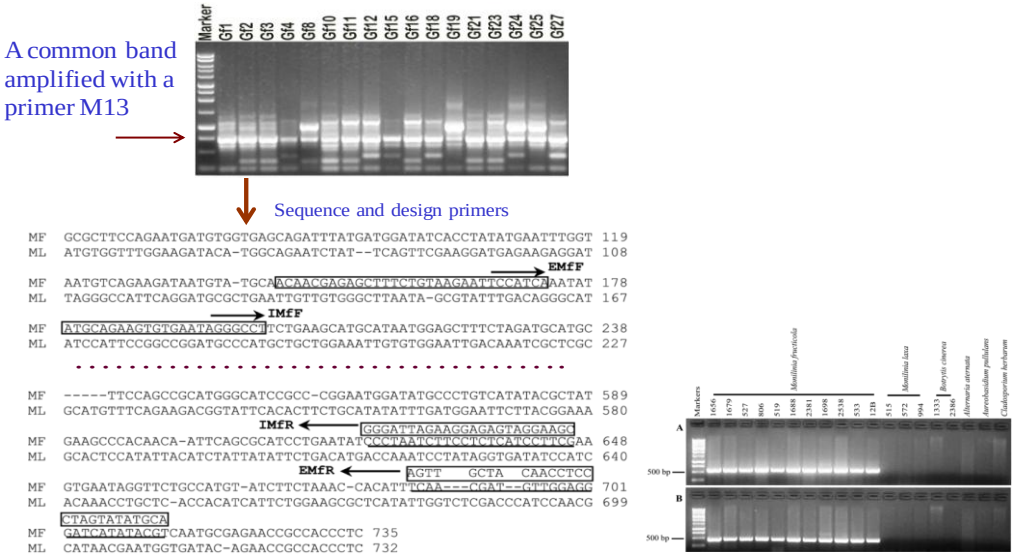
Procedure of RFLP



Steps of Real Time PCR



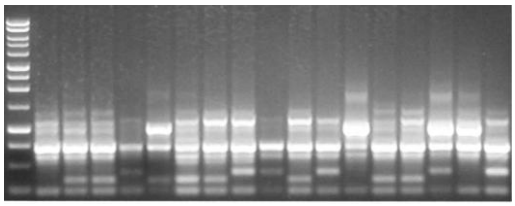
Sequence and design primers



A list of selected pathogens, markers, and authors for pathogen diagnosis

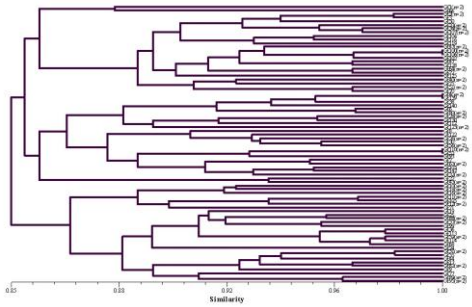
Pathogen	Marker method	Authors
Alternaria alternata	*AMT*-specific primers	Johnson, et al.
Plasmodiophora brassicae	from a rDNA section	Faggian, et al.
Rhizoctonia solani AG4 or 8	RAPD (Operon)	Brisbane, et al.
Erwinia carotovora subsp. *Atroseptica*	specific DNA-probe	De Boer, et al.
Clavibacter michiganensis subsp. *michiganensis*	DNA-probes from plasid-borne genes	Dreier, et al.
Stagonospora nodorum and Septoria tritici	r-DNA gene-specific primers	Beck and Ligon
Geminivirus subgroup III	Capsid protein gene sequences	Wyatt and Brown
Phytophthora cinnamomi	specific DNA-probe	Judelson, et al.
Pythium spp	5Sribosomal RNA gene specers	Klassen, et al.
Fusarium culmorum, F.graminearum, F. avenaceum	Oligonucleotide primers from RAPD	Schilling, et al.
Magnaporthe poae	Probes from genomic library of *M. poae*	Buting, et al.
Xanthomonas axonopodis pv. *citri*	a region of plasmid DNA by nested PCR	Hartung, et al.
Meloidogyne arenaria	DNA-probes from the plasmid library	Baum, et al.
Pythium ultimum	DNA-probes designed from ITS of rDNA	Levesque, et al.
Xanthomonas campestris pv. *phaseoli*	plasmid DNA fragment	Audy et al.
Xylella fastidiosa	prob from fragment of genomic DNA	Minsavage et al.
Verticillium tricorpus	ribosomal intragenic region	Moukhamedov

microsatellite primers used in PCR



•Five microsatellite primers, M13, $(AAG)_8$, $(AG)_8C$, $(GACA)_4$, and (AG)8C, were used in the PCR amplification.

Data analyses included:
•UPGMA tree development.
•Calculation of genetic diversity for each sampling group.
•Calculation of genetic identity and distance among sampling groups.



Ideal system for pathogenicity in molecular epidemiology

The ideal system

Pathogenicity pattern

	Variety					
Isolate	A	B	C	D	E	Race
a	-	+	+	-	+	1
b	+	+	-	+	-	2
c	-	-	+	-	+	3
d	+	+	-	-	-	4
e	-	+	-	+	-	5

Primer A

	Isolate				
	a	b	c	d	e
Race 2-related band →	-	-		-	
		-		-	
	-	-		-	-
	-	-	-	-	
	-	-	-		
				-	
			-		

Primer B

	Isolate				
	a	b	c	d	e
			-		
	-	-	-		
			-		-
	-	-			-
Race 4-related band →			-		
				-	
	-	-			
			-		-
	-		-		-

Molecular phenotypic pattern

Conclusions and future prospect

Since the recognition of *P. infestans* as a plant pathogen and also as the cause of famine and population displacement in Ireland in the mid 1800s, the biology, genetics and pathogenic strategies have been studied. However, relatively little research has explored *Phytophthora* diseases at the molecular level. The wealth of data comprising sequences involved in different developmental stages as well as infection stages should help in establishing expression profiles to understand mechanisms underlying different phenomena. There is a need however, to develop systematic gene disruption techniques to help elucidate the functional role of these genes. Evidently sequencing the genome of *P. infestans* will prove a valuable resource. Annotation of the genome of *P. infestans* will be facilitated by large unigene set generated from the ESTs as well as available physical and genetic maps. A complete genome sequence can be used for comparative analysis amongst *Phytophthora* species or with other organisms to decipher, for example the basic set of genes that makes *P. infestans* a plant pathogen. There is much hope that in the near future these resources will help us understand mechanisms underlying pathogenicity and host responses and ultimately lead to the development of improved strategies to control *P. infestans.* Undoubtedly, answers to some long-standing and important questions in fundamental and applied *P. infestans* research will emerge as the potential of modern genetic markers is realized, and as they are developed and exploited by the international research community. Fundamental to this is the release of *P. infestans* DNA sequence data. Of the methods discussed, SSRs appear to offer the greatest potential across a wide range of applications and should be developed further. Functional genomics is also characterizing the role of many novel *P. infestans* genes and the parallel tracking of neutral and functional markers will help to identify the forces driving pathogen evolution. Whichever marker systems are advanced, their potential will be maximized by the rapid public release of protocols and applications, ideally collated into a database alongside information on their map locations. The establishment of a European database comprising detailed information on *P. infestans* populations and their genetic characterization has already begun under the EUCABLIGHT project (www.eucablight.org). A comparison of the resolution and suitability of existing and newer DNA-based markers is also being undertaken on standard isolate collections to relate the 'old' and 'new' datasets. Cooperative approaches will be important in achieving the critical mass of detailed information necessary to reveal the driving forces and practical implications of population changes on this scale. Closer collaborations between specialists in the fields of plant pathology, epidemiology, population genetics/molecular ecology, *P. infestans* molecular biology and plant breeding are advocated to enable such progress. With increasing environmental and economic pressure to reduce agrochemical inputs, future sustainable management strategies ought to place more emphasis on host resistance (natural or 'engineered'). Their success will, however, hinge on understanding current diversity and predicting future responses of *P. infestans* populations to such resistance deployment. A population genetics approach that reveals the genetic structure of populations at both international and field scales and determines the extent of gene flow between populations and the balance between the forces of natural selection and chance effects of genetic drift and migration is essential to this understanding. As more markers are developed and the genome saturated, the approach will move towards the simultaneous analysis of many markers across

subsets, or even the whole genome, and a subsequent examination of linkage disequilibrium (LD), which has the power to separate locus-specific effects from those affecting the whole genome (Luikart *et al.*, 2003). Many questions remain: for example, the frequency of each mating type is important, but the cause of the marked spatiotemporal variation in mating-type ratios is unknown. Also, why, in some regions have both mating types coexisted for many years with little evidence of mating? Perhaps it relates to the compatibilities of the specific A1 and A2 mating-type strains within a region? What are the processes of host-pathogen coevolution in natural populations in South and Central America, and how do they differ from those in the 'artificial' *S. tuberosum*- and *Lycopersicon esculentum*-dominated agroecosystems? From a European perspective, are populations dominated by isolates introduced in the mid-1970s or have there been subsequent influxes? Is there any substructuring of European populations, or has long-range spread by wind and seed tubers created a random mosaic? To what extent and at what rate do local populations (if they exist) adapt to the environmental conditions, cultivars and management strategy in that region? How many samples are needed for an accurate reflection of the population structure? Also, in terms of breeding strategies, what is the selection pressure on different pathogen *Avr* genes and will some plant R genes be more useful than others for engineered resistance?

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