TOWARDS A GENOME SEQUENCE OF THE BROWN SPOT NEEDLE BLIGHT PATHOGEN (*Mycosphaerella dearnessii)* INFECTING LONGLEAF PINE

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Longleaf pine (*Pinus palustris* Mill.) is the dominant species in one of the most diverse terrestrial ecosystems in the temperate US. Acreage devoted to growing longleaf pine has diminished to less than three percent of that at the time of European settlement (Ware *et al.* 1993). America's Longleaf Restoration Initiative is currently coordinating an effort involving a number of agencies to increase the area occupied by longleaf pine forest to 8 million acres (8% of the original range). Widespread incidence of brown-spot needle blight is a potential threat that can hinder progress in this restoration program. This disease results from infection by the fungus *(Mycosphaerella dearnessii)*, which can disrupt development in longleaf seedlings. This disruption can slow growth, delay stand development, and cause an increase in seedling mortality (Siggers 1934). While prescribed fire is recommended management practice for minimizing disease incidence, its use is problematic for many land managers. The absence of a proper fire regime will result in inadequate management for this needle disease on many sites, suggesting that approaches other than prescribed fire are needed for establishing longleaf pine stands. At the same time, few resources exist for elucidating the mechanisms by which this fungus is able to infect pine species. Obtaining an understanding of the systems involved in the infection process should prove valuable in efforts to select and breed trees resistant to brown-spot needle blight. Here we outline research that has been initiated to address these shortcomings.

MATERIALS AND METHODS

Samples of *M. dearnessii* from 25 different longleaf pine seedlings were collected from the Harrison Experimental Forest near Saucier, MS. Lesions from infected needles were dissected and placed in potato dextrose agar (PDA). Examination of conidia from embedded lesions required careful observation of the cultures, to avoid transferring contaminating fungi. Acervuli produced from the lesions appeared as a black speck on the needle and was typically noticed within 10-14 days. After 14 days, contaminating fungi dominated the media. A sterile toothpick was manipulated to capture spores for microscopic identification (400X magnification), and sequentially transferred to clean media after positive identification. Cultures were then grown at 21 C for 21 days prior to DNA extraction. Genomic DNA was isolated from each of the cultures using a CTAB protocol. To achieve optimal DNA yield, fungal tissue was frozen in liquid nitrogen and macerated with a pestle and mortar. For DNA analysis, standard PCR conditions were sufficient for amplification. Annealing temperature for the microsatellite primers (MD1, MD2, MD9 isolated from *M. dearnessii* and provided by Josef Janoušek, Irene Barnes, Michael Wingfield), was 56 C, while the internal transcribed spacer (ITS) primers required an annealing

temperature of 51 C. Capillary electrophoresis was carried out on an ABI 3730xl DNA Analyzer to evaluate the PCR products. Sixteen unique haplotypes were identified and selected for testing vegetative compatibility. Each haplotype pair was tested on being compatible or incompatible, or possibly intermediate (Figure 1). To form the pairings, conidia from each haplotype was placed on PDA media 1 cm from another haplotype and allowed to grow for 21 days prior to scoring.

RESULTS AND DISCUSSION

A location within the historic range of longleaf pine was chosen to obtain infected needles (Boyer 1990). Upon isolation of the fungus, definitive identification was needed to confirm *M. dearnessii.* Universal fungal primers from the ITS region were employed to verify this identification. Three samples were chosen to sequence the ITS region and compare those results to the nucleotide database maintained by the National Center for Biotechnology Information (NCBI). Two samples exhibited 100% homology to the ITS region of *M. dearnessii*, while the other exhibited 99.8% homology. The ITS PCR products of these three samples were determined by capillary electrophoresis to be 580bp. All isolates of the fungus demonstrated a sequence length of 580bp of the ITS region, supporting our visual (microscopic) identification of *M. dearnessii.*

Microsatellite analysis revealed that each of the three markers studied showed polymorphism among our 25 isolates, and our collection contained 16 unique haplotypes. A representative culture of each haplotype was selected for vegetative incompatibility testing. Of the 120 different combinations observed, 61 (51%) were considered to exhibit intermediate compatibility (Figure 1). Due to a high proportion of intermediate compatibility combinations observed, these results suggest that multiple loci are involved in vegetative compatibility. A haplotype pair was interpreted as incompatible if a zone of exclusion was detected, so that the two cultures could not fully merge. With these criteria, 68 (57%) of the pairs were found to be incompatible, while 47 (39%) were judged as compatible.

The microsatellite results were additionally applied to select two isolates for whole genome sequencing. These two isolates were determined to be genetically distinct from each other, as a result of neither having a similar microsatellite marker allele. The genome of *M. dearnessii* is estimated to be around 50 Mb, based upon other *Mycosphaerella* genome sizes (Santana 2012, Goodwin 2011). Using this estimate, a single lane from an Illumina MiSeq will provide enough data to assemble two haploid individuals. Using paired-end reads, a strategy will be employed to essentially double the length of the reads, instead of producing a single 250bp read, paired-ends will merge two 250bp reads that overlap by 50bp, providing a continuous 400bp read. This strategy will produce 40X coverage of each haploid genome. These sequence data will be combined to assemble a draft genome, followed by gene predictions to obtain gene ontology (GO) associations. Overall, this information will be applied to studying pathogenesis of *M. dearnessii* on *P. palustris,* leading to improved measures for controlling the incidence of the

brown-spot needle blight and developing genetic resistant longleaf pines.

Haplotype	15	1	16	4	13	18	8	12	14	6	5	3	9	17	2	10
15		NA	I	C	I	I	C	C	C	C	C	C	C	I	C	C
1			I	C	I	I	I	I	C	I	I	I	C	NA	C	NA
16				I	I	I	I	I	I	I	C	C	I	C	I	I
4					I	I	I	I	C	I	NA	C	C	I	I	I
13						I	I	I	I	I	C	I	I	I	I	NA
18							I	I	I	I	C	I	C	I	C	C
8								I	I	I	C	I	C	I	I	C
12									C	C	I	I	I	C	C	C
14										C	I	C	C	C	I	C
6											I	I	C	I	I	C
5												I	C	I	C	C
3													C	I	I	I
9														I	C	I
17															C	I
2																C
10																

Figure 1. Vegetative compatibility matrix; illustrating all the combinations of haplotype pairs. The grey background represents intermediate compatibility. Incompatibility is represented by an I, while compatibility is represented by a C.

REFERENCES

Boyer, WD. 1990. *Pinus paulustris,* Mill. Longleaf pine. In *Silvics of North America,* technical coordinators R.M. Burns and B.H. Honkala. Conifers Washington, DC: USDA Forest Service.

Goodwin, SB et al. 2011. Finished genome of the fungal wheat pathogen *Mycosphaerella graminicola* reveals dispensome structure, chromosome plasticity, and stealth pathogenesis. *PLoS Genetics*. 7(6).

Santana, MF et al. 2012. Abundance, distribution and potential impact of transposable elements in the genome of *Mycosphaerella fijiensis*. *BMC Genomics*. 13:720.

Siggers, PV. 1934. Observations on the influence of fire on the brown-spot needle blight of longleaf pine seedlings. *Journal of Forestry*. 32:556-562.

Ware, S et al. 1993. Southern mixed harwood forest: The former longleaf pine forest. In Biodiversity of the Southern United States: Lowland Terrestrial Communities. pg. 447-493. *New York: John Wiley & Sons.*