Breeding Efforts to Improve Disease Resistance in Southern Pines

Gary Peter
Tania Quesada
Daniel Ence
Jason Smith
Matias Kirst

School of Forest Resources & Conservation
Pitch canker disease

• Caused by *Fusarium circinatum*
• Major disease of pines (most species)
  • Resinous lesions + high mortality
  • Economic losses to forest industry
  • Spreading globally
• Outbreaks occur at optimal environmental conditions
  • High temperature
  • High humidity
• Resistance is quantitative
  • Multiple genes involved
  • Heritability ~ 0.4
  • ~ 60% environmental effect
• Experimental tests easy to implement
• Genome sequences available for both host and pathogen

Source: Quesada et al., 2010
Pitch Canker Resistance

- CFGRP breeding programs use US Forest Service Resistance Screening Center
  - Each seedling is inoculated with spores under conditions to promote uptake
- Evaluate commercial selections for resistance
Pine health in the future: discovering biological drivers of disease ecology in a changing climate

T. Quesada, J. Smith - IFAS seed grant

Collaboration with USDA Resistance Screening Center, Asheville, NC

Objectives and proposed research:

Evaluate growth, sporulation, and pathogenicity of *F. circinatum* ecotopes from the entire host geographical range at increased temperatures.

Are some isolates more virulent than others?
*Fusarium circinatum* isolates from different sites showed phenotypic variations in culture.
Isolates were selected based on their response to temperature and geographical distribution.

Historically used by RSC for screening tests.
Pathogenicity tests USFS RSC

- 8 isolates – sent to Asheville
- 2 susceptible slash and 1 resistant loblolly OP families
- *F. circinatum* spore density:
  100,000 spores/ml
- 3 replicates
- 20 seedlings/replicate
- Lesion length measurements and survival recorded at 12 weeks after inoculation

Response at ~ 4 weeks after inoculation
(Photo: Sunny Lucas)
Isolate-driven mortality on susceptible and resistant controls

~60 plants inoculated per isolate and host family = 1440 plants

Total: 381 dead

Used by RSC for screening tests
Isolate pathogenicity on susceptible and resistant controls

Excludes dead

<table>
<thead>
<tr>
<th>Source</th>
<th>P-values</th>
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<tr>
<td>Rep</td>
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<td>Family * Isolate</td>
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<td>Rep * Family * Isolate</td>
<td>0.6096</td>
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Used by RSC for screening tests
Conclusions

• *F. circinatum* isolates from Florida and Georgia showed significant differences in pathogenicity.

• The recently-collected isolates showed greater pathogenicity than those routinely used at the RSC.

• New isolates are now used in screening tests at the RSC.
Fusiform Rust Resistance

• CFGRP breeding programs use field rust incidence in its selection of more resistant families.
  – Field rust works well to improve resistance when there is >20% incidence

• US Forest Service Resistance Screening Center
  – Each seedling is inoculated with spores under conditions to promote uptake
Resistance to fusiform rust

- Involves major genes
- Fr1 detected in loblolly pine progeny derived from Fr1/fr1 parent 17 (Wilcox et al., 1996) using RAPDs
- Validated in
  - CCLONES population (Kayihan et al., 2010)
  - Slash x loblolly hybrid (Huber & Amerson, 2011)
- Other resistance genes Fr2 – Fr8 also discovered (Amerson et al., 2005); some clustered with Fr1.
- No fine-mapping yet achieved.

<table>
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<tr>
<th>Pathogen</th>
<th>R</th>
<th>r</th>
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<tr>
<td>Avr</td>
<td>Resistance</td>
<td>Disease</td>
</tr>
<tr>
<td>avr</td>
<td>Disease</td>
<td>Disease</td>
</tr>
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</table>
Plant material and inoculation experiments

- **CCLONES** (population with known pedigree)
  - 70 full-sib families, 36 parents
  - Randomized incomplete block design
  - 5 clonal replicates

- Inoculation tests:
  - 1-gall and 10-gall spore mix
  - Gall score and gall length measured
  - 467 – 803 clones phenotyped and genotyped (4,853 SNPs)
Genomic selection approach

• Bayes Cπ more accurate method to predict rust score (Resende et al., 2012)

• Markers ranked according to %phenotypic variance explained by each marker

• Validation using BAMD on markers where %variance explained > 0.2.

• Single marker regression performed on half-sib families on significant SNPs:

  Full model: \[ Y = \mu + Xr + Zb + \varepsilon \]
  Reduced model: \[ Y = \mu + Xr + \varepsilon \]

  \( Y \) = phenotype
  \( \mu \) = population mean
  \( X, Z \) = incidence matrices
  \( r \) = replication
  \( b \) = SNP genotype

Resende et al., 2012
Comparative magnitude of SNP effects for rust gall score (red markers) and gall length (blue markers) obtained using Bayes $C_π$.
Significant SNPs overlapping between the one-gall and ten-gall score data

**Figure 2.** Venn diagram showing significant SNPs for rust resistance between gall score for 10 gall and one gall data that are summarized in Table 1. Of the total of 21 significant SNPs that were detected in the 10 gall inoculum test, four were also significant for the one gall inoculum at varying levels of significance, while 12 were only significant for the one gall inoculum.

Quesada et al., 2014 Forests 5:347-362
Single marker regression analysis on half-sib families

1-Gall Score

![Bar chart showing mean gall score for different genotypic classes (CC, CG, GG) for 2-7562-01-464.]

10-Gall Score

![Bar chart showing mean gall score for different genotypic classes (CC, CG, GG) for 2-7562-01-464.]

LOD Score

![Scatter plot showing LOD scores against mean gall scores for different families (17, 18, 20, 22).]

Quesada et al., 2014 Forests 5:347-362
## Results Combined – Fusiform rust

### 1 Gall Score

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Dataset</th>
<th>Mean Effect</th>
<th>CI</th>
<th>Scaffold</th>
<th>Position</th>
<th>Ref</th>
<th>Alt</th>
<th>Marker ID</th>
<th>LG</th>
<th>Seg 10–5</th>
<th>Putative Function</th>
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<td>99</td>
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<td>10733</td>
<td>G</td>
<td>T</td>
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<td>--</td>
<td>--</td>
<td>--</td>
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<td>V10922</td>
<td>PINEMAP</td>
<td>0.087</td>
<td>95</td>
<td>scaffold217054.2</td>
<td>13242</td>
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<td>A</td>
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<td>95</td>
<td>scaffold698284.2</td>
<td>8460</td>
<td>G</td>
<td>A</td>
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<tr>
<td>V56765</td>
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<td>95</td>
<td>tscaffold5830</td>
<td>60979</td>
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<td>C</td>
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<td>95</td>
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<td>480399</td>
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<td>A</td>
<td>0_5350</td>
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<td>Transcription factor IIA/ alpha/beta subunit</td>
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<td>Glycine hydroxymethyltransferase</td>
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<td>G</td>
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<td>T</td>
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<td>90</td>
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<td>C</td>
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<td>90</td>
<td>scaffold854030.2</td>
<td>100001</td>
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<td>G</td>
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<td>V19178</td>
<td>PINEMAP</td>
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<td>scaffold456449</td>
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<td>C</td>
<td>0_11406</td>
<td>7</td>
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</table>
Summary

- CCLONES → best characterized study in the FBRC
  - > 25 phenotypic traits
  - > 70,000 genotypic markers

- New genotyping process allows higher SNP density
- Association analyses with new SNP dataset produced equal or higher number of significant SNPs.
- When ADEPT2 SNPs and PINEMAP SNPs were analyzed together, some significant SNPs from ADEPT2 were also significant in the new dataset.
- Further characterization of significant SNPs is under way
SNP LOD Scores Map *Fr1* to a Candidate Gene

Figure 4 Identification of TNL candidate gene for Fr1. (A) Genome survey of rust resistance in segregating progeny of Fr1/fr1 *Pinus taeda* among clonally propagated half-siblings (upper) and full-siblings (lower). Bins with highest LOD scores contained SNP 2_5345_01 (*). (B) Translated gene model (G) on genome scaffold jcf7180063178873 is interrupted by three introns with sizes given in bp, previously available EST (E) containing SNP 2_5345_01 (*), fully assembled transcript Evg1_1A_all_V0_L3_3760_240252 from RNAseq (R) and the domain structure of the protein model (P).

Neale et al., Genome Biology, vol. 15:R59, 2014
Design of sequence capture probes for Fr1 mapping

Sequence-capture w/ three subsets of probes:
1. Probes near candidate Fr1 gene

2. Probes targeting putative R genes:
   – Annotation of gene models in genome
   – Annotation of putative R genes in transcriptomes

3. Probes evenly spaced throughout genome

   • ~ 10,000 probes in total (5,000 non-overlapping regions)

Leandro Neves, Jesse Breinholt
Rapid Genomic
Experimental Cross to Map *Fr1*

- **Fr1/fr1** Mother
- **fr1/fr1** Fathers

**Mother Tree**

**Open-pollinated Seedlings**

**Inoculation with CQF spores**

**Susceptible Seedlings**

**Resistant Seedlings**

**Sequence 150 of each + Sequence 32 megagametophyte samples**
Tracing *Fr1* Haplotype Through Breeding Experiment

- **Fr1*/fr1* Mother
- **Seedlings:** Inherited 2 Haplotypes
- **Megagametophytes:** Inherited 1 Maternal Haplotype (Either *Fr1* or *fr1*M)
- **Resistant Seedlings:** *Fr1* from mother, *fr1*P from fathers
- **Susceptible Seedlings:** *fr1*M from mother, *fr1*P from fathers
Expectation at the “True” Fr1 Locus

10-5 Mother Fr1/fr1  X  non 10-5 Fathers fr1/fr1

1:1 Fr1/fr1:fr1/fr1

Should also be true for loci linked to Fr1
# Fr1 Allele Frequency Expectations

<table>
<thead>
<tr>
<th>Allele</th>
<th>Source</th>
<th>Expected Freq. In Resistant Seedlings</th>
<th>Expected Freq. In Susceptible Seedlings</th>
<th>Expected Freq. in Megagam.</th>
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</thead>
<tbody>
<tr>
<td>Fr1 (resistant)</td>
<td>Maternal Fr1 haplotype</td>
<td>0.5 (All het.)</td>
<td>0</td>
<td>Between 0.32 and 0.68*</td>
</tr>
<tr>
<td>fr1\textsuperscript{M} (non-resistant)</td>
<td>Maternal non-Fr1 haplotype</td>
<td>0</td>
<td>0.5</td>
<td>Between 0.32 and 0.68*</td>
</tr>
<tr>
<td>fr1\textsuperscript{P} (non-resistant)</td>
<td>Paternal</td>
<td>&lt;0.5 (multiple different fathers)</td>
<td>&lt;0.5 (multiple different fathers)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*95% Confidence Interval of Binom(32,0.5)
Plan for Analysis

1. Candidate $Fr1, fr1$ Variants From Mega. Samples
2. Filter in Susceptible Samples
3. Candidate $Fr1$ Variants
4. Filter in Tolerant Samples
5. Candidate $Fr1$ Variants For Follow-up
• Candidate SNPs associated with fusiform rust resistance were identified using Bayes Cπ and were validated through association testing using BAMD.

• Highly-ranked SNPs were also significant in BAMD in the one-gall and ten-gall score datasets, with some exceptions.

• Markers linked to Fr1 were significant in the progeny of parent 17 (Fr1/fr1) in both one gall and 10 gall experiments. At least one other Fr genes was observed at the Fr1 locus.

• Other SNPs mapped to different Fr genes, such as Fr8 (LG10) and LG11.

• Markers associated with rust resistance may be used to guide breeding and selection of loblolly pine material.
Stem Terpenes: A Defense Against Boring Insects

![Stem Terpenes Image]

Number of Years of Southern Pine Beetle Outbreaks by County (1960-2000)

- Greater than 25
- 16 - 20
- 8 - 10
- 1 - 5

Map showing the distribution of Southern Pine Beetle outbreaks.

- Red: Greater than 25 years
- Orange: 16 - 20 years
- Yellow: 8 - 10 years
- Green: 0 - 1 year


Recent mortality of major western conifer beetles. (a) Map of western North America showing areas of major mortalities by three species. (b) Area of forested area affected by these three species over time. Data from the Canadian Forest Service, the British Columbia Ministry of Forests and Range, and the US Forest Service.
Stem Boring Insect-Fungal Defense

• Oleoresin defenses are key for resistance
• Oleoresin flow, resin canal number and wood terpene content are under quantitative genetic control
• Increased oleoresin flow is correlated with greater resistance
• CFGRP is just beginning to evaluate this trait for breeding
Conifer Terpenes & Resin Canal/Duct Organization

- **Primary** resin canal system in needles & non-woody tissues that are not a product of the cambium
- **Secondary** resin canal system in wood is a product of the cambium and has radial and axial canals
Stem terpene defenses against bark beetles

**Constitutive**

Bark beetle penetrates stem

*Physical barrier:*
Constitutive oleoresin flow

**Induced**

Wounding and fungi induce terpene synthesis

*Chemical defense:* terpenes toxic to bark beetles & pathogenic fungi

Immediately 0-7 days 2-4 weeks

Jasmonate and ethylene signaling

Wounding and fungi Induce new resin canals to form in the wood
Resin canal number and oleoresin flow is heritable in CCLONES.
Predicted first generation gains from breeding individuals from top 5% of genetic distribution

<table>
<thead>
<tr>
<th>Fold gain in trait means from current generation</th>
<th>Resin canal number</th>
<th>Wood diterpene content</th>
<th>Oleoresin flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breed to maximize individual traits</td>
<td>11%</td>
<td>8%</td>
<td>42%</td>
</tr>
<tr>
<td>Breed to maximize trait combination</td>
<td>8%</td>
<td>7%</td>
<td>27%</td>
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<tr>
<td>Breed to maximize oleoresin flow</td>
<td>5%</td>
<td>4%</td>
<td>42%</td>
</tr>
</tbody>
</table>
SNP predictive abilities of oleoresin flow in CCLONES

Black line = associated SNPs
Dotted line = all SNPs

Grey region = 95%
CI random SNPs

(a) Cuthbert, GA
N_{sig} = 65

(b) Nassau, FL
N_{sig} = 65

(c) Palatka, FL
N_{sig} = 55

(d) Across sites
N_{sig} = 73

number of loci included in prediction model
Three Levels of Engineering

**Development**
- **Goals**
  - Increase # of cells synthesizing terpenes
  - Increase storage
- **Approaches**
  - Discover regulators of resinosis
    - Inducers of new resin canal formation
    - Inducers of terpene synthesis

**Pathway**
- **Goals**
  - Increase flux
  - Increase efficiency of carbon conversion
- **Approaches**
  - Enzyme shunts that reduce carbon loss
  - Overexpression of rate limiting enzymes

**Enzyme**
- **Goals**
  - Alter terpene composition
  - Increase efficiency
- **Approaches**
  - Produce bisabolene in wood
  - Improve prenyl transferase and terpene synthases
Genetic

- We have identified 13 genes that when manipulated in loblolly pine can significantly increase wood terpene content
- We are breeding for increased oleoresin flow and yield in slash and loblolly pine

<table>
<thead>
<tr>
<th>Construct</th>
<th>Function</th>
<th>Avg % Increase</th>
<th>Best Line % Increase</th>
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<tbody>
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<td>1</td>
<td>MJ signaling</td>
<td>62</td>
<td>300</td>
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<td>2</td>
<td>DEG</td>
<td>62</td>
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<td>3</td>
<td>MJ signaling</td>
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<td>4</td>
<td>MJ signaling</td>
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<td>5</td>
<td>DEG</td>
<td>45</td>
<td>214</td>
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<tr>
<td>6</td>
<td>AG+DEG</td>
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<td>212</td>
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<td>7</td>
<td>MJ signaling</td>
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<td>8</td>
<td>DEG</td>
<td>35</td>
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<tr>
<td>9</td>
<td>AG+DEG</td>
<td>35</td>
<td>220</td>
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<tr>
<td>10</td>
<td>Ethylene syn.</td>
<td>35</td>
<td>228</td>
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<tr>
<td>11</td>
<td>Ethylene syn.</td>
<td>35</td>
<td>225</td>
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</table>
Conclusions

• The episodic nature of pitch canker outbreak and quantitative resistance has made it more difficult to justify breeding

• Fusiform rust resistance is done routinely in both species
  – Narrowing in of genes and markers for introgression

• Insect-fungal resistance is not bred for now but we are evaluating this trait.
  – Likely need to incorporate in next cycles of breeding
Identifying Maternal Haplotypes in Megagametophytes

• QC reads with cutadapt
  – 10bps from each end, min-quality: 30, minimum-length: 50bps

• Aligned to sequences (R genes and ref genome seqs) with probes
  – Aligned with Hisat2, default settings

• Called variants within probe regions with freebayes:
  – Megagameophyte samples: haploid setting
  – All other samples: diploid setting
Plan for Analysis

• Filter variants in megagametophyte samples:
  – Filters for missing data, biallelic, mean depth per sample, minor allele frequency (MAF)*
  – Candidate Fr1,fr1 variants

• Intersect candidate Fr1,fr1 variants from megagametophytes with susceptible samples.
  – Fr1 should be absent. Filter for MAF = 0.
  – Candidate Fr1 variants

• Intersect candidate Fr1 variants with resistant samples.
  – Expect MAF ~0.5, heterzygous variants
  – Expect up to ~30% “escapes” among the resistant samples
  – Candidate Fr1 variants for follow-up

*95% Confidence Interval of Binom(32,0.5). 0.32 < MAF < 0.68
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