Theor Appl Genet (2005) 110: 948–958
DOI 10.1007/s00122-004-1915-2

ORIGINAL PAPER

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Genetic dissection of fusiform rust and pitch canker disease traits in loblolly pine

Received: 18 July 2004 / Accepted: 16 December 2004 / Published online: 8 February 2005
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Abstract Loblolly pine (*Pinus taeda* L.) exhibits genetic resistance to fusiform rust disease (incited by the biotrophic fungus, *Cronartium quercuum* f. sp. *fusiforme*) and pitch canker disease (incited by the necrotrophic fungus, *Fusarium circinatum*). In this study, a total of 14,015 loblolly pine cuttings from 1,065 clones were screened in controlled greenhouse conditions to identify phenotypes of clones, families, and parents that guide a genetic dissection of disease traits associated with pitch canker and fusiform rust. A total of 23,373 phenotypic data points were collected for lesion length (pitch canker) and gall score, gall length, and gall width (fusiform rust). We verified heritable fusiform rust and pitch canker traits and calculated parental, clonal, and full-sib family rankings for both diseases. Genetic correlations revealed that traits associated with fusiform rust are genetically distinct from one another, and that the genetic mechanisms underlying pitch canker and fusiform rust resistance are independent. The disease phenotyping described here is a critical step towards identifying specific loci and alleles associated with fusiform rust and pitch canker resistance.

Communicated by D.B. Neale

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Introduction

Pinus species are both economically and ecologically important. Pines grown in the southeastern United States generate nearly half of the nation's pulpwood, with an annual harvest value of approximately $19 billion (McKeever and Howard 1996). Loblolly pine (*Pinus taeda* L.) is the most widely planted *Pinus* species in this region, averaging 74% of the annual seedling production (Carey and Kelley 1993). In addition to plantations, loblolly pine is the predominant species on 11.7 million ha of native forest (Baker and Langdon 1990), where it impacts the welfare of nearly 400 species of vertebrates (Schultz 1999).

Loblolly pine is a host for two endemic pathogens, *Cronartium quercuum* Berk. Miyable ex Shirai f. sp. *fusiforme* (Burdsall and Snow 1977), the inciting agent of fusiform rust disease, and *Fusarium circinatum* Nirenberg et O'Donnell (Nirenberg and O'Donnell 1998), the inciting agent of pitch canker disease. Fusiform rust is one of the most destructive fungal diseases in the southeastern United States, causing damage ranging from $25–$135 million per year (Cubbage et al. 2000). The major symptom of fusiform rust disease is the formation of stem galls that lead to decreases in survival, wood quality, and growth. Genetic variation in resistance at the family level has been demonstrated for fusiform rust (Kuhlman and Powers 1988; McKeand et al. 1999). Based on controlled inoculation studies carried out on specific loblolly and slash pine *Pinus elliottii* Engelm. var. *elliottii*) families, specific resistance—i.e., "gene-for-gene" interactions—has evolved (Powers 1980; Stelzer et al. 1997; Wilcox et al. 1996), as well as partial resistance in the form of short galls (Schmidt et al. 2000), which may be genetically distinct from specific resistance.

Pitch canker is also a significant, although more episodic, disease problem (Dwinell et al. 1985). Symptoms of pitch canker disease include resinous lesions on stems and branches that cause seedling mortality,

decreased growth rates, and crown dieback (Dwinell et al. 1985). A considerable amount of genetic variation for pitch canker resistance has been detected in loblolly pine families (Kuhlman et al. 1982) and clones (Dwinell and Barrows-Broaddus 1981); however, the genetic architecture of resistance is not well understood.

Our goal in this work was to obtain precise estimates of pitch canker and fusiform rust disease phenotypes expressed in loblolly pine. Precision was acquired by a combination of clonal propagation, which allows repeat observations of the same genotypes and is now feasible in loblolly pine (Frampton et al. 2000), testing of over one thousand pedigreed genotypes, and the use of a mixed linear model (GAREML) to adjust for environmental effects (Huber 1993). In this study, we identified traits, clones, families, and parents that guide a genetic approach to dissecting disease traits in loblolly pine. We verified that pitch canker and fusiform rust traits are heritable and identified the disease traits that are genetically distinct from one another. This work creates the baseline knowledge required for identifying genes that condition phenotypes of interest, either through genetic linkage analysis within defined pedigrees, or by association in populations of unrelated genotypes (Flint-Garcia et al. 2003; Jannink et al. 2001).

Materials and methods

Genetic material, plant propagation, and experimental design

The 63 loblolly pine families screened in this study were obtained from a circular mating design with some off-diagonal crossing (Fig. 1). Members of the Cooperative Forest Genetics Research Program at the University of Florida and the North Carolina State University–Industry Cooperative Tree Improvement Program provided the 32 parents and generated the full-sib families and clones screened in this study.

Forty-nine seeds from each full-sib family were germinated and grown into hedges for clonal propagation. Maintenance of hedges and propagation of cuttings is reported in Baltunis et al. (2005). In brief, cuttings were set in July 2001, assessed for rooting after 9 weeks, and clones with the highest rooting ability selected for this experiment. The number of clones within families and the number of ramets (i.e., rooted cuttings) for each clone was not equal, since families did not produce the same number of clones, and clones had different rates of rooting. Cuttings assigned to a greenhouse screen were chosen at random from the ramet pool of each available clone (Table 1). The screens were grouped according to the disease (fusiform rust or pitch canker). The fusiform rust screens were conducted using two types of inoculum (a one-gall mix or a ten-gall mix), whereas both pitch canker screens used a single inoculum. The experimental design was a randomized complete block with single-tree plots arranged in an alpha lattice with an incomplete block size of 20. The clones were replicated with a maximum number of five ramets per experiment. Ramets were pruned twice to stimulate synchronous elongation of multiple succulent shoots for inoculation. The initial pruning occurred in March 2002, 8 months after setting, by cutting back the shoots from 10–15 cm to 3–4 cm each. The second pruning occurred 6 weeks prior to inoculations for both pitch canker and fusiform rust screens; shoots were succulent and 5–8 cm in length at the time of inoculation. After pruning, all trees were fertilized weekly with Miracle-Gro 15-30-15 until inoculation.

ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1		16	17				18										19															
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3	34	23		19	19																											
4		89	37			15																										
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27		54																							31	32		20				
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30		12																										53				
31																																19
32																				61										4		

Fig. 1 A circular mating design was used to generate the plant material. Thirty-two parents were crossed following a circular design, and the resulting progeny was used as the material screened for this study. The *numbers in the cells above the diagonal* are the number of clones used from a given cross, and the *numbers below the diagonal* are the family identification numbers

Table 1 Summary of the four inoculation experiments reported in this study

Test[a]	No. of families	No. of clones	Range[b]	No. of ramets	No. of observations[c]
RSC pitch canker	63	1065	7–31	4,483	7,664
UF pitch canker	60	362	1–24	1,316	3,119
Ten-gall fusiform rust	63	1,360	17–31	5,473	11,395
One-gall fusiform rust	63	698	2–30	2,743	5,195

[a]*RSC* USDA Forest Service Resistance Screening Center, *UF* University of Florida
[b]Number of clones within families
[c]Number of observations exceeds the number of ramets because multiple shoots were assessed on a given ramet

Pitch canker: inoculations and data collection

The larger of the two pitch canker screens was conducted at the USDA Forest Service Resistance Screening Center in Bent Creek, North Carolina, and is referred to as the "RSC" screen in this manuscript. New growth (5–7 cm) was inoculated following the standard RSC protocol (Oak et al. 1987) with *F. circinatum* isolate S45 (Forest Pathology laboratory collection, University of Florida) at a density of 92,500 spores/ml. In brief, prior to spray inoculation, shoot tips were excised from two shoots on each ramet. After inoculation, plants were placed in a high-humidity chamber for 24 h, then transferred to a greenhouse and maintained at an average temperature of 20°C for 3 months.

The smaller of the two pitch canker screens was conducted at the University of Florida and is referred to here as the "UF" screen. Plants were pruned 6 weeks before inoculation with the same S45 isolate. One shoot tip per ramet was excised, and 1 μl of a 500-spores/μl solution was applied to the wound with a micropipette. All plants were incubated under high humidity for 24 h. The test was kept in the greenhouse for 36 days at an average temperature of 30°C.

Disease symptoms were measured at 90 days (RSC) and 36 days (UF). Shoot length and lesion length were measured (in millimeters) on one shoot chosen at random from each ramet at the RSC and on the single shoot inoculated per ramet at UF.

Both the RSC and UF pitch canker raw data sets included only one lesion-length and shoot-length measurement for each ramet. Prior to analysis, the data were standardized by experiment, using the phenotypic standard deviation calculated from the appropriate linear model for the screen.

Fusiform rust: inoculations and data collection

Plants were pruned twice before inoculation to stimulate elongation of multiple shoots per ramet. Both rust screens were inoculated at the RSC following standard protocols (Knighten 1988). The ten-gall test was inoculated at a density of 52,000 spores/ml with aeciospores pooled from a ten-gall collection obtained from a 6-year-old loblolly pine plantation near Lee, Florida (designated L-10-1-99, provided by Dr. Henry Amerson, NC State University) The one-gall test was inoculated at a density of 50,700 spores/ml with aeciospores isolated from a single gall obtained from a branch of slash pine family 84-57 near Callahan, Florida (designated #501, provided by Dr. Robert Schmidt, University of Florida).

Data were collected from both rust screens 6 months after inoculation. For each ramet with multiple shoots, the number of shoots with galls and the number of galls were counted and recorded. In addition, two shoots with single galls were randomly chosen to measure stem length, gall length, and gall width (in millimeters) for each ramet.

Data collected from both the ten-gall and one-gall screens were treated identically for gall measurements. Gall measurement values were averaged by ramet if there was more than one shoot with a single gall. Gall volume was calculated from gall length and gall width data, assuming a fusiform shape:

$$\text{Volume} = \frac{3}{4}\text{length} \times \text{width}^2$$

Ramets were scored as 0 (no gall) or 1 (at least one gall) for gall score. Ramets that did not form galls were not included in the gall length, width, and volume data. Gall length, width, and volume data sets were standardized using their respective phenotypic standard deviations calculated from the linear model.

Estimation of genetic parameters

Variance components and genetic parameters were estimated by GAREML (Huber 1993), which employs restricted maximum likelihood estimation (Patterson and Thompson 1971) and best linear unbiased prediction [(BLUP) Henderson 1973]. The same linear model was applied to the traits measured in all four disease screens since the experimental designs were identical. The linear model was:

$$y_{ijklm} = \mu + R_i + t(r)_{ij} + \text{gca}_k + \text{gca}_l + \text{sca}_{kl} + c(\text{family})_{klm} + r * f_{ikl} + e_{ijklm}$$

where:

- y_{ijklm} is the mth observation of the klth cross in the jth tray of ith rep.
- μ is the population mean.

- R_i is the fixed resolvable replication, $i = 1$–5.
- $t(r)_{ij}$ is the random variable tray incomplete block $\sim$NID$(0,\sigma^2_t)$, $j = 1$–21.
- gca_k is the random variable female general combining ability (GCA) $\sim$NID$(0,\sigma^2_{gca})$ $k = 1$–32.
- gca_l is the random variable male general combining ability $\sim$NID$(0,\sigma^2_{gca})$ $l = 1$–32.
- sca_{kl} is the random variable specific combining ability (SCA) $\sim$NID$(0,\sigma^2_{sca})$.
- $c(\text{family})_{klm}$ is the random variable clone within a family $\sim$NID$(0,\sigma^2_{c(\text{family})})$.
- $r{*}f_{ikl}$ is the random variable replication by family interaction $\sim$NID$(0,\sigma^2_{r*f})$.
- e_{ijklm} is the random variable error within the experiment $\sim$NID$(0,\sigma^2_e)$.

The narrow- (h^2) and broad-sense (H^2) heritabilities were calculated according to Falconer and Mackay (1996):

$$h^2 = \frac{4\hat{\sigma}^2_{gca}}{\hat{\sigma}^2_P} = \frac{\hat{V}(A)}{\hat{V}(P)}$$

$$H^2 = \frac{2\hat{\sigma}^2_{gca}+\hat{\sigma}^2_{sca}+\hat{\sigma}^2_{c(f)}}{\hat{\sigma}^2_P} = \frac{(\hat{V}(A)+\hat{V}(D)+\hat{V}(I))}{\hat{V}(P)}$$

where:

- $\hat{\sigma}^2_P$is the phenotypic variance.
- $\hat{V}(P)$is the total phenotypic variance.
- $\hat{V}(A)$is the additive variance.
- $\hat{V}(D)$is the dominance variance
- $\hat{V}(I)$is the epistatic variance.

To partition the broad-sense heritability derived above, we calculated the ratio of dominance variance to total phenotypic variance ($\hat{h}^2_D$) and the ratio of epistatic variance to total phenotypic variance ($\hat{h}^2_I$) using the following formulas:

$$\hat{h}^2_D = \frac{4\hat{\sigma}^2_{sca}}{\hat{\sigma}^2_P} = \frac{\hat{V}(D)}{\hat{V}(P)}$$

$$\hat{h}^2_I = \frac{\hat{\sigma}^2_{c(f)} - (2\hat{\sigma}^2_{gca} + 3\hat{\sigma}^2_{sca})}{\hat{\sigma}^2_P} = \frac{\hat{V}(I)}{\hat{V}(P)}$$

The broad-sense heritability of clonal means (H^2_C) and family means (H^2_F) were calculated using the formulae below:

$$\hat{H}^2_C = \frac{(2 * \hat{\sigma}^2_{gca}) + \hat{\sigma}^2_{sca} + \hat{\sigma}^2_{c(f)}}{(2 * \hat{\sigma}^2_{gca}) + \hat{\sigma}^2_{sca} + \hat{\sigma}^2_{c(f)} + (\hat{\sigma}^2_{r*f}/r) + (\hat{\sigma}^2_e/r)}$$

$$\hat{H}^2_F = \frac{(2 * \hat{\sigma}^2_{gca}) + \hat{\sigma}^2_{sca}}{(2 * \hat{\sigma}^2_{gca}) + \hat{\sigma}^2_{sca} + (\hat{\sigma}^2_{C(f)}/c) + (\hat{\sigma}^2_{r*f}/r) + (\hat{\sigma}^2_e/r * c)}$$

where r is the harmonic mean of ramets per clone, and c is the harmonic mean of clones per family.

Family deviations were predicted by summing the following BLUP estimates produced by GAREML: family deviation = predicted female value (gca_k) + predicted male value (gca_l) + predicted specific combining ability (sca_{kl}).

Genetic correlations

The genetic correlation between gall score and gall length at the parental, family, and clonal levels, and the correlation among screens within and across diseases were calculated on combined data sets by adding experiment × GCA (σ^2_{ge}), experiment × family (σ^2_{se}), and experiment × clone(family) ($\sigma^2_{c(f)e}$) interaction factors to the linear model and using the Type B genetic correlation formula (r_B, Yamada 1962):

$$(r_B)_{\text{Parental}} = \frac{\hat{\sigma}^2_{gca}}{(\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{ge})}$$

$$(r_B)_{\text{Family}} = \frac{2\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{sca}}{2\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{sca} + 2\hat{\sigma}^2_{ge} + \hat{\sigma}^2_{se}}$$

$$(r_B)_{C(F)} = \frac{2\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{sca} + \hat{\sigma}^2_{C(F)}}{2\hat{\sigma}^2_{gca} + \hat{\sigma}^2_{sca} + \hat{\sigma}^2_{c(f)} + 2\hat{\sigma}^2_{ge} + \hat{\sigma}^2_{se} + \hat{\sigma}^2_{c(f)e}}$$

Efficiency of using multiple ramets per genotype was calculated according to Huber et al. (1992):

$$\text{Efficiency} = \frac{1 - H^2}{r}$$

where r is the number of ramets per clone.

Results

The mating design shown in Fig. 1, coupled with clonal propagation, allowed predictions of clonal, family, and parental genotypic values as well as population-wise estimates of heritabilities and genetic correlations of disease traits for both pathosystems. A total of 27,373 phenotypic data points were collected for lesion length (pitch canker), gall score, gall length, and gall width (fusiform rust). We first present data on pitch canker phenotypes, followed by fusiform rust, and finally a comparison of pitch canker and fusiform rust resistance.

Pitch canker disease resistance is heritable

The pitch canker disease screens performed at UF and RSC resulted in 89% of the ramets (i.e., rooted cuttings) showing measurable disease symptoms in each screen. BLUP clonal values were predicted for each screen and the resulting distributions are shown in Fig. 2a. The consistency of the disease rates and the shapes of the distributions (i.e., skewed to the right) suggest that statistical comparisons between the RSC

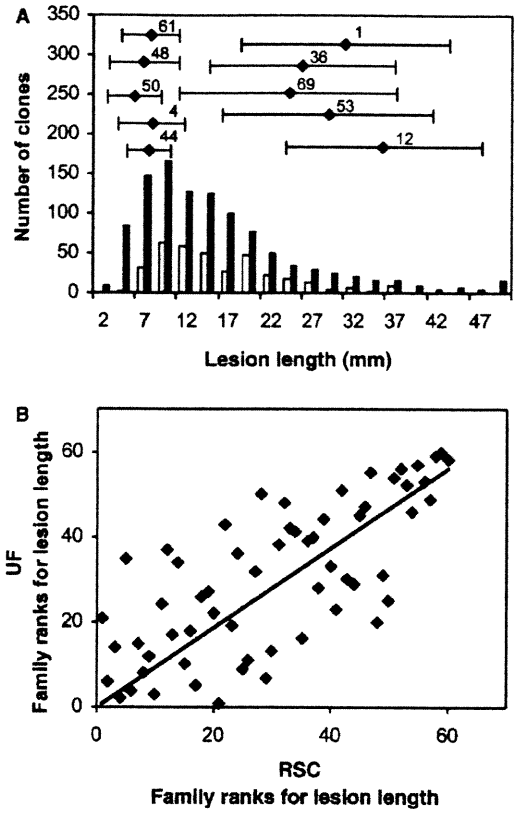

Fig. 2 Frequency distributions and genetic correlation for pitch canker lesion length. **a** Distribution of best linear unbiased prediction (BLUP)-predicted clonal values for the USDA Forest Service Resistance Screening Center [(*RSC*) *black*] and University of Florida [(*UF*) *white*] pitch canker screens. *Above the distributions* are the predicted means and standard deviations of the five most susceptible and resistant families identified by their family ID number. **b** Ranks based on BLUP-predicted family values for RSC and UF were plotted against each other (a rank of 1 is the most resistant and 63 the most susceptible). A least squares regression line is shown after being forced through the origin due to a non-significant intercept

and UF screens are appropriate. The genetic correlation between the RSC and UF screens was 0.88 at the parental level, 0.76 at the family level, and 0.69 at the clonal level. A scatter plot based on family ranks is presented in Fig. 2b and reflects the positive correlation between the two screens. Therefore, we conclude that parents, families, and clones performed consistently across screens.

After combining the data from the two screens, the five most resistant and the five most susceptible full-sib families were identified based on predicted family values and standard deviations for lesion length; these are indicated in Fig. 2a by their ID number from Fig. 1. The resistant tail contains families 50 and 48, which have parent 8 in common. The resistant tail also contains half-sib families 61 and 4, which share parent 32. Resistant family 44 is not related to any of the other resistant families in the tail (Fig. 1). The

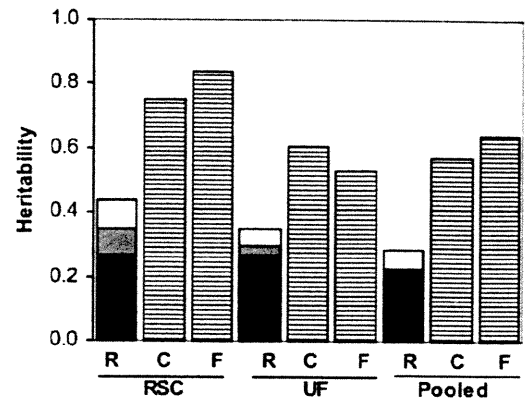

Fig. 3 Heritability estimates for pitch canker lesion lengths. The bar graph shows the heritabilities for individual ramets ($R=H^2$) and the broad-sense heritabilities for clonal ($C=H^2_C$) and family ($F=H^2_F$) means for the RSC, UF, and pooled data. Narrow-sense heritability [(h^2) *solid black*], epistatic heritability [(h^2_I) *solid gray*], and dominance heritability [(h^2_D) *white*] are stacked so that the *y-axis corresponding to the top of the bar* is the broad-sense heritability

susceptible tail is composed of three half-sib families. Susceptible family 12 has parent 30 in common with family 53. Family 12 also shares parent 2 with susceptible family 69. Susceptible families 1 and 36 have parent 17 in common. Families in the resistant tail and families in the susceptible tail did not have any parents in common, indicating no genetic relationships across the classes.

Two distinct inoculation procedures reveal similar heritabilities for lesion length

Using the RSC, UF, and pooled data, the heritabilities based on individual tree, family, and clonal means were calculated to determine how much of the variation in lesion length could be attributed to genetic variation and to determine the precision of the predicted clonal and family means. The broad-sense heritabilities for the clonal (H^2_C) and family (H^2_F) means were determined for both the individual and pooled pitch canker screens to evaluate the precision of the clonal and family means predicted above. H^2_C and H^2_F were greater for RSC (0.75) than for UF (0.61; Fig. 3), because the number of ramets per clone and the number of clones per family were approximately three times greater for the RSC screen compared to the UF screen (Table 1). Narrow-sense heritabilities (h^2) for both the RSC and UF datasets were 0.27. Broad-sense heritabilities (H^2) were similar for both the RSC (0.43) and the UF screens (0.37) (Fig. 3). When the RSC and UF data sets were pooled, heritabilities were not different from that calculated for each screen individually (Fig. 3). This is another indicator that results from the two screens were comparable.

Disease traits associated with fusiform rust are independently inherited

The two fusiform rust screens are characterized by the type of inocula used, either ten-gall or one-gall. There were 36% and 31% galled ramets for the ten-gall and one-gall screens, respectively. A disease incidence (referred to as "score") dataset was generated by designating disease-free ramets as 0 and galled ramets as 1. Fusiform rust screens for score are shown in Fig. 4a. The distributions for score in both screens follow a similar pattern, that is, there is a minor peak at a mean ~0.1, and the distribution is skewed to the right. In addition to disease incidence, gall length and gall width were measured for ramets with galls. In contrast to score, the predicted clonal means for gall length revealed a normal distribution for both fusiform rust screens (Fig. 4b). Because the distributions and overall disease incidences were similar, scaling prior to comparing the data from the two screens was not necessary for either trait.

Genetic correlations between the two screens were calculated for score and gall length in order to determine if inoculum type might impact trait expression. The genetic correlation for score was 0.80 at the parental level, 0.83 at the family level, and 0.86 at the clonal level, suggesting a general consistency in performance between the ten-gall and one-gall mixes. For gall length, the genetic correlation between the two screens was 1.00 at the parental level, 1.00 at the family level, and 0.76 at the clonal level, again indicating general consistency in performance between the two fusiform rust screens. Despite the high genetic correlations, we did observe "outlier" families that performed differently in the two screens, suggesting some potentially significant genotype by inoculum interactions (Fig. 4c, d).

Relationships among families with extreme phenotypes can reveal information regarding inheritance. For score, the predicted family values for the five most resistant and five most susceptible families are plotted on the graph in Fig. 4a, along with their within-family standard deviations and family ID numbers. The resistant tail contains two half-sib family groups, that is, families 26, 40, and 49 that have parent 9 in common, and families 52 and 53 that have parent 28 in common. The susceptible tail is composed of two families that are half-sibs, that is, families 3, 20, and 51 have parent 22 in common, and families 54 and 69 have parent 2 in common. Similarly for gall length, the five families with the shortest galls and the five families with the longest galls are shown above the distribution in Fig. 4b. The short gall-forming tail includes families 31 and 62 that have parent 25 in common. The remaining three families in this tail are unrelated. The five families with the longest galls comprise three half-sib families (13 and 29; 29 and 30; and 57, 13, and 57) that are related to one another through parents 21, 20, and 19, respectively. Family 15 is unrelated to the others. For both score and gall length, familial relationships within a given tail were common, whereas no such genetic relationships among families in opposing tails were observed. This is consistent with both score and gall length being heritable traits.

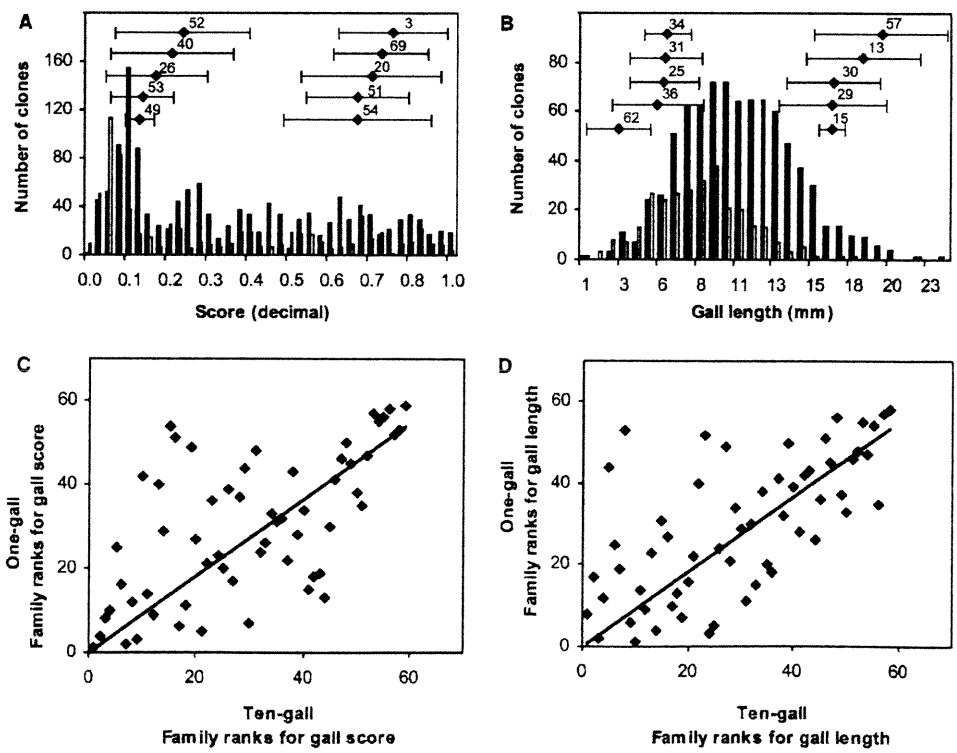

Fig. 4 Frequency distributions and genetic correlations for fusiform rust disease traits. Distribution of BLUP-predicted clonal values for gall score (**a**) and gall length (**b**) in the ten-gall inoculum (*black*) and one-gall inoculum (*white*) screens are shown. *Above the distributions* are the predicted values and standard deviations of the five most susceptible and resistant families identified by their family ID number. Ranks based on predicted family values for gall score (**c**) and gall length (**d**) (1 = resistant, 63 = susceptible) are plotted against each other. A least squares regression line is shown after being forced through the origin due to a non-significant intercept

To evaluate how much of the trait variation associated with fusiform rust can be attributed to genetic effects, heritabilities were calculated. Since the genetic correlations for score and gall length were high across inocula (Fig. 4c, d), data were pooled and used for heritability calculations. Gall score was consistently more heritable than gall length for the one-gall, ten-gall, and pooled datasets (Fig. 5a, b).

To determine whether the tendency to form galls (score) is related to the length of gall that is formed (gall length), genetic correlations were calculated and family ranks plotted for each trait (Fig. 5c). The lack of correlation between these traits indicates that gall length cannot be predicted from a family's tendency to form a gall and vice versa. In agreement with this, the estimated genetic correlation at the family level between score and gall length was very low (0.004). These results suggest different host genes control gall score and gall length.

Heritabilities for the other fusiform rust traits (gall width and gall volume) were lower than for score and gall length. The broad-sense heritabilities for gall width were calculated for the ten-gall ($H^2 = 0.05$) and one-gall ($H^2 = 0.14$) screens. Similar low values were obtained for gall volume ($H^2 = 0.05$ for ten-gall and $H^2 = 0.13$ for one-gall). The low heritability measures associated with gall width and gall volume suggest that these traits are affected more by environmental factors or measurement errors than are gall score and gall length.

Host genes underlying resistance to pitch canker and fusiform rust are independent

Necrotrophic (i.e., *F. circinatum*) and biotrophic (i.e., *C. quercuum)* pathogens have distinct life history properties. This implies that host genes underlying resistance may be different for diseases incited by necrotrophic and biotrophic pathogens. To determine whether host responses to *F. circinatum* and *C. quercuum* are independent, we computed the genetic correlations between lesion length (pitch canker) and the various gall characteristics (fusiform rust). There were no significant correlations between lesion length and gall length (Fig. 6), or between lesion length and gall score (data not shown). The estimated genetic correlation between lesion length and gall length were 0.00 at the parental level, 0.00 at the family level, and 0.02 at the clonal level. No genetic correlations were found between lesion length and gall volume or gall width (0.00 for all, data not shown). Together, these results imply that resistance to pitch canker and resistance to fusiform rust are controlled by different host genes.

Efficiency of using multiple ramets per genotype

Theoretically, if the number of ramets per genotype is high enough, heritability estimates based on clonal means will be 1. To describe the relationship between the number of ramets and H_C^2 for disease traits investigated in this study, the efficiencies (Huber et al. 1992) for increasing number of ramets per genotype were plotted against the number of ramets (Fig. 7), where efficiency is calculated as the average reduction in error per ramet. For the different disease traits, the error associated with a clonal mean decreases at different rates, depending on the number of ramets used to represent genotypes and

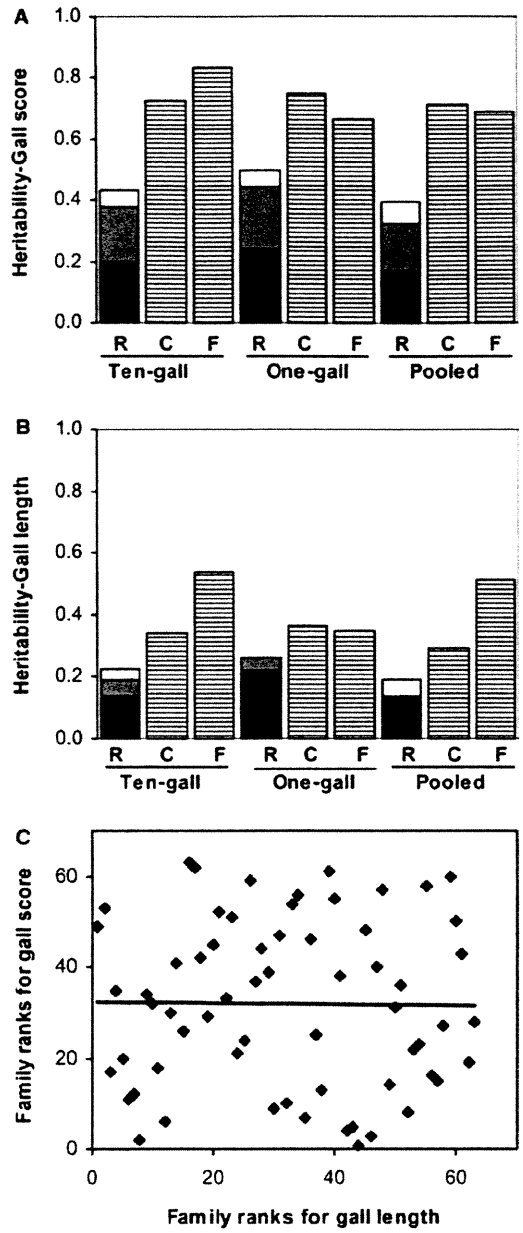

Fig. 5 Heritability estimates and family rank scatter plots for fusiform rust disease traits. Gall score (**a**) and gall length (**b**) heritabilities for R, C and F means are shown. Heritability estimates for the ten-gall and one-gall pooled screens are given for both traits. h^2 (*solid black*), h_I^2 (*solid gray*), and h_D^2 are stacked such that the *y-axis corresponding to the top of the bar* is the H^2. **c** Scatter plot of family ranks illustrates a lack of correlation between gall score and gall length traits (1 = resistant, 63 = susceptible)

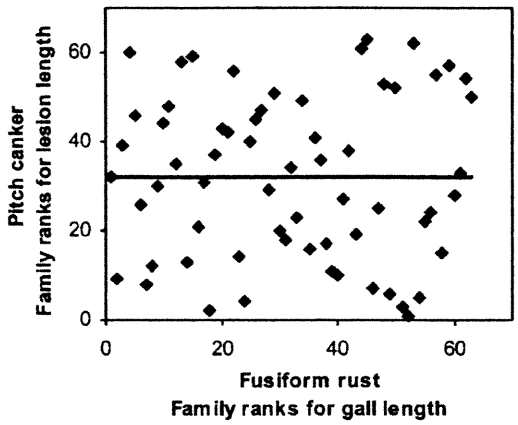

Fig. 6 No genetic correlation between pitch canker and fusiform rust resistance. Family rank–rank scatter plot based on predicted family means for pitch canker (lesion length) and fusiform rust (gall length), fitted with a least squares regression line (1 = resistant, 63 = susceptible)

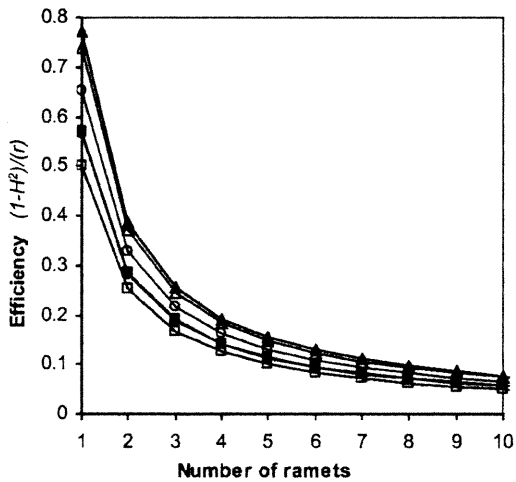

Fig. 7 Efficiency is inversely proportional to the number of ramets per genotype. Efficiency of using multiple ramets in the estimation of H_C^2 plotted against number of ramets for RSC-lesion length (*filled circles*), UF-lesion length (*open circles*), ten-gall-gall score (*filled squares*), one-gall-gall score (*open squares*), ten-gall-gall length (*filled triangles*) and one-gall-gall length (*open triangles*). Efficiency was calculated as $(1-H^2)$/(number of ramets)

H^2. An increase in the experiment size above ca. five ramets per clone does not appreciably increase the precision of heritability estimates, suggesting that future experiments of this type should be replicated to approximately the same extent as this study.

Discussion

Loblolly pine exhibits considerable variation in resistance to both fusiform rust (Kuhlman and Powers 1988; Powers and Zobel 1978) and pitch canker diseases (Dwinell and Barrows-Broaddus 1981; Kuhlman and Cade 1985). The pathogens that incite these diseases, the biotrophic fungus *C. quercuum* and the necrotrophic fungus *F. circinatum*, have distinct life history strategies, reflected in the contrasting disease symptoms visible on susceptible hosts. This study allowed a direct comparison of the host resistance mechanisms to these distinct pathogens in a common set of host genotypes. Consequently, it was possible to compare and contrast the genetic architecture of host responses to both pathogens.

Complex trait analysis requires a reliable estimation of phenotypic values for subsequent correlations with genotype. As a first step toward dissecting complex disease traits in loblolly pine, we undertook this study to evaluate a variety of disease phenotypes in a clonally propagated population generated via a circular mating design. Complex pedigree structures such as these can be useful for mapping QTL (Jannink et al. 2001).

Genetic variation for pitch canker resistance

Pitch canker resistance was continuously distributed across clones, suggesting that resistance may behave as a complex trait. Resistance to fungal necrotrophs is often inherited as a complex trait in crop species including maize (Bubeck et al. 1993) and rice (Wang et al. 1994). Another explanation for this continuous distribution is Mendelian inheritance of resistance within families that appears continuous when examined across families. If resistance were monogenic, some families would be expected to show a bimodal distribution for lesion length. To assess this possibility, we tested individual families for bimodal distributions of resistance. None of the within-family distributions was bimodal; all showed continuous distributions (data not shown). Since lesion length showed a continuous distribution within families across the entire study, we infer that pitch canker resistance is appropriate to analyze as a complex trait.

The repeatability of the pitch canker resistance screens was high, indicated by the high genetic correlation between the two screens, one of which was based on hand-inoculation in a warm environment (UF screen) and the other using established spray inoculation methods in a cooler environment (RSC screen). The stability of H^2 in the pooled dataset relative to the individual screens also supports this conclusion. We do not expect pathogenic variation to significantly change the resistance rankings of these genotypes, even though these experiments were performed by inoculating hosts with a single clonal isolate of *F. circinatum*. This is because there is little evidence for specific resistance in this pathosystem; families rank consistently when challenged with different fungal isolates (G. Blakeslee, personal communication). The facultative nature of this pathogen presumably creates little selection pressure for the evolution of gene-for-gene specificity in this pathosystem. Consequently, these clonal rankings may be robust across a broad range of pathogen isolates and predictive

of rankings expected in the clonal field trials established with these genotypes.

While narrow-sense heritability is an important metric for breeding applications, our use of clonally replicated material allowed additional heritability calculations (H^2, $H_C{}^2$, and $H_F{}^2$ values), which take advantage of the mating and propagation designs used in this study. H_C^2 is an appropriate metric for association and quantitative trait loci studies, because genotyping and phenotyping are both done at the clonal level. Accordingly, in the RSC screen (which involved the most genotypes of the two pitch canker screens) ca. 75% of the variation in lesion length at the clonal mean level was due to genetic variation. Therefore, we expect lesion length to be an appropriate phenotypic trait for future QTL identification.

Gall score and gall length are the most heritable fusiform rust traits

Our analysis of gall score (i.e., disease incidence) revealed a non-normal, right-skewed distribution with one major peak and several minor peaks.

The major peak of apparently "resistant" genotypes may reflect an overestimation of host resistance because of the use of rooted cuttings. Studies comparing the responses of seedlings to rooted cuttings have revealed that these two types of plant material behave differently in response to pathogen challenge, with rooted cuttings showing enhanced resistance (Foster and Anderson 1989; Frampton et al. 2000). This enhanced resistance phenomenon has been observed in other species and is often referred to as "age-dependent" resistance because the developmental stage of the infected organ is the key driver of resistance, over and above the action of specific resistance genes (Kus et al. 2002). As clonal host materials become more widely used in research and plantation forestry, our understanding of this phenomenon should improve.

Evidence for specific resistance in the loblolly pine–*C. quercuum* pathosystem has been obtained using genomic mapping (Wilcox et al. 1996) and by inference based on family rank changes in response to genetically distinct pathogen cultures (Kuhlman 1992; Powers 1980; Stelzer et al. 1997). Although the overall consistency among clonal performances in our two screens was high, we observed a few family and clonal rank changes for particular families and genotypes between the ten-gall and one-gall inoculations (see outliers in Fig. 4c), suggesting resistance genes in the host population interacted with specific pathotypes in the inocula. The families showing rank changes between the two inocula may provide a good starting point for identifying additional resistance genes in loblolly pine.

Gall length was normally distributed and heritable, although to a lesser extent than gall score. Gall length could only be measured on a subset of the population (i.e., on galled ramets), and this sampling effect may account in part for the reduced heritability estimates. Our rationale for measuring gall size characteristics was based on work in slash pine (Schmidt et al. 2000) suggesting that families exhibiting small (short) gall phenotypes were expressing partial resistance to fusiform rust, based on their lack of subsequent sporulation. Partial resistance may be a more durable form of resistance given that it is often race-nonspecific (Schmidt et al. 2000 and references therein). We observed continuous variation in gall length in loblolly pine and found no changes in the relative rankings of genotypes that formed galls in both screens as indicated by high genetic correlations (Fig. 4d). Thus, inoculum type did not appear to exert a major effect on gall length. Studies involving a number of defined pathogen cultures will be required to resolve the question of whether gall length is conditioned by (relatively late-acting) specific resistance factors, or if gall length is a complex trait, potentially involving multiple genes with small effects.

The relationship between gall score and gall length was of interest, because these are distinct phenotypes whose genetic relationship is not well understood. The lack of genetic correlation between gall score and gall length, and the lack of relatedness among families in the tail distributions for gall score and gall length, both suggest that distinct gene systems condition these two traits. Previous studies have revealed that mean gall length varies substantially in loblolly pine families phenotyped as "resistant" based on score (Kuhlman 1992), providing further support for the conclusion that gall score and gall length are conditioned by distinct genetic mechanisms. Future identification of QTL underlying gall length should help distinguish these loci from resistance genes known to be associated with gall score in loblolly pine (Wilcox et al. 1996).

Resistance to pitch canker and fusiform rust are under the control of two different mechanisms

The lack of genetic correlation between pitch canker resistance and fusiform rust resistance (as measured by gall score, or gall length) is consistent with distinct genetic architectures underlying host resistance to these two diseases. Biotrophic pathogens suppress host defenses because they require living host cells for nutrient uptake and survival. Hosts resistant to biotrophic pathogens often activate a localized cell death response to prevent spread of the pathogen (Thomma et al. 2001). In contrast, necrotrophic pathogens actively destroy host cells and utilize the released nutrients for survival. Therefore, a host cell death-response effective against biotrophic pathogens is postulated to benefit necrotrophic pathogens by increasing nutrient availability through accelerated host tissue destruction. We propose that resistance to the necrotrophic pathogen *F. circinatum* is mechanistically distinct from resistance to the biotrophic pathogen *C. quercuum* due to the differing strategies employed by the two pathogens to incite disease in the host. This is supported by gene-expression

array data, which revealed a lack of regulation of rust-associated genes after challenge by *Fusarium* (Morse et al. 2004). Although we identified families with excellent resistance to both diseases (Fig. 6), disease resistance to the two pathogens should be regarded as independent traits by breeders.

Phenotyping for disease trait dissection in loblolly pine

The work described in this manuscript has assigned specific phenotypic values to more than 1,000 loblolly pine genotypes, enabling the identification of genes and alleles that condition resistance through association studies. Genotyping and association studies are currently underway (ADEPT project Web site, Allele Discovery of Economically-important Pine Traits, http://dendrome.ucdavis.edu/ADEPT/) for candidate loci (Morse et al. 2004) thought to be involved in disease resistance in loblolly pine.

In this study, we increased the precision of phenotyping by using clonally propagated genotypes and mixed linear modeling to adjust for environmental effects. Increasing the number of ramets for a given clone will increase the clonal mean based heritability for use in linkage or association studies. However, there is a point of diminishing returns beyond which adding more ramets does not increase precision of phenotyping. This population was an excellent starting point to evaluate the heritabilities and relationships among disease traits. Furthermore, it should afford an opportunity to identify QTL by linkage and linkage disequilibrium (i.e., association) mapping, which has been proposed (Wu et al. 2002) and applied with success (Farnir et al. 2002; Meuwissen et al. 2002).

Acknowledgements This is journal article number R-10656 of the Florida Agricultural Experiment Station. The work was supported by USDA–CSREES–IFAFS (grant no. 2001-52100-11315) with in-kind contributions from the Forest Biology Research Cooperative at the University of Florida. We thank Carol Young, Katherine Smith, and Chris Dervinis for technical assistance, and Henry Amerson and Robert Schmidt for providing inoculum and helpful discussions. We gratefully acknowledge the support of many individuals for assistance with phenotyping at the Resistance Screening Center and UF. These people include the FBRC staff, especially Brian Roth, Brian Baltunis and Emilio Ancaya, staff from Sarah Covert's lab group, Keng Yen Lee, and members of the Davis lab group.

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