REVIEW

Markers, old and new, for examining *Phytophthora infestans* diversity

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Late blight, caused by *Phytophthora infestans*, is an ongoing threat to potato and tomato crop production worldwide and considerable fundamental and applied research is conducted with the long-term aim of improved disease control. Understanding the mechanisms, processes and rates of *P. infestans* 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 paper 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.

Keywords: haplotype, late blight, population genetics, potato, SNPs, SSRs

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 inoculum 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).

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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 & O'Sullivan, 1985) resulting in failures in disease control (Bradshaw & 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 & 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. These data include the type

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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* population biology. Understanding the relative contributions and rates of mutation, recombination, natural selection, gene flow, random genetic drift and migration (Burdon & Silk, 1997) to the generation and maintenance of variation in populations is important, yet such factors remain little studied (McDermott & 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 & Judelson, 1999; Whisson *et al*., 2001) and genetic (van der Lee *et al*., 1997) mapping, genomics (Kamoun *et al*., 1999; Birch & Whisson, 2001; Birch *et al*., 2003; Bos *et al*., 2003), and the functional analysis of genes involved in growth, development and plant infection (Birch & 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 & 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 (Table 1).

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 & 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

Table 1 Characteristics of an ideal marker system for the genetic analysis of *Phytophthora infestans*

Characteristic
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

further important goals of studies in South and Central America (e.g. Ordoñez *et al*., 2000; Flier *et al*., 2003). 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 & 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 & 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 (Goodwin *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 (Table 1). 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 are listed in Table 1. 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 fingerprinting, for example, discriminates isolates considered identical based on RG57 fingerprint (Purvis *et al*., 2001) and two SSR markers (Knapova & 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 & Clark, 1997) and genetic distances (Goldstein & 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 & 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

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. SSRs offer the greatest combination of required attributes for population analysis (see below and Table 1) 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 & 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 inoculum 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 & Yang, 1998), mitotic recombination (Goodwin, 1997), polyploidy (Tooley & Therrien, 1991) and aneuploidy (Carter *et al*., 1999) have all been reported in *P. infestans*. Phenotypic variation during clonal reproduction (Caten & 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). 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 & Shattock, 1991). The most studied of these phenotypic traits, and those that remain most informative, are mating type (Gallegly & Galindo, 1957), virulence (Malcolmson & Black, 1966) and fungicide resistance (Dowley & 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 & 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 R-gene-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 & 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 virulences 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 & 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 state that 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 & Cohen, 1996). Agar-based (Shattock, 1988) or *in vivo* testing of many isolates (e.g. Dowley & 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 & Cohen, 1996) or a fitness cost to metalaxyl resistance (e.g. Day & 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 & 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 (quinone 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 & 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 & Therrien, 1991) and antibiotic resistance (Shattock & 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 & Fry, 1998), but none of these characteristics has been systematically tested.

The importance and potential of genotypic markers

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 Table 1. 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 (Table 1). 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 & 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 time-consuming, 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 (see above) 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 & 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 & Shaw, 1998; Gavino & Fry, 2002). Marked regional variation in mtDNA haplotype frequency (Forbes *et al*., 1998; Griffith & 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 & 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 & 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 & 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 & 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 Table 1. With their high variability and dense distribution throughout the genome they have revolutionized the fields of molecular ecology and phylogeography (e.g. Goldstein & 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 & 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 & 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 & 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 & 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 & 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 & 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 (Table 1) 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 & 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 & Kohn, 2001a; Banke *et al*., 2004). Improved sequencing chemistry and reduced costs, combined with new concepts (Carbone & 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 long-range 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).

Conclusions, questions and future prospects

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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