Mycologia, 98(5), 2006, pp. 781–791.
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Population structure suggests reproductively isolated lineages of *Phaeocryptopus gaeumannii*

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Abstract: A survey of the genetic diversity and population structure of the Douglas-fir Swiss needle cast pathogen *Phaeocryptopus gaeumannii* was conducted with single-strand conformational polymorphisms (SSCP) to screen for variability in mitochondrial and nuclear housekeeping genes. Thirty host populations representing the natural range of Douglas-fir as well as locations where the tree was planted as an exotic were sampled. Sequencing of SSCP variants revealed that the method accurately detected both single nucleotide and indel polymorphisms. Sequence information was used to construct multilocus gene genealogies and to test various hypotheses of recombination (outcrossing) and clonality (selfing). We found that *P. gaeumannii* in the region of Oregon's Swiss needle cast epidemic exhibits strong multilocus gametic phase disequilibrium and is subdivided into two reproductively isolated sympatric lineages. Low genotypic diversity together with the presence of overrepresented genotypes in both lineages suggests a predominantly selfing reproductive mode. Genotypes of one lineage were found in isolates from a widespread geographic distribution, occurring throughout much of the Pacific Northwest as well as nonindigenous populations abroad that have historical reports of disease. Genotypes of the second lineage were detected only in isolates from Oregon's coastal region. Within the main epidemic area, abundance of this second lineage in young plantations appeared to be correlated with disease severity.

Key words: Ascomycota, foliage pathogen, forest pathology, population biology, population genetics

INTRODUCTION

Phaeocryptopus gaeumannii is an ascomycetous foliar parasite of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco). It grows as a haploid mycelium within needle tissue and sexual structures (pseudothecia), in which a brief diploid phase and meiosis occur, develop in stomata to release haploid ascospores in the spring. Asexual reproduction has not been observed but the fungus is capable of self-fertilization (Hood 1977, Winton 2001), but it is unknown whether it is capable of outcrossing. The fungus first was discovered in Switzerland in 1925 (Gäumann 1930) due to its association with Swiss needle cast disease, characterized by chlorosis, severe defoliation and growth reduction in Douglas-fir plantations. The fungus later was found to be widespread in western North America and was presumed to be endemic, although considered innocuous throughout the native range of its host in western North America (Boyce 1940).

Since its initial discovery Swiss needle cast has been reported in other locations where Douglas-fir has been cultivated outside of its native range (Boyce 1940), most recently in New Zealand (Hood and Kershaw 1975, Hood 1997). While the fungus apparently has long been a widespread component of the natural Douglas-fir mycobiota, Swiss needle cast disease has not been a significant problem in Pacific Northwest forests until recently, although the disease has been noted in Christmas tree plantations since the late 1970s (Hadfield and Douglas 1982). Since about 1990 a severe epidemic of Swiss needle cast has been observed in Douglas-fir forest plantations along the Oregon coast, particularly near the town of Tillamook, and disease severity is associated with abnormally high levels of *P. gaeumannii* (Hansen et al 2000, Manter et al 2005). Aerial surveys conducted annually since 1996 by the Oregon Department of Forestry indicate nearly 160 000 ha of Douglas-fir plantations in coastal Oregon are affected by this disease (Hansen et al 2000, A. Kanaskie, Oregon Department of Forestry, pers comm). Hypotheses proposed to account for the abrupt increase of this previously benign parasite include changes in forest management practices (Hansen et al 2000), climatic factors (Manter et al 2005) and the possible existence of a more virulent strain or race of the pathogen.

In attempting to reconcile the apparent harmlessness of *P. gaeumannii* on its host in western North America with its virulence on Douglas-fir planted in Europe, Boyce (1940) considered the possible existence of virulent races or strains of the pathogen. Since Boyce's (1940) original description of the

Accepted for publication 17 Jul 2006.
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disease the existence of a virulent *P. gaeumannii* strain has been postulated often but it never has been tested. Nothing is known about fundamental life history traits of the fungus such as its genetic structure, mating system or geographic differentiation. The tools of population genetics and molecular and evolutionary biology recently have demonstrated the power that explicit tests of reproductive mode can have on understanding the diverse life histories of fungal pathogens (Taylor et al 1999). The emerging theme is that reproductive mode (selfing or outcrossing) and reproductive morphology (sexual or asexual) are uncoupled to the extent that almost all fungi, including those with no known means of sexual reproduction, exhibit both clonal and recombining population structures in nature. Accordingly, in this paper, ''reproduction by recombination is defined as the production of progeny genomes that are mixtures of genetically different parental genomes, and reproduction by clonality is defined as the production of progeny genomes that are identical to the parental genome'' (Taylor et al 1999). These two models imply genetic outcomes that are amenable to empirical tests of *P. gaeumannii* populations.

We have used single-strand conformation polymorphism (SSCP; Orita et al 1989) to screen for DNA sequence variation at five loci in the *P. gaeumannii* genome. Hypotheses of clonality (selfing) and recombination (outcrossing) were evaluated with both population genetic and phylogenetic theory to test whether there is evidence of unrestricted gene flow throughout the entire range of the fungus or whether the species is geographically differentiated or includes reproductively isolated populations.

MATERIALS AND METHODS

Fungal sampling and isolation.—The locations of the 30 sampled host populations and the numbers of individual *P. gaeumannii* ascospores sampled from each are provided (TABLE I). Because ascospores are dispersed by wind and rain, a population was defined arbitrarily as a single plantation or stand of the Douglas-fir host. We sampled 17 such populations of the coastal form of Douglas-fir (*P. menziesii* var *menziesii*) within the native range of Douglas-fir in western Oregon and Washington. Ten of these were within the main epidemic area of the Oregon Coast Range (between Waldport and Astoria within 18 miles of the coast), four of which were from mature stands at least 80 y old. All other stands in the study were plantations aged 10–25 y. Seven of the 17 native coastal form populations were located outside the main epidemic area. One population from New Mexico represented a natural stand of the interior form of Douglas-fir (*P. menziesii* var. *glauca* (Beissn.) Franco). Because *P. gaeumannii* previously has caused disease only where Douglas-fir was planted as an exotic species, we also sampled 12 populations from outside the natural Douglas-fir range from locations where there had been reports of disease.

Trees selected for sampling were nonadjacent and haphazardly chosen within a stand with no regard to apparent disease severity. Branches with foliage bearing pseudothecia were collected in spring 1997, transported to the lab and prepared for isolation. Needles were examined for the presence of *P. gaeumannii* pseudothecia and absence of other common needle fungi *Stomiopeltis* sp. and *Rasutoria pseudotsugae*. Single-ascospore *P. gaeumannii* isolates were obtained by adhering 10–50 needles bearing only *P. gaeumannii* pseudothecia to lids of Petri plates containing water agar to allow ascospore discharge on the agar surface. Needles were selected arbitrarily from the most recent needle cohort (1996 or 1995) bearing mature pseudothecia. Samples were incubated in a moist chamber 3–5 d at 17 C and one individual ascospore per host tree was removed from the agar surface with a heat-drawn Pasteur pipette. Isolates were grown at 17 C for 3–5 mo on potato dextrose agar (Difco Laboratories, Detroit, Michigan). Single isolates were obtained from 5–21 trees per stand; a total of 402 isolates were tested.

DNA extraction and primer design.—Cultures were prepared for DNA extraction by scraping about 30 mg mycelium from the agar surface. Collected mycelium was placed into 2.0 mL microfuge tubes with 1.0 mm zirconia/silica beads (Biospec Products, Bartlesville, Oklahoma) and 1 mL CTAB extraction buffer (2% CTAB (cetyltrimethylammonium bromide), 100 mM Tris, pH 8.0, 20 mM Na_2EDTA pH 8, 1.4 M NaCl, 1% polyvinylpolypyrrolidone, 0.1% 2-mercaptoethanol) and shaken in a Mini-Beadbeater (Biospec Products) for 30 s at 5000 rpm. After mixing samples were incubated at 65 C for 2 h. The DNA was purified in 24:1 chloroform:isoamyl alcohol and further purified to reduce PCR inhibitors by passing the extract over QIAAmp Spin Columns (QIAGEN Inc., Valencia, California) according to the manufacturer's instructions.

Four isolates representing geographic extremes were chosen to evaluate portions of six nuclear protein-coding genes and one mitochondrial ribosomal gene for intraspecific variation. Genes encoding the products actin (ACT), α-tubulin (ATUB), β-tubulin (BTUB), calmodulin (CAM), chitin synthase 1 (CHS), translation elongation factor 1-alpha (TEF1), and the mitochondrial ribosomal small subunit (mtSSU) were chosen for initial examination because some regions, particularly introns, had shown variability within other species of Ascomycota (Carbone and Kohn 1999, Geiser et al 1998, Johannesson et al 2000, O'Donnell et al 1998). Selected regions were amplified and sequenced with these primer pairs (5′ to 3′): ACT1F, gtatgtcgaaggccggtttc and ACT1R, tagcagagcttctccttgatgtc for ACT, ATUB1F, ttgccagatcgccaactc and ATUB1R, agtggacgaaggcacgct for ATUB, T1 and T2 (O'Donnell and Cigelnik 1997) for BTUB, CAM2F, gartwcaaggaggccttctc and CAM2R, ttytgcatcatgagctggac for CAM, CS1F, gcttacgatgtayaaygaggacg and CS1R, cgagyttgtattcraarttytg for CHS, TEF1F, cgtaccatcgagaagttcga and TEF1R, tcaccagacttgat-

TABLE I. *Phaeocryptopus gaeumannii* sampling locations

Region[a]	Number[b]	Population	Location	N[d]
Australasia	1	New Zealand North Island	Rotorua	16
	2	New Zealand South Island	Dunedin	21
Europe	3	England	Dunheld, Perthshire	15
	4	France	Epinal	14
	5	Germany	Grosshansdorf	14
	6	Italy	Tosi	17
	7	Switzerland1	Horgen	8
	8	Switzerland3	Rapperswil	8
	9	Switzerland4	Schaffhausen	5
	10	Switzerland5	Zürich	21
U.S.-SW	11	New Mexico	Lincoln National Forest	5
U.S.-East	12	New York	Lansing	12
	13	Vermont	Burlington	17
U.S.-PNW-nonepidemic	14	Canby	Canby, OR	5
	15	Foster Dam	Sweethome, OR	9
	16	Gold Beach	Gold Beach, OR	18
	17	MacDonald Forest	Corvallis, OR	16
	18	Olympia	Olympia, WA	16
	19	Phipps	Elkton, OR	11
	20	Toledo	Toledo, WA	17
U.S.-PNW-nonepidemic	21	Bixby[c]	Beaver, OR	18
	22	Coal[c]	Nahalem, OR	18
	23	Drift Creek	Waldport, OR	17
	24	Edwards[c]	Tillamook, OR	11
	25	Juno Hill	Tillamook, OR	16
	26	Limestone	Beaver, OR	14
	27	Lower Stone	Tillamook, OR	8
	28	North Fork	Nahalem, OR	11
	29	Prairie[c]	Tillamook, OR	15
	30	Upper Stone	Tillamook, OR	9

[a] SW, Southwest, PNW, Pacific Northwest.
[b] Population identification number.
[c] Mature Stand > 80 y old.
[d] Number of individual ascospores sampled.

gaacttg for TEF1, and MS3F, gatgatggctctgattgaac and MS2 (White et al 1990) for mtSSU.

PCR reactions (50 μL) were performed in 1× enzyme buffer, 200 μM dNTP, 0.4 μM forward and reverse primers, 0.05 U/μL RedTaq DNA polymerase (Sigma, St Louis, Missouri) and 20–200 ng template DNA for 35 cycles of 60 s at 94 C, 60 s at 50–55 C, and 60 s at 72 C. After amplification PCR products were prepared for direct sequencing by isopropanol precipitation. Cycle sequencing on both strands was performed on an ABI model 377 fluorescent sequencer (PE Applied Biosystems Inc., Foster City, California) employing dye-terminator chemistry. Contigs were assembled and the overlapping chromatograms edited with the Staden software package (Staden 1996). Sequence alignments were generated with Clustal X (Thompson et al 1997) and compared for regions variable among the four tester isolates. Because they were polymorphic, BTUB, mtSSU, ATUB, CHS and CAM were selected for population-level SSCP analysis. For each gene, internal priming sites (200–500 base-pairs apart) were designed to span variable regions (TABLE II). The locus specific forward and reverse primers were extended at their 5′ ends with M13 universal sequencing primers. The M13 tails served as templates for a second, labeling reaction so that costs could be minimized by the purchase of only two fluorescently labeled primers (Boutin et al 1997).

SSCP analysis.—BTUB and mtSSU were amplified simultaneously in a multiplex reaction with all four primers at an equal 0.4 μM concentration. Then 0.5 μL of the PCR products were used as templates for a second, labeling PCR reaction containing M13(–21) and M13R primers, respectively labeled with the FAM and TET fluorescent dyes at their 5′ ends (Integrated DNA Technologies Inc., Coralville, Iowa). Labeled primer concentrations were limited to 16 nM to prevent excessive fluorescent signal. All PCR reactions were performed in 1× enzyme buffer, 200 μM dNTP, forward and reverse primers, 0.05 U/μL RedTaq DNA polymerase (Sigma, St Louis, Missouri), and

TABLE II. Loci and primers designed in this study and used for SSCP analysis of *Phaeocryptopus gaeumannii*

Locus	Primer Designation	Sequence[a] 5′→3′	Fragment Size (bp)
ATUB	ATUB2FM	M13(-21)-CGCCAAGACCTCTTCTACAT	163
	ATUB2RM	M13R-TGTTGAAGTCTGCGAAACAC	
BTUB	BTUB1FM	M13(-21)-GAAATGCTTGCAGGTCCACC	351
	BTUB4RM	M13R-CAGTATCCTCACTGCCATTG	
CAM	CAM3FM	M13(-21)-GTTATGCTGATCCAAACAGC	390
	CAM3RM	M13R-ACTCTTCCACAATCGAGACC	
CHS	CS3FM	M13(-21)-GTGTGATCAAGAACATCGAG	260
	CS4RM	M13R-AGTAGTGTACTCGTAAATGTG	
mtSSU	MS3FM	M13(-21)-GATGATGGCTCTGATTGAAC	469–490
	MS4RM	M13R-TATACGACATAGTCGATGCG	

[a] M13(-21) 5′-TGTAAAACGACGGCCAGT-3′, M13R 5′-CAGGAAACAGCTATGACC-3′

20–200 ng template DNA in a total volume of 6 μL. Thermal cycling was 35 cycles of 30 s at 94 C, 30 s at 52 C and 30 s at 72 C.

After PCR reactions were completed, 0.5 μL labeled products were denatured for 5 min at 95 C in 5.25 μL deionized formamide, 0.5 μL 0.3 N NaOH and 0.5 μL GeneScan-500 internal lane size standard (PE Biosystems). Samples were cooled on ice and loaded immediately onto a nondenaturing 12 cm 0.4× MDE (FMC Bioproducts, Rockland, Maine) gel amended with 10% glycerol. The first and last four lanes of each gel were loaded with the four tester isolates to serve as comparisons to identify both known and new alleles. Gels were run at 15 C at 20 W (constant limiting factor), 2000 V, 40 mA for 4.5 h on a Prism 377 Automated DNA Sequencer (PE Biosystems) set to GeneScan mode with filter set C. The gels were analyzed with GeneScan Analysis 2.0.2 software (PE Biosystems). To ensure that putative alleles inferred from patterns on SSCP gels (phenotypes) corresponded to unique nucleotide sequences, each allele was sequenced from at least three randomly chosen representatives. Where fewer than three alleles were found, all were sequenced.

Identical locus-specific amplification, labeling, SSCP and sequencing conditions as described above were used to assess variation at the ATUB, CHS and CAM loci, except that primer concentrations in the initial, locus-specific multiplex reactions were altered respectively to 0.13 μM, 0.4 μM, and 0.5 μM. This prevented shorter PCR products from depleting reagents before the longer, less efficient products could be synthesized.

Data analysis.—Because *P. gaeumannii* is presumed haploid with a potentially mixed reproductive mode (selfing and outcrossing), three multilocus permutation tests suitable for detecting recombination and population structure in haploid organisms were employed. All tests used the variable positions in DNA sequence data inferred from SSCP unless otherwise indicated. The multilocus index of association (I_A) was used to detect correlations between alleles at different loci (i.e. gametic disequilibrium, Brown et al 1980) to discern between the extremes of panmixia and clonality and to detect intermediate types of population structure such as an ''epidemic'' structure, in which one or a few genotypes are over-represented, as well as reproductive isolation between two or more otherwise recombining groups (Maynard Smith et al 1993).

The parsimony tree length permutation test (Burt et al 1996, Koufopanou et al 1997) was used to estimate the probability that, by chance, two or more groups of isolates did not share polymorphisms. The null hypothesis for both tests was random recombination. The distribution of alleles (defined as nucleotide linkage groups for each locus) in sampled populations was compared to a null distribution obtained by randomly shuffling alleles among isolates by means of the computer program Multilocus v1.2 (Agapow and Burt 2001). The null distribution was generated from 1000 randomized datasets. PAUP* 4.0b4 for Windows (Swofford 1999) was used to compute the tree lengths of most parsimonious trees for the observed data and for the randomized datasets.

The third permutation method used the partition-homogeneity test (Farris et al 1994, Huelsenbeck et al 1996) as implemented in PAUP* to test the compatibility of trees constructed from different loci. Here the null hypothesis was clonality and the sum of tree lengths of individual loci was compared to a null distribution of summed tree lengths in 1000 randomized datasets where nucleotide positions were shuffled randomly among loci.

RESULTS

Sequencing and genetic diversity.—Four SSCP phenotypes (putative alleles) were detected at the BTUB locus and five at mtSSU. Four of these (1 BTUB and 3 mtSSU) were not present in the initial four screening isolates and subsequently were discovered by SSCP. ATUB, CHS and CAM were each biallelic. All phenotypes were unambiguous and easy to score. More than one SSCP phenotype for a locus was not observed. Sequencing of 43 random phenotypes demonstrated that all variants corresponded to unique nucleotide sequences (GenBank accession numbers EF033159–EF033201). Henceforth we considered phenotypes to be alleles at a locus. There were three variable sites at BTUB, two at both ATUB

intron 2 and CAM intron 2, one at CHS and five at mtSSU, including a 21 base-pair indel (TABLE III). The indel was treated as a single character. All point substitutions were considered to be the result of single mutational events because no site had more than two different nucleotides among alleles.

Allele frequencies are provided (TABLE IV). There was no obvious geographic pattern to allelic distribution, except that many loci tended to be fixed (monomorphic) in localities where Douglas-fir is exotic. One BTUB allele was restricted to the three U.S. populations outside the contiguous native Douglas-fir range. Two rare alleles were found for the mitochondrial locus. One was observed in two mature stands within the epidemic area (Edwards and Prairie), while the second was found in a young plantation near Gold Beach, at the southern limit of sampling in Oregon.

Population structure.—Of 160 theoretically possible multilocus genotypes, only 10 were found (TABLE V). Of these two (AAAAA and AABAA) were found exclusively in the U.S. populations sampled outside the contiguous natural Douglas-fir range. Because these populations were small and geographically and environmentally isolated from the rest of the U.S. populations we reasoned that they lacked opportunity to outcross with other genotypes. Therefore these genotypes were excluded from tests of recombination. The most widely distributed genotypes (BABAB, BBBAB and BCBAB) shared identical nuclear alleles and differed only at the mitochondrial locus. At least one (but usually two or all three) of these genotypes were found in all of the populations outside North America and all of the Pacific Northwest populations except Gold Beach. The remaining five genotypes were found exclusively in the Pacific Northwest.

The two most common genotypes comprised 67% of the genotypes found in the entire dataset and 85% of the genotypes found in the epidemic area. That two genotypes were overrepresented is suggestive of an ''epidemic'' type population structure, which can bias explicit tests for recombination towards clonality (Maynard Smith et al 1993). Therefore the following tests for recombination were performed on a reduced dataset that was ''clone-corrected'' by the removal of duplicated genotypes.

We used the information on genotype distribution (TABLE V) to assume that those present in the coastal epidemic area had the opportunity for recombination, whether or not they were capable of doing so. After adjusting for overrepresented genotypes (clone-correction), we used two methods (I_A and the parsimony tree length permutation test) to test explicitly for deviations from recombination and the partition-homogeneity test to test for deviations from clonality. These tests employed information from only the BTUB, mtSSU, CHS and CAM loci because ATUB was fixed in all native populations.

Multilocus gametic disequilibrium, as assessed by the I_A test, differed from that expected in a single, recombining population ($P = 0.003$). A similar result was obtained upon repeating the analysis with allele state data instead of nucleotides partitioned into linkage groups ($I_A = 1.2$, $P = 0.002$, mean I_A of null distribution = 0.0).

The parsimony tree length permutation test also supported deviation from complete panmixia. Separate genealogies for each of the five loci were well resolved and of minimal length (i.e. no homoplasy). However the four most parsimonious trees based on data from all four loci (a total of eight informative sites) indicated extensive incompatibility among genealogies from different loci and subdivided genotypes into two reproductively isolated groups

TABLE III. Sequence variation in the BTUB, mtSSU, ATUB, CAM and CHS genes of *Phaeocryptopus gaeumannii* and the nucleotide positions (arranged vertically) at which alleles differ

Locus	Allele	Position				
			1	2		
		5	9	9		
BTUB		1	6	2		
	A	T	G	C		
	B	T	G	T		
	C	C	G	C		
	D	C	C	C		
				1	1	4
		2	5	2	2	2
mtSSU[a]		6	9	4	6	3
	A	C	1	A	T	G
	B	C	0	A	T	T
	C	G	1	A	T	G
	D	C	0	A	A	T
	E	C	0	T	T	T
		4	9			
ATUB		0	3			
	A	G	G			
	B	A	A			
		1	2			
		3	1			
CAM		9	7			
	A	C	A			
	B	T	G			
		1				
		4				
CHS		9				
	A	T				
	B	C				

[a] 21 base-pair insertion at position 61: 1, present; 0, absent.

TABLE IV. Allele frequencies at five loci for worldwide sampling of 30 *Phaeocryptopus gaeumannii* populations

	Locus and alleles														
	BTUB				mtSSU					ATUB		CHS		CAM	
Population	A	B	C	D	A	B	C	D	E	A	B	A	B	A	B
New Zealand North Island		1.00				0.94	0.06				1.00	1.00			1.00
New Zealand South Island		1.00				0.05	0.95				1.00	1.00			1.00
England		1.00					1.00				1.00	1.00			1.00
France		1.00			0.83		0.17				1.00	1.00			1.00
Germany		1.00			0.85		0.15				1.00	1.00			1.00
Italy		1.00			1.00		0.00				1.00	1.00			1.00
Switzerland1		1.00					1.00				1.00	1.00			1.00
Switzerland3		1.00			0.25		0.75				1.00	1.00			1.00
Switzerland4		1.00			0.60		0.40				1.00	1.00			1.00
Switzerland5		1.00					1.00				1.00	1.00			1.00
New Mexico	1.00				1.00						1.00	1.00		1.00	
New York	1.00				1.00					1.00		1.00		1.00	
Vermont	1.00				1.00					0.75	0.25	1.00		1.00	
Canby		1.00			0.20		0.80				1.00	1.00			1.00
Foster Dam		1.00			0.11		0.89				1.00	1.00			1.00
Gold Beach			1.00			0.83			0.17		1.00		1.00	1.00	
MacDonald Forest		0.94	0.06		0.19	0.06	0.75				1.00	0.94	0.06	0.06	0.94
Olympia		1.00			0.25	0.06	0.69				1.00	1.00			1.00
Phipps		0.27	0.73			0.82	0.18				1.00	0.27	0.73	0.73	0.27
Toledo		1.00			0.18		0.82				1.00	1.00			1.00
Bixby		0.59	0.41		0.12	0.41	0.47				1.00	0.61	0.39	0.39	0.61
Coal		0.39	0.61		0.06	0.67	0.28				1.00	0.39	0.61	0.61	0.39
Drift Creek		0.47	0.47	0.06	0.12	0.59	0.29				1.00	0.47	0.53	0.53	0.47
Edwards		0.36	0.64		0.09	0.64	0.18	0.09			1.00	0.36	0.64	0.64	0.36
Juno Hill		0.31	0.69		0.06	0.69	0.25				1.00	0.31	0.69	0.69	0.31
Limestone		0.86	0.14		0.14	0.21	0.64				1.00	0.86	0.14	0.14	0.86
Lower Stone		0.57	0.43			0.29	0.71				1.00	0.57	0.43	0.43	0.57
North Fork		0.64	0.36			0.45	0.55				1.00	0.64	0.36	0.36	0.64
Prairie		0.33	0.67		0.07	0.67	0.20	0.07			1.00	0.33	0.67	0.67	0.33
Upper Stone		0.56	0.44			0.44	0.56				1.00	0.56	0.44	0.44	0.56

(FIG. 1), hereafter designated Lineage 1 and Lineage 2. The most parsimonious trees from the combined genealogies were 11 steps in length, 3 longer than the minimum possible (1 step for each parsimony informative site = 8). Randomly shuffling the gene sequences among genotypes, leaving the linkage of nucleotides within loci intact, provided the null distribution for the recombination hypothesis. In 1000 such randomizations, 49 trees were found as short as or shorter than the observed most parsimonious trees. This provided suggestive evidence against recombination, but it could not be ruled out conclusively ($P = 0.049$).

The partition-homogeneity test was used to test the null hypothesis of clonality. In this case the sum of the individual locus tree lengths constructed from the observed data set (summed length = 8) was compared to summed tree lengths in randomized datasets, where nucleotide positions were shuffled randomly among loci. Trees constructed from the different loci were not incompatible at the 95% confidence level ($P = 0.072$, informative sites only, range of the summed tree lengths from randomized data was 8–11). Under these criteria the null hypothesis of clonal reproduction could not be rejected.

While geographic patterns of genotype distribution in the western United States were not obvious from the raw dataset, mapping the two lineages as separate entities was revealing (FIG. 2). Lineage 1 was the only lineage present in the four northern, interior populations and comprised the overwhelming majority (94%) of isolates from the MacDonald Forest stand in the eastern foothills of the Coast Range. It also was present in varying quantities in all populations in the epidemic area (31% to 86%). Conversely Lineage 2 was the only lineage present at the Gold Beach site on the southern Oregon coast and comprised the

TABLE V. Multilocus genotype frequencies in 30 worldwide populations of *Phaeocryptopus gaeumannii*. Loci are presented in the order BTUB, mtSSU, ATUB, CHS and CAM

	AAAAA	AABAA	BABAB	BBBAB	BCBAB	CBBBA	CCBBA	CDBBA	CEBBA	DBBBA	N_G[a]
New Zealand North Island				15	1						2
New Zealand South Island				1	18						2
England					15						1
France			10		2						2
Germany			11		2						2
Italy			17								1
Switzerland1					8						1
Switzerland3			2		6						2
Switzerland4			3		2						2
Switzerland5					21						1
New Mexico		5									1
New York	12										1
Vermont	12	4									2
Canby			1		4						2
Foster Dam			1		8						2
Gold Beach						15			3		2
MacDonald Forest			3		12	1					3
Olympia			4	1	11						3
Phipps				1	2	8					3
Toledo			3		13						2
Bixby			2		8	7					3
Coal			1	1	5	11					4
Drift Creek			2	1	5	8					4
Edwards			1	1	2	6		1		1	6
Juno Hill			1		4	11					3
Limestone			2	1	9	2					4
Lower Stone					4	2	1				3
North Fork				1	6	4					3
Prairie			1	1	3	9		1			5
Upper Stone					5	4					2
Total	24	9	65	24	176	88	1	2	3	1	

[a] number of genotypes.

majority of isolates (73%) from the site near the Oregon Department of Forestry D.L. Phipps Forest Nursery. The proportion of this lineage varied (14–69%) in sites within the epidemic area.

There were correlations between the proportions of the two lineages and Swiss needle cast disease severity measurements (taken from Winton et al 2003) at five young plantations within the epidemic area for which we had sufficient data (FIG. 3). For example canopy density, a measurement of defoliation, decreased

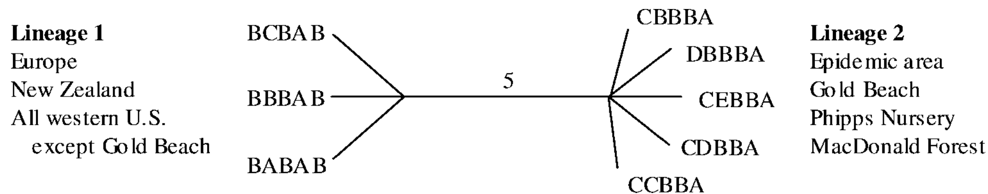

FIG. 1. Multilocus gene genealogy constructed from informative nucleotide positions in the BTUB, mtSSU, ATUB, CHS and CAM loci, respectively, for genotypes present in native Phaeocryptopus gaeumannii populations only. The genealogy collapsed onto a single branch subdividing genotypes into two reproductively isolated groups separated by five fixed nucleotide changes. All locations where these genotypes were found also are shown.

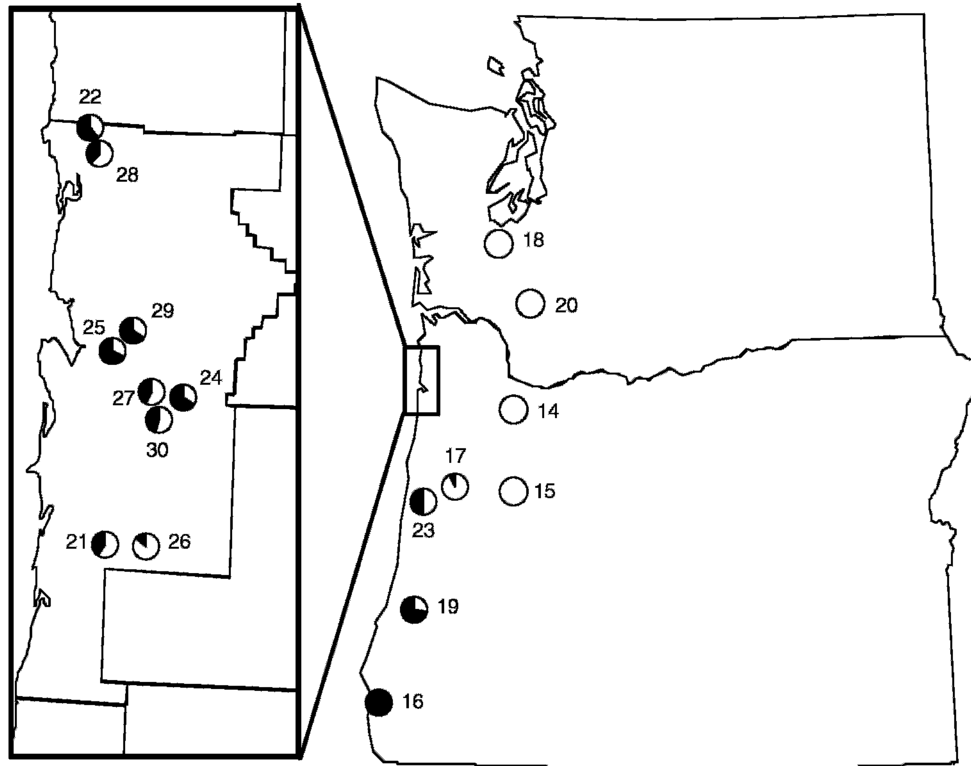

FIG. 2. Relative proportions of *Phaeocryptopus gaeumannii* Lineages 1 (○) and 2 (●) and their distribution in Oregon and Washington Douglas-fir stands. Population numbers and locations are provided (TABLE I). Inset shows Tillamook County, the area with the greatest incidence and severity of Swiss needle cast (Hansen et al 2000).

significantly as Lineage 2 contributed a greater proportion of the pathogen population of the stand ($P <$ 0.02, FIG. 3A). In addition there was a trend of increasing foliar discoloration with increasing proportions of this lineage ($P = 0.078$, FIG. 3B).

DISCUSSION

The observed SSCP patterns were consistent with the assumption that the vegetative mycelium of *P. gaeumannii* is haploid, as is typical of Ascomycota. While recombining diploid and dikaryotic fungi should have a significant number of heterokaryotes, we observed only one allele per locus for all of our isolates. Although a haploid genome is assumed for vegetative mycelium of most ascomycetes, neither *P. gaeumannii* nor any close relatives have had this assumption tested. Ploidy confirmation simplified our analysis in several respects. Alleles could be sequenced directly, with no need for cloning. In

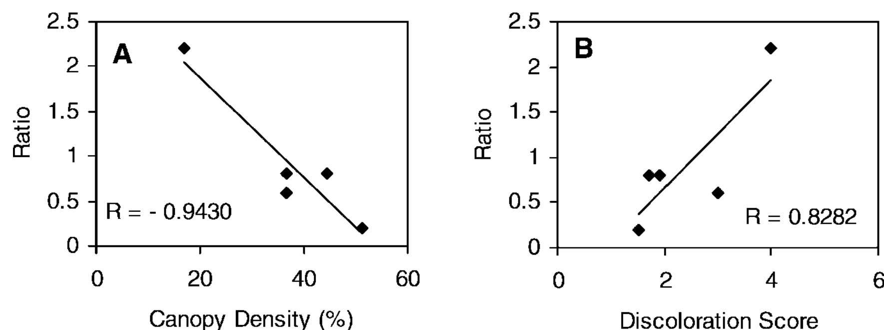

FIG. 3. Pearson correlations between the ratio of the two reproductively isolated lineages of *Phaeocryptopus gaeumannii* (Lineage 2 : Lineage 1) and the symptoms canopy density (A) and foliar discoloration (B) at five young Douglas-fir plantations within the area of the Tillamook epidemic.

addition, once sequences were available, intragenic recombination was ruled out based on the lack of homoplasy in individual gene genealogies. Tests for reproductive mode also were simplified because gene genealogies directly reflected the gametic phase of individuals.

That *P. gaeumannii* sexually reproduces has never been in question; there is direct evidence wherever Douglas-fir is grown. Its ability for self-fertilization was demonstrated by (Hood 1977) and (Winton 2001). However the role of recombination in the life history of the fungus, before this report, has never been addressed. In early phases of data acquisition and analysis it appeared that the Swiss needle cast outbreak in Oregon might simply be explained by high levels of genetic diversity and, by implication, recombination leading to genotypes more successful under current management or environmental conditions. However the use of population genetic and phylogenetic approaches for testing hypotheses on recombination and clonality revealed unexpected results that established a much more complicated picture.

Strong multilocus gametic disequilibrium indicated that *P. gaeumannii* in Oregon does not comprise a single, recombining population. Although the null distribution for unlinked loci in random mating populations is expected to be centered on zero, this analysis used sequence data wherein genes were permuted as linkage groups composed of the variable nucleotides for each locus. Thus the apparent disequilibrium in the null distribution (average = 0.6) actually reflects nucleotide site linkage within loci. Subtracting the average I_A of the null distribution from that observed, strong I_A among loci was estimated at 1.3.

The phylogenetic approach revealed that *P. gaeumannii* in Oregon is subdivided into two genetically differentiated groups that occur sympatrically in many coastal Douglas-fir stands, particularly within the region of the current Swiss needle cast outbreak. Multiple gene genealogies suggest that the two groups are reproductively isolated lineages that, by both biological and phylogenetic species concepts, constitute cryptic species. However recent admixture of the two lineages in coastal forests, combined with a low outcrossing rate, might alternatively explain the absence of recombined genotypes. Laboratory crosses could demonstrate whether the ability of the two lineages to mate has been lost, but only intensive, long-term monitoring would provide relevant evidence in natural circumstances.

One lineage (Lineage 1) is widespread, occurring throughout much of the natural range of the pathogen, and its host, in Oregon and Washington. This was the sole lineage in our sampling also found in Europe and New Zealand, two regions with historical reports of disease. Most of the populations sampled in these areas harbored at least two, and usually all three, genotypes of the lineage (TABLE V). The presence of one of these genotypes (BCBAB) in excess within the Oregon coastal epidemic area suggests reproduction by clonal processes. While the presence of overrepresented genotypes and association among alleles at different loci provide robust and significant evidence of clonal reproduction (Tibayrenc et al 1991), these features do not rule out recombination. Whether the divergence of genotypes within lineages was the result of recombination or mutation could not be resolved with our data. Because this lineage was the sole lineage found in four healthy stands at the northeastern limits of our sampling in the native Douglas-fir range, we tentatively propose that it is endemic there.

The second lineage (Lineage 2) comprised five genotypes and was found only in western Oregon. In most of these stands it was represented by only a single genotype (TABLE V). Maynard Smith et al (1993) refer to this as an epidemic population structure, distinguished by the occurrence of one or a few highly successful genotypes. By the same reasoning described above this lineage might be derived from the southern end of the Oregon coast gradually decreasing in abundance northward where it is replaced by Lineage 1. The next step is to confirm these proposed origins with an expanded latitudinal and longitudinal sampling strategy in the native range of Douglas-fir.

Both lineages were found, in varying frequencies, in the most severely diseased stands between Waldport and Astoria, Oregon. Attempts to correlate the relative abundance of the two lineages with stand-level disease severity met with limited success and implicated Lineage 2 in the Tillamook epidemic. An increase in the proportion of Lineage 2, represented largely by a single genotype, in stands was correlated with reduced canopy density and a trend of increasing discoloration, two measures of disease severity. Compared to Lineage 1 twice as many Lineage 2 isolates were obtained from a random sampling within the most severely diseased sites but only about half as many from relatively healthy stands.

Although the *P. gaeumannii* population from New Mexico is in a natural Douglas-fir stand, it is a small, isolated population on a mountaintop at the extreme eastern margin of the Douglas-fir range. These results are consistent with the hypothesis of reduced genetic diversity in small populations due to founder events and genetic drift. Genotypes from the New York and

Vermont populations also were relatively uniform. Presumably their geographic isolation from natural populations of *P. gaeumannii* is a barrier to gene flow and their genotypic uniformity reflects low diversity of the founding populations.

We demonstrated that the genetic diversity of the pathogen within Douglas-fir stands in Oregon's epidemic is not homogeneous and all the stands we sampled in the area harbored genotypes from both lineages. The correlations we observed between symptoms of Swiss needle cast and a specific strain or lineage of *P. gaeumannii* were suggestive but not conclusive. The sample size we had available to make these comparisons was small. In addition disease ratings, notoriously difficult for this pathogen, were obtained at the stand level rather than tree level. However there is substantial variation in the symptoms of disease among infected trees (Hansen et al 2000) and it might be help to determine how much of this variation can be explained by differences in pathogenicity of the two lineages or individual genotypes. Spore dispersal mechanisms and evidence of selfing suggests that individual trees are likely to be predominately infected by the same *P. gaeumannii* genotype. Future attempts at more detailed analyses of population structure seem crucial and should focus on the relative pathogenicity of the two lineages, their neighborhood sizes and how these variables correlate with tree-to-tree variability in disease expression in the forest.

SSCP proved efficient for screening both known and unknown single nucleotide polymorphism and indel events for more than 400 *P. gaeumannii* isolates. Although four PCR reactions and two gels were used to screen five genes for each individual, additional optimization of fragment lengths would have permitted all five genes to be screened in half the number of reactions and gels. In addition (Schuelke 2000) described a nested PCR method also using fluorescent M13 primers that combines the locus-specific and labeling reactions in a single run of the thermal cycler. Combining these approaches would reduce genotyping individuals to a single PCR setup and gel. This would allow either many more individuals or loci to be assayed and could significantly increase the statistical power of results.

ACKNOWLEDGMENTS

We thank Paolo Capretti, Roland Engesser, David Gernandt, Ottmar Holdenrieder, Ian Hood, Mike McWilliams, Michel Morelet, Derek Redfern, Richard Stephan, Richard Thum and Sandra Tracy for providing infected foliage samples. The Swiss Needle Cast Cooperative, a research consortium of industrial, federal and state landowners in Oregon and Washington, provided financial support.

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