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Article

Identification of Nine Pathotype-Specific Genes Conferring Resistance to Fusiform Rust in Loblolly Pine (*Pinus taeda* L.)

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Abstract: Nearly two decades of research on the host-pathogen interaction in fusiform rust of loblolly pine is detailed. Results clearly indicate that pathotype-specific genes in the host interacting with pathogen avirulence cause resistance as defined by the non-gall phenotype under favorable environmental conditions for disease development. In particular, nine fusiform rust resistance genes (Fr genes) are described here including the specific methods to determine each and their localization on the reference genetic map of loblolly pine. Understanding how these and other apparent Fr genes in loblolly pine and other rust-susceptible pines impact resistance screening, parental and progeny selection, and family and clonal deployment is an important area in forest genetics research and

operational tree breeding. The documentation of these Fr genes is a key piece of information towards gaining that understanding and ultimately improving breeding and deployment strategies.

Keywords: host-pathogen genetics; gene-for-gene interaction; resistance genes; *Pinus taeda*; *Cronartium quercuum* f.sp. *fusiforme*; fusiform rust; Fr genes; genetic markers

1. Introduction

Fusiform rust disease incited by the biotrophic, macrocyclic, heteroecious fungus *Cronartium quercuum* (Berk.) Miyabe ex Shirai f.sp. *fusiforme* (Cumm.) Burds. & Snow (*Cqf*) is endemic to oaks and pines of the southeastern United States. Damage on the telial or repeating host (*Quercus* subgenus *Erythrobalanus*, *i.e.*, red oaks) is confined to leaf spotting, whereas damage on the aecial or non-repeating host (subgenus *Pinus* section *Trifoliae* subsection *Australes*, *i.e.*, southern yellow pines) results in the formation of swollen galls on stems and/or branches of infected trees. These swellings can disrupt water flow and directly kill infected trees or weaken the stem and hence predispose them to future damage. Fusiform rust disease is a limiting factor in pine silviculture, both for loblolly pine (*Pinus taeda* L.) and slash pine (*Pinus elliottii* Engelm. var. *elliottii*), affecting both the quantity and quality of timber produced. Several studies have attempted to estimate the economic impact of losses due to fusiform rust disease [1–3]. Although estimates vary considerably depending upon the criteria used for devaluation, losses throughout the southeast are likely on the order of 100s of millions of dollars annually.

Foresters have long since recognized the presence of fusiform rust disease resistance in pines of the southeastern United States [4]. Since that time, testing of loblolly and slash pine families under natural field conditions or using artificial inoculation systems has led to the recognition of rust-resistant selections [5–7]. Undoubtedly, the development of artificial inoculation methods [8,9] has hastened recognition of rust-resistant loblolly and slash pine selections, but the genetic basis for this observed resistance has remained poorly understood. Frequent use of open-pollinated (OP) pine families and mixed genotype inoculum sources in both field (natural inoculation) and greenhouse (artificial inoculation) screening experiments typically led foresters to conclude that resistance to this pathogen was polygenic and breeding for rust resistance consequently utilized quantitative genetic models [10,11].

Jewell [12] based on a series of artificially inoculated trials, using expectedly diverse basidiospore inocula obtained from naturally infected oak leaves OP slash pine families from rust-free and rust-infected selections as well as full-sib families from rust-free and rust-infected slash pine selections, reported disease data summarized from several years of work. The OP families were useful for recognizing resistant and susceptible selections, and the full sib data led Jewell [12] to propose that resistance in slash pine was controlled by a dominant single gene, but later Jewell and Mallett [13], with additional disease data from full-sib seedlings from resistant selections 8-7 and 18-27, deemed resistance in slash pine to be more complex. Kinloch and Walkinshaw [14], in a reanalysis of an earlier study by Griggs and Walkinshaw [15] that used full-sib slash pine families from a five parent diallel cross (including resistant parents, 8-7 and 18-27 used in works [12,13]) challenged with basidiospore inocula derived from two single galls, reported fusiform rust resistance involving dominant single host

genes, two in that study, and based on the data hypothesized gene-for-gene interactions. Powers [16] and Kuhlman and Matthews [17] reported methods for the development of single-genotype *Cqf* isolates with the Kuhlman and Matthews work showing virulence variation among single-genotype isolates derived from the same single gall. Contemporaneous with and subsequent to Kuhlman and Matthews [17], the use of basidiospore inocula derived from single-genotype isolates has greatly advanced the understanding of the genetics of fusiform rust disease, and the hypothesis that this pathosystem largely conforms to a classic gene-for-gene model has yet to be rejected [18–25]. Recently, Nelson *et al.* [26] concluded that gene-for-gene interactions largely determine fusiform rust gall formation in loblolly and slash pine, with that conclusion being firmly supported for loblolly pine by the demonstration that host resistance gene *Fr1* and pathogen avirulence gene *Avr1* interact in a gene-for-gene fashion [27].

In this paper we used a gene-for-gene approach to provide an analysis of seven loblolly pine families challenged with inocula derived from five single-genotype isolates of the fusiform rust fungus, leading, along with the work of Wilcox *et al.* [19], to the identification and genetic mapping of nine pathotype-specific fusiform rust resistance genes (Fr genes). These results are discussed in the context of pathosystem biology, forest management, and tree improvement.

2. Materials and Methods

2.1. Host-Pathogen Interaction Matrix

To identify and map Fr genes we examined a matrix of data obtained for seven loblolly pine families inoculated with basidiospores derived from five single-genotype *Cqf* isolates. Classifications listed in the interaction matrix presented in Table 1 are based on a composite of data obtained from several different experiments performed over more than 15 years, by first author Henry V. Amerson and co-workers, especially E. George Kuhlman. Classifications are based on two criteria: the percentage of seedlings from a family that are galled by a particular isolate (discussed in more detail in Section 2.4 and in Nelson *et al.* [26]), and, for some matrix cells, DNA marker-phenotype association data (discussed in more detail in Section 2.5 and in Nelson *et al.* [26]).

Table 1. Host-pathogen interaction classification for seven loblolly pine families challenged with five different single-genotype isolates of *Cqf* (I = incompatible, C = compatible).

Host Families (Pine)	**Pathogen Isolates (*Cqf*)**				
	SC20-21	NC2-40	3327-13-3	NC2-36	0-5-32
Family 10-5	I^1	C	C	C	I^1 *
Family A	C	I^2	C	C	I^2
Family 152-329	C	I^3	I^3	C	I^3
Family 29R	C	C	I^4	I^4	C
Family B	C^5 I^9	C^5 C^9	C^5 C^9	I^5 C^9	C^5 I^9
Family C	I^6 I^7	C^6 C^7	I^6 C^7	C^6 I^7	C^6 C^7
Family D	I^8	C	C	C	C

* In families with a single heterozygous Fr gene the superscript numbers refer to the Fr gene responsible for the incompatible (I) interaction. In families with two heterozygous Fr genes the superscript designation denotes the compatibility status for each gene (*Fr5*, *Fr6*, *Fr7*, or *Fr9*).

2.2. Pine Families

Host materials were either full-sib families where female parents were the resistance source and male parents were highly susceptible, or open pollinated families where female parents were the resistance source. Although families of progeny are challenged with *Cqf*, the parental tree (in our case the maternal parent) carrying resistance is the genotype being characterized (as in a progeny test). The families were either progeny from selections that are property of privately-owned industrial tree improvement programs, or progeny from selections from public (USDA Forest Service and Georgia Forestry Commission) tree improvement programs. Private industry-owned selections have been assigned a generic single-letter code (except for selection 10-5, which was previously used to map *Fr1* [19]) to keep their identity anonymous. Publicly-owned materials are identified by their original tree improvement code.

2.3. Single-Spore/Single-Genotype Isolates and Inoculation Protocol

Pathogen lines were originally developed from individual aeciospores isolated from single galls on loblolly pine or slash pine as described by Kuhlman and Mathews [17]. Some lines have since been re-isolated from the original lines by bulking of spores derived from a single-uredinial-pustule (see SUP protocol Supplemental File 1), with all lines subjected to SSR (simple sequence repeat) marker evaluation [28,29] to assess single-genotype purity, and to provide a DNA fingerprint for future QA/QC. The geographic origins of the five isolates used are as follows: SC20-21 is from Colleton County, South Carolina; NC2-36 and NC2-40 are from the same gall sampled in Halifax County, North Carolina; 3327-13-3 is from central Georgia in the Baldwin County area and 0-5-32 is from Greene County, Georgia. These isolates were maintained as dikaryotic-urediniospore lines and used via oak inoculations to produce the haploid basidiospores needed for pine inoculations. Hence, when we speak of challenging pine progeny with a given single-genotype isolate, such as 0-5-32, this is a shorthand way of saying that the progeny were challenged with basidiospores derived from that given isolate. Greenhouse inoculations of pine seedlings (eight weeks post seed-sowing or approximately six weeks seedling age) were performed using the concentrated basidiospore inoculation system (CBS) of Matthews and Rowan [8], typically at a concentration of 100,000 basidiospores per mL, as recommended by Kuhlman *et al.* [22]. Inoculations were conducted at both the USDA Forest Service Southern Research Station in Athens, Georgia and at the USDA Forest Service Resistance Screening Center in Asheville, North Carolina. Inoculations typically utilized approximately 100 seedlings per family per inoculum, but varied from 65 to 200 seedlings per family per inoculum. Final disease assessments (no-gall *vs.* gall) were typically made at six to nine months post-inoculation.

2.4. Fr Gene Identification

The general methodology used to identify specific Fr genes was presented in detail in Nelson *et al.* [26], although much of the work reported here was completed prior to method formalization. This method is similar to that used in the cereal rust systems where a host-by-pathogen interaction is evaluated as incompatible (I) or compatible (C), although we inoculate segregating progeny instead of inbred lines (reviewed by J. Kolmer [30]). This fact required us to utilize genetic markers and mapping to

differentiate multiple Fr genes within families, but also allows the simultaneous mapping of the Fr gene loci (discussed in Section 2.5). In inbred systems, crosses of particular lines would need to be made to produce segregating progeny for genetic mapping.

Tentative host-pathogen classifications were initially based on the percentage of seedlings from a family that were galled by a particular single-genotype isolate. Going forward, we recommend that the tentative classification should not be accepted as final until subjected to genetic marker evaluation [26]; however, that has not necessarily been the case in some of the work covered here. Also, going forward as per Nelson *et al.* [26], high levels of infection (≥80%) should be classified as C and lower levels (≤65%) as I, while intermediate levels (between 65% and 80%) should be classified as ambiguous (A), in contrast to past practice. In past practice we did not focus on specific percent gall cut-offs for tentative I or C classifications. Instead, if a given percent gall value could represent the interaction of host resistance and pathogen avirulence (especially when the percent gall value might infer 1:1 segregation for resistance where the likelihood for Fr gene discovery is highest), the interaction was termed tentatively incompatible. If the percent gall value was considered too high to represent host resistance interacting with pathogen avirulence, the interaction was termed tentatively compatible. All I classifications (with one exception; see Section 3.2.6) and several of the C classifications noted in Table 1 were subjected to DNA marker evaluations to determine final compatibility status. Of the C classifications not subjected to marker investigation, none had less than 81% gall. The tentative classifications reported in this work were based on our subjective evaluations as noted above, and while that subjectivity now seems overly cumbersome, please note that this developmental work evolved into the current formalized criteria [26]. When percent gall values suggest 1:1 Fr gene segregation, this implies that one parent is heterozygous for the Fr gene or Fr/fr and the other parent is homozygous fr/fr. In the case of open-pollinated families, this implies that the female parent is heterozygous (Fr/fr) and the frequency of the resistance (Fr) allele is relatively low to non-existent in the pollen pool. In the case of an I interaction, an isolate must be homozygous for avirulence (Avr/Avr) to the corresponding Fr gene as heterozygous (Avr/avr) isolates cause a C interaction when using the CBS inoculation system [27]. In our analysis of the host selections and isolates that follows, when we say that an isolate is avirulent for a given Fr gene (*i.e.*, an incompatible interaction), we are saying that the isolate lacks the ability to cause disease in a host having the resistance (Fr) allele of the given Fr gene, and conversely if an isolate is called virulent against a given Fr gene (*i.e.*, a compatible interaction) we are saying that the isolate has the ability to cause disease in a host having the resistance (Fr) allele of the given Fr gene.

2.5. Fr Gene Mapping

In any one experiment, only a proportion of the cells (family-by-isolate combinations) are potentially informative with respect to mapping Fr genes, and these are cells where the interaction is classified as I. In these potentially informative cells, galled trees are expected to be homozygous (fr/fr) for the susceptible allele, while non-galled trees expectedly are heterozygous (Fr/fr) for resistance, where resistance is dominant to susceptibility. As noted in Section 2.4, in the case of I interactions the isolate must be homozygous for avirulence as heterozygous isolates result in a C classification. Scoring a large number of DNA markers for each inoculated seedling allows for tests of statistical association

(cosegregation analysis) to be made between the markers and the gall/no-gall disease phenotype. For our marker work, we relied primarily on random amplified polymorphic DNA (RAPD) markers that were used in all matrix families. RAPD protocols underwent multiple minor modifications over the many years of this work, but refer to Myburg *et al.* [31] for the most recently optimized protocols. Amplified fragment length polymorphism (AFLP) markers were utilized for only a single family × isolate combination. The AFLP protocol used was a modification of the protocols reported by Remington *et al.* [32] and Myburg *et al.* [33]. RAPD markers were named using the Operon manufacturer primer code (Operon Technologies Inc., Alameda, CA, USA) (e.g., J7), followed by the marker fragment size, approximated in base pairs (e.g., 470 bp), to yield the RAPD marker name, such as J7_470. AFLP marker names reflect the three selective nucleotides of the EcoR1 primer, the four selective nucleotides of the Mse1 primer, and the estimated fragment size in base pairs (e.g., marker ACC-CCTA550).

In pines, because the maternal contribution to a pine embryo in a given seed arises from the same megaspore that proliferates into the megagametophyte of that seed, we have typically used haploid megagametophyte DNA to analyze markers derived from the maternal parent. Most often, we have used a bulked segregant analysis (BSA) strategy [34], where DNA bulks from a tentatively I cell were assembled on the basis of gall *vs.* no-gall disease phenotypes and screened for differentially amplified markers to find markers potentially linked to Fr genes. In a second part of this strategy, the previously recognized markers were then assessed using a progeny array (individual seedling phenotypes and marker genotypes from the corresponding individual megagametophyte DNAs) from the tentatively I cell to support or reject linkage. Markers associated with phenotype at a LOD (logarithm of odds) score threshold ≥ 3.0 were considered to be significantly linked to an Fr gene (the trait locus involved in the interaction). Associations amongst several markers and the trait locus (Fr gene) were used to develop a genetic map of the Fr-gene chromosomal region in a given selection (parent) using MAPMAKER Macintosh version 2.0 [35] and for some selections Map Pop version 1.0 [36] was implemented. While the BSA strategy previously noted was our basic bulking strategy used to identify Fr-gene linked markers, BSA using megagametophyte DNA (bulked on the basis of an already-known genetic marker tightly linked with phenotype) was also often employed, and, infrequently, diploid DNA (bulked on the basis of disease phenotype) was used.

The power of this mapping approach is that once an Fr gene has been mapped within a specific pine selection the linked markers can be used to determine if tentative I interactions involving progeny of the same selection and different isolates are likely due to the same gene (significant marker-phenotype association) or to a different Fr gene (non-significant marker-phenotype association). However, it must be noted that given our current genetic map resolution we are not able to unambiguously distinguish the Fr gene in question from a different Fr gene that is tightly linked in the coupling phase. Nonetheless, as per Occam's razor (*i.e.*, the simplest explanation is preferred), when there is a significant LOD score we consider the Fr gene in question to be the same as the mapped Fr gene, thus minimizing the number of genes needed to explain observed percent gall values. For a tentative C interaction, a non-significant marker-phenotype association confirms virulence against the gene being investigated. Pertinent to the matrix in Table 1, in an I cell where a parental selection is known to carry two unlinked Fr genes (Family B) and incompatibility is conditioned by a single gene, a non-significant marker-phenotype association for the other gene in that cell indicates virulence with

respect to that particular gene. In Family C, where two Fr genes are tightly linked in repulsion in parent C and incompatibility in a cell is conditioned by one gene, phase-dependent marker-phenotype associations are required to assess isolate virulence or avirulence with respect to each gene.

Fr-linked markers were localized on the reference loblolly pine genetic map [37] by scoring and analyzing a sample of progeny from the same populations that were used to construct the reference map. Specifically, some Fr-linked markers from the current work were scored in the *base* (n = 78 scorable) and *qtl* (n = 81 scorable) pedigrees. These data were appended to the data sets provided by Echt *et al.* [37] (Tables 1 and 2 of Supplemental File 4 in Echt *et al.* [37]), and then subjected to genetic mapping using JoinMap 4.1 [38]. The mapping methodology was as described by Echt *et al.* [37], except that "fixed orders" were used for each of the three linkage groups (LG2, LG3, and LG10) containing Fr genes. The fixed orders corresponded to the orders of the loci on the corresponding reference map linkage groups [37], such that the best position for each Fr-linked marker was determined given the existing reference map.

3. Results

3.1. Fr Gene Identification

Although tightly coupled unknown Fr genes cannot be ruled out, and selection A (Family A, Table 1) does have at least one other fusiform rust resistance gene capable of conferring resistance apart from *Fr2* ([39] and see discussion Section 4.2), the families within the context of this matrix are segregating for one or two of the nine Fr genes identified here and are susceptible at all the other identified Fr genes. Table 1 displays the matrix of I and C classifications assigned to seven loblolly pine families inoculated with basidiospores obtained from five single-genotype isolates.

Focusing first on Families 10-5 and A and Isolates SC20-21 and NC2-40, the interpretation of the data in Table 1 is that Isolate SC20-21 is avirulent to a corresponding resistance gene (*Fr1*) that is segregating in Family 10-5. Wilcox *et al.* [19] previously identified and verified the existence of this pathotype-specific resistance gene via genetic mapping. Genetic mapping information for all the currently identified Fr genes is discussed in Section 3.2. Isolate NC2-40 is avirulent to a different Fr gene (*Fr2*) that is segregating in Family A, but virulent to gene *Fr1* segregating in Family 10-5, while Isolate SC20-21 is virulent to *Fr2* segregating in Family A. Although Family 10-5 is segregating for resistance at *Fr1* it is not segregating at *Fr2*, *i.e.*, all Family 10-5 progeny are susceptible at *Fr2*. Conversely, Family A is segregating for resistance at *Fr2* but is not segregating at *Fr1*.

Isolate 3327-13-3 is virulent to genes *Fr1* and *Fr2* based on its C interactions with Families 10-5 and A, but avirulent to another Fr gene (*Fr3*) that is segregating in Family 152-329. Isolate NC2-36 is virulent against genes *Fr1*, *Fr2*, and *Fr3* based on its C interactions with Families 10-5, A, and 152-329, but is avirulent to a new Fr gene (*Fr4*) that is segregating in Family 29R. Isolate NC2-36 is avirulent to an Fr gene in Family B, but this gene cannot be *Fr4* as Isolate 3327-13-3 is avirulent to *Fr4* but produces a C interaction when 3327-13-3 is used to challenge Family B. Thus, the Fr gene segregating in Family B and responsible for the observed I interaction with isolate NC2-36 must be a new Fr gene (*Fr5*). Isolate 0-5-32, which is avirulent to *Fr1*, *Fr2*, and *Fr3*, causes a C interaction with Family C, whereas Isolate SC20-21, which is virulent to *Fr2*, *Fr3*, *Fr4*, and *Fr5*, results in an I

interaction with Family C. Thus, Family C must contain a new source of resistance. Indeed, Family C is segregating for two new Fr genes (*Fr6* and *Fr7*) and there are two lines of evidence that support this observation. The first is that the level of galled progeny observed for the Family C × Isolate SC20-21 interaction is greatly below the ~50% level expected when families are segregating for only a single Fr gene. The second is that DNA markers used to examine Family C progeny interacting with isolates 3327-13-3 and NC 2-36 confirm the presence of two unique resistance genes (discussed in more detail in Section 3.2.6).

In Family D, the C interaction with Isolate 0-5-32 precludes *Fr1*, *Fr2*, or *Fr3* resistance and the C interaction with isolate 3327-13-3 further precludes *Fr4* and *Fr6* resistance while the C interaction with NC 2-36 eliminates the possibility of *Fr5* or *Fr7* resistance. Thus, Family D must be segregating for a new Fr gene (*Fr8*). Returning to Family B, a recent inoculation and marker investigations revealed the presence of a second (previously unaccounted for) Fr gene. This gene cannot be *Fr5*, as markers significantly linked with the *Fr5* gene do not explain the I interactions of Family B with isolates SC20-21 and 0-5-32 (*i.e.*, the *Fr5*-linked markers were not significantly associated with phenotype in these matrix cells). Isolate NC2-40 has I interactions with *Fr2* and *Fr3*, yet has a C interaction with Family B, thus this second Fr gene in Family B cannot be *Fr2* or *Fr3*. Likewise isolate 3327-13-3 has I interactions with *Fr4* and *Fr6*, yet has a C interaction with Family B, thus this second Fr gene in Family B cannot be *Fr4* or *Fr6*. Family B has an I interaction with 0-5-32 that is not due to *Fr5* yet 0-5-32 gives C interactions with *Fr7* and *Fr8*; thus the I interaction in Family B cannot be due to these genes. Isolates SC20-21 and 0-5-32 produce I interactions with *Fr1*, thus the I interactions with these isolates and Family B could be explained by *Fr1* resistance; however, marker investigation using an *Fr1*-linked marker (see Section 3.2.8) strongly suggests that *Fr1* is not responsible for this incompatibility. Therefore, these I interactions must be due to a ninth Fr gene (*Fr9*). In this manner nine unique pathotype-specific Fr genes have been identified in the matrix using interaction data and Fr-linked markers.

3.2. Fr Gene Mapping and Marker-Phenotype Analysis of I and C Classifications

3.2.1. *Fr1*

The first Fr gene to be discovered in loblolly pine was *Fr1*. This gene (*Fr1*) was identified and mapped segregating in progeny of selection 10-5 (*i.e.*, Family 10-5), which were challenged with Isolate SC20-21 [19,40]. Previous work [19,40] provided much of the basis for the consideration of *Fr1* in this manuscript. To date 20 RAPD markers are known to be significantly linked to *Fr1* in selection 10-5 (Table 2), and markers J7_470 (previously called J7_485 [19,40]) and AJ4_420 are the closest markers to *Fr1*. These two markers have both been extensively examined and are estimated to be ~1 to 2 cM away from *Fr1*. In selection 10-5 the band-present (+) allele of marker J7_470 is linked with the *Fr1* resistance allele (referred to as marker linked in coupling with the *Fr1* gene or, more simply, marker linked in coupling with *Fr1*), while the band-absent (-) allele of marker AJ4_420 is linked with the *Fr1* resistance allele (referred to as marker linked in repulsion with the *Fr1* gene or, more simply, marker linked in repulsion with *Fr1*). In selection 10-5, markers J7_470 and AJ4_420 are tightly linked to each other at a distance of ~1.3 cM, and although the exact position of the *Fr1* gene

relative to these markers has not been definitively determined, *Fr1* most likely resides in the interval between these markers. *Fr1*-linked marker analysis of the Family 10-5 × Isolate 0-5-32 cell, which was tentatively classified as I, suggests that *Fr1* is likely to be the gene responsible for the I classification within that cell. The Family 10-5 × Isolate 3327-13-3 cell was tentatively classified as compatible and *Fr1*-linked marker analysis failed to detect a significant association with phenotype confirming compatibility. The interaction of Family 10-5 progeny challenged with isolate NC2-36 was marker verified as compatible [19,40] and isolate NC 2-40 was also marker confirmed as virulent against *Fr1* resistance [22]. Following the discovery of *Fr1*, eight additional Fr genes (*Fr2–Fr9*), as previously noted and described here in Section 3.2, have been found in loblolly pine. Although the Fr genes are numbered 1–9, their numerical designation does not necessarily reflect the order in which they were discovered.

3.2.2. *Fr2*

Resistance gene *Fr2* in loblolly pine was identified and mapped segregating in progeny of selection A (Family A). Two different Family A matrix cells (A × 0-5-32 and A × NC 2-40) were tentatively classified as I based on the percentages of galled progeny observed. Both haploid and diploid DNAs, obtained from Family A progeny challenged by Isolate 0-5-32 were used for BSA to find markers significantly linked to *Fr2*. Including both RAPD and AFLP markers, a total of 12 markers (two more than mapped by Li [41]) significantly linked to phenotype (*i.e.*, the *Fr2* locus) in selection A have been identified (Table 2). The AFLP markers and RAPD marker AK6_850 reported by Li [41] were initially from Henry V. Amerson (unpublished data), with Li [41] adding additional AK6_850 data plus six additional RAPD markers (Table 2), as well as providing much of the basis for the consideration of *Fr2* in this manuscript. The two closest RAPD markers to *Fr2* are markers BD18_420 and AY12_1300, which are estimated to be linked with *Fr2* at ~7 and ~9 cM, respectively. BD18_420 is linked in coupling with *Fr2* and marker AY12_1300 is linked in repulsion. *Fr2*-linked marker analysis within the Family A × Isolate NC2-40 cell suggests that *Fr2* is likely to be the gene responsible for the I classification within this cell of the matrix. Three of the Family A matrix cells were tentatively classified as C and to date none have been investigated for marker-phenotype correlation.

3.2.3. *Fr3*

Resistance gene *Fr3* in loblolly pine was identified and mapped segregating in progeny of selection 152-329 (Family 152-329). Three different Family 152-329 matrix cells were tentatively classified as I based on the percentages of galled progeny observed. The Family 152-329 by Isolate NC2-40 cell was chosen for genetic linkage map construction of loblolly pine 152-329 [21]. A total of 10 RAPD markers were found to be significantly linked to *Fr3* (Table 2). Marker J7_1840 was most tightly linked with phenotype in the NC2-40 cell. The J7_1840 marker and several other linked markers based on the NC2-40 mapping were evaluated in the tentatively I cells involving Isolates 3327-13-3 and 0-5-32. All were significantly linked with phenotype in their respective cells, with J7_1840 again being the most tightly linked, suggesting that *Fr3* is likely to be the gene responsible for the I classification in these cells. The J7_1840 marker and phenotype data from all three cells were used to position *Fr3*. The J7_1840 marker is estimated to be ~10 cM away from *Fr3*, while the next closest marker, M18_655 (previously called M18_600 [21]), is ~13 cM away from *Fr3*. The *Fr3* locus maps to a

terminal position in the linkage group. Marker J7_1840 is linked in repulsion with *Fr3* and marker M18_655 is linked in coupling. Two matrix cells, those involving Family 152-329 progeny inoculated with Isolates SC20-21 and NC2-36, were tentatively classified as C. These cells were also investigated for marker-phenotype correlation and, as expected, none of the markers were significantly associated. Apart from the marker investigations involving isolates SC20-21 and NC2-36 and findings relating *Fr3* markers to the loblolly pine reference map [37], Jordan [21] is the basis for all other considerations of *Fr3* in this manuscript.

3.2.4. *Fr4*

Resistance gene *Fr4* in loblolly pine was identified and mapped segregating in progeny of selection 29R (Family 29R). Two Family 29R matrix cells (29R × 3327-13-3 and 29R × NC2-36) were tentatively classified as I, and three were tentative C cells. Megagametophyte DNAs from the Family 29R × Isolate 3327-13-3 cell were used for BSA to identify markers significantly linked with *Fr4*. A total of seven markers were found to be significantly associated with phenotype (Table 2). Currently, the two closest markers to *Fr4* are RAPD markers J7_1840 and F3_1490, which showed no recombination between each other in the mapping population and are estimated to be ~1.5 cM from *Fr4*. Marker F3_1490 is linked in coupling with *Fr4*, and marker J7_1840 is linked in repulsion; the same as noted for repulsion linkage of J7_1840 with *Fr3* in selection 152-329 (Section 3.2.3). Similar to the *Fr3* linkage group, *Fr4* also mapped to a terminal position. *Fr4*-linked marker analysis in progeny of Family 29R inoculated with Isolate NC2-36 suggests that *Fr4* is likely to be the gene responsible for the I classification within that cell. The 29R × NC2-40 cell was tentatively classified as a C interaction (although under the Nelson *et al.* [26] criteria it would have been classified as A, ambiguous), and *Fr4*-linked marker analysis yielded non-significant associations confirming the C classification. However, atypically for a C interaction, approximately half of the marker-designated resistant seedlings were galled, while nearly all marker-designated susceptible seedlings were galled. This gall/no-gall distribution within the marker-designated resistant seedlings is not characteristic of the interaction of a single heterozygous host gene and a corresponding pathogen gene carrying virulence. The basis of this distribution is not currently clear, but further investigation of the 29R × NC2-40 interaction seems warranted. *Fr4*-linked marker analysis in the other two Family 29R matrix cells (29R × SC20-21 and 29R × 0-5-32) that were tentatively classified as C interactions failed to detect a significant association with phenotype, as expected.

Table 2. Listing of RAPD and AFLP markers significantly linked at LOD ≥ 3.00 with fusiform rust disease resistance genes (*Fr1–Fr9*) in their respective loblolly pine selections (e.g., *Fr1* in selection 10-5). AFLP markers were used only in selection A. Map distance in cM is given for some of the closer markers for each Fr gene. Other markers (without cM designations) are listed with regard to proximity (order from top to bottom approximates near to far) to the Fr gene in the selection. Within a column marker names followed by a (+) have the band-present marker allele linked with the resistance allele, while marker names followed by a (-) have the band-absent marker allele linked with the resistance allele. Within a column markers preceded by the same superscript number were non-recombinant with each other in the mapping population. For markers followed by a superscript *, marker size (estimated in base pairs) is revised from previous citation (e.g., marker J4_565 was previously called J4_550). Listed marker sizes represent preferred usage. For *Fr1*, bold markers were previously reported (19, 40). For *Fr2*, the two bolded markers were not previously reported (41). For *Fr3*, all makers were previously reported (21).

Fr1 10-5	*Fr2* A	*Fr3* 152-329	*Fr4* 29R	*Fr5* B	*Fr6* C	*Fr7* C	*Fr8* D	*Fr9* B
J7_470+*	BD18_420+	J7_1840-	[1]J7_1840-	[1]B20_480-	G13_480-	G13_480+	[1]B20_480+	J7_1840+
~1-2 cM	~7 cM	~10 cM	~1.5 cM	~3 cM	< 6 cM	< 6 cM	~3 cM	~ 11 cM
AJ4_420-	ACC-CCTA550+	M18_655+*	[1]F3_1490+	[1]J18_800-	A20_1620-	A20_1620+	[1]A12_725-	K14_440+
~1-2 cM	~7 cM	~13 cM	K3_1530-	[1]J18_900+	< 6 cM	< 6 cM	BD12_1300-	~13 cM
[1]BF17_1100-	AY12_1300-	AP12_490-	D20_600-	J7_780+	J4_565-*	J4_565+*	AK19_840-	B11_780+
[1]BD16_830+	~9 cM	W13_915-*	D10_470+	[2]A6_350+	K3_1530+	K3_1530-	I10_370	A9_800+
[1]BH1_1300+	C13_580-	AT15_525-	C4_1750+	[2]D3_1250-*	AP11_415-	AP11_415+		AJ4_420-
AY14_410+	AK6_850-	N9_320-	G13_480-	[2]AK19_840-	K14_440-	K14_440+		G13_480-
[2]**D16_1180**+	[1]E12_1200+	[1]H20_400-		A6_1650-	J7_1840-	J7_1840+		
[2]**J4_565**+*	[1]ACG-CCTA550-	[1]I10_515+		A19_580+				
F13_780-	ACC-CCGA315+	[1]A12_420+						
J4_1000-	BB11_780+	D11_1350+*						
[3]**C12_1050**+	AZ18_850+							
[3]**C12_450**-	**ACG-CCAA258**-							
BF4_1100-	**ACG-CCAA532**+							
A9_625+								
D11_1350-*								
H20_400+								
H20_800-								
A19_500+								
K1_900-								
B8_650-								

3.2.5. *Fr5*

Resistance gene *Fr5* in loblolly pine was identified and mapped segregating in progeny of selection B (Family B). *Fr5* resistance in Family B has been evident since our first inoculation trial while resistance conditioned by a second, later discovered gene (*Fr9* fully considered later) in Family B was only evident in a more recent inoculation. The reason(s) for this discrepancy regarding *Fr9* is not currently clear. Nonetheless, collectively three Family B matrix cells, Family B × Isolates NC2-36, SC20-21, and 0-5-32, were tentatively classified as I. To identify markers significantly linked to *Fr5*, megagametophyte DNAs from the Family B × Isolate NC2-36 cell were subjected to BSA. A total of nine markers were found to be significantly associated with phenotype (Table 2). The three closest linked markers to *Fr5* were B20_480, J18_800, and J18_900. Markers B20_480 and J18_800 are linked in repulsion with *Fr5*, while J18_900 is linked in coupling with *Fr5*. These markers were non-recombinant among themselves in the mapping population and are estimated to be ~3 cM from *Fr5*. Although the Family B x Isolates 0-5-32 and SC20-21 interactions were tentatively classified as I, non-significant associations for *Fr5*-linked markers were observed. Therefore, we concluded that Family B carries an allele for resistance at another Fr gene. As discussed earlier, this gene has now been designated as *Fr9* (refer to Section 3.2.8).

3.2.6. *Fr6* and *Fr7*

Resistance genes *Fr6 and Fr7* in loblolly pine were identified and mapped segregating in progeny of selection C (Family C). The percentages of galled progeny for three different Family C matrix cells were tentatively classified as I. To identify markers significantly linked to phenotype, megagametophyte DNAs from the Family C × Isolate 3327-13-3 cell were subjected to BSA. Nine markers (subsequently reduced to eight) were found to be significantly associated with phenotype. Seven of the eight markers noted in the Family C × Isolate 3327-13-3 cell were also examined using megagametophyte DNAs from the Family C × Isolate NC2-36 cell and also found to be significantly associated with phenotype. Interestingly, while phenotype in these two matrix cells was associated with the same markers, the marker phase arrangements were reversed with respect to phenotype. For example, in the Family C × Isolate 3327-13-3 cell the band-absent allele for RAPD marker G13_480 was indicative of no-gall (the resistance allele) while the band-present allele was associated with gall (the susceptibility allele). Conversely, in the Family C × Isolate NC2-36 cell the opposite phase relationship was observed. This finding clearly indicates the presence of two different Fr factors where the resistance alleles are tightly linked in repulsion and interact differentially with isolates 3327-13-3 and NC2-36. The percent gall value (only 6%) from the Family C × Isolate SC20-21 cell (where the few galled progeny observed in this cell were interpreted as recombinant progeny which would lack resistance alleles at either gene) suggests that the resistance factors are two distinct genes rather than alleles of the same gene (although allelism cannot be ruled out as other factors such as a low level of contaminating virulence, if present in the inoculation could account for the few galls). Interpreting the few galled specimens as recombinants, the two resistance factors in selection C have been designated genes *Fr6* and *Fr7* with Isolate 3327-13-3 being avirulent against *Fr6* and virulent against *Fr7*, while Isolate NC2-36 was avirulent against *Fr7* and virulent against *Fr6*. Using the progeny from both the

3327-13-3 cell and the NC2-36 cell, a map containing the seven markers significantly associated with phenotype in each respective cell was generated for the resistance region. *Fr6* and *Fr7* (using the phenotype data from each of the two cells) were both placed in a ~6 cM region between their closest markers G13_480 and A20_1620 (Table 2). In light of the low percent gall value in the Family C × Isolate SC20-21 cell, markers were not assessed to confirm incompatibility. In the Family C × Isolate 0-5-32 matrix cell tentatively classified as C, non-significant marker-phenotype associations were observed confirming compatibility for both genes. The tentative C interaction for the Family C × Isolate NC 2-40 cell was not investigated with markers.

3.2.7. *Fr8*

Resistance gene *Fr8* in loblolly pine was identified and mapped segregating in progeny of selection D (Family D). Only the Family D × Isolate SC20-21 matrix cell was tentatively classified as I. To identify markers significantly linked to *Fr8*, megagametophyte DNAs for the Family D × Isolate SC20-21 cell were subjected to BSA. A total of five markers significantly associated with phenotype were found (Table 2). The two closest linked markers to *Fr8* are B20_480 and A12_725 which showed no recombination with each other and are estimated to be ≤3 cM from *Fr8*. Marker B20_480 is linked in coupling with *Fr8* while marker A12_725 is linked in repulsion. *Fr8*-linked marker analysis in the Family D × Isolate 0-5-32 cell tentatively classified as C confirmed compatibility, but none of the other tentative C interactions with Family D were marker investigated.

3.2.8. *Fr9*

In our previous consideration of Family B we noted that a second Fr gene, one other than *Fr5*, must be responsible for the tentative I classifications with Isolates SC20-21 and 0-5-32. As previously noted, genes *Fr2*, *3*, *4*, *6*, *7*, and *8* can be ruled out as candidates for the second gene, based on interaction classifications in the matrix cells, but given that both Isolates SC20-21 and 0-5-32 are avirulent against resistance gene *Fr1*, it was possible that the I classifications observed for these cells were due to *Fr1* resistance in Family B. However, as discussed in this section, *Fr1* does not appear to be the second gene in Family B, and the I classifications had to be due to a ninth gene termed *Fr9*. Marker investigations using the Family B × Isolate 0-5-32 matrix cell revealed a total of six markers significantly associated with phenotype (Table 2). Markers J7_1840 and K14_0440 were the two closest markers and were estimated to be ~11 and ~13 cM, respectively, from the *Fr9* locus, which mapped to a terminal position as previously seen with *Fr3* and *Fr4*, which also share the J7_1840 marker as a nearest marker (Table 2). Markers J7_1840 and K14_440 were both linked in coupling with *Fr9*. *Fr9*-linked marker analysis within the Family B x Isolate SC 20-21 matrix cell suggests that *Fr9* is likely to be the gene responsible for the observed I interaction in that cell. One of the six markers significantly associated with phenotype in the Family B × Isolate 0-5-32 cell was maker AJ4_420, which is linked with *Fr1* in selection 10-5 (Table 2). However, in selection 10-5 the AJ4_420 marker is one of the two closest markers to *Fr1* and is very tightly linked to *Fr1* at a distance of ~1 to 2 cM, while in selection B the AJ4_420 marker is nearly 30 cM from the resistance gene that we have termed *Fr9*. The very tight association of AJ4_420 with *Fr1* has been seen in multiple tests, so if *Fr1* were the second resistance gene in Family B, it should have been much closer to the AJ4_420

marker. The distance of AJ4_420 from the resistance gene in selection B and the closer proximity of four other markers (Table 2) was used to rule out *Fr1*, leaving us with our ninth resistance gene, *Fr9*. Neither of the two Family B matrix cells (Family B × Isolates 3327-13-3 and NC2-40) tentatively classified as C interactions for both *Fr5* and *Fr9* were subjected to marker-phenotype analysis. Further evidence that the second resistance gene in Family B could not be *Fr1* is provided by Isik *et al.* [42], where 10 field-collected inocula were used to challenge the families having resistance genes *Fr1* through *Fr9*. In Isik *et al.* [42] Table 4 shows that disease incidence values for progeny carrying the resistance allele of *Fr1* (these being designated R1 in that table) *vs.* those carrying only the *Fr9* resistance allele (these being designated r5R9 progeny in that table) were very different for multiple inocula and on average across the 10 inocula. That should not have been the case if *Fr1* and *Fr9* were one and the same. For correlative purposes, the Isik *et al.* [42] family designations F1, F2, F3, F4, F5&9, F6&7, and F8 respectively correspond to selections/families 10-5, A, 152-329, 29R, B, C, and D in the current work.

3.3. Genomic Localization of Fr Genes

One can gain understanding regarding homology among linkage groups in different parental selections by examining the groups for shared markers. Based on comparisons of shared markers amongst the individual Fr linkage groups of our studied selections (refer to Table 2), it is apparent that *Fr1*, *3*, *4*, *6*, *7*, and *9* all share markers, suggesting that these genes represent a linked cluster within the same linkage group. Also, *Fr5* and *8* share markers, suggesting they are clustered in a single linkage group, and based on marker comparisons with the full map of selection 10-5 [40] this linkage group differs from that of the *Fr1*, *3*, *4*, *6*, *7*, and *9* group. No *Fr2*-linked markers were shared with the other Fr genes, and the linkage group association of *Fr2* was unknown until an *Fr2*-linked marker (as covered in this section) was investigated in the loblolly pine reference mapping population. Prior to the discovery of *Fr9*, some of the Fr-linked RAPD markers from the *Fr1*, *3*, *4*, *6*, *7*, and *9* cluster, from the *Fr5* and *8* cluster, and from *Fr2* were scored in a progeny sample of the loblolly pine *base* and *qtl* reference pedigrees to determine their positions relative to the reference genetic map [37]. Table 3 shows assessed markers and the reference linkage group to which the various markers mapped. Placing these Fr-linked markers within the loblolly pine reference map shows that linkage group 2 contains the *Fr1*, *3*, *4*, *6*, *7*, and *9* cluster, that linkage group 3 contains *Fr2*, and that linkage group 10 contains *Fr5* and *Fr8* (Figure 1). Both *Fr4* and *Fr9* were placed based on shared markers as none of the *Fr4*-linked markers (Table 2) segregated in the reference pedigrees and *Fr9* was discovered after this mapping work was completed. This reference mapping effort clearly confirms our earlier linkage group observations concerning the *Fr1*, *3*, *4*, *6*, *7*, and *9* cluster, as well as *Fr5* and *Fr8*, and places *Fr2* in a third distinctly separate linkage group.

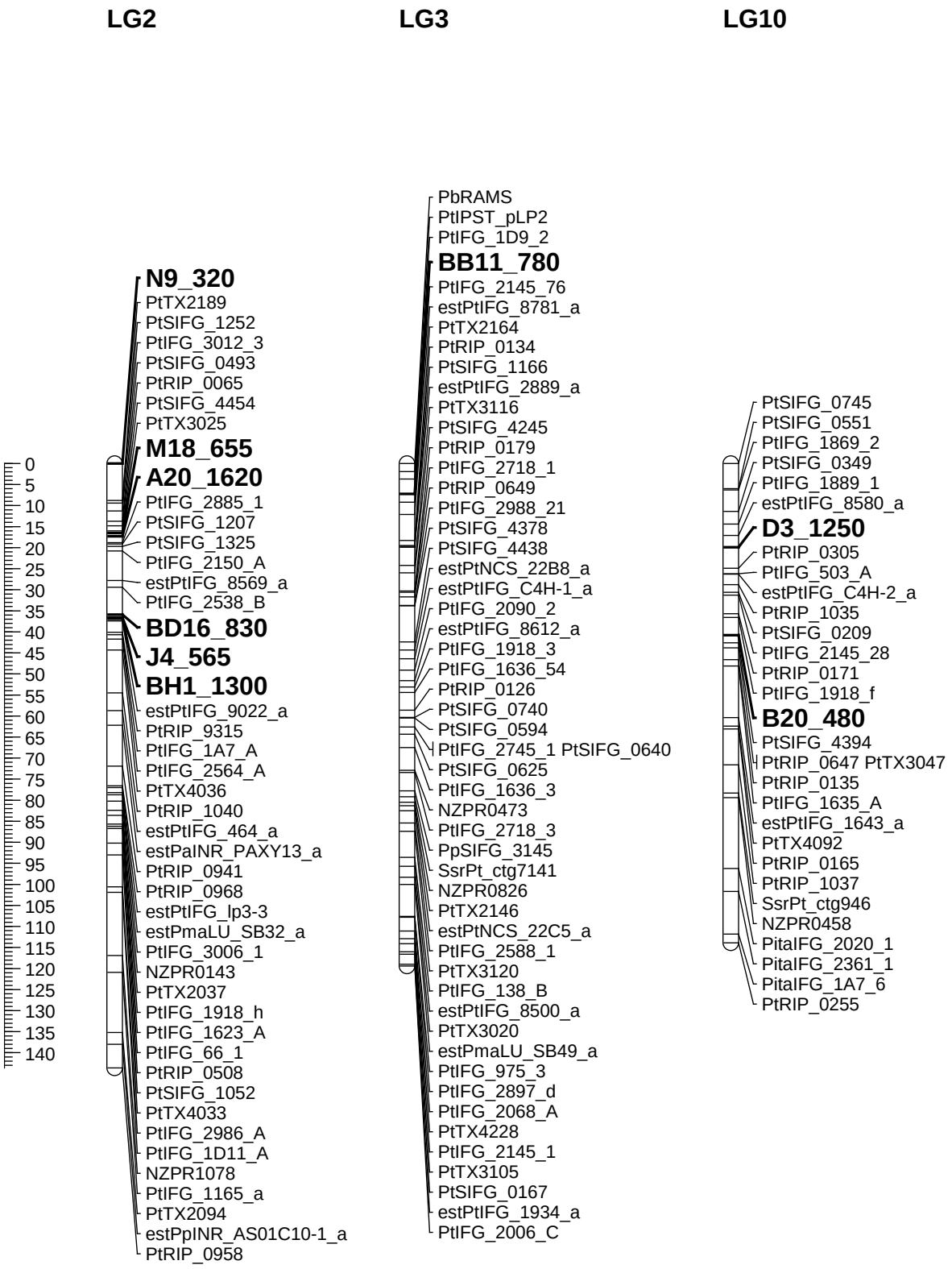

Figure 1. Loblolly pine reference genetic map linkage groups 2, 3, and 10 [37], showing the positions of the Fr gene-linked RAPD markers noted in Table 3. RAPD markers are in larger font and bold type.

Table 3. Loblolly pine reference genetic map [37] linkage group association of *Fr* gene-linked RAPD markers (from Table 2) investigated in the loblolly pine *base* and *qtl* mapping population.

RAPD Markers	Fr Gene/Cluster	Pedigree	Reference LG
BD16_830	*Fr1*, *Fr3*, *Fr4*, *Fr6*, *Fr7*, *Fr9*	*base*	2
BH1_1300	*Fr1*, *Fr3*, *Fr4*, *Fr6*, *Fr7*, *Fr9*	*base*	2
J4_565	*Fr1*, *Fr3*, *Fr4*, *Fr6*, *Fr7*, *Fr9*	*base*	2
N9_320	*Fr1*, *Fr3*, *Fr4*, *Fr6*, *Fr7*, *Fr9*	*base*	2
BB11_780	*Fr2*	*base*	3
B20_480	*Fr5*, *Fr8*	*base*	10
A20_1620	*Fr1*, *Fr3*, *Fr4*, *Fr6*, *Fr7*, *Fr9*	*qtl*	2
BD16_830	*Fr1*, *Fr3*, *Fr4*, *Fr6*, *Fr7*, *Fr9*	*qtl*	2
BH1_1300	*Fr1*, *Fr3*, *Fr4*, *Fr6*, *Fr7*, *Fr9*	*qtl*	2
M18_655	*Fr1*, *Fr3*, *Fr4*, *Fr6*, *Fr7*, *Fr9*	*qtl*	2
BB11_780	*Fr2*	*qtl*	3
D3_1250	*Fr5*, *Fr8*	*qtl*	10

4. Discussion

4.1. Fr Gene Identification and Genomic Mapping

Fr genes are discrete, apparently single major fusiform rust resistance genes involved in the regulation of gall formation (presence *vs.* absence). Furthermore, in order to be named/numbered (e.g., *Fr1* or *Fr3*, *etc.*) as a new Fr gene, the Fr gene in question has to be demonstrably different from previously named/numbered Fr genes. Through our family × isolate inoculation matrix and genetic mapping, we have shown that *Fr1–Fr9* are pathotype-specific and demonstrably different, and this has led us to commonly refer to Fr genes in general as pathotype-specific. Presumably, all Fr genes, given adequate investigation, would be recognizably pathotype-specific; however, the demonstration of pathotype specificity for an Fr gene is not a requirement. Notably, in the absence of recognized pathotype specificity, Quesada *et al.* [43] have reported the use of SNP (single nucleotide polymorphism) mapping techniques for loblolly pine, which can place potentially new Fr genes into linkage groups where none were previously known, thereby distinguishing them from previously known Fr genes. However, in the investigation of a given Fr gene, if the given gene in question maps similarly to a previously designated Fr gene (e.g., *Fr1*), is not known to be from the pedigree of the previous Fr gene, and shows the same disease interactions as the previous Fr gene when tested with the set of isolates previously used, then the gene in question should be designated the same as its apparent equivalent with the name being extended by a subscript X (e.g., $Fr1_x$). With our current technology, we cannot say with certainty if the two genes are indeed the same, but adhering to the Occam's razor principle they should be considered the same until shown otherwise [26]. In the event that subsequent testing differentiates the two genes, then the gene with the X designation would become a new named/numbered Fr gene.

The nine Fr genes reported here have been localized to three linkage groups on the loblolly pine reference genetic map (linkage groups 2, 3, and 10 [37]). Gaining better resolution regarding the positions of these genes relative to highly polymorphic markers such as SSRs and a large number of

biallelic markers such as SNPs is an important research objective moving forward. Higher resolution mapping of these and additional Fr genes will allow for more efficient integration of Fr gene testing into breeding programs by allowing the breeder to better predict the Fr gene status of candidate selections, possibly eliminating the need for costly artificial inoculation tests. In addition, the improved mapping will allow the Fr gene regions to be placed in the context of the newly released loblolly pine draft genome sequence [44] and this placement will provide a rich source of sequences for additional marker development and exploration of candidate gene function.

As a safeguard for future mapping efforts where the protocol involves the use of single-genotype isolates, one needs to have knowledge of pathogen purity and avoid pathogen contamination, where avirulence for the target gene(s) may be contaminated with some frequency of virulence for the same gene(s). We recommend that the single-genotype isolates (maintained as urediniospores) be monitored with a set of informative markers such as SSRs [28,29] for the presence of contamination and that contaminated lines be subjected to the SUP protocol (Supplemental File 1) for purification. Additionally, basidiospore inocula derived from single-genotype isolates should, prior to pine inoculation, also be assessed for contamination with informative markers, and discarded if contamination is observed. In addition, in tests where more than one single-genotype isolate is used, steps should be taken to minimize the possibility of cross-contamination during inoculations and incubations.

Six Fr-linked markers, all from the Fr *1*, *3*, *4*, *6*, *7*, and *9* cluster mapped to reference linkage group 2, clearly demonstrating the importance of this linkage group with regards to the genetics of fusiform rust resistance. However, it should be noted that the internal position of marker M18_655 in the reference mapping population differs from its placement in selection 152-329 [21], where marker M18_655 was located as a near terminal marker in the linkage group. The reason for this discrepancy is unclear, but irrespective of the differing placements for this marker, either placement is consistent with the inclusion of *Fr3* in reference linkage group 2. It is important to consider the consequences of finding the Fr genes clustered in two of the three linkage groups covered in this work. Initially, clustering will impact fine structure mapping and cloning efforts. If clustering turns out to be true for all or most Fr genes, knowledge of clustering could expedite the mapping of yet unidentified genes in new selections as many of the previously identified markers will prove useful for marker-phenotype association studies. However, clustering could prove both beneficial and at the same time potentially detrimental in regards to cloning Fr genes. Because a particular genomic region may contain multiple Fr genes, the likelihood of identifying and cloning at least one Fr gene is enhanced. However, determining the unique identity of a putative cloned Fr gene may prove problematic, especially if the putative gene is a currently unidentified Fr gene, *i.e.*, an Fr gene other than *Fr1–Fr9*. Also, Fr gene clustering patterns, be they the same or different, will have phylogenetic implications amongst the pine species that are hosts for *Cqf*. Furthermore, Fr gene clustering has strong implications for both resistance utilization and breeding, especially with regards to resistance pyramiding. For example, if a selection carried two unlinked heterozygous Fr genes it would be expected to transmit dual resistance to approximately 25% of its progeny. In contrast, if the two heterozygous Fr genes are tightly linked but in repulsion phase then it becomes very difficult for this selection to transmit both resistance alleles to its offspring, the rate of dual transmission being dependent upon recombination between the two genes.

4.2. Fr2 Clarification

The designation *Fr2* as used in this manuscript refers to the *Fr2* gene described in the current work (Results section), which differentially conditions resistance in Family A (Table 1). Prior to the detection of this *Fr2* gene, Wilcox [40], working with Family A progeny planted in a diallel field trial (challenged with ambient inoculum), detected significant associations between disease phenotype (gall *vs.* no gall) and two RAPD markers (A11_400 and A19_560). Although the two markers were both associated with phenotype, the markers themselves were unlinked, making it unclear whether the two markers flanked a single resistance locus or if two unlinked resistance loci were segregating. Even though Wilcox [40] did not give this resistance locus/loci an Fr designation, U.S. Patent #5,908,978 [45] utilized these data and named the locus *Fr2*.

Given the uncertainty of one locus *vs.* two as denoted by Wilcox [40], the *Fr2* designation in the patent was unwarranted, and until additional studies can resolve the issue of how many loci are involved and provide further insight into the resistance described by Wilcox in selection A, no numbered Fr designation should be assigned. However, we do know that the resistance detected in selection A [40] differs from the resistance that we now designate as *Fr2*, as our *Fr2* marker, AK6_850, was assessed in the same diallel samples used by Wilcox and found not to be significantly associated with the markers detected, or the disease phenotype observed in the Wilcox study (Henry V. Amerson unpublished data cited in [39]). Also, from our studies of Family A, it appears that the resistance observed in selection A by Wilcox [40] differs from all nine of the Fr genes designated in this paper. This is reflected by the finding that incompatible interactions seen for Family A progeny and two Isolates, 0-5-32 and NC-240, were significantly associated with the markers for our *Fr2* gene, while interactions with our other three isolates were compatible. This suggests that all five of the matrix isolates would carry virulence towards the resistance detected by Wilcox [40] in selection A and that would distinguish it from our current nine Fr genes. Once the issue of how many loci are involved in the resistance described for selection A [40] is resolved, we expect that would add to our current list of named Fr genes.

In addition to the *Fr2* designation noted in the patent [45] and prior to our detection of *Fr2* as described in this current work, Kong [46] used the designation *Fr2* for the naming of a fusiform rust resistance gene detected in slash pine. Kong [46] mapped the resistance gene in slash pine selection D4PC40 using progeny obtained from D4PC40 and a polymix of pollen from 10 rust-susceptible trees. Basidiospores used to challenge these progeny were derived from a mixed gall rust collection obtained from Louisiana. Three coded RAPD markers, G12A, B17A, and 324B were significantly associated with the gall/no gall phenotype trait locus that was named gene *Fr2* [46]. However, seemingly no comparisons of the resistance gene in slash pine selection D4PC40 with the originally mapped *Fr1* gene in loblolly selection 10-5 [19,40], the only published Fr gene at that time, were made. In the absence of evidence that this slash pine gene differed from *Fr1,* we argue that it should not have been named *Fr2* (*i.e.*, the name *Fr2* was inappropriate), because assigning the name *Fr2* implied that it was different from *Fr1*. The current authors are not aware of instances in the peer-reviewed literature where consideration of the *Fr2* gene did not refer to the *Fr2* gene defined in the results section of the present study.

4.3. Summation and Perspectives

In this paper we presented an interaction matrix of I and C classifications for seven loblolly pine host families challenged by each of five single-genotype isolates of the fusiform rust fungus. Interactions in the matrix (where all incompatible interactions conditioned by a single gene were confirmed by genetic markers) revealed nine pathotype-specific Fr genes residing in the seven host families. We described the experimental evidence for the presence and uniqueness of each Fr gene and developed and analyzed data showing their genomic localization within the loblolly pine reference genetic map.

Although nine different Fr genes have been identified and mapped in the seven host families used in the matrix, it is likely that additional Fr genes exist within loblolly pine. Currently (with additional loblolly pine selections and an additional *Cqf* isolate), we have data suggesting that at least four additional Fr genes exist, but these data were not presented as the Fr genes are as yet inadequately genetically mapped. Given the nine Fr genes that have been identified in the current work and data tentatively suggesting four additional Fr genes, this pathosystem may prove to be highly complex, similar to the cereal rust pathosystems reviewed by J. Kolmer [30]. In addition, because the pine-fusiform rust pathosystem has most likely coevolved for millions of years [47], the existence of numerous Fr genes seems probable.

Taken together, these data provide a much improved understanding of the genetic basis of the observed resistance (no gall *vs.* gall) within loblolly pine. Clearly much work needs to be done towards providing a reliable system for predicting the Fr gene status for any loblolly pine selection. However, the development, maintenance, and continued use of specific differential host families and single-genotype pathogen isolates are critical to further advancing our understanding of this pathosystem. The implications of correctly interpreting the pathosystem are critical both for tree breeding and seedling and clonal deployment in production forestry and for understanding the evolutionary dynamics of host-pathogen systems.

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Author Contributions

All authors participated in the research over the years. Henry V. Amerson led the research efforts, and Amerson, Thomas L. Kubisiak, and C. Dana Nelson conducted the analyses and prepared the manuscript. E. George Kuhlman developed and provided the *Cqf* single-genotype isolates, and several of the resistant loblolly pine selections were included based on his prior investigations. Kuhlman also conducted and evaluated many of the inoculations. Saul A. Garcia provided technical assistance and was substantially involved in many aspects of the work, especially genetic marker investigations.

Conflict of Interest

The authors declare no conflict of interest.

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