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# A genomic map enriched for markers linked to *Avr1* in *Cronartium quercuum* f.sp. *fusiforme*

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ABSTRACT

A novel approach is presented to map avirulence gene *Avr1* in the basidiomycete *Cronartium quercuum* f.sp. *fusiforme*, the causal agent of fusiform rust disease in pines. DNA markers tightly linked to resistance gene *Fr1* in loblolly pine tree 10-5 were used to classify 10-5 seedling progeny as either resistant or susceptible. A single dikaryotic isolate (P2) heterozygous at the corresponding *Avr1* gene was developed by crossing *Fr1* avirulent isolate SC20-21 with *Fr1* virulent isolate NC2-40. Bulk basidiospore inoculum derived from isolate P2 was used to challenge the pine progeny. The ability to unambiguously marker classify 10-5 progeny as resistant (selecting for virulence) or susceptible (non-selecting) permitted the genetic mapping of the corresponding *Avr1* gene by bulked segregant analysis. Using this approach, 14 genetic markers significantly linked to *Avr1* were identified and placed within the context of a genome-wide linkage map produced for isolate P2 using samples from susceptible seedlings.

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## 1. Introduction

Loblolly and slash pines are the most important intensively-cultured softwood species in the southeastern US Fast growth, amenability to intensive silviculture, and their high-quality lumber/pulp have made them the cornerstone of the US forest products industry. Fusiform rust disease incited by the biotrophic, macrocyclic, heteroecious fungus *Cronartium quercuum* (Berk.) Miyabe ex Shirai f.sp *fusiforme* (*Cqf*) is the single most important pathogen limiting pine productivity across the southeastern US, with current annual losses estimated in 2010 dollars (http://www.westegg.com/inflation/) to be in the range of US$70 million across five southeastern states to US$143 million south-wide (Powers et al., 1974; Anderson et al., 1986; Geron and Hafley, 1988; Cubbage et al., 2000).

Genetic resistance is the only economically feasible and ecologically acceptable means of controlling this disease in the forest environment (Kinloch and Walkinshaw, 1991). The prevailing view in the fusiform rust pathosystem is that disease is determined by the interaction of specific host resistance genes and their corresponding pathogen avirulence genes, presumably in a gene-for-gene manner (Kinloch and Walkinshaw, 1991; Kubisiak et al., 2005; Nelson et al., 1993, 2010; Wilcox et al., 1996). Haploid genetic analysis, afforded by the fact that the maternal contribution to a pine embryo in a given seed arises from the same megaspore that proliferates into megagametophyte tissue, was used to genetically map the first fusiform rust resistance gene (*Fr1*) using progeny from *Pinus taeda* L. parental selection 10-5 (Wilcox et al., 1996).

In this paper, we test the hypothesis that the *Fr1* resistance gene in *P. taeda* parental selection 10-5 interacts in a gene-for-gene manner with a single avirulence gene (*Avr1*) in *Cqf* isolate P2. We used bulked segregant analysis to map *Avr1* to a single genetic locus using DNA from haploid pycniospore progeny of P2 produced on diseased resistant (*Fr1*) 10-5 progeny. We reasoned that if *Fr1* was interacting with a single avirulence gene in P2, then markers could be identified that co-segregated with avirulence/virulence, and that those markers in turn would be linked to one another (see Section 2.6 for additional details of the approach).

The classical approach taken to map Avr genes in other pathosystems has been to create a pathogen cross that segregates for avirulence:virulence and then phenotype the progeny by inoculating known resistant and susceptible host differentials prior to identifying co-segregating genetic markers (for a recent example refer to Broggini et al. (2010)). Although this is the general approach we used here, because we are working with a host species that is not easy to clone or breed to homozygosity and a biotrophic pathogen that in its infective stage/form is difficult to propagate axenically, we developed a novel approach in which we collected

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fungal haplotypes from resistant hosts. Our approach used DNA markers previously known to be tightly linked to a single fusiform rust resistance gene (*Fr1*: Wilcox et al., 1996; Amerson, unpublished data) to classify the seedlings of a loblolly pine family as either resistant (i.e., selecting for fungal virulence) or susceptible (i.e., non-selecting). DNA extracted from pycniospore samples collected from diseased seedlings in the virulence-selected and non-selected classes were then used to identify genetic markers linked to the *Avr1* gene.

This experimental approach conclusively demonstrated the gene-for-gene interaction of *Fr1* and *Avr1* and implies that the significant progress made on mapping specific Fr genes in the host (Amerson et al., 2005; Jordan, 1997; Li, 2003; Wilcox et al., 1996; Amerson, unpublished data) can now be leveraged by identifying and mapping the corresponding Avr genes in the pathogen. This approach should aid map-based cloning efforts for *Avr1* as well as others, which will be facilitated by the current genome sequencing effort for this pathogen. Our practical goal is to identify Avr gene-specific markers that can be used to assess allele frequencies at many different Avr genes in natural populations, and enable land managers to more effectively deploy host resistance (Nelson et al., 2010). In this paper, we first describe the approach we used to identify DNA markers significantly linked to *Avr1* and second, how we placed *Avr1* within the context of a genome-wide genetic map for *Cqf* that will assist in the localization of additional Avr genes.

## 2. Materials and methods

### 2.1. Fungal isolates

Galled (rust diseased) loblolly pine seedlings that had been inoculated with basidiospores of *Cqf* isolates SC20-21 or NC2-40 were obtained from the USDA Forest Service Resistance Screening Center (RSC) in Asheville, NC and maintained under greenhouse conditions at the Harrison Experimental Forest in Saucier, MS. Isolate SC20-21 was previously known to be homozygous avirulent (*Avr1*/*Avr1*; Wilcox et al., 1996) and NC2-40 was known to be virulent and very likely homozygous as well (*avr1*/*avr1*) (Kuhlman et al., 1997). Prior to pycnial formation all galls were enclosed in protective cages made from mylar mesh and zip ties to exclude insects. A dikaryon was made by transferring pycniospores from galls incited by SC20-21 to the surface of sporulating galls incited by NC2-40 and vice versa. Cages were removed briefly to allow transfers and then reapplied and kept in place until all signs of pycniospore production had ceased. Aeciospores resulting from these transfers were harvested ~18 months later. DNA from spores were examined using eight *Cqf*-specific microsatellite markers (Kubisiak et al., 2004) that were known to be polymorphic between SC20-21 and NC2-40 (Kubisiak, unpublished data) to identify populations that contained both parental alleles, as this was indicative of successful cross fertilization/dikaryon formation. Candidate spore populations from two different galls were identified and used to initiate a series of single-urediniospore pustule (SUP)-derived isolates. One SUP isolate (P2) [with marker alleles from both parents] that was highly productive and yielded large quantities of urediniospores was chosen for mapping *Avr1* and for the development of a genome-wide genetic linkage map for *Cqf*.

### 2.2. Plant materials and fungal mapping population

Full-sib seedlings from a single control-pollinated loblolly pine family (10-5♀ × 4666-4♂) were used to generate virulence-selected and non-selected fungal mapping populations. Seed parent 10-5 is known to be heterozygous (*Fr1*/*fr1*) at the *Fr1* locus (Wilcox et al., 1996). Pollen parent 4666-4 is known to be highly susceptible to fusiform rust disease (Kuhlman, 1992) and homozygous for the recessive allele (*fr1*/*fr1*) at the *Fr1* locus (Kuhlman et al., 1997; Wilcox et al., 1996). A few days after seed germination, the megagametophyte was harvested while still attached to the cotyledons of each seedling and assigned a number that corresponded with the assigned progeny number. Megagametophytes were stored individually and frozen at −20 to −80 °C in numbered microcentrifuge tubes. To determine the genotypes of the progeny with respect to *Fr1*, megagametophyte DNA was isolated (Supplemental file 1) and analyzed using random amplified polymorphic DNA (RAPD) markers. RAPD reactions (formulation and assembly), PCR conditions, gel electrophoresis, DNA band visualization and scoring followed the protocol of Myburg et al. (2006). In loblolly pine selection 10-5 RAPD markers J7_0470 (previously called J7_485; Wilcox et al., 1996) and AJ4_0420 are tightly linked to each other at a distance of 1.3 cM (Amerson, unpublished data) and also tightly linked to the *Fr1* gene (Wilcox et al., 1996, Amerson, unpublished data) with *Fr1* most likely residing in the interval between these markers (Amerson, unpublished data), but precise positioning of *Fr1* awaits further study. Based on the assessment of megagametophyte DNA samples using these RAPD markers (J7_0470 and AJ4_0420) 493 inoculated seedlings were classified as *Fr1*/*fr1* resistant (+J7_0470, −AJ4_0420 marker genotype) and 521 were classified as *fr1*/*fr1* susceptible (−J7_0470, +AJ4_0420 marker genotype).

At 8 weeks post seed sowing, basidiospores derived from isolate P2 were used to challenge the seedlings at the RSC using the concentrated basidiospore system (CBS) (Matthews and Rowan, 1972) following standard RSC protocols (Knighten et al., 1988), except that the inoculum concentration was elevated to 100,000 basidiospores/ml. The inoculated seedlings were assessed for disease phenotype (gall present vs. gall absent) at 4.5 months post-inoculation, by which time >95% of seedlings were clearly galled. This suggested that isolate P2 was heterozygous for *Avr1* as it caused disease on seedlings classified as both *Fr1*/*fr1* resistant and *fr1*/*fr1* susceptible. Shortly after the disease assessment the seedlings were transferred from Ray Leach Super Cells into 1 gal pots and maintained under greenhouse or shadehouse conditions. Approximately 25% of the galled pine seedlings were artificially induced to produce pycniospores by placing them in a 10 °C incubator with a 12 h photoperiod for 2 weeks. These plants were subsequently returned to the greenhouse or shadehouse and pycnial drops were collected 2–3 weeks later. All other galled plants were maintained under greenhouse or shadehouse conditions until pycniospores were naturally induced that same fall (~1 year post-inoculation). In total, over 4000 pycnial droplets (equivalent to haploid fungal progeny of P2) were collected from galled seedlings. Individual pycnial droplets were harvested using a manual hand pipette and droplets were placed into microcentrifuge tubes and stored at −20 to −80 °C until use.

### 2.3. DNA extraction and assessment of single-genotype purity

DNA was isolated from pycniospore samples (a minimum of two drops per galled tree) using the protocol described in Supplemental file 2. Since multiple infections frequently occur on individual plants when using the CBS (Kubisiak et al., 2005) all samples were tested for single-genotype purity using the same eight *Cqf*-specific microsatellite markers previously determined as being heterozygous in isolate P2. Only samples that amplified a single P2 allele at each of the eight microsatellite loci and were collected from different galled trees, or were clearly unique genotypes as confirmed by genetic marker analysis where samples originated from the same galled tree, were retained for use in mapping studies. None of the samples used for mapping contained detectable amounts of contaminating pine genomic DNA as determined by PCR analysis using a chloroplast-specific primer pair (Taberlet