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PRIMER NOTE

Polymorphic microsatellite markers for the Douglas-fir pathogen *Phaeocryptopus gaeumannii*, causal agent of Swiss Needle Cast disease

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Abstract

Ten polymorphic microsatellite markers were isolated from *Phaeocryptopus gaeumannii*, causal agent of the Douglas-fir foliage disease Swiss Needle Cast. The primer sets were tested on 60 isolates that had, with more conservative markers, previously segregated into three reproductively isolated lineages comprising nine genotypes. The microsatellite data yielded 46 multilocus genotypes that segregated into three groups identical to the three lineages previously recovered. Microsatellite genotypic diversity ranged from 0.85 to 0.99 within lineages and was lowest where both pathogen and host are exotic.

Keywords: forest pathology, loculoascomycete, plant disease, plant pathogen, SSR

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Phaeocryptopus gaeumannii (Capnodiales, Ascomycota) is a haploid fungus that causes the foliage disease Swiss Needle Cast of Douglas-fir. The normally innocuous fungus has increased in abundance in Douglas-fir plantations in western Oregon, where it has been associated with damaging levels of disease since about 1990 (Hansen *et al*. 2000). By screening variable regions in five genes with single-strand conformation polymorphism (SSCP), Winton *et al*. (2006) found that *P. gaeumannii* in the region of Oregon's Swiss Needle Cast epidemic is subdivided into two reproductively isolated sympatric lineages. Genotypes of one lineage (Lineage 1) had a widespread distribution; they were found throughout the Pacific Northwest, where both the fungus and the host are native, as well as in non-indigenous populations in Europe and New Zealand. Genotypes of the second lineage (Lineage 2) were detected only in isolates from Oregon's coastal region. Among young plantations in the main epidemic area (along the northern Oregon coast), increased abundance of this second lineage appeared to be correlated with disease severity. A third lineage (Lineage 3) was found in small, geographically isolated non-native Douglas-fir plantations in the eastern USA. We developed microsatellite markers to study the population structure and recombination potential within lineages of *P. gaeumannii*.

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Genomic DNA was extracted from single ascospore *P. gaeumannii* isolates (Winton *et al*. 2006). Extracted DNA of one isolate was enriched for dinucleotides (AC_{13} and AG_{13}) or trinucleotides (ACG_6, AAC_6, ACC_8, AAG_8, and AAT_{12}) following a protocol modified from Hauswaldt & Glenn (2003). DNA was digested separately with the restriction enzymes *Rsa*I and *Bst*UI, ligated to SuperSNX linkers (Hamilton *et al*. 1999), hybridized with a mixture of the 3′-biotinylated microsatellite oligonucleotides, and captured on Dynabeads M-280 (Dynal Biotech). Captured DNA fragments were recovered by direct polymerase chain reaction (PCR) of the hybridized Dynabeads using SuperSNX24 as primer, purified with GeneClean Kit (Q-BIOgene), and re-hybridized with biotinylated oligonucleotides. The PCR product of the second enrichment was purified with GeneClean, cloned with the TOPO TA Cloning Kit (Invitrogen), and screened for recombinants. Positive colonies were amplified using M13 forward and reverse primers. Products of 500–1000 bp were sequenced with M13 forward primer using DYEnamic ET (Amersham Pharmacia Biotech) chemistry and an ABI 373 XL sequencer (Applied Biosystems). Sequences were automatically searched for microsatellites in SPUTNIK (http://cbi.labri.fr/outils/Pise/sputnik.html). Those containing microsatellites with more than six repeats were reverse sequenced with M13 reverse primer. Contigs were assembled and edited using the STADEN software package (Staden 1996). Microsatellite

Table 1 Characterization of 10 primer pairs that amplify microsatellite loci for *Phaeocryptopus gaeumannii*. Lineages (Lineage 1, Oregon, 20 isolates; Lineage 2, Oregon, 20 isolates; Lineage 3, New York and Vermont, 20 isolates) were reported in Winton *et al*. (2006). Sequences used to introduce sites for universal fluorescent primers are in italics. N_A, number of alleles; *H*, haploid gene diversity; range, size range (bp) of alleles

Locus (GenBank*)	Repeat motif in clone	Forward (F) and reverse (R) Primer sequence (5′–3′)†	N_A	Range	Lineage 1		Lineage 2		Lineage 3	
					N_A (*H*)	Range	N_A (*H*)	Range	N_A (*H*)	Range
Pgdi1 (EF372586)	$(CA)_{18}$	**F:** *M13F* TCCCCGCCTATATTTCTC **R:** *(FAM) M13R* CCGAATCGATTTGCTAGG	5	130–138	2 (0.34)	130–132	3 (0.57)	134–138	2 (0.10)	130–134
Pgdi2 (EF372587)	$(AG)_{26}$	**F:** *M13F* ATTCCAGAGCCATACCGTTG **R:** *(FAM) M13R* AGGTGGATGAGGGATGTTTG	11	263–287	4 (0.57)	277–283	4 (0.71)	267–273	4 (0.47)	263–287
Pgdi3 (EF372588)	$(TC)_{18}$	**F:** *M13F* GGGGATGCTGGAATGTATGT **R:** *(FAM) M13R* GCACATTGCTCAGTGCTCTC	6	370–385	4 (0.66)	370–401	3 (0.19)	370–376	2 (0.38)	279–381
Pgdi4 (EF372589)	$(GT)_{17}$	**F:** *M13F* GGCATCGCAGTCAACTTA **R:** *(FAM) M13R* CGAGCCGAACCTTTAGTT	9	478–528	3 (0.18)	482–486	6 (0.64)	505–528	2 (0.32)	478–482
Pgdi5 (EF372590)	$(AG)_4GA(AG)_5AA(AG)_3$ AAAGAA$(AG)_3$AAAGAC $(AG)_8$AAAGAC$(AG)_{11}$	**F:** *M13F* ATAGTATATTACACCAGG **R:** *(HEX) M13R* CAACAGCACATCGCAACA	7	376–443	3 (0.19)	400–443	4 (0.41)	376–411	1 (0.00)	421
PgTri1 (EF372592)	$GA(AG)_4GG(AG)_5AA(AG)_5GG(AG)_2$ $(CAT)_{22}(CTT)_{19}$	**F:** *M13F* TGGAGACCATTAACCCTGGA **R:** *(FAM) M13R* TTGGGAGGGTATTGAGGTTG	21	310–470	10 (0.83)	355–391	6 (0.76)	310–437	6 (0.49)	349–470
PgTri2 (EF372593)	$(GAC)_4[(GAA)_3(GAC)_3]_9$ $GAA(GAC)_2$	**F:** *M13F* AGGCAGAGAAGGGAGAGGAG **R:** *(HEX) M13R*TCTGCAAGACCGTCATCATC	12	301–442	8 (0.83)	350–442	3 (0.40)	301–338	3 (0.45)	331–387
PgTri6 (EF372594)	$(CAA)_{24}$	**F:** *M13F* CCCTTCCCAATCACTTCTCA **R:** *(NED) M13R* GGACTGCTTTGGGTGATGTT	24	304–464	11 (0.90)	332–464	11 (0.87)	304–354	4 (0.41)	323–382
PgTri7 (EF372595)	$(TTC)_8TTT(CTT)_7$	**F:** *M13F* ATGCTATCCCTCCCCAACTC **R:** *(FAM) M13R* TGCGAAGCGTGTAAATTCTG	11	415–473	4 (0.62)	424–439	4 (0.51)	415–430	5 (0.48)	418–473
PgTet1 (EF372591)	$(AATC)_{13}$	**F:** *M13F* CATCCGCTCCATTTCATTCT **R:** *(HEX) M13R* TGGCGACGGAGTTGATAATA	17	239–355	11 (0.81)	271–347	2 (0.10)	239–243	8 (0.66)	307–355

*GenBank accession number of sequenced clone.

†M13 tail sequences on 5′ end of primer: *M13F* (5′-TGTAAAACGACGGCCAGT-3′); *M13R* (5′-CAGGAAACAGCTATGACC-3′). Second round PCR utilized M13F and M13R, one of which was fluorescently labelled with FAM, HEX, or NED.

Table 2 The number of microsatellite multilocus genotypes (MLG) and genotypic diversity recovered from each of the SSCP lineages and multilocus genotypes previously reported (Winton *et al*. 2006)

SSCP Lineage	SSCP MLG	*N*	No. of microsatellite MLG	Lineage genotypic diversity
1	BABAB	7	7	
1	BBBAB	6	6	
1	BCBAB	7	6	0.99
2	CBBBA	15	11	
2	CDBBA	1	1	
2	CEBBA	3	1	
2	DBBBA	1	1	0.96
3	AAAAA	16	9	
3	AABAA	4	4	0.85

SSCP loci are presented in the order β–tubulin (BTUB), the mitochondrial ribosomal small subunit (mtSSU), α–tubulin (ATUB), chitin synthase 1 (CHS), and calmodulin (CAM).

locus-specific forward and reverse primers were designed using the program PRIMER 3 (Rozen & Skaletsky 2000) and an M13 universal primer sequence tail was added at the 5′ end of each primer pair (Table 1). Addition of the 5′-tail enabled use of a fluorescently labelled M13 primer in the PCR for cost-effective polymorphism screening of candidate primers pairs on the ABI 3100 sequencer (Boutin-Ganache *et al*. 2001).

Primer sets were tested on 20 isolates from each of three reproductively isolated lineages (populations) comprising nine SSCP genotypes (Winton *et al*. 2006). PCR amplifications were performed in 6.5-μL reaction volumes. PCR final concentrations were 1× PCR buffer with $MgCl_2$ (Sigma), 200 nM dNTPs, 0.2 μM M13-tailed microsatellite primer pairs (Table 1), 0.5 U RedTaq DNA polymerase (Sigma), and 20–50 ng DNA template. Separate reactions were conducted for each primer pair at the following thermal cycling parameters: 92 °C for 60 s followed by 30 cycles at 92 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. To fluorescently label the locus-specific products, 0.5 μL PCR product was re-amplified with 0.4 μM M13 primers, one of which was fluorescently labelled on the 5′ end (Table 1). Labelling reaction conditions were otherwise identical to the above except that only 25 cycles were performed at a 52 °C annealing temperature. PCR products were sized on an ABI 3100 sequencer utilizing GENOTYPER 2.5 (Applied Biosystems) and MapMarker1000 ROX size standard (BioVentures, Inc.).

Polymorphisms were found for all loci varying from five to 24 alleles per locus (Table 1). Haploid gene diversity (computed with GENALEX 6, Peakall & Smouse 2006) ranged from 0.00 to 0.90 per locus (mean for Lineage 1 = 0.59, Lineage 2 = 0.51, Lineage 3 = 0.37). Forty-six multilocus microsatellite genotypes were identified, as opposed to the nine more conservative SSCP genotypes recovered in Winton *et al*. (2006). STRUCTURE 2.1 analysis (Pritchard *et al*. 2000) differentiated the three lineages (K = 3) by assigning microsatellite genotypes into identical groupings (Table 2). Genotypic diversity was high within lineages and within each of the SSCP genotypes. Pairwise linkage disequilibrium (LD) within lineages was tested using MULTILOCUS version 1.2 (Agapow & Burt 2001). Significant LD was detected only in Lineage 1 between Pgdi2 and the other nine loci ($P < 0.02$).

Because *P. gaeumannii* is capable of self-fertilization, linkage disequilibrium was not unexpected. However, the absence of LD at nine of the 10 loci and high genotypic diversity suggests that recombination via outcrossing may provide a significant contribution to the population structure of this fungus. These microsatellite loci therefore appear be a suitable tool to estimate recombination rates within *P. gaeumannii* lineages and populations and for studies of fine-scale population structure. For example, it is unknown whether individual trees or needles are inhabited by one or several genotypes of the fungus.

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