



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 genet-

ic 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 Brogini 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

et al., 1991) and six fluorescently labeled loblolly pine nuclear microsatellite markers (Nelson et al., 2007). Of the samples deemed suitable for use in mapping studies, 290 were obtained from galled *Fr1/fr1* resistant trees (“virulence-selected” mapping population used to place the two most significantly linked markers with respect to *Avr1*) and 101 were obtained from galled *fr1/fr1* susceptible trees (“non-selected” mapping population used to develop a genome-wide genetic linkage map).

2.4. Random amplified polymorphic DNA (RAPD) marker identification

RAPD reactions were formulated as per Myburg et al. (2006) except that each reaction contained 1.25 ng of template DNA and 0.67 μ M of 10-mer oligonucleotide primer (MWG BioTech, Inc., High Point, NC). Reactions were loaded into flexible V bottom microtitre plates and overlaid with 25 μ L mineral oil. Microtitre plates were placed in preheated (85 °C) PTC-100 thermal cyclers (MJ Research, Waltham, MA) and covered with mylar film before amplification. Amplifications were conducted following the thermocycling profile of Myburg et al. (2006). Post amplification, 3 μ L of loading buffer (10 \times TAE, 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue) were added to each reaction well and products were electrophoresed in 2.0% (w/v) agarose gels and 1 \times TAE buffer (40 mM Tris base, 20 mM sodium acetate, 2.0 mM EDTA, glacial acetic acid to pH 7.2) for 3.5 h at 3 V/cm. After electrophoresis, gels were stained with ethidium bromide (0.4 μ g/ml in dH₂O) for 45 min, washed in dH₂O for 60 min and photographed using UV light. All candidate RAPD markers were given a subjective quality rating based on the intensity of amplification and the absence of co-migrating bands. Only high quality RAPDs were used for linkage mapping analyses. RAPD markers were named according to the manufacturer primer code (corresponding to the 10-mer primer responsible for their amplification), followed by a four-digit number indicating the fragment size in base pairs. The parental origin of the band-present allele was identified by prefix “sc” for isolate SC20-21 or “nc” for isolate NC2-40. When both alleles at a single locus were amplified by the same primer, the primer manufacturer code was given a prefix “c” for “codominant” but the individual products were scored separately.

2.5. Simple sequence repeat marker (SSR) identification

Amplification and detection of SSR markers was performed as described previously (Kubisiak et al., 2004; Burdine et al., 2007). Given that all polymorphic SSRs are codominant, the markers are only listed once with the parental origin identified by the prefix “b” for “both”.

2.6. Identification of RAPD and SSR markers linked to *Avr1* and markers useful for genome-wide genetic linkage mapping

RAPD and SSR markers linked to the *Avr1* gene were identified by a bulked segregant analysis (BSA) approach (Michelmore et al., 1991). To minimize the detection of false-positives, four independent bulks were constructed – two virulence-selected bulks and two non-selected bulks. Virulence-selected bulks each consisted of equal quantities of DNA obtained from 16 pycniospore samples collected from *Fr1/fr1* resistant trees. Non-selected bulks each consisted of equal quantities of DNA obtained from 16 pycniospore samples collected from *fr1/fr1* susceptible trees. All bulks were standardized to the same concentration. A total of 1200 RAPD 10-mers (primer sets A through BH; Eurofins MWG Operon) and 64 SSR primer pairs were screened against the four bulks allowing candidate *Avr1*-linked markers to be identified that were polymorphic between both bulk sets. Given their dominant inheritance, the only informative RAPD marker phase for identifying candidate

markers was when the band-present RAPD allele was in coupling with the *Avr1* avirulence allele. Thus, 10-mer primers that generated a PCR product in only the non-selected bulks were considered as candidates for further analysis. To identify additional candidate RAPD markers linked in repulsion phase with *Avr1*, as well as RAPD and SSR markers that could be used for the production of a genome-wide linkage map, products amplified from eight *Cqf* samples (four from each of the virulence-selected and non-selected mapping populations) were examined. The same 1200 RAPD 10-mer primers and 64 SSR primer pairs used for BSA were screened against the eight samples and segregating markers were identified. Candidate markers potentially linked to the *Avr1* gene were further characterized by genotyping 48 samples from each of the virulence-selected and non-selected mapping populations. Markers linked to *Avr1* were identified as those that were significantly ($P \leq 0.05$) distorted from 1:1 segregation in samples from the virulence-selected mapping population but not significantly distorted in samples from the non-selected mapping population. Using only the samples from the virulence-selected mapping population, a logarithm of the odds (LOD) ratio of 3.0 was arbitrarily chosen as the minimum threshold for declaring significant linkage to *Avr1*.

2.7. Amplified fragment length polymorphism (AFLP) marker identification

Amplified fragment length polymorphism (AFLP) reactions were performed essentially as described by Vos et al. (1995). Restriction-ligation and pre-selective amplification reactions were performed using the Ligation and Pre-selective Amplification Kit for Small Plant Genomes (Applied Biosystems Inc., Foster City, CA) according to the manufacturer’s instructions, with the exception that pre-selective reactions were diluted 1:5 in TE (0.1) buffer (20 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Selective amplification reactions were performed in a volume of 10 μ L that contained 1.5 μ L of 1:5-diluted pre-selective reaction, 0.5 μ L of 0.1 μ M EcoRI selective primer, 0.5 μ L of 0.5 μ M *MseI* selective primer and 7.5 μ L of AFLP Amplification Core Mix (Applied Biosystems Inc., Foster City, CA). Reactions were amplified according to the manufacturer’s instructions and a DNA Engine thermal cycler (Bio-Rad, Hercules, CA) was used for all amplification steps. EcoRI and *MseI* selective primers were obtained from Applied Biosystems Inc. (Foster City, CA) or Eurofins MWG Operon (Huntsville, AL). All EcoRI selective primers were fluorescently labeled with one of three dye-labels (FAM, NED or JOE). The University of Florida’s Interdisciplinary Center for Biotechnology Research Genetic Analysis Laboratory provided fragment length analysis services and samples were run on an ABI3730 DNA Analyzer (Applied Biosystems Inc., Foster City, CA) using GeneScan 600 LIZ size standard (Applied Biosystems Inc., Foster City, CA). Data were analyzed using GeneMarker V1.70 software (SoftGenetics, LLC, State College, PA). AFLP markers used for mapping were named according to the primers used in product amplification (as shown in Table 1) followed by a four-digit number indicating the fragment size in base pairs. The parental isolate origin of the band-present allele was identified by prefix “sc” or “nc”. When both alleles at a single locus were amplified by the same primer combination, the primer manufacturer code was given a prefix “c” for “codominant” but the individual products were scored separately.

2.8. Development of sequence characterized amplified region (SCAR) markers linked to *Avr1*

Two RAPD markers significantly linked to *Avr1* were converted into polymorphic SCAR markers (Paran and Michelmore, 1993). Products cut from gels were purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA) according to

Table 1
AFLP primer combinations selected for genetic mapping of *Cronartium quercuum* f.sp. *fusiforme*.

EcoRI primer	EcoRI 3' selective bases	MseI primer	MseI 3' selective bases	Polymorphic fragments amplified
E1	TG	M2	CAC	4
E2	TC	M6	CTC	4
E2	TC	M11	CC	7
E3	AC	M11	CC	8
E4	TT	M5	CTA	4
E5	AT	M2	CAC	2
E5	AT	M11	CC	5
E6	TA	M7	CTG	3
E6	TA	M8	CTT	3
E6	TA	M11	CC	6
E7	AG	M2	CAC	4
E7	AG	M6	CTC	6
E7	AG	M7	CTG	7
E7	AG	M9	CG	4
E7	AG	M11	CC	9
E8	AA	M1	CAA	2
E8	AA	M3	CAG	6
E8	AA	M7	CTG	7
E8	AA	M8	CTT	3
E8	AA	M22	CAATA	1
E9	A	M6	CTC	2
E9	A	M7	CTG	5
E10	T	M9	CG	4
E11	C	M3	CAG	10
E11	C	M4	CAT	4
E11	C	M5	CTA	10
E11	C	M7	CTG	6
E11	C	M8	CTT	9
E11	C	M11	CC	9
E12	G	M4	CAT	1
E12	G	M5	CTA	2
E12	G	M11	CC	1
E13	GA	M2	CAC	2
E13	GA	M6	CTC	5
E13	GA	M8	CTT	4
E14	GT	M4	CAT	7
E14	GT	M11	CC	5
E15	GC	M4	CAT	3
		Total		184

manufacturer instructions. DNA fragments were ligated into the pGEM-T vector (Promega, Madison, WI) and selected plasmids were prepared using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Dye-terminator sequencing services were provided by Arizona State University DNA Sequencing Unit. For one RAPD marker, it was necessary to obtain additional flanking sequence to develop a polymorphic marker and this was obtained using the GenomeWalker Universal kit and Advantage 2 Polymerase mix (Clontech, Mountain View, CA) according to the manufacturer's instructions. DNA sequences were used to design PCR primers (Table 2) that amplified polymorphisms between parental isolates NC2-40 and SC20-21.

2.9. Linkage analysis and genome-wide genetic map construction

Each marker was tested for goodness-of-fit to the expected Mendelian segregation ratio (1:1) using chi-square (χ^2) analysis.

Table 2
Polymorphic SCAR markers developed from RAPD markers significantly linked to *Avr1*.

Source of primer sequences	Forward primer name	Forward primer sequence 5'–3'	Reverse primer name	Reverse primer sequence 5'–3'	Product size (bp)	Annealing Temperature (°C)	Marker type
BB07_0750	Cqf18	CTGCATTGGATGTGGACCATTC	Cqf20	GTGTTACTCAGAGTCACGGAG	365 and 668	58	Co-dominant
AZ14_0900 AZ14_1100	Cqf38	CACGGCTTCCCAACATACAC	Cqf41	CACGGCTTCCATCGATTGGC	903, ~1000 and ~1100	55	Dominant

Markers with significant transmission ratio distortion ($P < 0.05$) were initially excluded from preliminary mapping analyses, but were added to the dataset once a preliminary framework was established. The software package MAPMAKER/EXP version 3.0 (Lincoln et al., 1992) was used to investigate genetic linkages and construct a genome-wide linkage map. The dataset was duplicated and recoded to allow the detection of markers in repulsion phase. Linkage groups were established using a minimum LOD threshold of 4.0 and a maximum recombination distance of 40 cM. Genetic distances were calculated using the Kosambi mapping function. If a putative linkage group consisted of fewer than nine loci, an exhaustive search for the “best” marker order was performed using the COMPARE command. For linkage groups consisting of nine or more markers, the BIG LODS command was used to identify tightly linked markers that could be ordered and set as a fixed order bin. This made exhaustive searches more manageable. Marker orders were further verified using the RIPPLE command and a maximum window size of eight loci. Once a “best” order was determined, potentially spurious genotypes, i.e., suspect double-recombinants or highly recombinant progeny were identified using the GENOTYPES command with error detection “on”. All spurious genotypes were re-verified from archived RAPD gel images or AFLP electropherograms and the data (if changed) were subject to re-analysis. The software MapChart 2.1 (Voorrips, 2002) was used to produce a graphical representation of the genetic linkage map.

3. Results

3.1. Identification of RAPD and SSR markers linked to *Avr1*

A total of 30 candidate *Avr1*-linked RAPD markers were identified among the products produced after screening 1200 individual 10-mer primers against the two virulence-selected and two non-selected bulks. In addition, seven candidate RAPD markers linked in repulsion phase with the *Avr1* avirulent allele were identified after screening the 1200 RAPD primers against four individuals from each of the virulence-selected and non-selected pycniospore populations. No candidates were found among the 64 SSR markers screened.

The 37 candidate RAPD markers were further characterized for linkage to *Avr1* using 48 virulence-selected and 48 non-selected pycniospore samples as outlined in Section 2.6. Five of the 30 candidate RAPD markers identified using BSA were significantly linked to *Avr1* and an additional 48 samples from each mapping population were genotyped. Based on 96 virulence-selected samples, only two samples were recombinant between *Avr1* and marker sc_BB07_0750 (LOD score 12.34) and eight samples were recombinant between *Avr1* and markers sc_AO13_1275, sc_AO13_1300, sc_AZ14_0900 and sc_AZ14_1100 (LOD score 8.47). Since the two recombinants observed for sc_BB07_0750 were different from the eight observed for the other four RAPDs, the *Avr1* locus was tentatively placed in the interval between sc_BB07_0750 and the other four RAPDs. Only one of seven candidate RAPD markers potentially linked in repulsion phase with the *Avr1* avirulence allele was significantly linked. Four recombinants were observed for RAPD marker nc_AV08_2100 (LOD = 8.47). As these four recombinant

samples were the same as those observed previously for RAPD markers sc_AO13_1275, sc_AO13_1300, sc_AZ14_0900, and sc_AZ14_1100, no additional genotyping of virulence-selected samples was performed.

To more precisely determine the location of *Avr1*, SCAR marker Cqf18.20 (synonymous with RAPD marker sc_BB07_0750) and SCAR marker Cqf38.41 (synonymous with RAPD marker sc_AZ14_0900) were assayed on a total 290 samples from the virulence-selected mapping population (original 96 plus an additional 194 samples). Four recombinant samples were observed for marker Cqf18.20 and 21 recombinants for marker Cqf38.41. *Avr1* was again placed in the interval between these markers. Marker Cqf18.20 (sc_BB07_0750) is estimated to be on one side of *Avr1* at a distance of 1.38 cM and marker Cqf38.41 (sc_AZ14_0900) is estimated to be on the opposite side at a distance of 7.24 cM (refer to LG III in Fig. 1). It should be noted that since *Avr1* was positioned based on the virulence-selected mapping population while the genome-wide marker map was developed from the non-selecting mapping population, the 1.38 cM and 7.24 cM values given here differ slightly from those depicted in the map shown in Fig. 1.

3.2. Identification of RAPD and SSR markers for constructing a genome-wide genetic linkage map

Of 1200 RAPD primers screened against four individual samples from each of the virulence-selected and non-selected pycniospore

populations, 143 primers produced 208 markers that met our quality rating criteria. Among these 143 primers, 92 RAPD primers produced one polymorphic band, 39 produced two polymorphic bands and 12 primers produced three or more. Segregation data was obtained for a total of 101 samples from the non-selected pycniospore population. Of the 64 SSR primer pairs tested, 34 were found to be polymorphic and segregation data was obtained for 93 of the 101 samples used for RAPD analysis.

3.3. Identification of AFLP markers linked to *Avr1* and for genome-wide genetic linkage mapping

AFLP templates were prepared using two bulks containing equal quantities of DNA from samples of the non-selected pycniospore population that either had band-present alleles for both markers sc_BB07_0750 and sc_AZ14_0900 (an *Avr1* avirulent bulk), or had band-absent alleles for markers sc_BB07_0750 and sc_AZ14_0900 (an *avr1* virulent bulk). In addition, AFLP templates were prepared using NC2–40 and SC20–21 DNA. These four templates were screened using 16 fluorescently labeled *EcoRI* primers (one or two 3' selective nucleotides) and 14 *MseI* primers (two or three 3' selective nucleotides) in 206 primer combinations and between 0 to 67 fragments were amplified and scored per primer pair (data not shown). Eight AFLP fragments produced by seven different primer pairs were amplified differentially between the *Avr1* avirulent and *avr1* virulent bulks, suggesting possible linkage to the *Avr1*

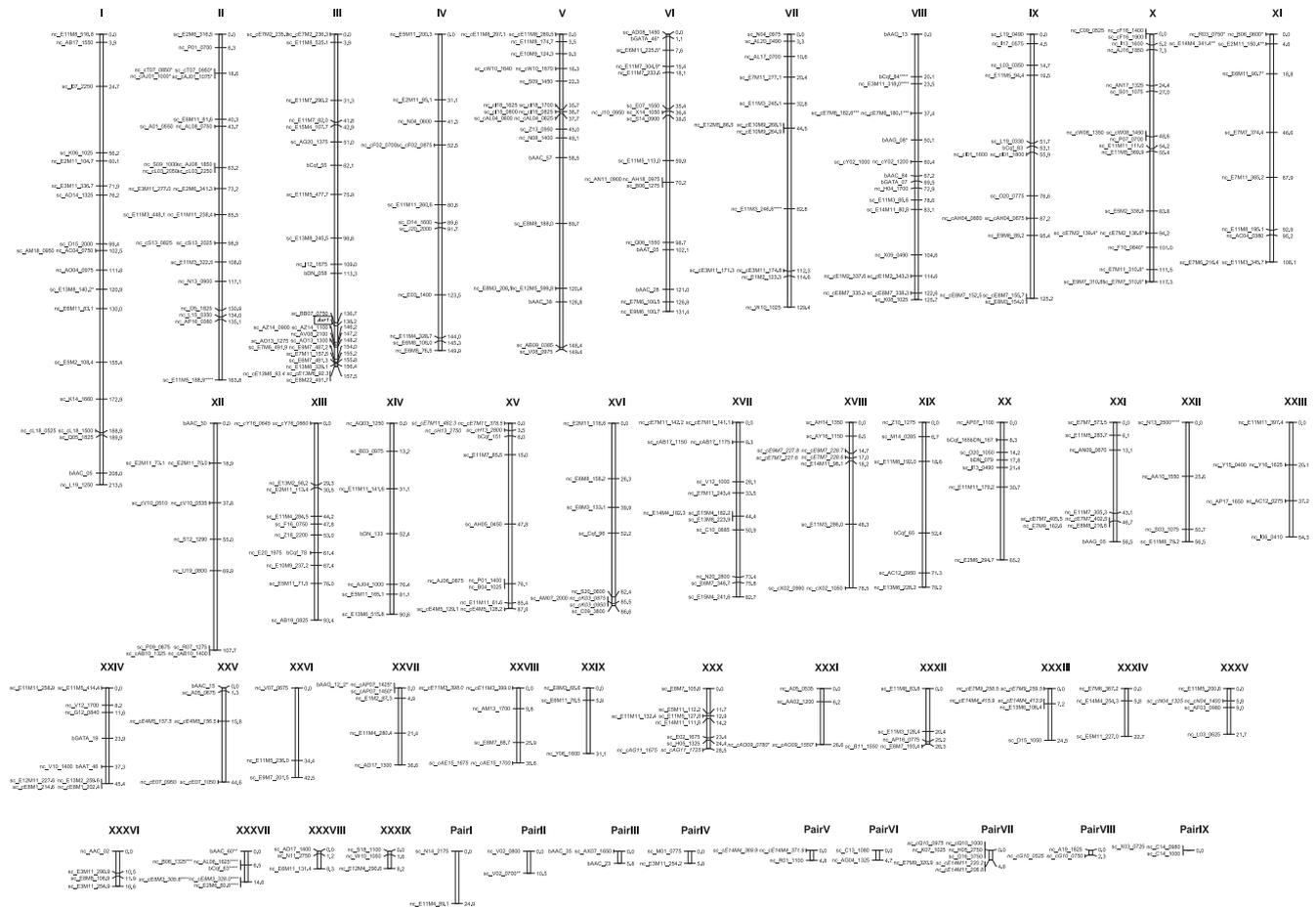


Fig. 1. Genetic map for isolate P2 of *Cronartium quercuum* f.sp. *fusiforme* based on segregation of markers in 101 pycniospore samples. The map consists of 421 markers (312 unique loci) linked at minimum of LOD 4.0 and maximum distance of 40 cM. Dominant markers inherited from isolate SC20–21 are identified by a prefix “sc” and those from isolate NC2–40 are identified with a prefix “nc”. Codominant RAPD and AFLP markers are indicated by a prefix “c” and highlighted in *italic* font. The *Avr1* locus is identified in bold font and placed within a rectangle. The *Avr1* locus was placed in its most likely position based on its known linkage with adjacent markers when using only samples derived from *Fr1/fr1* resistant trees.

locus. Thirty-one primer pairs that produced the greatest number of differentially amplified fragments between NC2-40 and SC20-21 were selected for mapping (Table 1). Segregation data for 184 AFLP markers were obtained for 93 of the 101 samples from the non-selected pycniospore population that were used for RAPD and SSR analysis.

3.4. Genome-wide genetic linkage map for *Cqf* isolate P2

The 208 RAPDs, 34 SSRs, and 184 AFLPs were used to generate a genome-wide genetic map for *Cqf* isolate P2 using the methods described in Section 2.8. Of the 426 markers for which segregation data were obtained, 40 were significantly distorted from the 1:1 segregation expected for a single genetic locus at $P \leq 0.05$. Markers with significant segregation distortion were initially excluded from preliminary mapping analyses, but were added later once a framework was established. In total, 421 of the 426 markers mapped to

312 loci at a LOD of 4.0 and maximum distance of 40 cM with the final genetic map consisting of 39 groups (three or more loci) and nine pairs. The number of markers per group ranged from 3 to 27, with a median of 9. The number of unique loci per group ranged from 3 to 21, with a median of 6. The genetic distance spanned per linkage group ranged from 8.2 to 213.8 cM, with a median of 65.2 cM. The genetic distance spanned per locus/pair ranged from 0.0 cM (three markers mapped to a single locus) to 24.9 cM, with a median of 4.8 cM. Altogether the 39 groups and 9 pairs spanned 3006.4 cM (Table 3 and Fig. 1). The six RAPD markers significantly linked to *Avr1* and the eight AFLPs differentially amplified between *Avr1* avirulent and *avr1* virulent bulks were all found to map to linkage group III. The 40 markers (33 loci) with transmission ratio distortion at $P \leq 0.05$ were found to map to 11 groups and 1 pair (I, II, VI, VII, VIII, X, XI, XXII, XXVII, XXXI, XXXVII, and Pair II). In a majority of the cases, the distorted markers were located distally (groups II, X, XI, XXII, XXVII, XXXI and Pair II), and one linkage

Table 3
Marker distribution across the *Cronartium quercuum* f.sp. *fusiforme* genome.

Linkage group	Map length (Kosambi cM)	Total # markers	Total # of unique loci	Average spacing between unique loci	Largest interval	Total # of marker loci with distorted segregation ^a
I	213.5	20	18	11.9	31.5	1
II	163.8	25	15	10.9	28.7	5
III	157.5	27	21	7.5	27.4	0
IV	149.9	12	11	13.6	31.8	0
V	149.4	22	16	9.3	31.2	0
VI	131.4	18	15	8.8	28.5	3
VII	129.4	13	10	12.9	38.3	1
VIII	125.7	19	15	8.4	21.5	5
IX	125.2	15	11	11.4	32.2	0
X	117.3	19	13	9.0	28.4	6
XI	108.1	11	8	13.5	27.8	5
XII	107.7	11	6	18.0	37.8	0
XIII	93.4	12	10	9.3	29.3	0
XIV	90.6	7	7	12.9	21.3	0
XV	87.8	13	8	11.0	32.8	0
XVI	86.6	9	7	12.4	30.2	0
XVII	82.7	13	9	9.2	22.5	0
XVIII	78.5	10	7	11.2	30.2	0
XIX	78.2	6	6	13.0	33.8	0
XX	65.2	8	7	9.3	34.5	0
XXI	56.5	9	6	9.4	30.0	0
XXII	56.5	4	4	14.1	25.6	1
XXIII	54.3	6	4	13.6	20.1	0
XXIV	45.4	11	6	7.6	13.4	0
XXV	44.6	6	4	11.2	28.8	0
XXVI	42.5	3	3	14.2	34.4	0
XXVII	36.6	6	4	9.2	16.5	0
XXVIII	35.6	6	4	8.9	16.1	0
XXIX	31.1	3	3	10.4	25.2	0
XXX	28.5	9	7	4.1	11.7	0
XXXI	26.6	4	3	8.9	20.4	2
XXXII	26.3	5	4	6.6	20.4	0
XXXIII	24.5	6	3	8.2	17.3	0
XXXIV	22.7	3	3	7.6	16.9	0
XXXV	21.7	5	4	5.4	12.7	0
XXXVI	16.6	4	4	4.2	10.5	0
XXXVII	14.6	7	3	4.9	8.1	7
XXXVIII	8.3	3	3	2.8	7.1	0
XXXIX	8.2	3	3	2.7	6.6	0
Pair I	24.9	2	2	12.5	24.9	0
Pair II	10.5	2	2	5.3	10.5	1
Pair III	5.8	3	2	2.9	5.8	0
Pair IV	5.8	2	2	2.9	5.8	0
Pair V	4.8	3	2	2.4	4.8	0
Pair VI	4.7	2	2	2.4	4.7	0
Pair VII	4.6	8	2	2.3	4.6	0
Pair VIII	2.3	3	2	1.2	2.3	0
Pair IX	0	3	1	0.0	0.0	0
Total	3006	421	312	NA	NA	40
Avg. spacing (unique loci)	9.6					

^a Codominant markers were counted as unique markers.

group (group XXXVII) was composed entirely of distorted markers.

4. Discussion

Avr genes have been mapped in a number of ascomycetes (Broggini et al., 2010; Kema et al., 2002; Kuhn et al., 2006; Lai et al., 2007; Luna-Martínez et al., 2007; Pedersen et al., 2002; Pongam et al., 1998; Zhong et al., 2002), basidiomycetes (Dodds et al., 2004; Zambino et al., 2000), and oomycetes (May et al., 2002; Sicard et al., 2003; van der Lee et al., 2004; Whisson et al., 1995). For all of these species the mapping of Avr genes has been facilitated by a capacity to control the genotype(s) of both host and pathogen, as well as the ability to readily propagate clonal lineages of the pathogen. In the fusiform rust pathosystem the pine host is not easy to clone and breeding to homozygosity is simply not a feasible option given long generation times and high genetic loads (Remington and O'Malley, 2000). In addition, the infective stage/form of the fungus is quite difficult to culture axenically (cf. Amerson and Mott, 1978). Thus, a novel approach for mapping Avr genes in *Cqf* was devised. Our approach capitalized on information about DNA markers known to be tightly linked to a single resistance gene (*Fr1*; Wilcox et al., 1996) to classify the seedlings of a single host family as either resistant (i.e., selecting for virulence) or susceptible (i.e., non-selecting). This innovative approach allowed for the genetic mapping of the corresponding *Avr1* gene by BSA as proposed in Kubisiak et al. (2005). Virulence-selected pycniospore samples collected from diseased *Fr1*-containing trees were used to estimate the degree of linkage between the *Avr1* gene and co-segregating genetic markers, whereas non-selected pycniospore samples collected from diseased trees that lacked *Fr1* resistance were used to construct a genome-wide genetic linkage map. We produced a local genetic map surrounding the *Avr1* avirulence gene for the pine fusiform rust fungus *Cronartium quercuum* f.sp. *fusiforme* that contains 14 closely linked markers and have shown that *Avr1* resides in an interval of 8.62 cM, flanked by the markers *Cqf18.20* (sc_BB07_0750) and *Cqf38.41* (sc_AZ14_0900). This will provide an excellent foundation for future identification of the *Avr1* gene by map-based cloning.

Using BSA, 14 markers were identified that were significantly linked to *Avr1* in *Cqf* isolate P2. The *Avr1* gene was localized to a specific genetic interval with the closest genetic marker estimated to be only 1.38 cM away. We constructed a genome-wide genetic linkage map for *Cqf* isolate P2 comprising 421 markers that mapped to 312 unique loci. The map consisted of 39 linkage groups and nine linkage pairs and covered a total of 3006 cM. Markers significantly linked to *Avr1* were found to map to *Cqf* linkage group III. Doudrick et al. (1993) previously constructed a preliminary genetic linkage map for the single-urediniospore *Cqf* isolate WLP-10-2.SSU, where segregation data were collected for 106 RAPD markers with 54 being distributed among eight groups and 12 pairs covering 291 cM. Unfortunately, only one or possibly two candidate RAPD markers appear to be common between the WLP-10-2.SSU and P2 mapping populations based on the 10-mer used for amplification and estimated band size. Given this, no attempts were made to determine the possible homology of these candidate markers to draw conclusions as to what groups and pairs may be syntenic between the two maps. Currently, there is no karyotype information specifically for *Cqf*. However, assuming that *Cqf* has 18 chromosomes as has been observed for other closely related rust species in the order Pucciniales (Boehm and Bushnell, 1992; Boehm et al., 1992) our map should still be considered preliminary. Thus, linkage group number designations can be considered tentative and associations between groups are expected to change as more markers are added to the map. Regardless, the linkage map presented here represents a significant improvement over the

previously published map for the study of genome structure and organization, and for mapping additional Avr genes in the P2 genome.

In order to avoid transmission ratio distortion, especially around *Avr1*, the progeny set used for constructing the genome-wide linkage map for *Cqf* isolate P2 consisted of pycniospore samples collected exclusively from the non-selecting *fr1/fr1* susceptible host class. Nevertheless, 40 markers (33 loci) with significant segregation distortion were observed. This is more than twice the number expected by chance (5% of 312 loci = 15.6). There was no significant difference between the parental isolates in contribution of markers having distortion. A majority of the distorted markers mapped to the terminal ends of groups and one linkage group was composed entirely of distorted markers. This may partially account for the large number of groups and pairs observed. Although the eight screening samples gave >99% probability ($=1 - (0.5)^7$) of detecting segregation useful for mapping, this would not have been the case around regions experiencing transmission ratio distortion and would have resulted in genomic regions devoid of markers.

Marker loci exhibiting transmission ratio distortion suggest the presence of distorting genetic factors in these regions. Transmission ratio distortion has been reported for a number of fungal and oomycete species and has been attributed to several factors such as scoring errors (Xu and Leslie, 1996), bands of the same size originating from unrelated sequences (Jurgenson et al., 2002), bias in the collection of spores used for mapping (Kerrigan et al., 1993; Larraya et al., 2000; Lind et al., 2005), differential viability of progenies used for mapping (Xu and Leslie, 1996), chromosomal rearrangements (Nitta et al., 1997; Gale et al., 2005), and interspecific crossing (De Vos et al., 2007). What is likely to be causing transmission ratio distortion in *Cqf* isolate P2 can only be speculated. Differential viability as a direct result of meiotically driven chromosomal length polymorphism is one possible mechanism. Flow cytometry-based estimates of genome size for isolates SC20-21 and NC2-40, the parents of isolate P2 mapped in this study, were shown to be significantly different as were genome size estimates for individual progenies of P2 (Anderson et al., 2010). Another possible mechanism is host selection resulting from currently uncharacterized Fr gene:Avr gene interactions. Nine different marker defined Fr genes are currently known and have been mapped in loblolly pine (Amerson et al., 2005; Jordan, 1997, Amerson, unpublished data) and *Cqf* isolate P2 may be heterozygous for as many as seven additional Avr genes (in addition to *Avr1*) that correspond to known Fr genes (Amerson, unpublished data). Although selection 10-5 carries resistance at only one of these Fr genes and 4666-4 is homozygous susceptible at all of these genes, we cannot rule out that selection 10-5 or 4666-4 may harbor resistance at Fr genes that have yet to be characterized.

The physical size of the *Cqf* genome is of interest for map-based cloning and genome sequencing efforts. Using flow cytometry the physical size of the *Cqf* genome has been estimated to be approximately 90 Mb (Anderson et al., 2010). Assuming that *Cqf* has 18 chromosomes, that each unlinked marker accounts for 80 cM, and that the ends of linkage groups not covering true telomeric regions each account for 40 cM, we estimate the recombinational-based genome size of *Cqf* to be approximately 5800 cM. This is close to the 5100 cM estimate obtained previously for *Cqf* by Doudrick et al. (1993). Given an average physical genome size of 90 Mb and a recombinational map size of 5800 cM, the physical size to genetic distance is estimated to be 15.5 kbp/cM. Although physical to genetic distance estimates have been found to vary considerably across fungi and oomycete species, this estimate is similar to those reported for *Cryphonectria parasitica* 14.5 kbp/cM (Kubisiak and Milgroom, 2006), *Cryptococcus neoformans* 13.2 kbp/cM (Marra et al., 2004), and *Heterobasidion annosum* 11.1 kbp/cM (Lind et al., 2005). Assuming that the relationship between physical to

genetic distance is constant within as well as between *Cqf* chromosomes, marker *sc_BB07_0750* may be as close as 21.4 kbp from *Avr1*. Thus, a map-based positional cloning approach of this gene appears very favorable (Sweigard et al., 1995) especially in light of the fact that the *Cqf* genome sequence will soon be obtained through the Department of Energy's Joint Genome Institute Community Sequencing Program (<http://www.jgi.doe.gov/sequencing/csseqplans2010.html>).

To our knowledge, the study reported here is the first report of the genetic mapping of an *Avr* gene conferring race- or pathotype-specificity in a basidiomycete fungus infecting a forest tree species. The approach is novel, taking advantage of what was previously known about the molecular genetics of the pathosystem. A similar approach could be used to map *Avr* genes in other forest tree pathogens where gene-for-gene interactions have been suggested (Kinloch and Dupper, 2002) and genetic markers tightly linked to specific host resistance genes have been identified (Cervera et al., 1996; Harkins et al., 1996; Jorge et al., 2005; Liu et al., 2006; Liu and Ekramoddoullah, 2008; Newcombe et al., 1996; Tabor et al., 2000; Villar et al., 1996; Yin et al., 2004). The mapping and cloning of specific *Avr* genes in fungi pathogenic to forest tree species provides a unique opportunity to better understand the dynamic between host resistance and fungal virulence in natural populations.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.fgb.2010.09.008](https://doi.org/10.1016/j.fgb.2010.09.008).

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