INSIGHTS INTO THE EPIDEMIOLOGY AND DISPERSAL CAPABILITIES OF *LEPTOGRAPHIUM WAGENERI* VAR. *PSEUDOTSUGAE* WITHIN AND BETWEEN YOUNG DOUGLAS-FIR STANDS IN WESTERN OREGON

Patrick I. Bennett[1,2*] and Jared M. LeBoldus[2,3]

Introduction

Black stain root disease (BSRD) is a vascular wilt that causes mortality in several conifer species (Wagener & Mielke 1961, Harrington & Cobb 1987). It is caused by the fungus *Leptographium wageneri*, which can spread via root contact and is also vectored by insects (Witcosky & Hansen 1985, Witcosky et al. 1986). Douglas-fir (*Pseudotsuga menziesii*) is host to a specific variety of the fungus, *L. wageneri* var. *pseudotsugae* (Harrington & Cobb 1987). The fungus is vectored by two species of weevil (*Pissodes fasciatus*, *Steremnius carinatus*) and the Douglas-fir root beetle (*Hylastes nigrinus*) (Witcosky & Hansen 1985, Witcosky et al. 1986). The incidence and severity of BSRD in young Douglas-fir plantations in the Pacific Northwest has intensified in recent years. This disease is emerging as a serious threat to the health and productivity of young trees in managed forest stands in Oregon. The population structure of the BSRD fungus as it relates to epidemiology has not been investigated previously. It is not currently known whether local or long-distance dispersal is more important for the establishment of new BSRD infections, and the relative genetic diversity of *L. wageneri* var. *pseudotsugae* within and between stands has not been described. This study was designed to address these gaps in knowledge and may provide insights into the emergence and spread of BSRD. The specific objectives were to: 1) sequence genomes of *L. wageneri* var. *pseudotsugae* isolates collected in western Oregon Douglas-fir plantations; 2) identify single nucleotide polymorphisms (SNP) to assign genotypes; and 3) compare population structure and diversity among plantations, and among stands within plantations, to make inferences about epidemiological characteristics such as dispersal and colonization.

Methods

Wood samples showing characteristic staining were collected from the root crowns of 107 symptomatic *P. menziesii* trees in 11 stands across three spatially disjunct timber plantations in western Oregon (Figure 1). Additionaly, six isolates of *L. wageneri* var. *wageneri* were collected from two single-leaf pinyon pine (*Pinus monophylla*) stands in the San Bernardino National Forest in southern California. All field samples were kept on ice and returned promptly to the lab, and isolations were performed within 14 days following collection. Stained wood pieces were surface sterilized and plated on a selective medium consisting of 1.5% malt agar supplemented with 100 ppm streptomycin and 200 ppm cycloheximide (CSMA) (Harrington 1992, Jacobs & Wingfield 2001). Cultures were incubated for 2-4 weeks. Fungi displaying morphological characteristics consistent with *L. wageneri* were isolated onto 2% malt extract agar (MEA) and incubated for an additional 2-4 weeks. Mycelium from the MEA plates was then transferred to 2% liquid malt extract broth and grown for 2-4 weeks prior to the extraction and purification of genomic DNA.

*[1]USDA Forest Service, Forest Health Protection, Northern and Intermountain Regions, Missoula, Montana, USA. *patrick.bennett@usda.gov. [2]Department of Botany and Plant Pathology, Oregon State University Corvallis, OR, USA. [3]Department of Forest Engineering, Resources, and Management (FERM), Oregon State University, Corvallis, OR, USA*

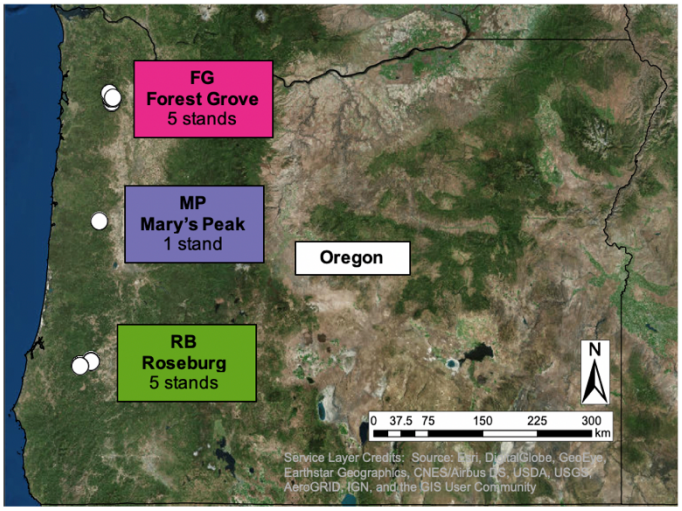

Figure 1: *Map showing the locations of the three Douglas-fir plantations in western Oregon (FG, MP, RB) from which* L. wageneri *var.* pseudotsugae *isolates were collected. A total of 47 isolates were collected from five stands at the FG plantation, 10 isolates were collected from one stand at the MP plantation, and 50 isolates were collected from five stands at the RB plantation.*

Whole genome sequencing was performed for all isolates with an Illumina HiSeq 3000 with 150 bp paired-end reads. All DNA extractions, library preparation, and Illumina sequencing were performed by the Center for Genome Research and Biocomputing at Oregon State University in Corvallis, Oregon. The Illumina reads were aligned to a reference genome of *L. wageneri* var. *pseudotsugae* type strain CMW154, and SNPs were identified to assign individual genotypes. The R statistical computing platform (R Core Team 2018) was used along with the package vcfR (Knaus & Grunwald 2017) for quality filtering. The filtering protocol masked all SNP variants with < 10x sequencing coverage, all samples with > 55% missing data, and all variants with > 20% missing data. A series of population genetic analyses were performed in R with *poppr 2.8.1* (Kamvar et al. 2014, Kamvar et al. 2015) and *adegenet* (Jombart 2008, Jombart et al. 2010).

Results

After quality filtering, the final dataset included 112,577 genome-wide SNPs. The branch topology and support values in the neighbor-joining dendrogram suggested that the two *L. wageneri* varieties are highly differentiated and that no gene flow occurs between them (Figure 2). The *L. wageneri* var. *pseudotsugae* isolates collected from three plantations in western Oregon represented two distinct genetic clusters. One cluster contained only isolates collected at the FG plantation, while the other consisted of a mixture of isolates from the three plantations (Figure 2).

The scatter plot from a discriminant analysis of principal components (DAPC) (Jombart et al. 2010) revealed some overlap between the clusters representing the three plantations. This indicates that some of the isolates from the three plantations have similar genotypes (Figure 3A). The DAPC genotype composition plot (Figure 3B) indicated that the genotypes of isolates collected in the MP and FG plantations have a high probability of membership in the cluster represented by the RB

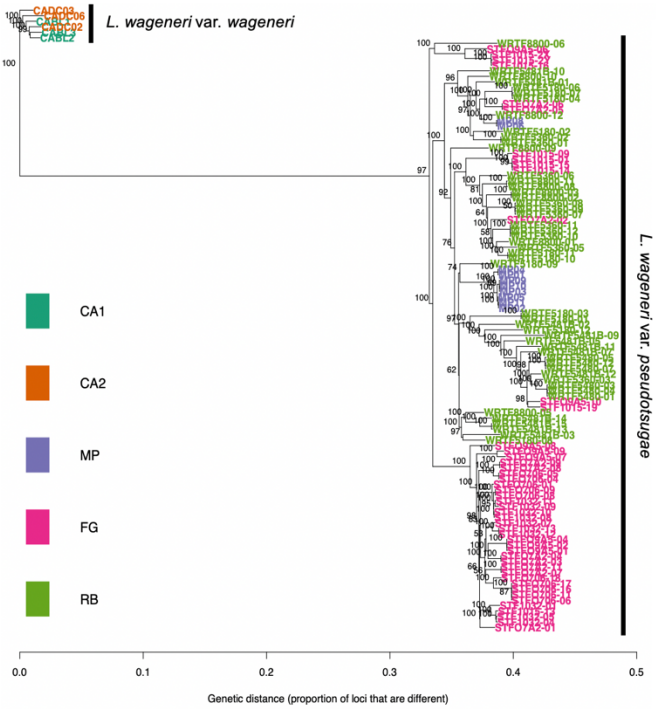

Figure 2: *Genetic distance (neighbor-joining) tree constructed with 112,577 genome-wide SNPs from 107 isolates of* L. wageneri *var.* pseudotsugae *and six isolates of* L. wageneri *var.* wageneri. *Branch support values were calculated from 100 bootstrap replicate trees.*

isolate genotypes. This suggested that the isolates collected from the MP and FG plantations are more similar to those collected in the RB plantation than they are to the other isolates collected in the MP and FG plantations. These results were supported by genetic differentiation (G'_{ST}) (Hedrick 2005) values calculated pairwise among each of the plantations. The isolates from the MP and FG plantations were more strongly differentiated than the isolates from the FG and RB plantations or from the MP and RB plantations (Table 1).

An analysis of molecular variance (AMOVA) assessed the spatial distribution of genetic diversity within and between plantations. The results were consistent with the other analyses in that they indicated that most of the genetic variation (53.37%) occurred within stands (i.e. stands are a diverse and heterogeneous mixture of isolates) (Table 2). The remaining genetic variation occurred between stands within plantations (19.84%) and between plantations (26.79%) (Table 2).

Table 1: *A standardized genetic differentiation measure (G'_{ST}) calculated pairwise for the three plantations sampled. This statistic is equal to 0 when all alleles are shared among populations and 1 when no alleles are shared.*

	MP	**FG**	**RB**
MP	0	-	-
FG	0.228	0	-
RB	0.119	0.156	0

Table 2: *Analysis of molecular variance (AMOVA) table showing the partitioning of genetic variance among levels of a population hierarchy. The phi statistic (φ) is an estimate of genetic differentiation. The P-value for each phi statistic was calculated from 999 permutations of a randomization test.*

Hierarchical Level	**Variance (%)**	**ϕ**	**P ***
ϕ_{ST} (between plantations)	26.787	0.268	0.002
ϕ_{SS} (between stands within plantations)	19.842	0.271	0.001
ϕ_{IS} (within stands)	53.370	0.467	0.002

Discussion

This study examined genetic differentiation between two of the three host-specific *L. wageneri* varieties and compared genetic structure and variation within and among 11 stands in three separate Douglas-fir plantations in western Oregon (Figure 1). The SNP genotypes representing isolates from the varieties of *L. wageneri* occurring on single-leaf pinyon pine (*P. monophylla*) and Douglas-fir (*Pseudotsuga menziesii*) were highly differentiated due to a lack of gene flow (Figure 2) reflecting their evolutionary divergence. Whether these groups represent separate species or should be maintained as varieties of the same species should be investigated further with phylogenetic analyses.

Although sexual reproduction has not been observed in the Douglas-fir variety of *L. wageneri*, there was more genetic variation present in populations of *L. wageneri* var. *pseudotsugae* than would be expected from asexual reproduction alone. Other sources of variation could include migration, diversifying selection,

genetic drift, or random mutation. The three plantations sampled in western Oregon were approximately 300 km (186 mi) apart, but some of the isolates collected at each of the plantations had similar genotypes suggesting that they were closely related. This indicates migration as a source of variation within plantations. The co-occurrence of isolates from all three plantations in a single genetic cluster in the neighbor-joining dendrogram (Figure 2), overlap between DAPC clusters (Figure 3A), posterior membership probabilities (Figure 3B), and moderate genetic differentiation among some of the plantations (Tables 1 and 2) are all consistent with gene flow occurring between plantations. Another explanation could be that the *L. wageneri* populations collected in the separate plantations came from a common source population.

Natural dispersal by insect vectors over the 300 km distance between the FG and RB plantations is unlikely. The weevil *S. carinatus* is flightless, while the Douglas-fir root beetle (*H. nigrinus*) and *P. fasciatus* are capable of flight (Jacobi 1992), but their dispersal capabilities are not known. It is more likely that the similarity of genotypes across western Oregon plantations is due to the fact that the Douglas-fir host has a continuous distribution in this region, and thus spatially disjunct timber plantations may be interconnected by shorter-distance insect flights in a sort of "stepping-stone" fashion (Linde et al. 2002). Overall, the evidence suggests that the insect-mediated dispersal of *L. wageneri* between stands has a greater influence on the genetic structure of its populations than local vegetative spread.

The apparent migration among plantations could also result from human-mediated migration of *L. wageneri* or its insect vectors. It is not known whether infested trees, logs, soil, or equipment may be moving between these plantations or whether these plantations share a common source such as a nursery that may be infested with *L. wageneri* or its vectors. These possibilities should be investigated in future studies.

Migration between stands within plantations has resulted in high stand-level genetic variation (Table 2). The variation observed within stands is not consistent with a single infection and subsequent vegetative spread via root contact, but rather suggests that an infected stand is a heterogeneous mix of isolates that are often distantly related. This is most likely the result of multiple independent infections occurring in each stand, and reflects the importance of insect-mediated dispersal relative to spread via root contact. This might be expected in younger Douglas-fir stands where adjacent trees are spaced far enough apart such that their root systems have not yet come into contact. The stand-level diversity also reflects the diversity of *L. wageneri* present in surrounding stands and in the insect vector populations that serve as the sources of inoculum for each BSRD infection. Future studies of *L. wageneri* diversity and epidemiology could be facilitated by trapping insect vectors and analyzing the genetic variation of *L. wageneri* isolates on individual insects.

It is important to consider the fact that insect vectors regularly transport *L. wageneri* between stands when planning timber harvest activities. For instance, it is known that activities such as thinning as well as soil compaction resulting from roads and skid trails attract the insects that vector *L. wageneri* (Witcosky et al. 1986, Hessburg et al. 2001, Kelsey & Joseph 1998). Considering the fact that the vectoring of *L. wageneri* from outside a stand seems to be much more important than vegetative spread within young Douglas-fir stands, managers may choose to keep these impacts to a minimum to avoid attracting vectors from nearby BSRD infection centers. Given an estimate of the dispersal capabilities of *L. wageneri* vectors, managers may also be able to better locate timber harvest and management activities away from stands that are known to be infested with *L. wageneri* and its vectors.

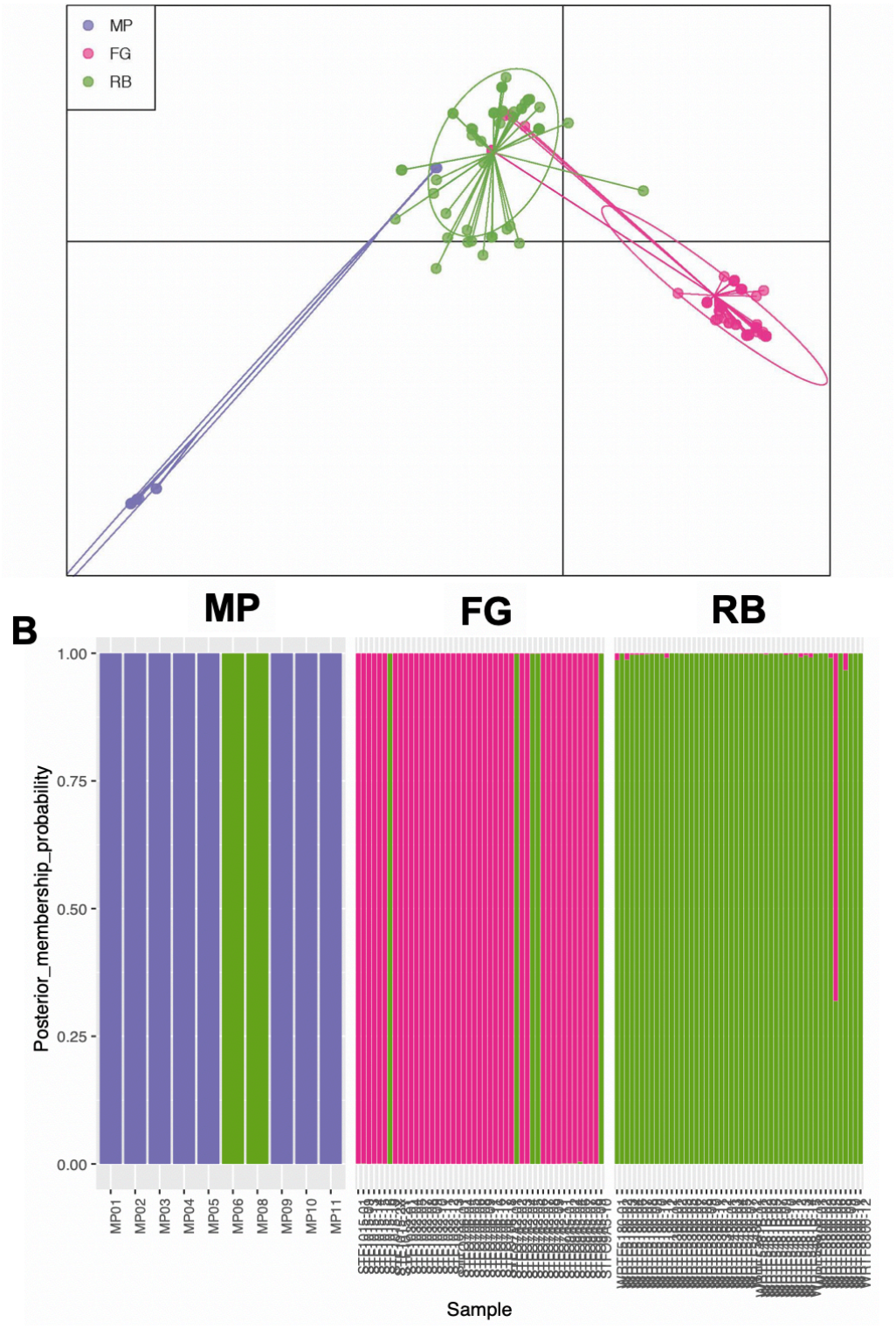

Figure 3: *Discriminant analysis of principal components (DAPC).* ***A)*** *Scatter plot showing clustering among isolates of* L. wageneri *var.* pseudotsugae *collected from three Douglas-fir plantations in western Oregon.* ***B)*** *Genotype composition plot showing the posterior membership probability of each isolate collected in the three plantations.*

Acknowledgements: The authors thank S. McMurtrey, R. Lenz, and A. Bouche for assisting with the field work for this study. Special thanks to T. Duong and M. Wingfield at FABI for providing the reference genome for *L. wageneri* var. *pseudotsugae*. K. Carter and M. Dasenko at the Center for Genome Research and Biocomputing kindly provided technical support. The authors also thank A. Leon and J. Browning at Weyerhaeuser Co., and B. Reding at Stimson Lumber Co., for providing access to their forest lands and support in the field. Funding was provided by the U.S. Forest Service Forest Health Protection (FHP) Region 6, Weyerhauser Co., and the Agricultural Research Foundation (ARF).

Literature Cited

Harrington, T.C., Cobb Jr., F.W. (1987). *Leptographium wageneri* var. *pseudotsugae*, var. nov., cause of black stain root disease on Douglas-fir. *Mycotaxon, 30, 501–507.*

Harrington, T.C., 1992. *Leptographium*, in: Singleton, L.L., Mihail, J.D., Rush, C.M. (Eds.), *Methods for Research on Soilborne Phytopathogenic Fungi*. St. Paul, Minnesota: American Phytopathological Society Press. p. 129–133.

Hedrick, P.W. (2005). A standardized genetic differentiation measure. *Evolution, 59(8), 1633–1638. https://doi.org/10.1111/j.0014-3820.2005.tb01814.x*

Hessburg, P.F., Hansen, E.M. (1986). Mechanisms of inter-tree transmission of *Ceratocystis wageneri* in young Douglas-fir. *Canadian Journal of Forest Research 16(6), 1250–1254. https://doi.org/10.1139/x86-221*

Hessburg, P.F., Goheen, D J., Koester, H. (2001). Association of black stain root disease with roads, skid trails, and precommercial thinning in Southwest Oregon. *Western Journal of Applied Forestry, 16(3), 127–135. https://doi.org/10.1093/wjaf/16.3.127*

Jacobi, W.R. (1992). Potential insect vectors of the black stain root disease pathogen on Southern Vancouver Island. *Journal of the Entomological Society of British Columbia, 89, 54–56.*

Jacobs, K., Wingfield, M.J. (2001). *Leptographium Species: Tree Pathogens, Insect Associates, and Agents of Blue-Stain*. St. Paul, Minnesota: APS Press. 207 p.

Jombart, T. (2008). Adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics, 24(11), 1403–1405. https://doi.org/10.1093/bioinformatics/btn129*

Jombart, T., Devillard, S., Balloux, F. (2010). Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics 11, 94. https://doi.org/10.1186/1471-2156-11-94*

Kamvar, Z.N., Brooks, J.C., Grunwald, N.J. (2015). Novel R tools for analysis of genome-wide population genetic data with emphasis on clonality. *Frontiers in Genetics, 6, 208. https://doi.org/10.3389/fgene.2015.00208*

Kamvar, Z.N., Tabima, J.F., Grünwald, N.J. (2014). Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ 2, e281. https://doi.org/10.7717/peerj.281*

Kelsey, R.G., Joseph, G. (1998). Ethanol in Douglas-fir with black-stain root disease (*Leptographium wageneri*). *Canadian Journal of Forest Research, 28(8), 1207–1212. https://doi.org/10.1139/x98-087*

Knaus, B.J., Grünwald, N.J. (2017). vcfr: a package to manipulate and visualize variant call format data in R. *Molecular Ecology Resources, 17(1), 44–53. https://doi.org/10.1111/1755-0998.12549*

Linde, C.C., Zhan, J., McDonald, B.A. (2002). Population structure of *Mycosphaerella graminicola*: from lesions to continents. *Phytopathology, 92(9), 946–955. https://doi.org/10.1094/PHYTO.2002.92.9.946*

R Core Team. (2019). R: *A Language and Environment for Statistical Computing. R Foundation for Statistical Computing*, Vienna, Austria. https://www.R-project.org/

Wagener, W.W., Mielke, J.L. (1961). A staining-fungus root disease of ponderosa, jeffrey, and pinyon pines. *Plant Disease Reporter, 45, 831–835.*

Witcosky, J.J., Hansen, E.M. (1985). Root-colonizing insects recovered from Douglas-fir in various stages of decline due to black-stain root disease. *Phytopathology, 75, 399-402. https://doi.org/10.1094/Phyto-75-399*

Witcosky, J.J., Schowalter, T.D., Hansen, E.M. (1986). *Hylastes nigrinus* (Coleoptera: Scolytidae), *Pissodes fasciatus*, and *Steremnius carinatus* (Coleoptera: Curculionidae) as vectors of black-stain root disease of Douglas-fir. *Environmental Entomology, 15(5), 1090–1095. https://doi.org/10.1093/ee/15.5.1090*