EVIDENCE FOR CLIMATE AS A DRIVER OF GENETIC DIVERGENCE IN NATIVE POPULATIONS OF THE DOUGLAS-FIR SWISS NEEDLE CAST FUNGUS *PHAEOCRYPTOPUS GAEUMANNII*

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INTRODUCTION

Swiss needle cast (SNC) is a Douglas-fir foliage disease caused by the fungus *Phaeocryptopus gaeumannii* Rohde (Petrak). The spore-bearing structures of this fungus (pseudothecia) occlude the stomata in Douglas-fir needles resulting in an inhibition of gas exchange and photosynthesis, premature foliage loss, and reduced growth (Hansen et al., 2000; Manter et al., 2000). The volume growth reductions inflicted by SNC can exceed 50% in severely diseased stands (Maguire et al., 2011). Since the 1990s, the disease has continued to intensify in coastal low-elevation forests in the Pacific Northwest due to a combination of factors, including forest management practices that have resulted in extensive young Douglas-fir plantations in the coastal Sitka spruce vegetation zone (Hansen et al., 2000; Shaw et al., 2011), and a climate that is conducive to *P.gaeumannii* infection (Hansen et al., 2000; Lee et al., 2013, 2016; Manter et al., 2005; Rosso and Hansen, 2003; Stone et al., 2007, 2008; Watt et al., 2010, 2011).

The possibility that a more aggressive pathogen strain or race may have contributed to the emergence and intensification of SNC was the focus of early studies of the population genetics of *P. gaeumannii.* That research revealed that the Oregon *P. gaeumannii* population is subdivided into two reproductively-isolated lineages (designated Lineage 1 and Lineage 2) that may constitute phylogenetic species (Winton et al., 2006). Lineage 1 was found to be widespread in northwestern North America (i.e. the native range of Douglas-fir) as well as where Douglas-fir was planted as an exotic in the eastern U.S.A., Europe, and New Zealand. Lineage 2, however, was only found in the SNC "epidemic zone" in the northwestern Oregon Coast Range (Winton et al., 2006). Initial epidemiological studies seemed to implicate Lineage 2 in the recent intensification of SNC in western Oregon. It was found in the highest abundance where SNC was most severe in the western Coast Range, and was absent at sites further inland where SNC was less severe. Forest stands in which both Lineages were present and where the proportion of Lineage 2 was greater than Lineage 1 had greater foliage loss and more severe foliage discoloration (Winton et al., 2006). Compared to Lineage 1, the recovery of Lineage 2 isolates was twice as likely in severely diseased stands, and only half as likely in stands rated as healthy (Winton et al., 2006).

The current study aimed to assess the spatial genetic variation in *P. gaeumannii* across a disease gradient in the western Oregon and Washington Coast Ranges in relation to climatic variables. The goal was to identify factors that might influence SNC disease severity and pathogen population structure along this gradient. The specific objectives of this study are to 1) map the spatial distributions of the two *P. gaeumannii* lineages in relation to SNC disease severity in the western Oregon and Washington Coast Ranges, and 2) apply a multivariate statistical ordination to examine relationships between the genetic structure of *P. gaeumannii* populations, SNC disease severity, climate, and geography in this region.

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METHODS

This study analyzed multilocus SSR genotypes (MLGs) for nine polymorphic microsatellite markers (Winton et al., 2007) in a sample of 549 *P. gaeumannii* isolates collected from 14 sites in the Oregon State University Swiss Needle Cast Cooperative plot network in northwestern Oregon and southwestern Washington in 2014 (Bennett and Stone, 2016; Ritóková et al., 2016), as well as 304 isolates collected from nine sites in western Washington in 2015 (Table 1, Figure 1). Foliage sampling, fungal isolations, culturing, DNA extraction, PCR amplification, and microsatellite genotyping were performed using the methods described in (Bennett and Stone, 2016). Multilocus genotypes were analyzed to determine the relative abundances of both *P. gaeumannii* lineages recovered from each of the 23 sites in western OR and WA. Maps depicting the distributions of the two lineages in this region were superimposed on SNC aerial survey maps from surveys conducted by the Oregon Department of Forestry (ODF), the USDA Forest Service, and the Washington Department of Natural Resources (Ramsey et al., 2015; Ritóková et al., 2016) (Figure 1).

Foliage retention was estimated using the methods described in (Ritóková et al., 2016) for each of the trees from which our isolates were collected, and the average foliage retention index (AFR) was calculated by averaging the values for three canopy sections from each tree.

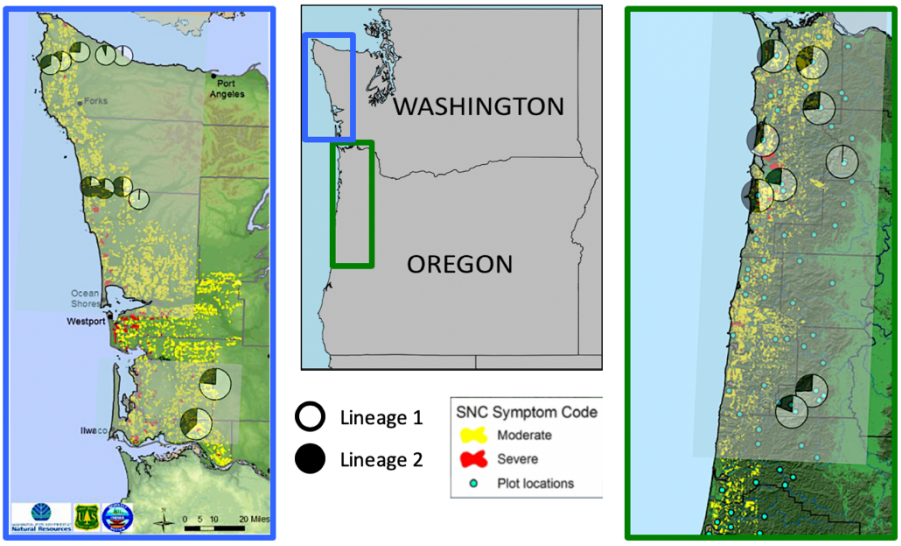

Figure 1. Aerial survey maps showing SNC disease with overlay showing the relative proportions of the two crypic *P. gaeumannii* lineages recovered from each site. Left: Washington SNC aerial survey 2015 map (Ramsey et al. 2015). Southern sites sampled in 2014, Olympic Peninsula sites sampled in 2015. Right: Oregon aerial survey map, sites sampled from SNCC plot network in 2014 (Ritóková et al. 2016).

A non-metric multi-dimensional scaling (NMS) ordination was used as a non-linear approach to depict the genetic differentiation between sampling sites based on allele frequencies. Two separate NMS analyses were performed, one that included all sites sampled in 2014 and 2015, and one that only included the sites sampled in 2014 for which SNC severity data were available. For the analysis that included all 23 sample sites, a 23 x 212 matrix of sample sites x allele frequencies was used as the main matrix. For the analysis that included only the 2014 sample sites, a 14 x 188 matrix of sample sites x allele frequencies was used. Both NMS analyses were based on Euclidean distance measures, and were conducted in PC-ORD 7 (McCune and Mefford 2016).

The environmental matrices consisted of climatic and geographic variables associated with each site, and were used for the joint-plot overlay to visualize statistical correlations with P. gaeumannii allele frequencies. These matrices included geographic coordinates (latitude and longitude in decimal degrees), elevation, and nine climate variables that have been used in predictive models of SNC severity and P. gaeumannii abundance (Manter et al., 2005; Stone et al., 2007, 2008; Watt et al., 2010). Time-series values for the climatic variables were obtained for each of the sample sites from the PRISM data explorer (http://www.prism.oregonstate.edu) (Table 2). The secondary matrix for the NMS with sites sampled in 2014 included a column corresponding to

the average foliage retention index (AFR). The matrices also included a column of values corresponding to the relative proportion of Lineage 2 isolates recovered from each of the sample sites. The joint-plot overlay is displayed as a series of radiating vectors, with the direction of the vector corresponding to the statistical correlation with a given NMS axis, and the length of the vector corresponding to the strength of the relationship (r, Pearson correlation coefficient).

Table 1. Summary of sample sizes, numbers of multilocus genotypes (MLG), and diversity statistics for the 2014 and 2015 sample collection years.

Year	Sites	Trees	Isolates	MLG	Gene Diversity (H_{exp})[a]	Genotypic Diversity (H)[b]
2014 (NW OR, SW WA)	14	70	549	332	0.802	5.54
2015 (NW WA)	9	45	304	227	0.798	5.21
Total	23	115	853	559	0.809	6.06

Diversity statistics calculated for rarefied sample size of 8 isolates.
[a]Nei's 1978 Gene Diversity.
[b]Shannon-Weiner Diversity Index (H).

Table 2. Climatic, geographic, and disease variables corresponding to the abbreviations used in NMS joint plot overlay in Figure 3.

Variable (units)	Abbreviation
Latitude (decimal degrees)	Lat
Longitude (decimal degrees)	Long
Elevation (feet)	Elev (ft)
January 2014 minimum temperature (˚C)	Jan Tmin
Winter 2014 mean average temperature (November 2013-March 2014) (˚C)	Tmean Winter
August 2014 maximum temperature (˚C)	Aug Tmax
Summer 2014 maximum temperature (May-September 2014) (˚C)	Summ. Avg. T
Summer 2014 mean temperature (May-September 2014) (˚C)	Summ. Tmean
May-July 2014 mean average dewpoint temperature (˚C)	MADT
May-July 2014 maximum vapor-pressure deficit (kPa)	Max VPD
Relative proportion of Lineage 2 isolates in sample site (Number of Lineage 2 Isolates/Total Number of Isolates)	PL2
Disease severity- Average foliage retention	AFR

RESULTS

An overlay of the maps showing the relative proportions of *P. gaeumannii* Lineages 1 and 2 at each of the sample sites on the SNC aerial disease survey maps from Oregon and Washington revealed a strong association between Lineage 2 and SNC symptom severity. Generally, sites nearest the coast had the highest proportions of Lineage 2 and the most severe SNC symptoms (Figure 1). The multilocus SSR genotype data also revealed that Lineage 2 in Washington has a similar coastal distribution to the sites in northwestern Oregon, and also exhibits a similar relationship with SNC disease severity. Sites further inland, where Lineage 2 is absent, have

little or no SNC symptoms (Figure 1). The southwestern-most site in the Olympic peninsula sampling, near Queets, had the highest relative proportion of Lineage 2 of any site in our sampling distribution (PL2 = 0.774).

In both of the NMS ordinations, two axes accounted for most of the variation in allele frequencies (cumulative variance explained = 0.875 (Figure 2A), and 0.917 (Figure 2B)). The alleles of one SSR locus (PgDi1) contributed to the distribution of sample sites along Axis 1. For example, in the NMS that included all sites sampled in 2014 and 2015, the PgDi1 allele 96, which is exclusive to Lineage 1, had a very strong negative relationship with NMS Axis 1 (r = -0.959), while PgDi1 alleles 100 and 102, which are exclusive to Lineage 2, had strong positive correlations with NMS Axis 1 (r = 0.719, r = 0.602 for PgDi1 100 and 102, respectively) (Figure 2) (Winton et al., 2007). This resulted in an arrangement of sample sites along NMS Axis 1 according to the relative proportions of the two lineages present in the sample site. This also resulted in the separation of coastal and inland sample sites along this axis according to the lineage distributions. Longitude was strongly correlated with this arrangement, as sites nearer to the coast generally had greater proportions of Lineage 2 (and thus higher frequencies of SSR alleles associated with this lineage), while Lineage 2 was generally recovered at low frequencies from the sample sites further inland.

In the NMS that included all sites, the proportion of Lineage 2 had the strongest relationship with NMS Axis 1 (PL2, $r = 0.878$), and longitude had the second strongest relationship with this axis (Long, $r = -0.477$) (Figure 2A, Table 3). May-July 2014 dew point temperature had positive relationships with both NMS axes (MADT, NMS Axis 1: $r = 0.426$, NMS Axis 2: $r = 0.378$), and covaried with mean winter temperature on both axes (Tmean Winter: $r_{Axis\ 1} = 0.386$, $r_{Axis\ 2} = 0.313$) (Figure 2A, Table 3). January 2014 minimum temperature had the strongest relationship with NMS Axis 2 (Jan Tmin, $r = 0.447$). August maximum temperature (Aug. Tmax, $r = -0.549$), Summer mean temperature (Summ. Tmean, $r = -0.383$), Summer average maximum temperature (Summ. Avg. Tmax, $r = -0.409$), and May-July maximum vapor pressure deficit (Max VPD, $r = -0.402$) were all negatively correlated with NMS Axis 2 (Figure 2A, Table 3).

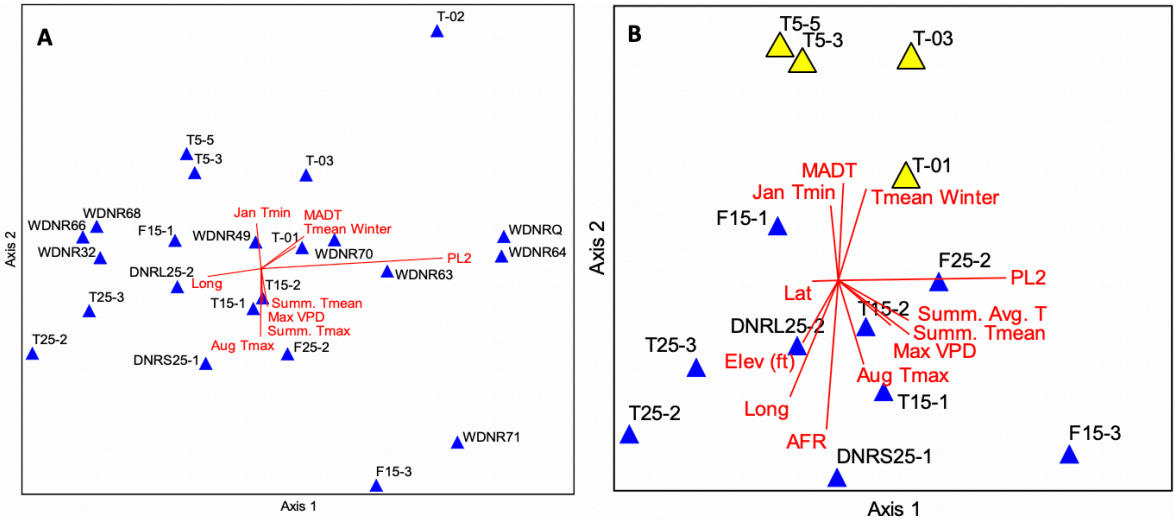

Figure 2. Joint plots from non-metric multidimensional scaling (NMS) ordination of sampling sites in multilocus SSR allele frequency space. The plots are overlaid with vectors representing environmental and geographic variables, and the relative proportion of Lineage 2 (PL2) recovered from the site (Table 2). Only variables with correlation coefficient (r) greater than 0.200 are shown. A) Sites sampled in northwestern Oregon and southwestern Washington in 2014 and northwestern Washington in 2015. Cumulative variance explained = 0.875, final stress = 11.135. B) Sites sampled in 2014 only, with a vector representing an average foliage retention index (AFR) estimated from 5 trees at each site. Cumulative variance explained = 0.917, final stress = 9.036. The yellow triangles in (B) represent sites in the original SNC epidemic zone near Tillamook, Oregon.

Table 3. Pearson coefficients (r) for correlations between the NMS axes and each of the variables used in the joint plot overlay in Figure 2, Table 2.

	2014 + 2015 (Figure 2A)		2014 only (Figure 2B)	
Variable	**Axis 1**	**Axis 2**	**Axis 1**	**Axis 2**
Lat	0.121	0.106	-0.347	-0.048
Long	-0.477	-0.188	-0.470	-0.736
Elev (ft)	NA	NA	-0.404	**-0.539**
Jan Tmin	-0.139	0.447	-0.191	**0.589**
Tmean Winter	0.386	0.313	0.356	**0.651**
Aug Tmax	-0.036	**-0.549**	0.343	**-0.625**
Summ. Avg. Tmax	0.166	-0.409	**0.566**	-0.430
Summ. Tmean	0.090	-0.383	**0.569**	**-0.501**
MADT	0.426	0.378	0.150	**0.672**
Max VPD	0.025	-0.402	0.490	-0.458
PL2	**0.878**	0.219	**0.873**	0.101
AFR	NA	NA	-0.229	**-0.831**

For the NMS that included only the 2014 sample sites, the proportion of Lineage 2 also had the strongest relationship with NMS Axis 1 (PL2, $r = 0.873$), and a similar relationship with Longitude also was observed (Long, $r = -0.470$). Summer maximum temperature and mean summer temperature were correlated with both axes (Summ. Avg. Tmax, $r_{Axis\ 1} = 0.566$, $r_{Axis\ 2} = -0.430$; Summ. Tmean, $r_{Axis\ 1} = 0.569$, $r_{Axis\ 2} = -0.501$). Elevation also had negative correlations with both axis (Elev (ft.), $r_{Axis\ 1} = -0.404$, $r_{Axis\ 2} = -0.539$). The average foliage retention index had a very strong negative correlation with Axis 2 (AFR, $r = -0.831$). Variables corresponding to winter temperature and mean dew point temperature had strong positive correlations with Axis 2, while variables associated with summer temperature and vapor pressure deficit had strong negative correlations with this axis (Figure 2B, Table 3).

DISCUSSION

The geographic distribution of *P. gaeumannii* Lineage 2 in the western Coast Ranges in Oregon and Washington appears to be correlated with SNC severity, as assessed via aerial survey. In general, SNC symptoms are most severe where both lineages coexist within 20 miles of the coast. Further inland, Lineage 2 is exceedingly rare and SNC symptoms are less severe or absent (Figure 1). Two sites at the southern end of our sampling distribution did not fit this trend, as the relative proportion of Lineage 2 at these sites were 0.20-0.25, but these sites are outside of the SNC epidemic zone and did not exhibit visible symptoms of SNC. Overall, these trends are in agreement with the findings of Winton et al. (2006), which suggested that sample sites with greater abundances of Lineage 2 also had greater SNC severity.

The multivariate non-metric multidimensional scaling (NMS) analysis revealed strong spatial genetic differentiation between inland and coastal sample sites. This approach allowed the strength of the relationships

between the environmental variables and the observed allele frequency variation to be visualized. The geographic distribution of allele frequency variation was highly correlated with mean winter temperature, mean summer maximum temperature, maximum vapor pressure deficit, and mean average dew point temperatures in the year prior to sampling. These results corroborate previous models of SNC severity that included these variables, and provide further statistical support for their importance as predictors of disease severity (Lee et al., 2013, 2016; Manter et al., 2005; Stone et al., 2007, 2008; Watt et al., 2010, 2011). Foliage retention was lowest at sites where Lineage 1 and Lineage 2 coexisted near the coast in Tillamook County, Oregon. These sites had the highest winter average temperature and the highest January minimum temperature, as well as the highest spring/summer dew point temperatures. These sites also had low summer temperatures and low vapor pressure deficits. Foliage retention was much higher at the higher elevation inland sites where the winters were cold, summers were hotter and drier, and Lineage 2 was absent.

The results of the NMS ordinations suggest that climate may be a potential driver of genetic diversification in *P. gaeumannii* populations. The *P. gaeumannii* isolates collected from the original SNC epidemic zone exhibited a unique allelic signature suggesting that some adaptation to local climate, or natural selection for advantageous genotypes, has occurred in this region. Whether these genotypes are more aggressive, or the observed distributions of SNC severity is simply the result of greater *P. geaumannii* abundance and needle colonization due to a conducive climate, should be the focus of future studies. This system could provide a model for answering a variety of biological questions relating to the influence of climate in structuring fungal populations, and the role of climate change as a driver of fungal forest pathogen emergence.

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