Genetic Diversity and the Presence of Two Distinct Groups in *Ophiostoma clavigerum* Associated with *Dendroctonus ponderosae* in British Columbia and the Northern Rocky Mountains

S. Lee, R. C. Hamelin, D. L. Six, and C. Breuil

First and fourth authors: Department of Wood Science, University of British Columbia, 2424 Main Mall, Vancouver, BC V6T 1Z4, Canada; second author: Forest Pest Biotechnology, Laurentian Forestry Centre, Natural Resources Canada, 1055 rue du PEPS, Quebec City, QC, G1V 4C7, Canada; third author: Department of Ecosystem and Conservation Science, College of Forestry and Conservation, University of Montana, Missoula 59812.

Accepted for publication 4 April 2007.

ABSTRACT

Lee, S., Hamelin, R. C., Six, D. L., and Breuil, C. 2007. Genetic diversity and the presence of two distinct groups in *Ophiostoma clavigerum* associated with *Dendroctonus ponderosae* in British Columbia and the northern Rocky Mountains. *Phytopathology* 97:1177-1185.

The sapstaining fungal pathogen *Ophiostoma clavigerum* is associated with the mountain pine beetle (*Dendroctonus ponderosae*), which is currently the most destructive forest pest in North America. The genetic diversity of *O. clavigerum* populations collected from five sites in Canada and two sites in the United States was estimated with amplified fragment length polymorphism (AFLP) analysis. Genomic DNA from 170 *O. clavigerum* isolates was digested with *EcoRI* and *PstI* and amplified

with six primer sets. A total of 469 AFLP markers consisting of 243 monomorphic and 226 polymorphic loci were scored. The overall genetic diversity of the *O. clavigerum* population was low ($H_s = 0.0531$) and the differentiation of the seven *O. clavigerum* populations was moderate ($\Phi = 0.143$). Genetic distances among the populations were not significantly correlated with geographic distance ($r = 0.3235$, $P = 0.074$). Two genetically distinct groups in the *O. clavigerum* populations were shown by unique AFLP profiles and the unweighted pair group method with arithmetic averages. Further work to characterize biological differences between the two groups will be needed to confirm whether cryptic species are present in the *O. clavigerum* population.

Ophiostoma clavigerum (Robinson-Jeffrey & Davidson) Harrington is a sapstaining ascomycete associated with bark beetles and pines in western North America. *O. clavigerum* is haploid through most of its life cycle, but is transiently diploid when it reproduces sexually. Like many other ophiostomatoid fungi, it produces asexual and sexual spores in a slimy mass that can be easily dispersed by insects (8). *O. clavigerum* is dispersed by two bark beetle (Curculionidae, Scolytinae) vectors, *Dendroctonus ponderosae* and *D. jeffreyi* (22,24,33).

The two beetles are sister species and thus genetically very closely related. However, they infest different host trees and except for small areas of sympatry, inhabit different geographic ranges. *D. ponderosae* primarily infests *Pinus contorta* and *P. ponderosa*, but it is relatively polyphagous, infesting most pines within its range except *P. jeffreyi*. In contrast, *D. jeffreyi* is monophagous and attacks only *P. jeffreyi*. While *D. ponderosae* occurs in British Columbia (BC) and Alberta in Canada and across the western portion of the United States, *D. jeffreyi* inhabits a relatively limited area in the United States (California) and Mexico (Baja California) (4,23,28).

Both beetles can cause major outbreaks. Currently, *D. ponderosae* is active in BC and the northern Rocky Mountains of the United States. The current Canadian outbreak is the most severe ever recorded for this beetle. As of 2006, 9.2 million hectares of *P. contorta* forests in BC had been infested (4).

In addition to *O. clavigerum*, *D. ponderosae* also carries *O. montium*, an *O. minutum*-like species, *Leptographium longi-*

clavatum, and *Entomocorticium* spp. (10,11,22,26,33). Several potential benefits of the fungi to the beetles have been proposed. These include overcoming tree defenses (3), reducing sapwood moisture content (19,30), and providing nutrients for developing larvae and adult reproduction (1,23,32).

Among the fungi associated with *D. ponderosae*, *O. clavigerum* appears to be the most virulent pathogen to *P. contorta*. It grows at the leading edge of infested sapwood, disrupts water transport, and can kill trees when inoculated at high densities (26,34,35). However, neither the variance in pathogenicity nor the genetic diversity of *O. clavigerum* isolated from *D. ponderosae* has been characterized. Six and Paine (25) reported that *O. clavigerum* isolated from *D. jeffreyi* in the United States possesses low genetic diversity. However, these results cannot be extrapolated to *O. clavigerum* associated with *D. ponderosae* because fungal and beetle populations may have coevolved in host-specific ways and *D. ponderosae* and its associated *O. clavigerum* inhabit a much broader geographic range with several geographically isolated populations that may affect levels of genetic variation.

Amplified fragment length polymorphism (AFLP) analysis is a powerful analytical tool for population genetic studies (29). AFLP is a dominant DNA fingerprinting marker and suitable for genetic studies for haploid fungi. AFLP analysis can produce a large number of markers using relatively few primers and is more reproducible than random amplified polymorphic DNA (RAPD) analysis (13,15). It is also more suitable for investigating differentiation within and among populations than allozyme analysis, which has lower power to resolve variation (14). Furthermore, the data generated by neutral AFLP markers are less biased than selective markers such as pathogenicity phenotype (14).

In this work, we examined the genetic structure of the *O. clavigerum* population associated with *D. ponderosae* and *P. con-*

Corresponding author: C. Breuil; E-mail address: colette.breuil@ubc.ca

doi:10.1094/PHYTO-97-9-1177

© 2007 The American Phytopathological Society

torta in seven outbreak regions in Canada and the United States. AFLP markers from 170 isolates were analyzed to (i) investigate genetic diversity in the *O. clavigerum* population and (ii) determine whether *O. clavigerum* exists as a single panmictic meta-population across the wide range of the epidemic area or as genetically distinct subpopulations.

MATERIALS AND METHODS

Sampling and identification of *O. clavigerum* isolates. *O. clavigerum* was isolated from five sites in Canada (Banff, Fort St. James, Houston, Manning Park, and Williams Lake) and two sites in the United States (Hellroaring and Hidden Valley) during 2002 and 2003 (Fig. 1; Table 1). The longest distance between sampling sites spanned more than 1,400 km (between Fort St. James and Hellroaring). Ten trees (*P. contorta* var. *latifolia* Engelm.) per Canadian site, standing approximately 5 m apart, were randomly harvested (Table 1). All sampled trees had been successfully attacked by *D. ponderosae* during the previous year. From each tree, one bolt (60 cm long) was cut at breast height and debarked as described by Lee et al. (11). One to six *O. clavigerum* strains were isolated from the phloem of each bolt. In the United States, *D. ponderosae* was captured with three Lindgren traps baited with *D. ponderosae* pheromone (Pherotech International Inc., Delta, BC, Canada) at each site. Traps were spaced

approximately 50 m apart in stands with heavy beetle infestations. Each trap likely collected beetles from many trees. One fungal strain was isolated from mycangia of each *D. ponderosae* as described by Six and Paine (25) (Table 1).

The purity of the *O. clavigerum* isolates used for identifications and DNA analysis was ensured by single-spore isolation. Identification of the *O. clavigerum* isolates was based on the conidiogenous structures described by Robinson-Jeffrey and Davidson (21) and Lee et al. (9) and confirmed by *O. clavigerum*-specific polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) markers (9).

The *O. clavigerum* isolates B6, B9, B13, and B26, which were characterized as Group 2 in this study, were deposited at the University of Alberta Microfungus Collection and Herbarium and were registered as UAMH 10627, UAMH 10628, UAMH 10629, and UAMH 10630, respectively.

DNA isolation. Two plugs were removed from the edge of an actively growing colony and placed on 2% malt extract agar (MEA) overlaid with cellophane sheath. After 3 to 4 days of incubation at room temperature, mycelia were harvested aseptically and kept at -80°C for as many as 30 days before DNA extraction. To yield high quality DNA, nonpigmented mycelia were harvested. Approximately 50 to 60 mg of frozen mycelia per isolate was ground in mortars and pestles with liquid nitrogen. Genomic DNA was isolated following the protocol of Zolan and Pukilla (36) with a modification as follows: the grounded mycelia were transferred to 0.7 ml of CTAB buffer (2.5% cetyltrimethylammonium bromide, 1% PVP-40, 1.4 M NaCl, 0.02 M EDTA, 0.1 M Tris-HCl, and 0.5% β-mercaptoethanol freshly added) in a 2-ml microtube (Sarstedt, Montreal, QC, Canada). The mixture was vortexed for 15 s, incubated at 60°C, and then treated twice with 700 μL of chloroform and isoamyl alcohol (vol/vol = 24:1). The solution was gently rotated for 10 min at room temperature and centrifuged at 12,000 rpm (Eppendorf Centrifuge 5417R; Hamburg, Germany) for 5 min to collect the top aqueous layer. DNA was precipitated with ice-cold isopropanol and resuspended in 300 μl of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). RNA was digested with 3 μl of Rnase A (10 mg/ml; Sigma-Aldrich, Oakville, ON, Canada) and incubated overnight at room temperature. The chloroform extraction was repeated one more time. DNA was precipitated by adding 100 μl of 5 M sodium acetate (pH 5.2) and 725 μl of ice-cold 100% ethanol. The pellet was washed with 500 μl of ice-cold 70% ethanol, air dried for 5 to 25 min avoiding over drying, dissolved in 20 to 46 μl of water depending on the pellet size, and incubated at room temperature overnight. The quality and concentration of DNA were assessed using 1.2% agarose gel and a spectrophotometer (Ultraspec3000; Pharmacia Biotech, Amersham, Piscataway, NJ). Only high-quality genomic DNA of which absorbance ratio at 260/280 nm was between 1.8 and 2 was used for AFLP reactions.

AFLP. For analysis of AFLP markers, we used a Li-Cor automated sequencer (Li-Cor Inc., Lincoln, NE) and followed a protocol modified by the Genetic Data Centre at the University of British Columbia (7) from Remington et al. (20). Genomic DNA (500 ng) was digested with *EcoRI* and *PstI* at 37°C and was

Fig. 1. Map showing the seven sampling sites of *Ophiostoma clavigerum* in Canada (Fort St. James, Houston, Williams Lake, and Manning Park in British Columbia; Banff in Alberta) and the United States (Hellroaring in Idaho; Hidden Valley in Montana).

TABLE 1. Sampling sites, sources of isolation, and number of fungal isolates in each location

Site no.	Location	Longitude, latitude	Date sampled	Source	No. of source	No. of fungal isolates
1	Banff, AB, Canada	51°10'N, 115°34'W	July, 2003	<i>Pinus contorta</i> infested by <i>Dendroctonus ponderosae</i>	10 trees	24
2	Fort St. James, BC, Canada	53°50'25"N, 124°31'41"W	July, 2003	<i>P. contorta</i> infested by <i>D. ponderosae</i>	10 trees	28
3	Hellroaring, ID, USA	44° 2' N, 114°51'W	Summer, 2002	<i>D. ponderosae</i>	28 beetles	28
4	Hidden Valley, MT, USA	47°23'15"N, 115°18'15"W	Summer, 2003	<i>D. ponderosae</i>	21 beetles	21
5	Houston, BC, Canada	54°08'N, 126°40'W	July, 2003	<i>P. contorta</i> infested by <i>D. ponderosae</i>	10 trees	22
6	Manning Park, BC, Canada	49°11'35"N, 120°35'05"W	June, 2003	<i>P. contorta</i> infested by <i>D. ponderosae</i>	10 trees	29
7	Williams Lake, BC, Canada	52°01'35"N, 122°31'27"W	August, 2003	<i>P. contorta</i> infested by <i>D. ponderosae</i>	10 trees	18

ligated with *EcoRI* and *PstI* primer adaptors (7) at 37°C overnight. Ligation mixture was preamplified with six pairs of primers having a single selective nucleotide (*EcoRI* + T/*PstI* + T, *EcoRI* + T/*PstI* + A, *EcoRI* + C/*PstI* + G, *EcoRI* + G/*PstI* + A, *EcoRI* + G/*PstI* + G, and *EcoRI* + G/*PstI* + T) (Table 2). The PCR parameters for preamplification were: (i) 94°C for 1 min; (ii) 28 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s; and followed by (iii) 72°C for 5 min (PTC-100; MJ Research, Waltham, MA). The final selective amplification was carried out in a 10- μ l PCR containing preamplified mixture and three primers: a standard M13 forward primer with an infrared label, one of the selective *PstI* (+1) primers used in the corresponding preamplification, and one of the selective *TecoRI* (+1) primers, which has the same selective nucleotide as the corresponding *EcoRI* (+1) primer used in preamplification. The PCR cycling parameters for the final reaction were: (i) 94°C for 1 min; (ii) three cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s; (iii) 12 cycles with the annealing temperature decreasing by 0.7°C per cycle, the other conditions remained the same; (iv) 22 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s; and (v) extension at 72°C for 1 min. After the PCR amplification, 3 μ l of formamide loading buffer was added to each 10- μ l amplified product and denatured at 95°C for 3 min. One microliter of each sample was loaded on 6% sequencing polyacrylamide gel (0.4 mm thickness, 25 cm length) with 62 wells and electrophoresed in TBE buffer at 70 W, 2,000 V for 3.5 h. The amplified band profiles were visualized and automatically recorded with the Li-Cor 4200 autosequencer. Bands were scored with SAGA-MX software (Li-Cor Inc.) and through manual editing. When scoring the AFLP profiles, it was assumed that comigrating bands on a gel were the same loci with identical sequences. To ensure reproducibility, three independent reactions were repeated with the DNA of 15 randomly chosen isolates and the same six pairs of primers.

Data analysis. Population data analysis was conducted on 170 isolates except for the haplotype analysis (see below). Allele frequency, proportions of polymorphic loci, and Nei's (16) unbiased genetic diversity index (H_s) were calculated with tools for population genetic analyses (TFPGA, Version 1.3, Miller, Northern Arizona University, Flagstaff). Haplotypes were determined by pairwise differences with Arlequin (S. Schneider, D. Roessli, and L. Excoffier, (2000) Version 3.0, University of Geneva, Geneva, Switzerland). For the inference of haplotypes, 40 isolates with missing data were excluded from the dataset because they biased pairwise comparisons. An analysis of molecular variance (AMOVA) was conducted with Arlequin to partition variances into hierarchical structures such as among groups, among populations, among populations within groups, within groups, and within populations (5). The analysis also estimated the genetic differentiation (Φ) among populations with F statistics. With the analysis, a matrix of population pairwise F_{ST} values was generated as well (31). To estimate the genetic similarity among the seven *O. clavigerum* populations, Nei's unbiased genetic distances (16) between populations were calculated and a dendrogram using the unweighted pair group method with arithmetic

averages (UPGMA) was generated with TFPGA. The confidence level of branches in the UPGMA tree was estimated with bootstrapping values (6) using 1,000 randomizations. To investigate the correlation between genetic and geographic distances, a Mantel test was performed with TFPGA. The distance between sampling sites was estimated with ArcMap (Version 9.1, ESRI Inc., Redlands, CA). Fine level cluster analysis, in which each *O. clavigerum* isolate was considered as a single unit, was achieved by constructing an UPGMA tree with PAUP* version 4.0b (Swofford, Sinauer Associates, Sunderland, MA) using the Nei and Li coefficient (17). To statistically support the branches within the UPGMA tree, bootstrapping values were determined by employing 1,000 randomizations. To examine the level of asexual reproduction versus sexual reproduction, the indices of multilocus association, I_A and r_d (2,12), were computed with MultiLocus (Version 1.3, Agapow and Burt, Imperial College, Berkshire, UK). The significance of I_A was tested by comparing the observed value with the expected values from 100 randomized datasets under the null hypothesis of free recombination. Linkage disequilibrium (LD) was also measured with an Exact test using Arlequin. The LD (%) was given as the number of disequilibrium values significant at $P < 0.05$ over the total number of pairwise comparisons between polymorphic loci.

RESULTS

Genetic polymorphisms, heterozygosity, and haplotypes. A total of 175 fungal isolates were collected and initially identified as *O. clavigerum* on the basis of morphology. Among them, five isolates did not generate the *O. clavigerum*-specific bands (238 and 490 bp long) by the PCR-RFLP marker based on the β -tubulin gene (9) and were reidentified as *L. longiclavatum* or *L. terebrantis* by this marker and morphology. Therefore, only 170 isolates with confirmed identification were used to generate AFLP profiles. Each primer set generated 59 to 88 scorable loci with sizes varying from 64 to 660 bp (an example gel is shown in Fig. 2). The reproducibility test indicated that the markers were highly reproducible, with 92% replication of the same banding patterns. A total of 469 loci were scored; of these, 243 were monomorphic and 226 were polymorphic. One hundred and ninety three polymorphic loci had allele frequencies between 0.01 and 0.99 and the remaining 33 were rare alleles (Table 3). One hundred polymorphic loci with allele frequencies between 0.05 and 0.95 were used as a dataset for most of the analyses.

Some polymorphic alleles were not randomly distributed throughout the geographic area studied. Thirty-eight markers were observed only in the populations of the Rocky Mountain Region (Banff, Hellroaring, and Hidden Valley) and not in the BC populations (Fort St. James, Houston, Manning Park, and Williams Lake) (Table 4). In contrast, six markers were observed exclusively in the BC populations.

Nei's unbiased heterozygosity (H_s) values over all loci in seven *O. clavigerum* populations ranged from 0.040 to 0.075 and averaged 0.053 (Table 5). In general, higher values of heterozygosity and percentage of polymorphic loci were observed in the Rocky

TABLE 2. Primers used in the amplified fragment length polymorphism analysis

Adapters and primers	DNA sequence
<i>EcoRI</i> + C	5'-GACTGCGTACCAATTCC-3'
<i>EcoRI</i> + G	5'-GACTGCGTACCAATTTCG-3'
<i>EcoRI</i> + T	5'-GACTGCGTACCAATTCT-3'
<i>TecoRI</i> + C	5'-CACGACGTTGTAAAAACGACGACTGCGTACCAATTCC-3'
<i>TecoRI</i> + G	5'-CACGACGTTGTAAAAACGACGACTGCGTACCAATTTCG-3'
<i>TecoRI</i> + T	5'-CACGACGTTGTAAAAACGACGACTGCGTACCAATTCT-3'
<i>PstI</i> + A	5'-GACTGCGTACATGCAGA-3'
<i>PstI</i> + G	5'-GACTGCGTACATGCAGG-3'
<i>PstI</i> + T	5'-GACTGCGTACATGCAGT-3'

Mountain populations than in the BC populations. The Hidden Valley population, located in the middle-eastern Rocky Mountains (Fig. 1), showed the highest level of *Hs* (0.075), whereas the Fort St. James population collected from the most northern part of the epidemic area had the lowest level of *Hs* (0.040). The values of *Hs* over polymorphic loci ranged from 0.173 (Fort St. James) to 0.270 (Banff), with an average of 0.215 (Table 5). The diversity of haplotypes in the *O. clavigerum* population was high. A total of 128 haplotypes were identified in 130 isolates using polymorphic loci ($0.05 < \text{allele frequency} < 0.95$). Two isolates from Fort St. James had the same haplotype. One haplotype was found at two sites: Banff and Hellroaring.

AMOVA. AMOVA analysis performed on seven *O. clavigerum* populations showed that 85.7% ($P < 0.001$) of the genetic variability was due to differences among individual isolates within populations (Table 6). The remaining variability resulted from differences among the seven populations (14.3%, $\Phi = 0.143$, $P < 0.001$). To further investigate the differentiation among the geographical populations, all isolates from the seven populations were pooled and resorted into two distinct geographic groups:

Fig. 2. An amplified fragment length polymorphism analysis profile of *Ophiostoma clavigerum* generated with the primer combination *EcoRI* + *T/PstI* + A. Lane 1: marker; lanes 2–17: HT7–22, the *O. clavigerum* isolates collected from Houston; lanes 18–37: HV1–19, *O. clavigerum* collected from Hidden Valley. Note distinct profiles in lane 25 (HV4), lane 28 (HV7), and lane 31 (HV10).

Rocky Mountain Region and BC Plateau. A high proportion of the variance was still attributed to differences among individual isolates within populations (83.6%, $P < 0.001$), whereas the differences between groups contributed only 5.8% to the total genetic variability ($\Phi = 0.058$, $P = 0.031$). The genetic differentiation observed between the isolates sampled from *P. contorta* and *D. ponderosae* was not significant ($\Phi = -0.006$, $P = 0.520$) (Table 7).

Pairwise comparison of the populations and cluster analysis. The matrix of pairwise F_{ST} indices suggested that the Banff and Fort St. James populations were the most differentiated ($F_{ST} = 0.222$, $P < 0.01$), whereas the Houston and Fort St. James populations were the least differentiated ($F_{ST} = 0.082$, $P < 0.01$) (Table 8). The matrix of population pairwise F_{ST} indices was calculated from the analysis of molecular variance.

A UPGMA tree based on Nei's unbiased genetic distances among populations showed a similar trend to that observed in the pairwise F_{ST} analysis (Fig. 3). The UPGMA dendrogram showed that the Houston and Fort St. James populations were the most similar and formed one cluster with a strong bootstrapping value (97%). The other two clusters consisted of the Rocky Mountain populations and the central to southern BC populations and were supported by relatively low bootstrapping values (61 and 42%, respectively).

The general association of genetic distance to geographic distance was evaluated with the Mantel test. The test showed that Nei's unbiased genetic distances were not significantly correlated with spatial distances ($r = 0.324$, $P = 0.074$).

Fine level cluster analysis. A UPGMA tree resolving each individual isolate showed that the *O. clavigerum* population has two genetically distinct groups (Fig. 4). The clearly separated two clusters were supported with strong bootstrapping values; 93% for Group 1 and 100% for Group 2. Most *O. clavigerum* isolates belonged to Group 1, whereas Group 2 contained only nine isolates, which included five isolates from Banff (B5, B6, B9, B13, and B26), three isolates from Hidden Valley (HV4, HV7, and HV10), and one isolate from Hellroaring (HR25). Twenty markers, which represent 53% of the Rocky Mountain population-specific markers (20 of 38), were observed only in the isolates belonging to Group 2 (Table 4). Similarly, all of the isolates in Group 1 (but none of the isolates in Group 2) had the GG₃₅₄ allele.

Linkage disequilibrium and indices of association. Linkage disequilibrium estimated for the seven populations was relatively high at 27.5% and the index of association was significantly different from zero, indicating nonrandom association among loci across the entire populations ($I_A = 4.20$ and $r_d = 0.044$, $P < 0.01$). The high index of association may indicate reproductive isolation of *O. clavigerum* Group 1 from Group 2. Indeed, each group had much lower indices of association when considered separately (Group 1: $I_A = 1.29$ and $r_d = 0.016$, $P < 0.01$; Group 2: $I_A = 0.94$ and $r_d = 0.018$, $P < 0.01$). However, the indices were significantly different from zero for both groups.

DISCUSSION

Prior to the AFLP analysis, the identity of all fungal isolates was confirmed with the PCR-RFLP marker that we developed to

TABLE 3. Number of monomorphic and polymorphic loci observed in the amplified fragment length polymorphism analysis profiles of 170 *Ophiostoma clavigerum* isolates with the six selected primer combinations

Primer sets	Total loci	Monomorphic loci	Polymorphic loci	Polymorphic loci (5 < % < 95)
<i>EcoRI</i> + C / <i>PstI</i> + G	88	62	26	12
<i>EcoRI</i> + G / <i>PstI</i> + A	85	60	25	18
<i>EcoRI</i> + G / <i>PstI</i> + G	78	44	34	18
<i>EcoRI</i> + G / <i>PstI</i> + T	73	17	56	16
<i>EcoRI</i> + T / <i>PstI</i> + A	86	27	59	18
<i>EcoRI</i> + T / <i>PstI</i> + T	59	33	26	18
Total number	469	243	226	100

TABLE 4. Polymorphic alleles specific to the Rocky Mountain populations (BF, HR, and HV) or the British Columbia (BC) populations (HT, St. J, MP, and WL) of *Ophiostoma clavigerum*

Locus ^b	Allele frequency ^a							
	Rocky Mountains				BC			
	Total <i>n</i> = 170	BF <i>n</i> = 24	HR <i>n</i> = 28	HV <i>n</i> = 21	HT <i>n</i> = 22	St. J <i>n</i> = 28	MP <i>n</i> = 29	WL <i>n</i> = 18
CG_672	0.031	0.143	0.000	0.095	0.000	0.000	0.000	0.000
CG_579	0.055	0.238	0.038	0.143	0.000	0.000	0.000	0.000
CG_443	0.055	0.238	0.038	0.143	0.000	0.000	0.000	0.000
CG_335	0.049	0.238	0.038	0.095	0.000	0.000	0.000	0.000
CG_302	0.049	0.238	0.038	0.095	0.000	0.000	0.000	0.000
CG_266	0.055	0.238	0.038	0.143	0.000	0.000	0.000	0.000
CG_260	0.049	0.238	0.038	0.095	0.000	0.000	0.000	0.000
CG_235	0.055	0.238	0.038	0.143	0.000	0.000	0.000	0.000
CG_155	0.055	0.238	0.038	0.143	0.000	0.000	0.000	0.000
CG_135	0.067	0.238	0.038	0.238	0.000	0.000	0.000	0.000
GG_451	0.067	0.333	0.000	0.143	0.000	0.000	0.000	0.000
GG_408	0.146	0.292	0.480	0.238	0.000	0.000	0.000	0.000
GG_357	0.049	0.208	0.000	0.143	0.000	0.000	0.000	0.000
GG_296	0.043	0.208	0.000	0.095	0.000	0.000	0.000	0.000
GG_253	0.055	0.250	0.000	0.143	0.000	0.000	0.000	0.000
GT_651	0.012	0.000	0.000	0.095	0.000	0.000	0.000	0.000
GT_559	0.012	0.000	0.037	0.048	0.000	0.000	0.000	0.000
GT_436	0.055	0.333	0.000	0.048	0.000	0.000	0.000	0.000
GT_284	0.048	0.208	0.037	0.095	0.000	0.000	0.000	0.000
GT_213	0.012	0.000	0.095	0.000	0.000	0.000	0.000	0.000
GT_83	0.012	0.000	0.000	0.095	0.000	0.000	0.000	0.000
GA_642	0.012	0.000	0.000	0.095	0.000	0.000	0.000	0.000
GA_586	0.036	0.167	0.000	0.095	0.000	0.000	0.000	0.000
GA_533	0.054	0.208	0.038	0.143	0.000	0.000	0.000	0.000
TA_587	0.056	0.182	0.038	0.200	0.000	0.000	0.000	0.000
TA_571	0.031	0.136	0.038	0.050	0.000	0.000	0.000	0.000
TA_543	0.043	0.182	0.038	0.100	0.000	0.000	0.000	0.000
TA_530	0.031	0.136	0.038	0.050	0.000	0.000	0.000	0.000
TA_429	0.031	0.136	0.038	0.050	0.000	0.000	0.000	0.000
TA_373	0.049	0.182	0.038	0.150	0.000	0.000	0.000	0.000
TA_304	0.056	0.227	0.038	0.150	0.000	0.000	0.000	0.000
TA_285	0.012	0.000	0.038	0.050	0.000	0.000	0.000	0.000
TA_268	0.068	0.182	0.077	0.250	0.000	0.000	0.000	0.000
TT_685	0.059	0.227	0.038	0.143	0.000	0.000	0.000	0.000
TT_625	0.039	0.045	0.115	0.095	0.000	0.000	0.000	0.000
TT_314	0.059	0.227	0.038	0.143	0.000	0.000	0.000	0.000
TT_168	0.046	0.227	0.038	0.048	0.000	0.000	0.000	0.000
TT_154	0.059	0.227	0.038	0.143	0.000	0.000	0.000	0.000
CG_168	0.037	0.000	0.000	0.000	0.000	0.143	0.000	0.111
CG_106	0.031	0.000	0.000	0.000	0.136	0.071	0.000	0.000
GA_405	0.083	0.000	0.000	0.000	0.227	0.214	0.103	0.000
GA_249	0.054	0.000	0.000	0.000	0.000	0.000	0.172	0.222
TA_251	0.037	0.000	0.000	0.000	0.136	0.074	0.036	0.000
TA_242	0.049	0.000	0.000	0.000	0.000	0.074	0.214	0.000

^a The polymorphic loci with allele frequencies between 0.01 and 0.99 are listed. BF, Banff; HR, Hellroaring; HV, Hidden Valley; HT, Houston; St. J, Fort St. James; MP, Manning Park; WL, Williams Lake.

^b The amplified fragment length polymorphism analysis markers for the loci written in bold were observed only in *O. clavigerum* group 2.

TABLE 5. Genetic diversity indices of Rocky Mountain populations (BF, HR, and HV) and British Columbia populations (HT, St. J, MP, and WL) comprising 170 *Ophiostoma clavigerum* isolates

Population ^a	<i>H_s</i> ^b		Polymorphic loci (%) ^c	
	Over all loci	Over polymorphic loci	0.05 < frequency < 0.95	0.01 < frequency < 0.99
BF	0.070 (0.045)	0.270 (0.207)	19.4	22.6
HR	0.053 (0.050)	0.214 (0.234)	13.9	23.9
HV	0.075 (0.064)	0.267 (0.237)	24.6	32.1
HT	0.043 (0.048)	0.182 (0.211)	12.6	15.8
MP	0.045 (0.047)	0.193 (0.225)	12.8	16.2
St. J	0.040 (0.041)	0.173 (0.201)	10.9	13.5
WL	0.046 (0.048)	0.203 (0.236)	14.3	14.3
Average	0.053 (0.049)	0.215 (0.221)	15.5	19.8

^a BF, Banff; HR, Hellroaring; HV, Hidden Valley; HT, Houston; MP, Manning Park; St. J, Fort St. James; WL, Williams Lake.

^b *H_s*: Nei's (1987) unbiased genetic diversity index for each population over all loci, including monomorphic and rare alleles, or over polymorphic loci with allele frequencies greater than 0.05 and less than 0.95. The numbers in brackets are for Group 1 only (see discussion).

^c The percentage of polymorphic loci with allele frequencies between 0.05 and 0.95, or between 0.01 and 0.99, over the total 469 loci.

differentiate *O. clavigerum* from morphologically similar *Ophiostoma* and *Leptographium* species. Given this, we assumed that all isolates examined in this analysis were a single species, *O. clavigerum*.

Although only isolates identified as *O. clavigerum* were used for AFLP and the genetic diversity in the population was low, the data strongly suggest that two genetically distinct groups are present in *O. clavigerum*. Group 1 and Group 2, which possessed clearly different AFLP profiles, were further differentiated by UPGMA. However, it is not clear whether the two groups are simply subpopulations of the same species or two different sympatric species. Unfortunately, we could not test for mating between Group 1 and Group 2 because we were unable to obtain the sexual structures of this fungus on MEA or *P. contorta* sapwood blocks after pairing many isolates derived from the ascospores of a single teleomorph of *O. clavigerum*. Although we have no biological evidence that Group 1 and Group 2 are two different species, the following data indicate that *O. clavigerum* might be a species complex containing reproductively isolated cryptic species. First, a large number of AFLP markers were observed only in Group 2, suggesting that gene flow between Group 1 and Group 2 was limited. When the two groups were treated as two subpopulations of the *O. clavigerum* population in AMOVA, the differentiation between the two groups was very high ($\Phi = 0.587$,

Fig. 3. UPGMA (unweighted paired group method) tree based on Nei's unbiased genetic distance among seven *Ophiostoma clavigerum* populations. Genetic distance was computed from 100 polymorphic loci with allele frequencies between 0.05 and 0.95. The bootstrapping values above the branches were inferred from 1,000 replicates. Bar indicates Nei's unbiased genetic distance. BF, Banff; HR, Hellroaring; HT, Houston; HV, Hidden Valley; MP, Manning Park; St. J, Fort St. James; and WL, Williams Lake.

TABLE 6. Analysis of molecular variance (AMOVA) of amplified fragment length polymorphism markers for 170 isolates of *Ophiostoma clavigerum* collected from seven sites in British Columbia (BC) and the Rocky Mountains^a

Source of variation	df	SSD	Variance components	Percentage of variation (%)	<i>F</i> statistics (Φ)	<i>P</i>
Analysis for seven populations ^b						
Among populations	6	293.31	1.62	14.3	0.143	<0.001
Within populations	163	1581.49	9.70	85.7	...	...
Total	169	1874.80	11.32	...	...	...
Analysis for two groups of populations ^c						
Between groups	1	96.73	0.68	5.8	0.058	0.031
Among populations within groups	5	196.57	1.23	10.6	0.112	<0.001
Within populations	163	1581.49	9.70	83.6	0.164	<0.001
Total	169	1874.80	11.61	...	...	...

^a AMOVA was performed on a dataset including all 469 loci. df, degree of freedom; SSD, sums of squared deviations; *P*, probability of obtaining larger or equal values of variance components; and Φ by chance, determined by 1,023 permutations.

^b The seven populations were treated as one group (Banff, Hellroaring, Hidden Valley, Houston, Fort St. James, Williams Lake, and Manning Park).

^c The two groups were: group 1, Rocky Mountain Region (Banff, Hellroaring, and Hidden Valley); group 2, BC (Houston, Fort St. James, Williams Lake, and Manning Park).

TABLE 7. Analysis of molecular variance (AMOVA) of amplified fragment length polymorphism analysis markers for two *Ophiostoma clavigerum* subpopulations: one subpopulation was sampled from *Pinus contorta* and the other from *Dendroctonus ponderosae*^a

Source of variation	df	SSD	Variance components	Percentage of variation (%)	<i>F</i> statistics (Φ)	<i>P</i>
Analysis for two groups of populations ^b						
Among groups	1	46.31	−0.07	−0.6	−0.006	0.520
Among populations within groups	5	247.00	1.65	14.6	0.146	<0.001
Within populations	163	1581.49	9.70	86.0	0.140	<0.001
Total	169	1874.80	11.29	...	...	...

^a AMOVA was performed on a dataset including all 469 loci of 170 *O. clavigerum* isolates. df, degree of freedom; SSD, sums of squared deviations; *P*, probability of obtaining larger or equal values of variance components; and Φ by chance, determined by 1,023 permutations.

^b The two groups were: group 1, populations isolated from *D. ponderosae* (Hellroaring and Hidden Valley); group 2, populations isolated from *P. contorta* (Banff, Houston, Fort St. James, Williams Lake, and Manning Park).

TABLE 8. Population pairwise *F*_{ST}^a indices among seven *Ophiostoma clavigerum* populations comprising 170 isolates from analysis of molecular variance

Population ^b	BF	HR	HT	HV	MP	St. J	WL
BF	...	...	...	...	...	...	...
HR	0.090	...	...	...	...	...	...
HT	0.160	0.144	...	...	...	...	...
HV	0.085	0.101	0.101	...	...	...	...
MP	0.205	0.170	0.148	0.105	...	...	...
St. J	0.222	0.193	0.083	0.157	0.171	...	...
WL	0.176	0.129	0.181	0.108	0.117	0.110	...

^a All *F*_{ST} values in the table were significant at *P* < 0.01 (*P*, probability of obtaining larger or equal values determined by 1,023 permutations).

^b BF, Banff; HR, Hellroaring; HT, Houston; HV, Hidden Valley; MP, Manning Park; St. J, Fort St. James; WL, Williams Lake.

Group 1 and Group 2 ($I_A = 1.29$ and 0.94 , respectively) were much lower than the index computed from the total population ($I_A = 4.20$). If populations were genetically isolated, a comparison of individual fungi taken from different populations would be like

a comparison of clonal organisms because they would not recombine (27).

If *O. clavigerum* contained cryptic species, we should consider each species separately in the analyses to avoid biased results. Group 2 contained only nine individuals while Group 1 contained 161 isolates. Excluding Group 2 isolates from the analysis lowered the heterozygosity of the *O. clavigerum* population only slightly ($H_s = 0.049$ versus $H_s = 0.053$; Table 5), but did not change which were the most and least heterozygous populations (Hidden Valley and Fort St. James, respectively). The Mantel test with only Group 1 showed no significant correlation between genetic and geographic distances ($r = 0.383$, $P = 0.069$), as with both Group 1 and Group 2. A UPGMA tree for Group 1 showed a topology similar to that of the tree for the two groups. The northern BC populations (Houston and Fort St. James) formed a separate clade, while the Rocky Mountain and southern BC populations were in the same clade (data not shown). Unfortunately, the number of individuals in Group 2 was too small to conduct a thorough population analyses. A great number of individuals belonging to Group 2 need to be isolated to compare Group 1 and Group 2 populations.

D. ponderosae, one of the two vectors of *O. clavigerum*, is an insect native to temperate conifer forests of North America and is a natural disturbance agent in *P. contorta* ecosystems. An endemic *D. ponderosae* population can expand to an epidemic level when permitted by environmental factors (mild temperatures and abundant mature hosts) (18). The current *D. ponderosae* outbreak in Canada is the third such incident in BC in the last 100 years. The first occurred near Kootenay National Park and the Chilcotin Plateau in the central region of BC in the 1930s. The second, in the late 1970s and early 1980s, was again located in the Kootenay National Park and Chilcotin Plateau region. The current outbreak started near Tweedsmuir Park in east-central BC in 1993. Primarily because of continuous warm winters, this epidemic has spread much further north and east than the previous outbreaks, reaching Houston, Fort St. James, and the Rocky Mountain Region, where *D. ponderosae* might have been present at an endemic level.

We found both Groups 1 and 2 of *O. clavigerum* in the Rocky Mountain Region, but only Group 1 in BC Regions we surveyed. Given that the epidemic is spreading from central BC to northern BC as well as eastward into the inland Rocky Mountain Region, it is possible that Group 2 isolates belong to the original endemic population in the Rocky Mountain Region, while the Group 1 isolates have been recently introduced into this region by the eastward spreading beetles from central BC.

In conclusion, this work has provided strong evidence that two distinct groups are present in *O. clavigerum* in the regions sampled. While gene flow between the two groups appears to be limited, it is not yet clear whether Group 2 is a cryptic species. Characterizing the two groups more fully requires establishing a mating test and expanding the phylogenetic analyses. To better understand the current beetle epidemic, further investigations are needed of the two groups' physiological characteristics, effects on host beetle fitness, and pathogenicity to host trees. Group 1 may be more prevalent because it is a more virulent pathogen, more competitive, or more beneficial to the beetles. Group 2, which was found only in the Rocky Mountain Region, may be better adapted to colder conditions. An investigation with more isolates from the expanding epidemic in Canada and from more areas in the United States, as well as isolates from different host trees, would provide a better understanding of the genetic structure of *O. clavigerum*.

LITERATURE CITED

- Adams, A. S., and Six, D. Temporal variation in mycophagy and prevalence of fungi associated with developmental stages of the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Curculionidae, Scolytinae). *Environ. Entomol.* 36:64-72.
- Agapow, P. M., and Burt, A. 2001. Indices of multilocus linkage disequilibrium. *Mol. Ecol. Notes* 1:101-102.
- Berryman, A. A. 1972. Resistance of conifers to invasion by bark beetle-fungal associations. *Bioscience* 22:598-602.
- BC Ministry of Forests and Range. 2006. Forest Practices Branch. Forest health conditions in British Columbia. SB764.C3595 Internet (ISSN 1715-0167).
- Excoffier, L., Smouse, P., and Quattro, J. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* 131:479-491.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.
- Goodwillie, C., Ritland, C., and Ritland, K. 2006. The genetic basics of floral traits associated with mating system evolution in *Leptosiphon* (Polemoniaceae): An analysis of quantitative trait loci. *Evolution* 60:491-504.
- Harrington, T. C. 1993. Biology and taxonomy of fungi associated with bark beetles Pages 37-51 in: *Beetle-Pathogen Interactions in Conifer Forests*. T. D. Schowalter and G. M. Filip, eds. Academic Press, San Diego.
- Lee, S., Kim, J. J., and Breuil, C. 2003. A PCR-RFLP marker distinguishing *Ophiostoma clavigerum* from morphologically similar *Leptographium* species associated with bark beetles. *Can. J. Bot.* 81:1104-1112.
- Lee, S., Kim, J. J., and Breuil, C. 2005. *Leptographium longiclavatum* sp. nov., a new species associated with the mountain pine beetle, *Dendroctonus ponderosae*. *Mycol. Res.* 109:1162-1170.
- Lee, S., Kim, J. J., and Breuil, C. 2006. Fungal diversity associated with the mountain pine beetle, *Dendroctonus ponderosae* and infested lodgepole pines in British Columbia. *Fungal Divers.* 22:91-105.
- Maynard, J., Smith, N. H., O'Rourke, M., and Spratt, B. G. 1993. How clonal are bacteria? *Proc. Natl. Acad. Sci. USA* 76:5269-5273.
- McDonald, B. 1997. The population genetics of fungi: Tools and techniques. *Phytopathology* 87:448-453.
- McDonald, B., and McDermott, J. 1993. Population genetics of plant pathogenic fungi. *Bioscience* 42:311-319.
- Mueller, U., and Wolfenbarger, L. 1999. AFLP genotyping and fingerprinting. *Tree* 14:389-394.
- Nei, M. 1978. Estimation of average heterozygosities and genetic distance from a small number of individuals. *Genetics* 89:583-590.
- Nei, M., and Li, W. H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* 76:5269-5273.
- Paine, T. D., Raffa, K. F., and Harrington, T. C. 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Ann. Rev. Entomol.* 42:179-206.
- Reid, R. W. 1961. Moisture changes in lodgepole pine before and after attack by mountain pine beetle. *For. Chron.* 37:368-375.
- Remington, D. L., Whetten, R. W., Liu, B.-H., and O'Malley, D. M. 1999. Construction of an AFLP genetic map with nearly complete genome coverage in *Pinus taeda*. *Theor. Appl. Genet.* 98:1279-1292.
- Robinson-Jeffrey, R. C., and Davidson, R. W. 1968. Three new *Europhium* species with *Verticicladiella* imperfect states on blue-stained pine. *Can. J. Bot.* 46:1523-1527.
- Six, D. L. 2003. A comparison of mycangial and phoretic fungi of individual mountain pine beetle. *Can. J. For. Res.* 33:1331-1334.
- Six, D. L. 2003. Bark beetle-fungus symbioses. Pages 97-114 in: *Insect Symbiosis*. K. Bourtzis and A. M. Thomas, eds. CRC Press, Boca Raton, FL.
- Six, D. L., and Paine, T. D. 1997. *Ophiostoma clavigerum* is the mycangial fungus of the Jeffrey pine beetle, *Dendroctonus jeffreyi*. *Mycologia* 89:858-866.
- Six, D. L., and Paine, T. D. 1999. Allozyme diversity and gene flow in *Ophiostoma clavigerum* (Ophiostomatales: Ophiostomataceae), the mycangial fungus of the Jeffrey pine beetle, *Dendroctonus jeffreyi* (Coleoptera: Scolytidae). *Can. J. For. Res.* 29:324-331.
- Solheim, H. 1995. Early stages of blue-stain fungus invasion of lodgepole pine sapwood following mountain pine beetle attack. *Can. J. Bot.* 73:70-74.
- Taylor, J. W., Geiser, D. M., Burt, A., and Koufopanou, V. 1999. The evolutionary biology and population genetics underlying fungal strain typing. *Clin. Microbiol. Rev.* 12:126-146.
- U.S. Department Agriculture. 2001. Mountain pine beetle. *Forest Insects and Disease Leaflet 2*. Online publication. February 1, 2007.
- Vos, P., Hogers, R., Bleeker, M., Van Reijans, M., de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting *Nucleic Acids Res.* 23:4407-4414.
- Webb, J. W., and Franklin, R. T. 1978. Influence of phloem moisture on brood development of the southern pine beetle (Coleoptera: Scolytidae).

31. Weir, B. S. 1996. Genetic Data Analysis II: Methods for Discrete Population Genetic data. Sinauer Associates, Inc., Sunderland, MA.
32. Whitney, H. S., Bandoni, R. J., and Oberwinkler, F. 1987. *Entomocorticium dendroctoni* gen. et sp. nov. (Basidiomycotina), a possible nutritional symbiote of the mountain pine beetle in lodgepole pine in British Columbia. Can. J. Bot. 65:95-102.
33. Whitney, H. S., and Farris, S. H. 1970. Maxillary mycangium in the mountain pine beetle. Science 167:54-55.
34. Yamaoka, Y., Hiratsuka, Y., and Maruyama, P. J. 1995. The ability of *Ophiostoma clavigerum* to kill mature lodgepole-pine trees. Eur. J. For. Pathol. 25:401-404.
35. Yamaoka, Y., Swanson, R. H., and Hiratsuka, Y. 1990. Inoculation of lodgepole pine with four blue-stain fungi associated with mountain pine beetle, monitored by a heat pulse velocity (HPV) instrument. Can. J. For. Res. 20:31-31.
36. Zolan, M., and Pukilla, P. 1986. Inheritance of DNA methylation in *Coprinus cinereus*. Mol. Cell. Biol. 6:195-200.