Genetic Variability of Canadian Populations of the Sapstain Fungus *Ophiostoma piceae*

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ABSTRACT

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Genetic diversity was studied in seven Canadian populations of *Ophiostoma piceae*, the most prevalent sapstain fungus in Canadian softwoods. A total of 239 single-spore isolates were recovered following a systematic survey of sapstain fungi in logs and lumber at seven selected sawmills in six Canadian provinces (British Columbia, Alberta, Saskatchewan, Ontario, Québec, and New Brunswick). Sampling was carried out on five commercially important softwood species: balsam fir (*Abies balsamea*), white spruce (*Picea glauca*), black spruce (*Picea mariana*), jack pine (*Pinus banksiana*), and lodgepole pine (*Pinus contorta* var. *latifolia*). The A and B mating types occurred at equal frequency ($MAT\ A/MAT\ B = 1.00:1.13$) over all populations. Pseudo-allelic frequencies were estimated at each of 24 putative genetic loci by scoring for presence or absence of random amplified polymorphic DNA

fragments generated by five primers. A total of 237 haplotypes were found among the 239 isolates, revealing a high level of genotypic diversity among isolates. Total gene diversity ($H_T = 0.414$) was mostly attributable to diversity within populations ($H_S = 0.369$). Thus, only 11.2% of the total variability was attributable to frequency differences among populations. An analysis of molecular variance revealed that most genetic variability occurred within subpopulations within mills (84.3%; $P < 0.001$), whereas low but statistically significant levels of genetic differentiation were also observed among subpopulations within populations (5.4%; $P < 0.001$) and among populations (10.3%; $P < 0.001$). Estimates of Nei's genetic distances were not correlated with geographic distances among sampling locations ($r = -0.092$; $P = 0.310$), although principal component analysis indicated that subpopulations located east of Saskatchewan were grouped on the same side of the second principal component axis. Overall, results suggest moderate genetic differentiation of *O. piceae* in Canada, which is consistent with the observation that sexual reproduction is frequently observed in this fungus.

Sapstain is defined as a blue or black discoloration of sapwood caused by penetrating fungi. Because the color that these fungi impart to the wood is frequently a bluish gray, the stain is often referred to as blue stain (13). Softwood and hardwood lumber are susceptible to sapstain during processing and storage. Sapstain fungi can infect sapwood through log ends, bare patches where bark has been damaged by handling, insect damage, and seasoning checks (8). Damage to bark and wood caused during harvesting further increases the opportunities for fungal attack (43). Certain wood species, such as most species of *Pinus*, are particularly prone to sapstain (8,37,51).

Sapstain fungi cause either superficial discoloration or penetrating stains but, unlike decay fungi, they generally do not alter the structural properties of wood (35,37). Nevertheless, the forest products industry worldwide suffers considerable losses in revenue due to fungal stains. Of the protective methods that have been used to prevent wood discoloration in lumber, the two most important are kiln drying and chemical treatments. Kiln drying reduces the moisture content of the wood below 20% but it is costly and does not protect rewetted wood from reinfection. Wood protection through chemicals is no longer a fully viable option because of legislated restrictions and public concern. Therefore, new approaches must be developed in order to prevent or control sapstain (1).

The Ophiostomatoid fungus *Ophiostoma piceae* (Münch) H. & P. Sydow, belongs to the Ascomycetes and is ubiquitous on wood of a wide variety of mostly coniferous species throughout the world. In Canada, it was suggested that *O. piceae* was the most frequent sapstain fungus isolated from stained softwood lumber (37,38). A more recent study confirmed that *O. piceae* was by far the most common sapstain fungus found in coniferous logs and lumber in Canadian sawmills (44).

Ophiostoma piceae is haploid through most of its life cycle and can reproduce both sexually and asexually by perithecia and synnemata, respectively. It is heterothallic with two mating types designated A and B (6). *O. piceae* is closely related but genetically distinct from *O. quercus*, which is almost exclusively found on hardwood species (6). The two species had earlier been considered as a single taxon until a closer analysis of their mating reactions (6), temperature growth responses (7), synnematal size (17), random amplified polymorphic DNA (RAPD) polymorphisms (17,33), and ribosomal DNA gene sequences (25) suggested otherwise. Kim et al. (25) recently described the development of two pairs of internal transcribed spacer-derived primers for the specific detection of *O. piceae* and *O. quercus* in stained wood by the polymerase chain reaction (PCR).

The development of DNA-based markers has provided powerful tools for population genetic studies of plant pathogenic fungi. Analysis of RAPD polymorphisms (45,47) has become increasingly popular for estimating genetic diversity in natural populations (11,16,18,19,40). RAPD markers offer the advantages of technical simplicity and the ability to provide large numbers of polymorphic loci. Analysis of RAPD variation is particularly well suited for genetic studies of haploid fungi such as

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O. piceae, because there is no loss of genetic information caused by the dominant inheritance of RAPDs. However, RAPD analysis of this species in particular and of Ophiostomatoid fungi in general has been so far limited. For example, previous studies by Halmschlagel et al. (17) and Pipe et al. (33), while showing the occurrence of RAPD differences between *O. piceae* and *O. quercus*, did not include any systematic analysis about the genetic diversity of these fungi whose population structure thus remains unknown.

The objective of this study was to describe the population structure of *O. piceae* in Canada. This species was selected because it accounted for nearly 50% of all sapstain fungi isolated from softwood logs and lumber sampled in a recent survey (44). RAPD polymorphisms from 239 isolates were analyzed in order to (i) estimate gene and genotype diversity of *O. piceae*; and (ii) determine whether *O. piceae* occurs as a single metapopulation or as genetically distinct subpopulations characterized by different hosts or geographical locations. A preliminary report has been presented elsewhere (15).

MATERIALS AND METHODS

Sampling and identification of fungal isolates. A fungal survey at seven sawmills across Canada was carried out in 1997 (Table 1; 44). Briefly, logs and boards from the following softwood species were sampled: lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.), jack pine (*Pinus banksiana* Lamb.), white spruce (*Picea glauca* [Moench] Voss), black spruce (*Picea mariana* [Mill.] B.S.P.), and balsam fir (*Abies balsamea* [L.] Mill.). Fungal hyphae and fruiting bodies of *Ophiostoma* spp. were aseptically removed from stained sapwood and transferred onto petri plates containing 2% malt extract agar (MEA) (Oxoid, Nepean, Canada) amended with 100 mg/ml of cycloheximide (21). Inoculated plates were incubated at room temperature. Each organism was purified to a single spore culture within 1 week. Isolates of *O. piceae* were identified by their morphology (41,48,49) and by mating tests with known tester strains. A total of 239 *O. piceae* isolates were thus identified and analyzed. All isolates from the same mill were regarded as one population, whereas isolates from a given host species within the same mill were regarded as one subpopulation. Thus, the 239 isolates were divided into 7 populations consisting of 15 subpopulations.

Sexual mating tests. Controlled crosses were carried out on solid medium composed of 30 g of Bacto agar (Difco Laboratories, Detroit) per liter of distilled water supplemented with 50 g per liter of white pine (*Pinus strobus* L.) sawdust. Pine sawdust was autoclaved separately and added to the medium immediately before pouring plates (9-cm petri dishes). A 6-mm mycelium plug from each of the two tester isolates with known

mating types (*MAT* A/H2181, *MAT* B/H2009; from J. F. Webber, Forest Research, U.K.) was placed in the center of two separate sets of plates. Four days later, five isolates with unknown mating type were placed 2 cm apart from the *MAT* A tester in one plate and the *MAT* B tester in the complementary plate. Every mating was replicated at least twice. Plates were sealed with Parafilm and kept at room temperature (20 to 22°C) under normal daylight. Formation of perithecia was recorded 4 weeks after plating the isolates. Mating occurred exclusively with only one of the tester isolates. Sexual mating tests were performed on all 239 isolates.

DNA extraction and PCR amplification. DNA was extracted from mycelium by a modification of the protocol described by Kim et al. (25). Isolates of *O. piceae* were grown on MEA covered with a layer of cellophane sheet (Bio-Rad Laboratories, Hercules, CA). Mycelium was harvested, divided among 2-ml screw cap microfuge tubes (approximately 200 mg per tube), and stored at -80°C. Prior to extraction, tubes were put on ice and 300 µl of extraction buffer (50 mM Tris-HCl at pH 8.5, 50 mM EDTA, and 3% sodium dodecyl sulfate) was added. The mycelium was crushed for 1 min with an electric drill with a stainless steel bit that was cleaned and sterilized between isolates. Drilling was repeated four times. Then, 150 µl of 3 M sodium acetate, pH 5.2, was added, and the tubes were finger-vortexed. The tubes were kept at -20°C for 20 min and centrifuged at 13,000 rpm for 10 min. The upper phase was pipetted into 1.5-ml microfuge tubes, and the DNA was precipitated by adding an equal volume of isopropanol before incubating at room temperature for 10 min. The DNA was pelleted by centrifuging for 10 min at 13,000 rpm and washed with 70% ethanol, after which the pellet was dried and resuspended in Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). DNA concentration was estimated by comparing the band intensity of samples on agarose gel with a known amount of lambda DNA standard. DNA was diluted depending on its concentration and stored in TE buffer at -20°C.

DNA was amplified in a thermal cycler (9600; Perkin-Elmer Cetus, Norwalk, CT). Amplifications were carried out in volumes of 12 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 100 mM each dNTP (Pharmacia Biotechnology Inc., Baie d'Urfé, Canada), 0.04 unit per µl of *Taq* DNA polymerase (Boehringer Mannheim, Laval, Canada), 0.1 to 0.5 ng of genomic DNA, and 0.2 mM oligonucleotide primer (Operon Technologies, Alameda, CA). Amplification conditions were as follows: 20 cycles of 15 s at 94°C (denaturation), 15 s at 35°C (annealing), and 1.5 min at 72°C (extension); 25 cycles of 15 s at 94°C, 15 s at 35°C, and 1.5 min at 72°C followed by 5 s of auto-extension per cycle; and 10 min at 72°C. All amplifications were done at least twice to ensure reproducibility of results. Only reproducible bands were used for analysis. Amplification products

TABLE 1. Mill locations across Canada, wood species sampled, and number of isolates sampled

Mill	Location (latitude-longitude)	Subpopulation	Host species	No. of isolates
Prince George	53°55'00"N	BC1-WS	White spruce	15
(British Columbia)	122°45'00"W	BC1-LP	Lodgepole pine	15
Okanagan Falls	49°21'00"N	BC2-WS	White spruce	15
(British Columbia)	119°34'00"W	BC2-LP	Lodgepole pine	15
Blairmore	49°36'00"N	AL-WS	White spruce	15
(Alberta)	114°26'00"W	AL-LP	Lodgepole pine	15
Big River	53°50'00"N	SA-WBS	White spruce, black spruce	30
(Saskatchewan)	107°01'00"W	SA-JP	Jack pine	14
Kirkland Lake	48°09'00"N	ON-BS	Black spruce	15
(Ontario)	80°02'00"W	ON-JP	Jack pine	15
St-Émile	46°52'00"N	QC-BS	Black spruce	15
(Québec)	71°20'00"W	QC-JP	Jack pine	15
		QC-BF	Balsam fir	15
Plaster Rock	46°54'14"N	NB-WS	White spruce	15
(New Brunswick)	67°23'27"W	NB-BF	Balsam fir	15

were separated by electrophoresis on 1% agarose plus 0.5% synergel (Diversified Biotech, Boston) in 0.75× Tris-phosphate-EDTA (TPE) buffer and were visualized by fluorescence following ethidium bromide staining.

In a preliminary test, 121 primers were screened using six isolates (one from each of the six provinces) to detect DNA polymorphisms. Most primers revealed polymorphisms but did not show clear banding patterns. Primers that generated reproducible polymorphisms and clear banding patterns were used to amplify DNA from all the isolates. The five 10-mer primers satisfying these criteria were OPA9 (5'-GGGTAACGCC-3'), OPA13 (5'-CAGCACCCAC-3'), OPC1 (5'-TTCGAGCCAG-3'), OPE16 (5'-GGTGACTGTG-3'), and OPQ18 (5'-AGGCTGGGTG-3'). Amplification products were identified by the name of the primer and the size of the amplicon.

Data analysis. All polymorphic RAPD fragments were scored as putative loci with 2 alleles, one allele indicating the presence of a fragment and the other the absence of a fragment. The data set was compiled as a matrix of 239 isolates and 24 RAPD polymorphisms. Marker frequency was calculated for each of the seven populations. When determining allelic frequencies, it was assumed that each fragment represented a unique, bi-allelic locus. The possibility that for a given primer, two fragments different in size might represent allelic length mutations was addressed by verifying whether such fragments were mutually exclusive, i.e., never occurred together in the same haploid isolate. This situation was never observed. For each locus, heterogeneity of the marker frequency between sawmills was calculated by the likelihood G test (SAS Institute, Cary, NC). Allelic diversities were estimated by Nei's diversity parameters (29) with corrections for sample size (31). The population structure was studied by dividing the total genetic diversity (H_T) into the genetic diversity of individuals

relative to their subpopulation (H_S) and the genetic diversity between subpopulations (D_{ST}). Genetic differentiation among populations (G_{ST}) was measured as D_{ST}/H_T . This analysis was favored because it was designed for both diploid and nondiploid organisms (29). The variance associated with the G_{ST} value was calculated by 1,000 bootstrap samplings over loci with software developed and provided by J. Beaulieu (Canadian Forest Service, Sainte-Foy, QC).

An analysis of molecular variance (AMOVA) (12) was carried out using a matrix of Euclidean distances between all pairs of haplotypes (24). The analysis partitioned the total variance into hierarchical components (among populations, among subpopulations, and within subpopulations). A measure of genetic differentiation was obtained by F statistic analogs (Φ statistics). The variance components and Φ statistics were tested statistically by nonparametric randomization tests with 1,000 repetitions. All analyses were conducted with the WINAMOVA version 1.55 program provided by L. Excoffier (Department of Anthropology and Ecology, University of Geneva, Switzerland).

The RAPD and *MAT* data set was also subjected to principal component analysis using SAS for Windows software (release 6.12; SAS Institute). The first two principal components were plotted to visualize the grouping of samples. Nei's unbiased genetic distances (30) were measured between all pairs of subpopulations. Relationships between subpopulations were investigated by constructing a phylogenetic tree with the phenetic approach of the unweighted pair group method with arithmetic average (UPGMA) using the Tools for Population Genetic Analysis (TFPGA) software (version 1.3; M. Miller, Northern Arizona University, Flagstaff). Genetic distance data were examined for possible correlation with geographic distances by performing a Mantel test between the genetic and geographic distance matrices.

TABLE 2. Frequency of marker alleles and genetic diversity of seven Canadian populations of *Ophiostoma piceae*

Locus	Frequency of the marker allele ^a							Genetic diversity ^b							
	BC1 ^b	BC2	AL	SA	ON	QC	NB	All populations				Without SA and ON			
	<i>n</i> = 30	<i>n</i> = 30	<i>n</i> = 30	<i>n</i> = 44	<i>n</i> = 30	<i>n</i> = 45	<i>n</i> = 30	Pr	<i>H</i> _S	<i>H</i> _T	<i>G</i> _{ST}	Pr	<i>H</i> _S	<i>H</i> _T	<i>G</i> _{ST}
OPA9 ₅₂₅	1.000	0.733	0.933	1.000	0.867	0.800	0.833	**	0.198	0.211	0.060	*	0.230	0.242	0.052
OPA13 ₈₃₀	1.000	0.900	0.733	0.841	0.300	0.711	0.733	**	0.303	0.381	0.203	**	0.283	0.303	0.064
OPA13 ₉₀₀	0.367	0.167	0.200	0.477	0.600	0.444	0.100	**	0.400	0.448	0.108	*	0.358	0.383	0.064
OPA13 ₁₁₉₀	0.600	0.233	0.400	0.386	0.567	0.467	0.367	ns	0.478	0.493	0.031	ns	0.470	0.488	0.036
OPA13 ₁₂₁₀	0.167	0.067	0.167	0.500	0.400	0.244	0.267	**	0.356	0.385	0.075	ns	0.297	0.300	0.009
OPA13 ₁₄₀₀	0.467	0.567	0.467	0.705	0.467	0.422	0.433	ns	0.497	0.502	0.009	ns	0.509	0.501	0.000
OPA13 ₁₅₉₀	0.700	0.733	0.767	0.932	0.767	0.689	0.467	**	0.380	0.403	0.058	ns	0.432	0.444	0.027
OPA13 ₁₆₅₀	0.767	0.667	0.833	0.955	0.933	0.511	0.600	**	0.334	0.374	0.107	ns	0.425	0.441	0.036
OPA13 ₁₇₅₀	0.700	0.467	0.767	0.909	0.633	0.444	0.600	**	0.424	0.459	0.077	ns	0.464	0.485	0.043
OPA13 ₂₀₅₀	0.667	0.667	0.767	0.955	0.867	0.511	0.467	**	0.377	0.421	0.106	ns	0.463	0.476	0.028
OPC1 ₅₈₀	0.733	0.600	0.700	1.000	1.000	0.889	0.567	**	0.291	0.340	0.142	*	0.408	0.424	0.037
OPC1 ₆₄₀	0.467	0.367	0.200	0.818	0.500	0.333	0.367	**	0.440	0.494	0.109	ns	0.452	0.456	0.008
OPC1 ₈₀₀	0.600	0.367	0.200	0.477	0.533	0.311	0.333	*	0.461	0.483	0.045	ns	0.441	0.465	0.051
OPC1 ₈₂₀	0.233	0.733	0.167	0.250	0.100	0.400	0.300	**	0.365	0.431	0.152	**	0.397	0.467	0.149
OPC1 ₁₀₉₅	0.233	0.600	0.500	0.591	0.667	0.467	0.267	**	0.464	0.501	0.073	*	0.459	0.488	0.058
OPC1 ₁₃₀₀	0.333	0.566	0.500	0.341	0.467	0.600	0.433	ns	0.494	0.499	0.011	ns	0.496	0.503	0.012
OPE16 ₁₂₀₀	0.967	0.933	0.900	1.000	1.000	0.622	0.567	**	0.196	0.248	0.211	**	0.274	0.324	0.154
OPE16 ₁₄₀₀	0.367	0.300	0.300	0.614	0.467	0.511	0.167	**	0.450	0.477	0.058	ns	0.430	0.444	0.033
OPQ18 ₇₀₀	0.333	0.400	0.433	0.114	0.433	0.200	0.300	**	0.419	0.434	0.034	ns	0.445	0.447	0.006
OPQ18 ₈₆₀	0.367	0.733	0.633	0.159	0.000	0.756	0.600	**	0.359	0.499	0.281	*	0.448	0.475	0.058
OPQ18 ₉₅₀	0.967	1.000	0.933	0.977	1.000	0.778	0.733	**	0.143	0.160	0.108	**	0.191	0.209	0.086
OPQ18 ₁₀₂₀	0.633	0.267	0.533	0.932	0.967	0.578	0.433	**	0.371	0.473	0.214	ns	0.481	0.503	0.043
OPQ18 ₁₁₉₀	0.567	0.300	0.433	0.955	0.600	0.422	0.233	**	0.414	0.502	0.175	ns	0.464	0.479	0.032
OPQ18 ₁₂₁₀	0.067	0.533	0.500	0.046	0.000	0.222	0.067	**	0.247	0.327	0.244	**	0.328	0.403	0.185
MAT A	0.333	0.500	0.500	0.432	0.433	0.555	0.500	ns	...	...	...	ns	...	...	...
Average	...	...	...	...	...	...	...	...	0.369	0.414	0.112	...	0.402	0.423	0.053
SE	...	...	...	...	...	...	...	...	...	...	0.016	...	...	...	0.009

^a BC1 = British Columbia (Prince George), BC2 = (Okanagan Falls), AL = Alberta (Blairmore), SA = Saskatchewan (Big River), ON = Ontario (Kirkland Lake), QC = Québec (St-Émile), and NB = New Brunswick (Plaster Rock). Only random amplified polymorphic DNA data were used for estimating allelic diversity. Mating-type frequencies were calculated for each population but were not used in genetic diversity analyses.

^b H_S , genetic diversity of individuals relative to their subpopulation; H_T , total genetic diversity; G_{ST} , genetic diversity of subpopulations relative to their population; Pr, G test heterogeneity of allele frequencies among populations; * significant at $P < 0.05$; ** significant at $P < 0.01$; ns, not significant; and SE, standard error.

The software Progciciel R (Beta 3.0; A. Vaudor, Université de Montréal, Canada) was used to calculate the product-moment coefficient, r , which is related to the Mantel statistic, Z . The statistical significance of the resulting correlation coefficient was tested by performing 1,000 random permutations of the data set and calculating the proportion yielding values that were greater than or equal to the observed correlation coefficient. Finally, user-defined contrasts between subpopulations were analyzed using TFPGA.

RESULTS

Colonies of *O. piceae* growing on MEA exhibited a broad range in their pigmentation from a light tint to a dark brown. However, the growth rate of the colonies on MEA was uniform (data not shown). Each isolate crossed with one of the two *O. piceae* testers used in mating tests. The mating types occurred at equal frequency ($MAT\ A/MAT\ B = 1.00:1.13$; $\chi^2 = 0.941$, $P > 0.10$, not significant [ns]) over all populations. Furthermore, $MAT\ A$ and B occurred in equal frequencies within each of the seven populations (Table 2).

The five RAPD primers used to amplify genomic DNA in 239 isolates generated 24 polymorphisms. Between one and nine fragments were consistently amplified for each primer, ranging in size from 525 to 2,050 bp (Table 2). The matrix of RAPD data revealed the occurrence of a high level of genotypic diversity in *O. piceae*, while 237 different haplotypes were observed among the 239 isolates analyzed. With the exception of RAPD markers OPQ18₈₆₀ and OPQ18_{1,210}, which were not observed in the population from Ontario, all other markers were present in every population. No marker was unique to one or a few populations; thus, the alleles occurred over the near-4,000 km range. A few loci (OPA9₅₂₅ and OPA13₈₃₀ in Prince George, British Columbia [BC]) were invariant for the marker allele within a population. However, most loci were polymorphic both within and among populations, and some populations (Alberta, Québec, and New Brunswick) were polymorphic at all loci. None of the primers

amplified a fragment specific to the host species or regional origin of an isolate.

The frequency of 21 loci varied significantly ($P < 0.05$) among the seven populations (Table 2). Marker OPA13₈₃₀ was found on average in 30% of the samples from Ontario, but was present in 100% of the samples from Prince George (BC). Marker OPQ18₈₆₀ was present in 76% of the samples from Québec but was absent from Ontario. Markers OPC1₅₈₀ and OPE16_{1,200} were fixed in Saskatchewan and Ontario but were polymorphic in the other populations. Most of these genetic differences were accounted for by frequency differences between the Saskatchewan and Ontario populations and the other populations, because when those two populations were omitted from the analysis, the frequency of only 10 loci varied significantly at $P < 0.05$ and of five loci at $P < 0.01$ (Table 2). In addition, marker frequencies from Saskatchewan and Ontario populations were higher than those from populations located east and west, for 18 loci.

Computations of gene diversity indicated that diversity within populations accounted for most of the total genetic diversity, whereas genetic diversity between populations represented only a small proportion of the total diversity. Adjusted values of genetic diversity within populations (H_S) ranged between 0.143 and 0.494 and averaged 0.369, accounting for 89% of the total genetic diversity ($H_T = 0.414$). The proportion of the total genetic diversity attributable to the population differentiation (G_{ST}) ranged from 0.011 to 0.281 and averaged 0.112 over all loci (Table 2). When population structure was analyzed while omitting the Saskatchewan and Ontario populations, G_{ST} ranged between 0 and 0.185 and averaged 0.053. The G_{ST} value between these two provinces was very low (0.040). Furthermore, differences among isolates from different host species revealed a low level of genetic differentiation ($G_{ST} = 0.058$). Analysis of contrasts between subpopulations isolated from spruce ($n = 7$) or pine ($n = 6$) indicated that host tree species had very little effect overall (results over 24 loci, 48 df: $\chi^2 = 57.76$, $P = 0.158$, ns). Results from principal component analysis (Fig. 1) confirmed this general trend, although it was noted that the three subpopulations from jack pine were grouped on the same side of the first principal component axis.

Computation of Φ statistics by AMOVA confirmed that most (85.0%) of the diversity occurred within populations, with 15.0% differentiation among populations (data not shown). A more detailed analysis based on a nested AMOVA (Table 3) indicated that total genetic diversity was mostly attributable to diversity within subpopulations from the same mill (84.3% of the total variance, $P < 0.001$). However, the analysis also revealed low but statistically significant levels of genetic differentiation among subpopulations within populations (5.4% of the total variance, $P < 0.001$) as well as among populations (10.3% of the total variance, $P < 0.001$).

Estimates of Nei's unbiased genetic distances among subpopulations ranged from 0.007 to 0.326 (Table 4). Low distance estimates were observed among subpopulations from Saskatchewan and Ontario (0.007 to 0.075). Low estimates were also observed between some geographically distant subpopulations from other provinces, such as between those isolated from white spruce in Alberta and from balsam fir in New Brunswick (0.050).

Fig. 1. Principal component analysis with 24 random amplified polymorphic DNA markers for 15 Canadian subpopulations of *Ophiostoma piceae*. Components 1 and 2 accounted for 36.8 and 12.8% of the variation, respectively.

TABLE 3. Nested analysis of molecular variance of random amplified polymorphic DNA haplotypes for 239 isolates of *Ophiostoma piceae* from seven Canadian populations grouped into 15 subpopulations^a

Source of variation	df	SSD	MSD	Variance component	F statistics (Φ)	% Variance components	P
Among populations	6	158.48	26.41	0.520	0.103	10.29	<0.001
Among subpopulations within a population	8	68.26	8.53	0.275	0.061	5.44	<0.001
Within subpopulations	224	954.63	4.26	4.262	0.157	84.27	<0.001

^a df, degrees of freedom; SSD, sums of squared deviations; MSD, mean squared deviations; P, probability of obtaining equal or larger value determined by 1,000 randomizations of the treatments.

Of the 105 distance estimates computed between all 15 subpopulations, the highest 21 values always involved one subpopulation from either Ontario or Saskatchewan and one subpopulation from one of the remaining regions. Genetic distances between the subpopulation isolated from lodgepole pine at the Okanagan Falls mill (BC2-LP) and subpopulations from either Saskatchewan or Ontario were particularly high, ranging from 0.253 to 0.326.

The UPGMA cluster analysis of Nei's unbiased distances (Fig. 2) confirmed that the four subpopulations from Ontario and Saskatchewan were genetically closer to each other (95% homology) than to subpopulations from other regions, with which they showed a lower (84%) level of homology. Results from 1,000 bootstraps indicated that this node, which was supported in 100% of the resampling, was the strongest in the dendrogram. In contrast, clustering of subpopulations from British Columbia, Alberta, Québec, and New Brunswick was associated with low bootstrap values (3 to 59%). Comparison of the genetic and geographic distance matrices did not reveal any correlation between the two data sets. The product-moment correlation coefficient was slightly negative ($r = -0.092$) but not significantly different from zero ($P = 0.310$).

The principal component analysis confirmed that subpopulations from Saskatchewan and Ontario were genetically related; the four of them occurred on the same side of the first principal component axis (Fig. 1). Interestingly, all subpopulations located east of Saskatchewan (i.e., from Ontario, Québec, and New Brunswick) were grouped on the same side of the second principal component axis. This group also included the Prince George subpopulation isolated from lodgepole pine (BC1-LP). The subpopulations isolated from white spruce in Okanagan Falls (BC2-WS) and Blairmore (AL-WS) were almost indistinguishable from each other. Subpopulations isolated from balsam fir in Québec (QC-BF) and in New Brunswick (NB-BF) were also very similar to each other.

DISCUSSION

Analyses of RAPD polymorphisms in 239 isolates of *O. piceae* from different regions and coniferous hosts across Canada revealed the occurrence of high levels of gene and genotype diversity but relatively low genetic differentiation among subpopulations sampled from different coniferous hosts and distributed spatially over nearly 4,000 km. Although principal component analysis showed that subpopulations from jack pine were loosely grouped along the same side of the first principal component axis, the occurrence of specific genotypes of *O. piceae* associated with

distinct coniferous host species was not supported by analysis of gene diversity nor by analysis of contrasts. This confirms a recent observation by Yang (50) who cross-inoculated 28 *O. piceae* isolates from the 1997 survey (44) on wood wafers of jack pine, white pine, white spruce, and balsam fir and reported that most individuals isolated from one softwood species could grow on any of the wood species tested. Likewise, subpopulations east of Saskatchewan were grouped along the second principal component axis, but comparison of the genetic and geographic distance matrices by a Mantel test did not support the hypothesis of isolation-by-distance among the 15 subpopulations studied. Therefore, we conclude that there is no evidence that there may be a significant barrier to gene flow operating in *O. piceae* in Canada.

A combination of high genetic variability and low genetic differentiation is usually observed in populations of fungi that reproduce sexually. Examples for fungal species associated with trees include North American populations of *Armillaria gallica* (36), *Chondrostereum purpureum* (16), and *Cronartium quercuum* f. sp. *fusiforme* (19), as well as European populations of *Crumenulopsis sororia* (10) and *Fomitopsis pinicola* (23). In these species, the occurrence of sexual incompatibility systems favoring outcrossing and the presence of efficient mechanisms for long-distance spore dissemination appear to be driving forces for deploying genetic variability and homogenizing populations through extensive gene flow.

O. piceae reproduces both sexually and asexually by means of ascospores produced in perithecia and by conidia produced at the tip of synnemata, respectively. It is a bipolar, heterothallic species in which sexual exchange only takes place between individuals carrying different alleles (A or B) at a single mating-type locus. The two mating types occurred at equal frequency over all populations analyzed in this study, allowing for random mating. Both ascospores and conidia are embedded in sticky mucilage and are thus easily dispersed by vectors but not by air (9). Overall, our data suggest that the population genetics of *O. piceae* follow the model that is common for sexually reproducing fungi, in which most of the genetic variability occurs within populations (or subpopulations). This differs sharply from patterns reported for the few other Ophiostomatoid species analyzed so far. For example, Zambino and Harrington (52) compared isozyme polymorphisms in three different host-specific varieties of *Leptographium wagnerii* (= *Verticicladiella wagnerii*). They computed low gene diversity estimates for each variety but high genetic differentiation ($G_{ST} = 0.86$) between them. More recently, Six and Paine (39), who also studied isozyme polymorphisms, reported low gene diversity and high genetic differen-

TABLE 4. Nei's genetic distance for 15 subpopulations of *Ophiostoma piceae* isolated from coniferous hosts in Canada^a

Subpopulation	BC1-WS	BC1-LP	BC2-WS	BC2-LP	AL-WS	AL-LP	SA-WBS	SA-JP	ON-BS	ON-JP	QC-BS	QC-JP	QC-BF	NB-WS	NB-BF
BC1-WS	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
BC1-LP	0.036	...	...	...	...	...	...	...	...	...	...	...	...	...	...
BC2-WS	0.078	0.090	...	...	...	...	...	...	...	...	...	...	...	...	...
BC2-LP	0.143	0.146	0.065	...	...	...	...	...	...	...	...	...	...	...	...
AL-WS	0.072	0.079	0.049	0.041	...	...	...	...	...	...	...	...	...	...	...
AL-LP	0.063	0.088	0.080	0.143	0.086	...	...	...	...	...	...	...	...	...	...
SA-WBS	0.079	0.073	0.151	0.253	0.146	0.135	...	...	...	...	...	...	...	...	...
SA-JP	0.082	0.079	0.172	0.326	0.167	0.144	0.016	...	...	...	...	...	...	...	...
ON-BS	0.085	0.085	0.155	0.274	0.121	0.104	0.051	0.030	...	...	...	...	...	...	...
ON-JP	0.100	0.066	0.175	0.274	0.136	0.150	0.075	0.056	0.007	...	...	...	...	...	...
QC-BS	0.112	0.084	0.092	0.130	0.099	0.107	0.163	0.196	0.147	0.134	...	...	...	...	...
QC-JP	0.071	0.066	0.082	0.146	0.112	0.062	0.107	0.131	0.117	0.109	0.051	...	...	...	...
QC-BF	0.151	0.095	0.092	0.067	0.049	0.150	0.212	0.257	0.206	0.191	0.068	0.139	...	...	...
NB-WS	0.093	0.078	0.082	0.089	0.061	0.103	0.208	0.246	0.193	0.184	0.045	0.070	0.057	...	...
NB-BF	0.071	0.095	0.127	0.109	0.050	0.147	0.192	0.229	0.179	0.177	0.112	0.157	0.071	0.081	...

^a Computed from 24 random amplified polymorphic DNA loci. BC1 = British Columbia (Prince George), BC2 = (Okanagan Falls), AL = Alberta (Blairmore), SA = Saskatchewan (Big River), ON = Ontario (Kirkland Lake), QC = Québec (St-Émile), and NB = New Brunswick (Plaster Rock); and WS = white spruce, LP = lodgepole pine, WBS = white spruce, black spruce, JP = jack pine, BS = black spruce, and BF = balsam fir.

tiation ($G_{ST} = 0.51$) for 10 Californian populations of *O. clavigerum*, a species associated with the mycangia of the Jeffrey pine beetle (*Dendroctonus jeffreyi*).

Because caution is necessary when comparing genetic data generated by different types of markers and taking into account the fact that isozymes are more conserved than RAPDs, we suggest that differences between the results mentioned previously and those reported here for *O. piceae* may be accounted for, to some extent, by the differences in the biology of the fungal populations investigated. The populations of *L. wagnerii* and *O. clavigerum* both appear to reproduce almost exclusively by asexual means in nature and have limited geographic distribution (western North America) and host range. In fact, the populations of *O. clavigerum* studied by Six and Paine (39) may represent an extreme case of specialization in an Ophiostomatoid fungus because they are found exclusively on a monophagous bark beetle, which colonizes a host species whose distribution is limited to the area between northern Mexico and northern California. In contrast, *O. piceae* is an ubiquitous saprobe with a broad host range encompassing several coniferous species, reproduces sexually to a large extent in nature, and appears to be vectored rather indiscriminately by various species of phoretic mites, nematodes, and insects (9,26,42).

A total of 237 different haplotypes were identified among the 239 isolates studied. This very high level of genotype diversity further suggests that *O. piceae* can be considered indigenous to Canada, or was introduced early on upon European settlement, possibly more than once and from several different sources. Although genetic relationships between populations of *O. piceae* from different continents are not known, fungal species that have been introduced to North America during the last century tend to exhibit reduced genetic diversity. This is particularly evident for exotic species whose sexual stage is either absent (e.g., *Sirococcus clavignenti-juglandacearum* [14]) or has not been observed in North America (e.g., European race of *Gremmeniella abietina* [20]), but this is also true for outcrossing species that produce sexual fruiting bodies in the field. For example, North American populations of the introduced *Nectria coccinea* var. *faginata* are genetically far more homogenous than populations of the native *N. galligena* (27,34). Low genetic diversity was also reported for North American populations of *O. novo-ulmi*, the Dutch elm disease fungus, which is phylogenetically closely related to *O. piceae* (4,32). These *O. novo-ulmi* populations appear to be partially clonal, because they are dominated by few vegetative compatibil-

ity groups (5) and mitochondrial genotypes (22). Milgroom and Brasier (28) have suggested that restricted recombination or selection against recombinant genotypes may account for the low diversity found in *O. novo-ulmi*. This is clearly not happening in *O. piceae* because virtually every isolate represented a unique genotype.

When Nei's genetic distances were estimated and further analyzed by UPGMA, it was obvious that the subpopulations from the Ontario and Saskatchewan sawmills, separated by 1,980 km, were genetically closer to each other than to other subpopulations, which, in some cases, were separated by shorter distances, e.g., 690 km between the Alberta and Saskatchewan mills, or 670 km between the Ontario and Québec mills. Furthermore, if the Ontario and Saskatchewan populations were omitted from the data set, the calculated G_{ST} was reduced by half (0.053 for the remaining five populations, compared with 0.112 for all populations). We believe the overall results from our analyses do not provide sufficient evidence for rejecting the hypothesis that the Ontario and Saskatchewan populations are part of the same metapopulation as populations from the other provinces. However, the actual status of the Saskatchewan and Ontario populations is intriguing. Moreover, results from the population analysis of *O. piceae* are mirrored to some extent by results from the analysis of species composition at each of the mills sampled in 1997 (44). Whereas the latter indicated that none of the 13 species of sapstain fungi identified occurred exclusively in a particular region or wood species, it nevertheless suggested that slight differences occurred among the sites. Thus, *O. piceae* was recovered less frequently in Ontario and Saskatchewan, where it accounted for 43.3 and 24.7% of all isolates, respectively, than in most other sites (60.5 to 84.0% of all samples isolated), with the exception of the Okanagan Falls mill (37.8%). On the other hand, *O. minus* was isolated more frequently in Ontario and Saskatchewan (24.1 and 27.7%, respectively) than anywhere else (0.0 to 5.3%). In order to determine whether the Ontario and Saskatchewan populations differ significantly from other Canadian populations, it may be necessary to sample additional locations such as mills in western Ontario or in Manitoba (located between Saskatchewan and Ontario).

Phenotypic diversity was also observed in *O. piceae*, as evidenced by the broad variation in the pigmentation of colonies grown on MEA medium. Among the 239 isolates tested, several produced colonies showing little or even no discernible pigmentation. Albino phenotypes have been reported in *O. piliferum* (3,53) and *O. pluriannulatum* (46) following controlled laboratory crosses between wild-type-pigmented strains. The recovery of albino strains, in which the production of melanin is inhibited, provides opportunities for the genetic and molecular analysis of pigmentation in Ophiostomatoid fungi as well as for the elucidation of the biological role of melanin in these species. Moreover, albino mutants of otherwise pigmented Ophiostomatoid fungi are potential candidates as biocontrol agents against sapstain. For example, an albino mutant of *O. piliferum*, marketed as Cartapip-97, has been reported to significantly reduce colonization of logs and lumber of red pine (2) and aspen (46) by wild-type sapstain fungi.

The study reported here confirmed that *O. piceae* is a genetically variable species (17,33). In addition, the analysis of 24 RAPD loci in 239 haploid individuals collected following a systematic survey conducted across Canada in 1997 allowed us to estimate gene and genotype diversity, study population structure, and show that most of the genetic diversity occurred within subpopulations.

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Fig. 2. Dendrogram generated by unweighted pair group method with arithmetic average analysis of Nei's unbiased genetic distances among 15 Canadian subpopulations of *Ophiostoma piceae*. Genetic distances were computed from 24 random amplified polymorphic DNA loci. Bootstrap values were calculated from 1,000 bootstraps. Only bootstrap values of 50% or greater are shown.

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