SELECTION OF RAPD MARKERS FOR INVESTIGATION OF GENETIC POPULATION STRUCTURE IN FUSIFORM RUST FUNGUS INFECTING LOBLOLLY PINE

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Abstract--Research to determine patterns of genetic differentiation among and within field populations of *Cronartium quercuum* f. sp. *fusiforme* using RAPD markers is currently underway in the molecular genetics laboratory at the Southern Institute of Forest Genetics. Fungal tissue was collected as a drop of spermatia or scrapings of a discrete hymenium from a single gall on each tree sampled at a location. Collections were made on twenty or more loblolly pines at 25 geographic locations, widely dispersed throughout the natural range of this host species. DNAs are presently being extracted from these tissue samples. The extracted DNAs are being amplified using the polymerase chain reaction (PCR) and 10-mer oligonucleotide primers to produce RAPD products that have potential for use as genetic markers. From bulked samples, twenty-one such RAPD markers have been identified that show consistent, clear band separations and polymorphisms that closely correspond to those produced by genetic markers previously shown to segregate as Mendelian factors in a C. *q. fusiforme* population derived from a single urediniospore culture. It is likely, however, that some of our extractions contain contaminant DNA, from host trees and also possibly from insects and other fungi. This extrinsic DNA might be amplified by PCR in addition to the targeted DNA. In this paper, we describe techniques that are being employed to provide reasonable assurance that the RAPD markers we use for analysis of allele and haplotype frequencies are C. *qe fusiforme* allelic segregants.

Keywords: *Cronartium quercuum* f. sp. *fusiforme,* genetic diversity, Mendelian segregation, polymerase chain reaction.

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INTRODUCTION

Pathogenic fungi are continually challenged to adapt to their environments, especially to that environmental component that is their host species. Those pathogens that attack forest tree species are no exception. Most are confronted by an array of environmental factors and infect host species that are genetically variable. Because they are constantly subjected to changes in their environments, these fungi must maintain the capability to evolve in order to survive. To have the ability to respond to such challenges, pathogen species must harbor a reservoir of genetic variation. Amounts of such variability as well as its distribution among and within populations are governed by evolutionary forces, working singly or in combination. These forces include genetic mutation, mating behavior, gene flow between populations, natural selection and population size effects. Ultimately, population geneticists that study pathogens of forest trees, hope to determine which of these factors are important in shaping genetic variability within most, if not all, of these fungal species.

An appropriate first step to determine how evolutionary forces affect pathogen populations is to study their genetic population structure (McDonald 1997). In the context of population genetics, population structure refers to the amount and distribution of genetic variability among and within populations and subpopulations. It is strongly influenced by the evolutionary history of the populations and provides information about their capacity to change in response to new evolutionary pressures. Information about the population structure of a pathogen is therefore needed to guide development of effective control measures for use in management of its pathosystem.

Fusiform rust disease caused by *Cronartium quercuum* (Berk.) Miyabe ex Shirai lisp. *fusiforme*, a heteroecious rust fungus, is a major threat to the health of loblolly pine *(Pinus taeda* L.) forests in southern United States. At present, the primary control measure consists of planting resistant trees developed through tree breeding. In these breeding efforts, however, control of the attacking pathogen's genetic constitution is not feasible and the genetic variability existing among and within the attacking populations is not known. As a consequence, little information is available regarding the nature of the resistances being deployed.

Currently, almost nothing is known about the genetic population structure for this pathogen. Several investigations, however, have uncovered evidence that suggests considerable genetic variability exists within this formae speciales. In studies of traits related to ability to cause disease, both gall development and percent infection were found to vary among collections originating from separate galls (Snow et al. 1975; Powers et al. 1977; Powers 1980; Kuhlman and Matthews 1993). Using RAPD markers in a preliminary study of population structure of *Ce qe fusiforme* infecting loblolly pine, Hamelin et al. (1994) obtained results that indicate populations occurring east of the Mississippi river might be genetically differentiated from those coming from regions west of the river. Regional differention, however, accounted for only a small portion of the allele frequency variation observed; most of the genetic variation occurred within regions. These findings indicate that a more comprehensive study of population structure, based on neutral markers, is needed to clarify and further quantify patterns of genetic

differentiation that exist in *C. q. fusiforme* attacking loblolly pine. Such an investigation, using RAPD markers, is now underway at the Southern Institute of Forest Genetics.

SAMPLING METHODOLOGY

Fungal tissue was collected from galls on individual trees at 25 widely separated locations. Populations sampled were approximately 100 miles apart on a grid that spans most of the natural range of loblolly pine with major incidence of fusiform rust disease. For 23 of these populations, samples were taken from single galls on 20 trees. In each of the two other populations, Athens, GA and Saucier, MS, infections on more than 50 trees were sampled. In addition, multiple collections were made on some galls sampled in the Athens, GA population, so genetic variation within galls resulting from field infections may be detected if present.

Tissue was collected from each gall as a single drop of spermatia or as scrapings from a discrete hymenium. Spermatia in a single drop of fluid are believed to be produced by a single spermagonium and therefore to result from mitotic divisions originating from a single haploid mycelium (Mims and Doudrick 1996). Tissue obtained from a hymenial layer was taken from a restricted area (less than 10 mm^2) of a single hymenial zone occurring under the bark covering the surface of a gall. Each of these collections also is considered to be made up of cells derived from a single mycelium. Scrapings were not taken from a larger area because results obtained by Doudrick et al. (1993a) demonstrate that spermatial drops collected more than 10 mm apart can contain spores with different genetic constitutions, indicating that they are derived from mycelia produced by different infections.

Since we sampled tissue before the initial phase of sexual reproduction occurred, inferences drawn from our genetic analysis apply to the gametothallic haploid *C. qe fusiforme* populations that infect populations of loblloly pine. An advantage to concentrating effort on the gametothallic phase of the fungal life cycle is that multiple-locus haplotype frequencies can be studied in addition to allele frequencies. This makes it feasible to test for linkage disequilibrium, thereby gaining an additional analytical tool that may be used to help decipher genetic variation patterns.

RAPD MARKERS

Our individual tissue samples contain only small amounts of *Ce q. fusiforme* biomass, consequently our genetic analysis must, by necessity, be based on molecular markers produced by polymerase chain reaction (PCR) methodology. RAPD markers resulting from PCR amplified DNA were used successfully by Doudrick et al. (1993a) to study genetic polymorphisms in a population of *Ce q. fusiforme.* Methods developed in that research are readily available, making RAPDs the markers of choice for our investigation. Accordingly, using modifications of techniques described by those workers, DNA currently is being extracted from our samples and amplified using PCR employing 10-mer oligonucleotide primers to produce RAPD products. The resulting products are then separated by electrophoresis on

agarose gels and subsequently revealed by ethidium bromide staining and UV irradiation.

To perform analyses that will properly detect patterns of genetic variation that exist, we need marker polymorphisms that manifest consistent band profiles across a variety of genetic backgrounds and furthermore, that represent alleles which show Mendelian inheritance at individual segregating genetic loci. Such RAPD genetic polymorphisms are indicated by band presence or absence gels and are caused by nucleotide sequence variations or are products of DNA insertions or deletions between primer attachment sites (Clark and Lanigan 1993). An advantage that comes with analysis of haploid individuals is that we are not faced with the problem of dominance phenotypic expression that is troublesome when RAPD markers are used in genetic analysis of diploid eukaryotes or fungal heterokaryons.

MARKER ANALYSES

As a first step in the process to identify markers that satisfy the required criteria, we screened RAPD polymorphisms produced from bulked samples representing 24 of our populations. Each of these bulked lots contained DNA from three galls that are components of one of the population samples. Twenty one potential markers were identified in this screening process. These markers were polymorphic and showed clear, consistent bands that closely correspond to RAPD genetic markers that either have been included on a genetic map constructed for a segregating population of C. *qe fusiforme* (WLP-10-2.SS1) infecting loblolly pine (Doudrick et al. 1993b) or were found to be unlinked to the mapped loci. Both the mapped and unmapped markers were shown by those workers to demonstrate 1:1 band presence, band absence ratios in accordance with Mendelian segregation. Of our potential markers, six have bands that match those of loci mapped to four of the eight identified linkage groups, six match loci that mapped to linked pairs of loci and nine corresponded to loci that have not yet been assigned to a group or a linked pair.

To be reasonably sure that markers we use in our analysis are identical to genetic loci segregating in WLP-10-2.SS1 (WLP-10) and are not conflicted by spurious band polymorphisms, several check procedures are necessary in our DNA assays. A combined sample of WLP-10 DNA will be included in all of our assays to provide a standard for comparison purposes. Also all of the potential markers we have selected will be tested for authenticity by Southern hybridization analysis using DNA of the corresponding matching RAPD products from WLP-10 as probes. Those markers that fail to produce the expected hybridization pattern will be excluded from our genetical analysis. Moreover, there are two potential scoring problems that we must contend with; DNA contamination and PCR artifacts. Both can be eliminated as sources of error by Southern hybridization analysis.

Some of our extractions may contain contaminant DNA. In samples taken from hymenial tissue, pine host tissue might have been inadvertently collected along with fungal tissue. To check for this possibility, a bulked sample of loblolly pine DNA will be included in each of our RAPD assays. Markers found to have bands that migrate similar to amplified products produced by pine DNA sequences will be excluded from our analysis. In addition, samples obtained from

drops of spermatia may include DNA from insects or alien fungi. Such extrinsic DNA, however, is expected to be at low concentrations in our samples and to be only weakly amplified if at all because of competition for primers from the more highly concentrated target *C. q. fusiforme* DNA. This latter source of contamination is not expected to result in scoring problems. Samples that yield anomalous band profiles, that might be caused by either form of contamination will be discarded.

RAPD assays are sometimes subject to aberrant migration patterns that can lead to misclassification of phenotypes if caution is not used in scoring. This drawback to use of RAPD analysis is well known and can usually be traced to amplification irregularities or to nearly identical migration behavior of nonhomologous fragments. The review by Bachmann (1994) and discussion by Smith et al. (1994) describe the causes in detail and suggest procedures to be followed for elimination of mistakes. We are particularly concerned with two RAPD product detection problems that have surfaced in some of our assays. The first stems from competition for primers between annealing sites and is signaled by the appearance of one or more intensely bright bands. When such intense bands are observed but a RAPD product is not detected for the marker of interest, we can not determine whether the product is actually present but at such a low amplification that it can not be detected by ethidium bromide staining, or if it, in fact, is absent. The second problem involves comigrating nonhomologous products of similar but not equal molecular weight. Scoring mistakes arise when the product of interest is absent but its comigrating nonhomologue is present.

Both of the aforementioned detection problems can be resolved by preparing Southern blots using target RAPD products from WLP-10 as probes. The detection limit for Southern based systems (.01 ng) can be as high as 1000 times greater than that realized with ethidium staining. Furthermore, to insure that our genetic analyses are based upon data collected using rigorous methods, all samples yielding questionable assays will be subjected to Southern hybridization analysis using appropriate target DNA from WLP-10 as probes. Those that fail to yield expected hybridization patterns will be disregarded.

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