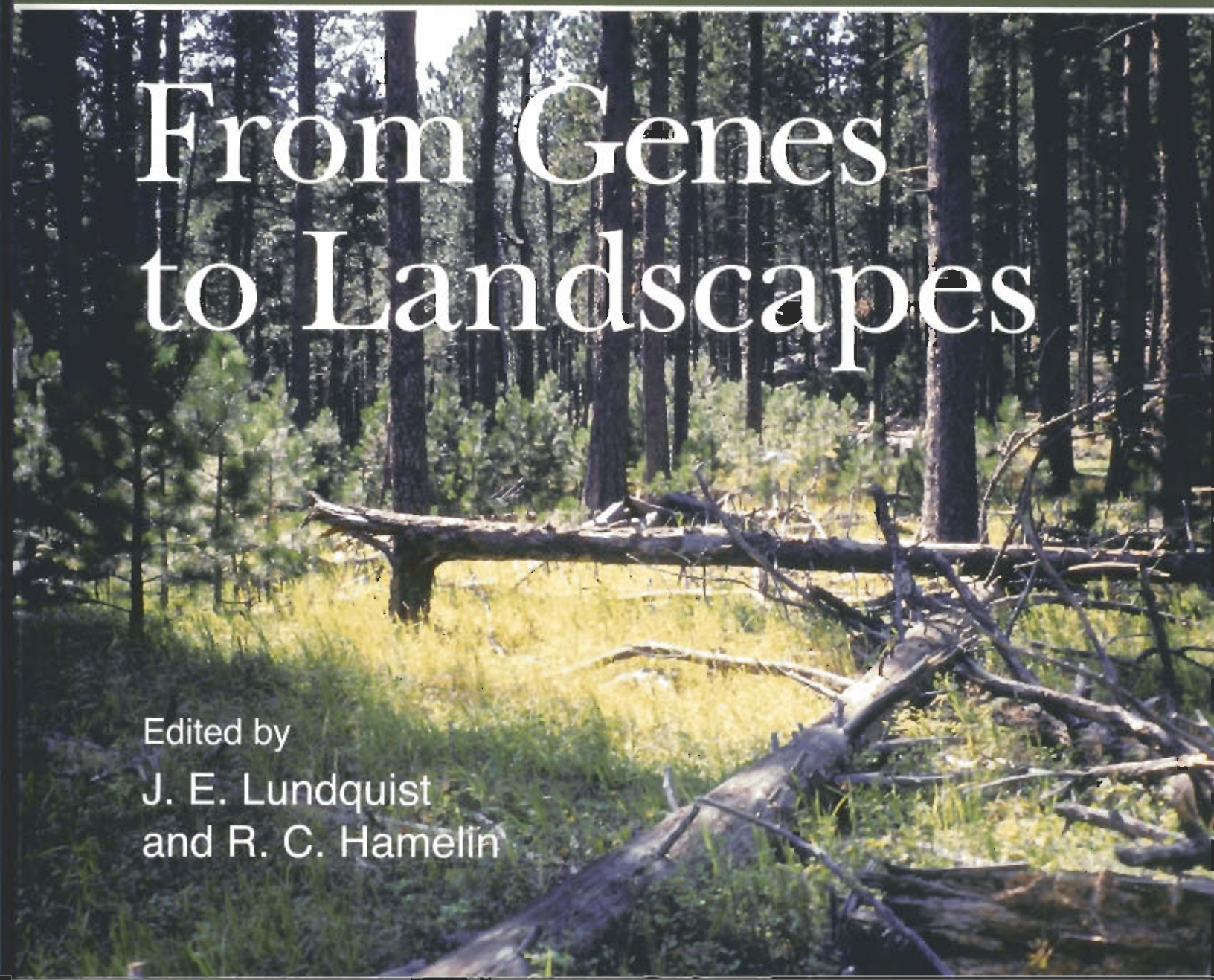
FOREST PATHOLOGY

From Genes to Landscapes



Edited by
J. E. Lundquist
and R. C. Hamelin

Neal B. Klopfenstein

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The American Phytopathological Society
St. Paul, Minnesota U.S.A.

Front cover: root disease pocket in a mature forest in the Black Hills. (Courtesy of John E. Lundquist)

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CHAPTER 2

Application of Molecular Genetic Tools to Studies of Forest Pathosystems

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Introduction

The use of molecular genetics in forest pathology has greatly increased over the past 10 years. For the most part, molecular genetic tools were initially developed to focus on individual components (e.g., pathogen, host) of forest pathosystems. As part of broader forest ecosystem complexes, forest pathosystems involve dynamic interactions among living components (e.g., pathogens, hosts, antagonists, hyperparasites, endophytes, epiphytes, symbionts, etc.), as well as responses to their surrounding biophysical environments. These biotic and abiotic components shape and influence overall pathosystem functions through effects at the molecular level (e.g., genetic selection, gene expression, gene flow, etc.). By exploiting molecular tools and by broadening our studies of forest pathosystems to its various components, our understanding of forest pathosystems will be improved.

These tools, including genetic markers, and more recently, genomics approaches, provide powerful approaches for molecular diagnosis, genetic mapping, DNA fingerprinting, and studies of phylogenetics, population genetics, and hybridization within forest pathosystems. Genetic studies of pathogens, hosts, and their interactions with biophysical environments are primary topics of forest pathosystem studies that employ diverse molecular genetic tools. This chapter provides a brief introduction of DNA-based genetic markers, examples of their application in forest pathosystems, and their potential integration into disease management. Because most recent molecular studies in forest pathology have focused solely on the pathogen, pathogen studies provide the predominant information for this chapter. However, the exciting potential to simultaneously examine the pathogen and host components is also discussed.

Genetic Markers

Several genetic marker systems have been applied to characterize individuals or populations of pathogens and hosts. This chapter primarily addresses DNA-based markers because of their versatility, reliability, and application for genetic studies of both host and pathogen. The resolving power and usefulness of DNA-based markers has improved as survey methods have moved from single-locus analysis of a few individuals to automated high-throughput technologies that provide multiplex genotyping and allow hundreds of markers to be genotyped for thousands of individuals. Examples of DNA-based genetic markers (hereafter referred to as molecular markers) include: 1) Restriction Fragment Length Polymorphisms (RFLPs), 2) Polymorphisms among specific products of the Polymerase Chain Reaction (PCR), 3) Simple Sequence Repeats (SSRs, also known as Microsatellites), 4) Random Amplified Polymorphic DNAs (RAPDs), 5) Amplified Fragment Length Polymorphisms (AFLPs), 6) Single-Nucleotide Polymorphisms (SNPs), and others. Each type of molecular marker has unique qualities and limitations (Glaubitz and Moran 2000). The choice of a marker system depends on the biological questions that are being asked.

RFLPs, the earliest DNA fingerprinting method, have been used extensively. RFLP markers are robust and co-dominant and can therefore be used to distinguish if individuals are homozygous or heterozygous for an allele. This advantage can be especially important for the study of fungi with a dominant diploid or dikaryotic phase, such as basidiomycetes and oomycetes. In addition, RFLPs give an indication of gene copy number and presence of gene families -- information that other approaches often do not reveal. This information can be meaningful since it has been shown that gene copy numbers can vary even within a genus. Detection of gene copy numbers is generally not

practical using standard PCR-based techniques, a limitation that can lead to artifacts (Tsai *et al.* 1994). However, genomic RFLPs require a large amount (e.g., 5-10 μg) of high-quality DNA for each assay, and pure cultures are typically required prior to analysis. This requirement usually precludes the use of RFLPs for small samples collected directly from natural settings, which may require some form of DNA amplification or use of mixed samples.

PCR-based markers have largely replaced the use of RFLPs in genetic studies. Fingerprinting techniques, such as RAPDs generate DNA profiles from template DNAs. RAPDs are fast, inexpensive, relatively simple to use, require small amounts of DNA (e.g., 10-25 ng), and do not necessitate prior knowledge of the genome sequence. They have been used extensively for population and mapping studies of fungi. However, RAPDs have suffered from a lack of reproducibility, and also generate mostly dominant markers, which must be considered in data interpretation (Hamelin *et al.* 1995, Lynch and Milligan 1994).

AFLP is another PCR-based fingerprinting method that is increasingly being used because of its ability to generate large numbers of markers and availability of "ready-to-use" kits that optimize procedures for most organisms (Vos *et al.* 1995). Although the assay is methodologically fairly complex, AFLPs can generate substantially more markers than most other fingerprinting methods including RAPDs. In addition, AFLP markers are generally more reproducible than RAPDs. Another notable advantage of AFLPs is that the assays can be run on automated sequencers and much of the analysis can be done using high-throughput methods (Fig. 2.1). Large datasets assembled in this way are popular for mapping where a large number of markers are needed. As with RAPDs, however, AFLP markers are mostly dominant, although some markers can be converted into co-dominant markers if pedigree information is available. As with RFLPs, AFLPs are relatively expensive, but require a more moderate amount of high quality DNA (e.g., 50-500 ng) compared to RFLPs.

SSRs are powerful markers for fingerprinting studies because of the large number of alleles per locus and the high level of statistical power that can be achieved when allele frequencies are known in populations. SSRs require only a small amount of DNA (e.g., 10-50 ng) per assay and yield co-dominant markers, since it is the size (i.e., number of repeats, for example $(CA)_n$) of the SSR region that is assayed. However, previous sequence information is usually necessary to design primers for amplification of specific SSR loci. Since new alleles with more or fewer repeats are generated at high frequency within the SSR regions, some questions have been raised, about the use of SSRs in allele genealogy. Absolute fragment size as scored in microsatellite assays was found to be an unreliable indicator of allele genealogy (Orti *et al.* 1997). Clearly, caution is necessary when analyzing microsatellites, and sequence data on flanking region increases the reliability of the assay.

New genomics platforms are making it possible to develop high-throughput genotyping methods that allow 100,000's of genotypes to be analyzed within days. These genotyping platforms can be aimed at the detection of single nucleotide polymorphisms (SNPs), which are purported to be the most frequent type of polymorphism within genomes. An advantage of SNP genotyping is that genes with known functions can be genotyped, and genome-wide sequence databases can be generated at the population level. Therefore, genetic polymorphisms that are associated

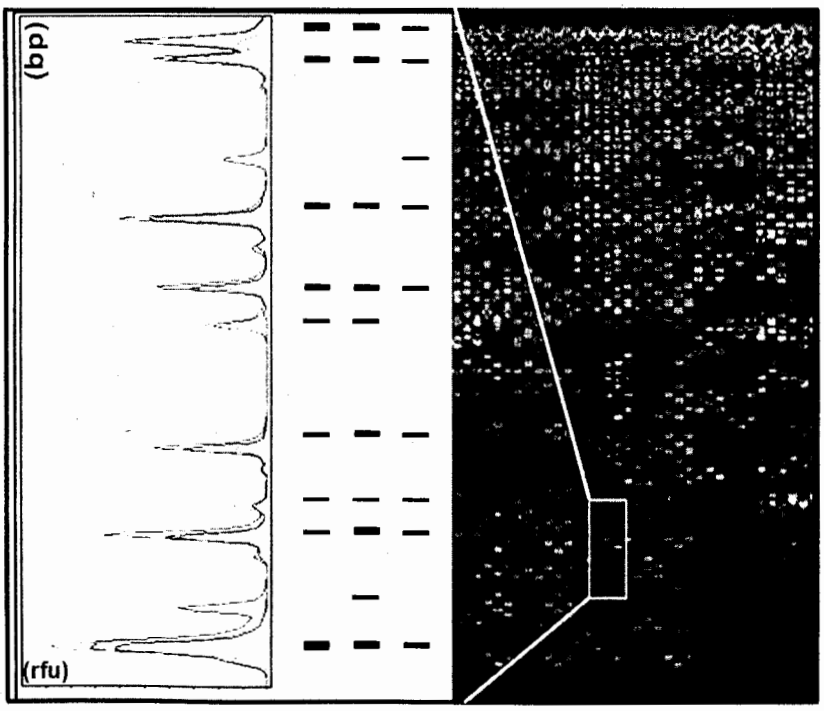


Figure. 2.1. An example of Amplified fragment length polymorphism (AFLP) gel image (right) of different genets (vegetative clones) of *Armillaria* spp. The size (bp) and intensity (rfu) of bands can be converted into a chromatogram (left), which can be extracted to a spread sheet using GeneScan Analysis software (Applied Biosystems, Foster City, CA). Large datasets generated by this method are useful for genetic mapping and other applications (see text for details).

with important adaptive traits can be analyzed and correlated with phenotypes.

An important consideration for selecting appropriate molecular markers is the availability of host or pathogen DNA. For example, molecular markers generated by RFLPs and AFLPs require large to moderate quantities of DNA. The quantity of pure DNA that can be practically acquired is limited for certain fungal pathogens/symbionts; some are obligate biotrophs (e.g., *Cronartium* spp., *Erysiphe* spp.) or grow very slowly, while metabolites may interfere with DNA isolation from other fungi (e.g., *Armillaria* spp.) (Gang and Weber 1995, Rogers *et al.* 1989).

The advent of DNA amplification allows the use of minute amounts of starting DNA for marker analyses. Therefore, investigators can now study pathogens and host-pathogen interactions in pathosystems where such analyses were previously infeasible. One of the most exciting aspects of DNA amplification techniques is the possibility to generate genetic data directly from environmental samples without the need for culturing. However, this has been complicated in the past by the variability in the starting material and the presence of inhibitors in the DNA extracts. A factor that can allow work with such difficult material is the use of improved DNA extraction techniques (Flynn and Niehaus 1997, Zambino 2002). Higher yields of DNA with fewer inhibitors will be essential for working with many types of environmental samples.

In general, the choice of molecular markers for studying forest pathosystems depends upon the study objectives, the resources available (e.g., funding, facilities, skills of personnel), amount of DNA that can be extracted, and pre-existing genome information (Lundquist and Klopfenstein 2001). Based on current trends, it appears that molecular marker technology will continue to increase in overall utility and accessibility. Markers specific for use at various hierarchical levels, including species, race, and even individuals, could be used concomitantly in genotyping studies by multiplexing reactions. Concurrently, DNA sequence information should become increasingly available, as sequences of entire genomes of selected microbes are completed (see Microbial Genome Sequencing Project, http://www.csree.usda.gov/fo/fundreview.cfm?fonum=1108). Such developments should provide target areas for developing markers that can also contribute to improved understanding of microbial diversity, identification of microbes that play roles in diverse ecosystems, and understanding of microbial impacts on productivity, sustainability, and succession of forests.

Several studies have successfully applied molecular markers to 1) study forest pathogen diversity (Doudrick *et al.* 1993, Goggioli *et al.* 1998, Gosselin *et al.* 1999, Hamelin 1996, Hamelin *et al.* 1994, Hamelin *et al.* 1995, Hantula *et al.* 1998, Högberg *et al.* 1999, La Porta *et al.* 1997, Lilja *et al.* 1998, Marçais *et al.* 1998, Rogers *et al.* 1999, Schulze 1999, Schulze *et al.* 1997), 2) identify forest pathogens (Anderson *et al.* 1998, Fischer and Wagner 1999, Frontz *et al.* 1998, Johannesson and Stenlid 1999, Hamelin *et al.* 1996, Hamelin *et al.* 2000, Rizzo *et al.* 2002), 3) monitor spread and establishment of forest pathogens (Hamelin *et al.* 1998b, Pappinen *et al.* 1996), 4) determine phylogenetic relationships of forest pathogens (Anderson and Stasovski 1992, Coetzee *et al.* 2000, Hamelin and Rail 1997, Harrington *et al.* 2001, Piercey-Normore *et al.* 1998, Vogler and Bruns 1998, Zhang and Blackwell 2001, Zhou and Stanosz 2001), and 5) identify the origin of pathogenesis-related genes of forest pathogens (Et-Touil *et al.* 1999).

Molecular Diagnostics—Pathogen Diagnostics

In recent years, molecular biology has contributed a series of diagnostic tools that have proved invaluable for detecting and monitoring forest pathogens and beneficial microbes. Although antibody-based, protein, or isozyme diagnostic tools have been used with some success in the past (Petrini *et al.* 1990, Powers *et al.* 1989), DNA-based molecular markers have become the tool of choice. Because their sensitivity results in the detection of pathogens without the need for culturing, molecular diagnostics are frequently replacing other biochemical techniques.

Ash Yellows is a tree disease caused by a phytoplasma that remained unidentified until molecular methods allowed its detection and monitoring. This phytoplasma cannot be cultured, and its diagnosis has been complicated in the past. Ash Yellows has caused noticeable symptoms of decline in white ash (*Fraxinus americana*) since the 1960's. Molecular surveys have shown that Ash Yellows is much more prevalent and damaging in the northeastern U.S.A. than had been previously known (Sinclair *et al.* 1996). This example of a pathogen that cannot be cultured poses an intriguing question: how many weak pathogens are present on trees, possibly weakening them, slowing their development, or rendering them more susceptible to other agents? The ubiquitous tobacco mosaic virus may be such a pathogen. It has been detected in black spruce and other conifers in forested areas far from agricultural crops (Jacobi *et al.* 1998).

In some situations, a correlation can be found between virulence or aggressiveness and DNA profiles. Two pine pathogens, *Sphaeropsis sapinea* (the cause of diplodia tip blight of pines) and *Gremmeniella abietina* (the cause of Scleroderris canker of pines), can be categorized for aggressiveness using methods of DNA characterization, such as PCR-based RFLPs and RAPDs (Bernier *et al.* 1994, Blodgett and Stanosz 1999, Hamelin *et al.* 1993, Smith and Stanosz 1995, Stanosz *et al.* 1996). However, such correlations between DNA profiles and phenotypes usually require extensive association studies, and if detecting a locus-specific marker, segregation and mapping studies (Et-Touil *et al.* 1999). In the case of Scleroderris canker, such association is related to the fact that the more aggressive European pathogen race is made up of clones covering large geographic areas (Hamelin *et al.* 1998b).

Molecular diagnostics can be particularly useful for distinguishing closely related species that may differ in pathogenicity. Species of the genus *Armillaria* can vary dramatically in their effects on forest ecosystems, from aggressive pathogenicity to beneficial saprophytism. To assess the impact of *Armillaria* species on forest health, it is critical that species are identified correctly. Identification of unknown *Armillaria* isolates commonly involves interfertility or somatic incompatibility tests (Anderson and Ullrich 1979, Darmono *et al.* 1992, Hintikka 1973, Korhonen 1978). Both approaches employ extensive pairings against standard strains that can require laborious culture and mating experiments (Guillaumin *et al.* 1991). Molecular genetic methods (PCR-RFLP) have been developed to allow identification of *Armillaria* species directly from minute amounts of DNA obtained by scraping mycelia (Harrington and Wingfield 1995). This method has since been widely applied in surveys of *Armillaria* species (e.g., Banik and Burdsall 1998, Banik *et al.* 1996, Kim *et al.* 2000, Volk *et al.* 1996, White *et al.* 1998) (Fig. 2.2).

Many of North America's most devastating forest diseases have resulted from introductions of species or races of exotic pathogens (http://www.spfnic.fs.fed.us/exfor/). Molecular genetic tools can be used to detect introduced pathogens, and non-native strains of native species. For example, recently, the prevalence and severity of powdery mildew [*Erysiphe* (Sect. *Microsphaera*) *pulchra* and *Phyllactinia guttata*] disease on dogwood (*Cornus* spp.) has greatly increased throughout the southeastern U.S.A. Factors contributing to the sudden emergence of the disease are unknown (Mmbaga *et al.* 2000). However, Mmbaga *et al.* (2000) have reported that *E. pulchra* isolates from *Cornus florida* in Tennessee and *C. kousa* in Japan have virtually identical sequences of internal transcribed spacer (ITS) region. They raise a critical question: was this powdery mildew pathogen introduced to the U.S.A. with *C. kousa* when intensive efforts were underway to identify sources of resistance to anthracnose (caused by *Discula destructiva*) from diverse geographic areas (Mmbaga *et al.* 2000)?

Gremmeniella abietina causes serious disease (Scleroderris canker) of conifers in Europe, Asia, and North America (Karlman *et al.* 1994, Laflamme and Lachance 1987, Ohman 1966, Yokota 1975). Asian, European, and North American races of this pathogen have been recognized (Dorworth and Krywienczyk 1975). The European race was discovered in North America in the 1970s and can cause mortality in mature susceptible trees, while the North American race is typically confined to seedlings and the lower portion of trees (Dorworth *et al.* 1977). It is quite difficult to distinguish the European and North American races due to their morphological similarity. However, Hamelin *et al.* (2000) have developed a PCR-based method

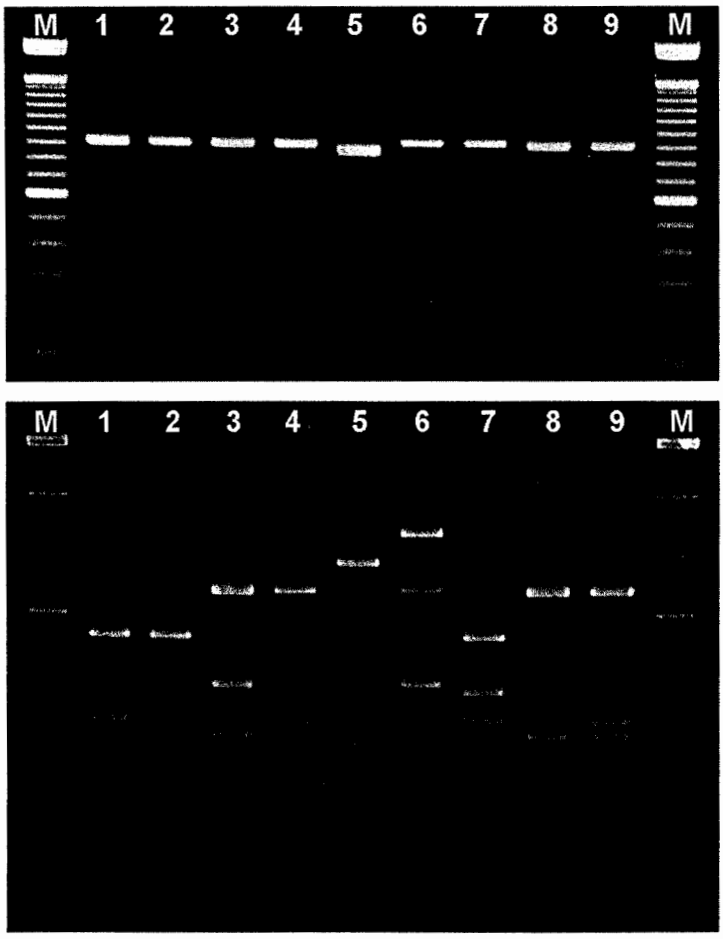


Figure 2.2. An example of pathogen diagnostics using PCR-RFLP. (Top) PCR amplified products (intergenic spacer region) of nine North American *Armillaria* species. (Bottom) PCR-RFLP band patterns of nine North American *Armillaria* species IGS-1 digested with *Alu* I. Lanes 1: *A. ostoyae*, 2: *A. gemina*, 3: *A. calvescens*, 4: *A. sinapina*, 5: *A. mellea*, 6: *A. gallica*, 7: *A. nabsnona*, 8: North American Biological Species (NABS) X, and 9: NABS XI. Size markers (M) are shown on far left and far right.

for identifying races in which a portion of the ITS region is amplified, and then cut with restriction endonucleases to produce race-specific RFLPs. This PCR-based method is especially practical because it can detect latent infections by the fungi.

Recently, a previously undescribed disease of oaks (*Lithocarpus densiflorus*, *Quercus agrifolia*, *Q. kelloggii*) in California, now known as sudden oak death (SOD), was shown to be caused by *Phytophthora ramorum* based on morphological and molecular (ITS sequence) characteristics (Rizzo *et al.* 2002). It is currently suspected that this pathogen was introduced from Europe. The continuing expansion of the geographic area and plant hosts affected by *P. ramorum* raises concerns about potential spread to oak forests in the eastern U.S.A. (Rizzo *et al.* 2002) and Canada (L. Cree, Canadian Food Inspection Agency, personal communication). This pathogen was discovered in 2003 in Oregon, Washington, and B.C., Canada. Further monitoring of the spread and impact of this pathogen on the many forest ecosystems will be necessary to develop management strategies. Surveys are underway in the U.S.A. and Canada to determine the extent of dissemination of *P. ramorum*. These surveys are using both microbiological isolation methods and DNA detection using PCR of ITS, COI, COII, and β-tubulin sequences. Interestingly, these new introductions correspond to the European mating type, which was previously absent from North America. This finding using molecular approaches can have extremely important consequences in terms of finding the origin of these new introductions and on the application of quarantine programs. These findings have impact as well on disease management programs and may be critical for limiting future introductions.

Phylogenetic Studies

Taxonomic uncertainty adversely impacts research in forest pathology and control of forest diseases. A lack of clearly identified and recognizable taxonomic groups can also have significant regulatory impacts, for example when quarantines are involved. DNA sequencing and analysis can contribute to clarification of taxonomic and evolutionary relationships among taxa. DNA sequence and molecular marker data provide meaningful information for addressing phylogenetic and systematic questions about species relatedness, species origins, and parentage. Such studies have addressed taxonomic questions of *Armillaria* species (Anderson and Stasovski 1992, Coetzee *et al.* 2000, Piercey-Normore *et al.* 1998, Terashima *et al.* 1998), *Botryosphaeria* species (Zhou and Stanosz 2001), the *Ophiostoma piceae* complex and the Dutch elm disease fungi (Harrington *et al.* 2001), *Ceratocystis laricicola* and *C. polonica* (Harrington *et al.* 2002), dogwood anthracnose fungus (*Discula destructiva*) (Zhang and Blackwell 2001), pine stem rust fungi (*Cronartium* and *Peridermium* species) (Vogler and Bruns 1998), *Gremmeniella* species (Hamelin and Rail 1997), and others.

The DNA region used in phylogenetic studies can influence the results. Although most phylogenetic studies have used genes in the ribosomal DNA (large subunit and small subunit, ITS, and intergenic spacer), new studies routinely include other coding genes, such as the β-tubulin gene and α-elongation factor. In some cases, the incongruity among datasets from different sequenced regions has significantly altered our perception of relationships, and can be indicative of recombination (O'Donnell *et al.* 1998).

The power of DNA sequence phylogeny has generated numerous debates over its use in defining species. Several species concepts have been proposed. Recently, the biological species concept (BSC), based on limits to outcrossing, and the phylogenetic species concept (PSC), based on degree of divergence in DNA or other traits, have been compared (Avise and Wollenberg 1997). Theoretically, it was argued that convergence between the two concepts is expected since genetic isolation should lead to accumulation of mutations and eventually phylogenetic differentiation. Although the debate is far from over, when PSC was applied to the taxonomy of *Fusarium* spp., congruence was generally found between the PSC and BSC (O'Donnell *et al.* 1998).

With the increasing amount and availability of genomic data, we are no longer limited to only a few genes in phylogenetic studies. The recent advent of phylogenomics has allowed evolutionary studies using entire metabolic pathways or genomes (Sicheritz-Ponten and Andersson 2001). In addition, phylogenetic studies can now be conducted that examine relationships of biochemical pathways within and among microbial species. Phylogenetic approaches can also be used to study biodiversity and co-evolution, while providing a better understanding of evolutionary relationships among components in forest pathosystems.

Hybridization

Hybridization has long been documented in plants and animals. But, hybridization has been virtually ignored in plant pathology because intermediate morphological characters, which can be observed in many hybrids or introgressed individuals, are difficult to observe in plant pathogens. The presence of intermediate DNA profiles or DNA profiles comprising markers from two closely related species can now facilitate the identification of such hybrids.

The potential for hybridization is a critical issue in forest pathology. Hybridization between pathogens is currently a major concern because of its potential to generate individuals with new pathogenic traits (Brasier 2000). Examples of fungal hybridizations that have produced altered pathogenicity include Melampsora rust caused by *Melampsora xcolumbiana*, a natural hybrid of *M. medusae* and *M. occidentalis* (Newcombe *et al.* 2000, Newcombe *et al.* 2001), Dutch elm disease fungi caused by *Ophiostroma novo-ulmi*, which may contain a pathogenicity gene introgressed from *O. ulmi* (Et-Touil *et al.* 1999, Konrad *et al.* 2002), and

new disease on *Alnus* species in Europe caused by a hybrid of *Phytophthora* species (Brasier *et al.* 1999). In another recent study, Kim *et al.* (2001) used a combination of molecular tools (PCR-RFLP and flow cytometry) to confirm hybridization between biological species of *Armillaria*. More studies are needed to fully understand the impacts of hybridization among forest pathogens. Molecular tools will be important in these efforts.

Population Genetics

Molecular markers are useful for characterizing species, populations, and individuals at various spatial scales. Gene flow or migration, mode of reproduction, population size, and selection are some of the population attributes that are estimated by measuring genetic parameters (Hartl and Clark 1997). Hypotheses can be tested and inferences can be drawn regarding potential barriers to gene flow, sources of epidemics, biotic or abiotic effects on population structure, means of dispersal, etc. It is, nevertheless, important to keep in mind that most of the inferences will come from indirect measurements and that multiple factors can result in a given observation. For example, asexual reproduction, inbreeding, or "hitchhiking" of tightly linked genes can result in linkage disequilibrium.

Molecular markers have been used to characterize genetic diversity of forest pathogens. For example, Frontz *et al.* (1998) used PCR-RFLPs to show the relative similarity among various cultures and species of *Armillaria* isolated from various tree species in Pennsylvania, U.S.A. Hamelin *et al.* (1998a) used RAPD markers to show that white pine blister rust (*Cronartium ribicola*) spermogonia were of the same genotype within each canker, and that the fine-level genetic structure of white pine blister rust populations was relatively homogeneous. Goggioli *et al.* (1998) used isozymes and RAPDs to examine diversity among selections of intersterility groups (ISG) F, S, and P of *Heterobasidion* at various locations throughout Italy. Hamelin *et al.* (1998b) examined the geographical pattern of genetic diversity of *Gremmeniella abietina* var. *abietina* in Canada and U.S.A. to determine that Scleroderris canker was likely the result of multiple introductions of a European race into North America. La Porta *et al.* (1997) used RAPDs to indicate that genetic variation in ISG S isolates of *Heterobasidion annosum* across Italy was clinal. Milgroom *et al.* (1996) used RFLPs to characterize the population structure of *Cryphonectria parasitica*, the cause of chestnut blight, among isolates from China, Japan, North America, and Europe, and found that this pathogen was probably introduced to North America via Japan, not China or Europe as originally postulated.

Pathogens can impact the genetic structure of natural host populations (Anagnostakis 1992, Holah *et al.* 1997, McDonald *et al.* 1998). Oak wilt disease (caused by *Ceratocystis fagacearum*) has affected the genetic structure of live oak (*Quercus fusiformis*) populations in Texas (McDonald *et al.* 1998). The oak wilt pathogen itself represents either a relatively recent introduction to North America or a recent speciation event, as indicated by low level variation in both mitochondrial DNA and the nuclear genome (Kurdyla *et al.* 1995). A comparison of two natural western white pine (*Pinus monticola*) populations from local geographic areas showed that the population exposed to low pressure from blister rust (caused by *Cronartium ribicola*) had higher polymorphism and heterozygosity than the population that had experienced high mortality due to blister rust (Kim *et al.* 2003). In addition, the population from low blister-rust pressure had twice as many unique alleles as the blister rust-selected population based on AFLPs markers. More detailed descriptions of population genetics of pathogens-hosts and their interactions are presented elsewhere (Richardson *et al.*, Chapter 3, this volume).

Population Genomics

The availability of complete genomes and high-throughput genotyping methods are changing how population genetics is approached (Kwok 2001). Sequence data can now be collected at the population level (Clark and Otto 2002). Gene genealogies can be constructed to better understand population structures and allelic relationships (Anderson and Kohn 1998). Genomic studies and large-scale genotyping provide direct access to the genomes of naturally occurring microbes without the need for extensive laboratory analyses or culturing (DeLong 2002). These data will yield information on genome content, diversity, population biology, and evolution in natural microbial populations. The power of comparative genomics for investigating the evolutionary processes that shape genomic landscapes and the evolution of functional sequences is just beginning to unfold.

Microarrays containing hundreds or thousands of gene probes provide a powerful new tool to survey genome-wide patterns of genetic variation or gene expression at individual or population levels (Gibson 2002). Such methods allow contributions of various environmental factors, spatial distribution, and other variables to be analysed in relation to genetic variability or gene expression. The ability to study simultaneously entire metabolic pathways at the population level will generate extremely insightful information.

Genetic Mapping

One of the early uses of molecular markers has been the generation of genetic maps. Such maps are generated by various genetic markers, which can be correlated to phenotypic traits (e.g., disease resistance, virulence), thereby helping to detect the genetic basis of phenotypic traits. The correlation between molecular markers and phenotypic traits allows the development of early selection systems (marker-assisted selection) to identify trees with desired characteristics.

Phenotypic and genetic variance in resistance to *Septoria populicola*, the cause of leaf spot, was studied using a

genetically mapped, hybrid *Populus* pedigree. The majority of resistance variation could be explained by two quantitative trait loci (QTLs) (Newcombe and Bradshaw 1996). Similar studies were used to localize genes for resistance to Melampsora rust using RAPD and AFLP markers (Cervera *et al.* 1996, Newcombe *et al.* 1996, Tabor *et al.* 2000, Zhang *et al.* 2001) (Fig. 2.3). In sugar pine (*Pinus lambertiana*) and loblolly pine (*P. taeda*), RAPD markers were tightly linked to a single dominant gene conferring resistance to white pine blister rust (caused by *Cronartium ribicola*) (Devey *et al.* 1995) and fusiform rust (caused by *Cronartium quercuum*) (Wilcox *et al.* 1996), respectively. Other workers identified genetic markers linked to black leaf spot (caused by *Stegophora ulmea*) resistance in Chinese elm (*Ulmus parvifolia*) (Benet *et al.* 1995).

Molecular genetic mapping projects are underway with *Populus*, *Juglans* (Fjellstrom and Parfitt 1994), *Pinus* (Ahuja *et al.* 1994, Changxi and Yeh 2001, Costa *et al.* 2000, Devey *et al.* 1999, Echt and Nelson 1997, Nelson *et al.* 1993, 1994, Remington *et al.* 1999, Sewell *et al.* 1999), *Picea* (Binelli and Bucci 1994), *Eucalyptus* (Bundock *et al.* 2000, Gratapaglia and Sederoff 1994), *Larix* (Arcade *et al.* 2000), *Taxus* (Göçmen *et al.* 1996), and species of other forest tree genera. Recently, Cervera *et al.* (2001) completed dense linkage maps of three *Populus* species (*P. deltoides*, *P. nigra*, and *P. trichocarpa*) based on AFLP and SSR markers. In addition, the entire genome of *Populus* is currently in the process of being sequenced (http://genome.jgi-psf.org/poplar0/poplar0.home.html), which will generate a powerful tool for validating the maps. As genetic mapping technologies continue to improve, the use of molecular markers will become more efficient and widespread for selection of trees with increased disease resistance and genetic diversity (Cervera *et al.* 1997, 1999).

Genetic maps of forest pathogens may also be helpful when designing breeding strategies by identifying pathogenicity genes, avirulence (*Avr*) genes, and genes involved in the disease process. For example, genomic mapping of the Dutch elm disease pathogens (*Ophiostoma ulmi* and *O. novo-ulmi*) is already underway using a combination of molecular methods (DNA sequencing, PCR, DNA electrophoresis, restriction enzyme digestion, SSCP-Single Stranded Conformation Polymorphism) (Dusabenyagasani *et al.* 2000). These efforts are directed toward isolating a pathogenicity gene *Pat1* (Et-Touil *et al.* 1999) and manipulating other genes of interest.

Gene mapping has had its limitations; however, maps have been particularly difficult to compare among different pedigrees within a species (Dewar *et al.* 1997). Genetic maps usually have not led to the cloning of genes, often the

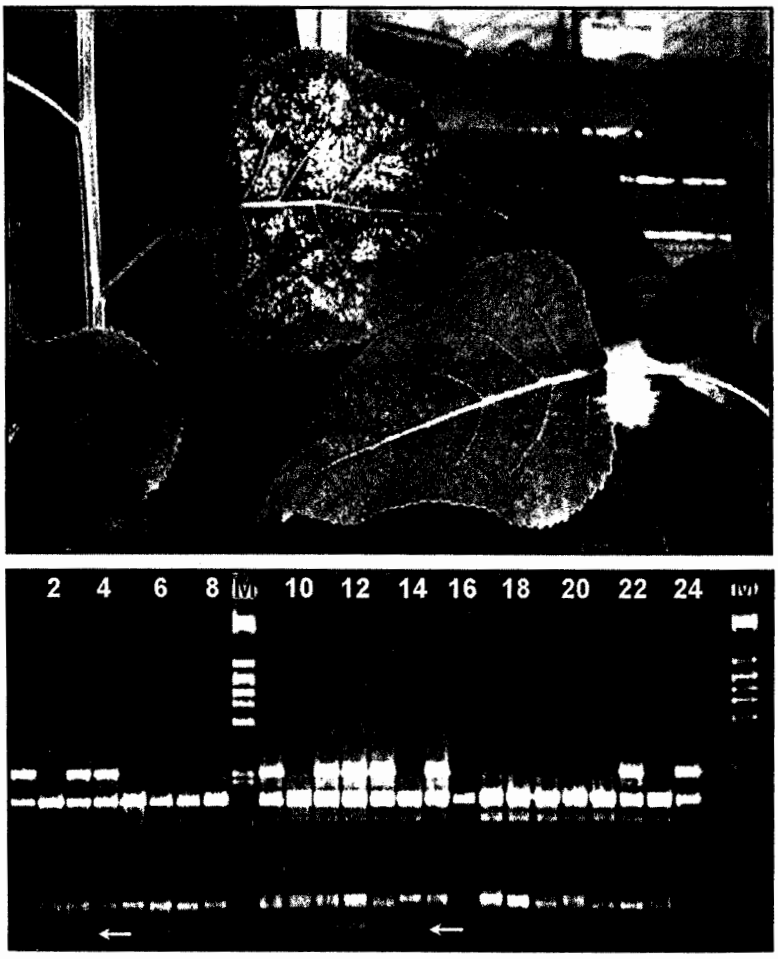


Figure 2.3. An example of marker-assisted selection using RAPD markers. (Top) Resistant (left) and susceptible (right) individuals of *Populus deltoides* inoculated with leaf rust (*Melampsora medusae*) Courtesy of G.M. Tabor). (Bottom) Primer OPG10 produced a polymorphic band ($OPG10_{340}$) that distinguished the resistant, susceptible parents, and progenies from both parents against leaf rust in *P. deltoides* family. Lanes 1: resistant parent, 2: susceptible parent, 3: bulked DNA from resistant progenies, 4: bulked DNA from susceptible progenies, 5-14: resistant progenies, 15-24: susceptible progenies, and 25: negative control. Arrow indicates the marker, $OPG10_{340}$. Size markers (M) are shown at middle and far right (Tabor *et al.* 2000).

ultimate target of many mapping projects. Mapped markers can also be used in marker-aided-selection programs. Although such breeding programs are reported in agricultural pathosystems, there are few examples in forest pathology. In addition, with the new platforms for complete genome sequencing now available, many mapping projects have been supplanted by genome sequencing projects. It is to be expected that mining of such genomes will yield valuable information on the biological processes specific to forest pathogens.

Integration of Molecular Genetics into Forest Disease Management and Future Applications

The new molecular tools have given forest pathologists new insights into the biology and epidemiology of pathogens, their hosts, and their interactions. Although much new knowledge can be gained, efforts must be devoted to integrate this new information in the management of pathosystems. Molecular approaches combined with the ability to precisely position the site of collection of samples result in advantages over conventional methods. The ability to genotype environmental samples (i.e., determine characteristic genetic profiles) can lead to the precise identification of species, races, and/or individuals without isolation. This can help us identify newly introduced species more rapidly, such as in the case of SOD, and perhaps allow eradication and prevent spread. It can also give us needed information about source of origin and routes of spread. In some cases, molecular methods can help differentiate between pathogenic and nonpathogenic races or species. Such information is important for effective management of forest diseases, and to prevent catastrophic episodes such as the Dutch elm disease, white pine blister rust, and chestnut blight epidemics. Molecular tools are currently proving to be instrumental in attempts to track and eradicate the SOD epidemics in the U.S.A. and Canada. Regulatory agencies in the U.S.A. and Canada are using PCR-based detection and identification methods to confirm identification of *P. ramorum*, monitor its host and geographic range, and determine the mating types and potential origin. Ultimately these data will help shape regulations, including the movement of plant/soil materials and the enforcement of quarantines.

Breeding programs can also benefit from the information generated by these approaches. For example, population structure can help define epidemiological units, which are an important consideration for selections used by tree breeders. Conversely, barriers to gene flow can be identified that indicate the presence of genetically distinct populations (Hamelin *et al.* 2000). In the case of white pine blister rust, the east-west divide in the population structure should be considered not only by pine breeders but also by the *Ribes* industry, which may provide a bridge between these distinct populations. In addition, disease-resistant host trees might be identified and selected at an early stage for effective breeding and restoration programs. Also, tree plantations might be designed to contain sufficient genetic diversity to avoid strong selection pressure on pathogen populations (Miot *et al.* 1999). Such approaches could reduce the likelihood of increasing pathogen aggressiveness.

Recently, new approaches that incorporate molecular markers technology, spatial modeling, and Geographic Information System (GIS) have been suggested for managing forest diseases at the landscape level (Klopfenstein *et al.* 2001, Lundquist and Klopfenstein 2001, McDonald *et al.* 2003). Because the genetic architecture of hosts and associated microbes exhibit spatial patterns of variation across the biophysical environment, it is essential to shift the focus of management to a landscape level. The genetic structure and geographic distribution of pathogens, beneficial organisms, and host-tree populations can be compared to site attributes such as temperature, moisture, soil properties, fire history, topographic characteristics, and management practices. Genetic data are gathered from individual organisms, but these individuals are components of spatially distributed populations. Using spatial modeling and GIS, genetic patterns can be mapped and modeled along with abiotic and biotic environmental variables. This approach can provide novel insights into interactions between genetics and environment, and can potentially serve as a means of predicting important patterns in organisms that are controlled or impacted by environment. Such analyses should help in the development of predictive models that will contribute to forest disease risk assessment. Although these goals are not yet fully realized at the practical level, theoretical approaches reflect the predictive power and management sophistication that should characterize resource management in the future.

In summary, development of molecular genetic approaches will continue to improve for such applications as 1) molecular diagnostics, particularly to identify introductions or emerging races of forest microbes or pathogens, 2) identifying pathogenicity genes, *Avr* genes, and other genes of interest for effective breeding and disease management programs, 3) monitoring gene flow, hybridization, and environmental adaptation in pathogens and host-tree species; and 4) integrating spatial modeling and GIS to allow evaluations at the landscape level. One of the great challenges in the future will be to integrate genetic, environmental, and landscape data in searchable databases that will provide the users with useful information. In the future, one can envision that the use of molecular genetic tools will likely be extended to many pathogens as well as beneficial microbes in forest pathosystems, and will greatly expand our understanding of forest pathosystem processes.

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