Genetic Diversity Between and Within Cankers of the White Pine Blister Rust

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I thank E. Pouliot for technical support in the laboratory; L. Côté for field collections; N. Isabel and L. Bernier for critically reviewing this article; and L. Excoffier for kindly providing the AMOVA software free of charge.
Accepted for publication 26 April 1996.

ABSTRACT

Hamelin, R. C. 1996. Genetic diversity between and within cankers of the white pine blister rust. Phytopathology 86:875-879.

The hierarchical structure of genetic diversity was studied in the monokaryotic haploid (spermatia) and the dikaryotic (aeciospores) phases of the white pine blister rust fungus, *Cronartium ribicola*. Multiple samples of aeciospores and spermatia were collected on cankers in three plantations of eastern white pine, *Pinus strobus*, situated more than 200 km apart. Of the 20 random amplified polymorphic DNA (RAPD) markers scored, 11 were either fixed or nearly fixed, and the remaining markers were polymorphic. Eighty-nine percent of the cankers sampled contained aecidia with more than one RAPD profile. Partitioning of the total genetic diversity by an analysis of molecular variance revealed that 69 to 74% ($P < 0.001$) of the genetic variability was attributable to the sampling of different aecidia within cankers, while genetic diversity between cankers within sites and between sites made up 22 to 24% ($P < 0.001$) and 2.7 to 6.7% ($P = 0.027$ to 0.001), respectively, of the total diversity. Surprisingly, different RAPD profiles were also found between spermatial samples within cankers. The presence of several monokaryotic haploid genotypes within a single canker raises the possibility of outcrossing within cankers and might explain previous inconclusive results concerning heterothallism in this fungus. On cankers with monomorphic spermatia, 75% of the aecidia were the result of outcrossing, i.e., at least one of the RAPD loci had the null allele in the spermatia but the marker allele in the aecidia. Because of the small number of polymorphic markers studied, this should be considered an underestimate of outcrossing.

Additional keywords: AMOVA, PCR.

The tree pathogen *Cronartium ribicola* J. C. Fischer ex Rabenh. causes white pine blister rust, a devastating disease of five-needle pines. This pathogen was introduced into North America at the beginning of this century (26) and has since been spreading throughout a large portion of the range of five-needle pines. In eastern North America, where a large proportion of old-growth white pines (*Pinus strobus* L.) has been harvested, reforestation with white pine is severely hampered by this disease.

Since a marker linked to a gene for resistance to white pine blister rust has been mapped in *P. lambertiana* (4), the possibility of transferring this gene to other five-needle pines can be envisaged. However, rust phenotypes virulent to trees carrying a hypersensitivity resistance gene have already been found in some parts of California (14,15,16). It is, therefore, important to further our knowledge of the population biology and epidemiology of this pathogen to better understand and predict its evolution and migration.

Although informative, traditional epidemiological studies on *C. ribicola* have been conducted (27,28,29), many important questions concerning the epidemiological role of the different spore stages remain unanswered. DNA fingerprinting has shown some promise for answering these questions. For example, almost 90% of the genetic diversity in aeciospores of *C. ribicola* were found within single plantations or natural stands, but genetic differentiation was very low between geographic areas (8). That finding was consistent with the hypothesis that extensive gene flow is taking place between *C. ribicola* populations separated by over 1,000 km or, alternatively, that those populations share a common recent ancestor.

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Publication no. P-1996-0606-02R

The role of spermatia in dikaryotization has been proposed based on microscopy studies (24), but the epidemiological significance of spermatia is uncertain. It is assumed that nuclei are united when spermatia carried by insect vectors germinate on spermogonia to produce dikaryotic mycelium and aeciospores. The production of dikaryotic aeciospores was greatly reduced when cankers were sealed prior to spermatization, and it was suggested that *C. ribicola* was heterothallic (12). However, in most studies of heterothallism, some aeciospores were produced on sealed cankers (10,12,23), suggesting that *C. ribicola* might be homothallic (10).

The fine structure of genetic variability is an aspect of population genetics and epidemiology that has received little attention in tree rusts. In fact, the smallest sampling unit has often been single cankers in inoculation and isozyme studies of fusiform rust (22) and white pine blister rust (3). However, variation in virulence was found among single aeciospore isolates within galls of *C. quercuum* f. sp. *fusiforme* (17) and in a genetically inherited lesion type in *C. ribicola* (23). This can be important when assessing rust virulence, since avirulent genotypes might be masked by virulent ones in a bulked inoculum.

The objectives of the present study were to i) measure the distribution of genetic diversity in *C. ribicola* at the following hierarchical levels: within cankers, between cankers within sites, and between sites; and ii) assess the genetic contribution of the fertilizing spermatia by comparing DNA profiles in the unspermatized canker and in the dikaryotic aeciospores resulting from the spermatization.

MATERIALS AND METHODS

Sampling. Three eastern white pine plantations, St. Cyprien (CY), Plessisville (PL), and St. Alexis (SA), separated by over

200 km were selected based on earlier results indicating that their rust populations were polymorphic (8). Each site was visited in early May 1994, and a minimum of 50 trees with cankers were tagged, labeled, and mapped. Trees were sampled following a systematic pattern (e.g., the closest infected tree was selected every 20 m) to provide the best possible representation of the rust population. The final numbers of cankers used in the analysis were 44 at SA, 45 at PL, and 49 at CY. We estimated that over 75% of the active cankers present in the plantations were sampled.

Aeciospores were collected starting on May 15, 1994, after blisters started appearing, but before they ruptured, to avoid airborne contamination or cross-contamination between aecidia. At least three single aecidia were sampled individually on each canker by rupturing the aecidium with the tip of a sterile scalpel and harvesting the aeciospores into a 1.5-ml Eppendorf microtube. All samples were placed in a desiccator containing a silica-based desiccant, lyophilized, and stored at –80°C.

Starting during the first week of July, all cankers were revisited to collect the monokaryotic spermatia. For each previously sampled canker, three individual spermatial drops were collected with a Pasteur pipette by rupturing the unopened spermatial blisters around the cankers. When such unopened blisters were not present, an exuded spermatial drop was collected. Each spermatial drop constituted a sample. The spermatial nectar was pipetted into a 1.5 M sorbitol solution and kept at –80°C.

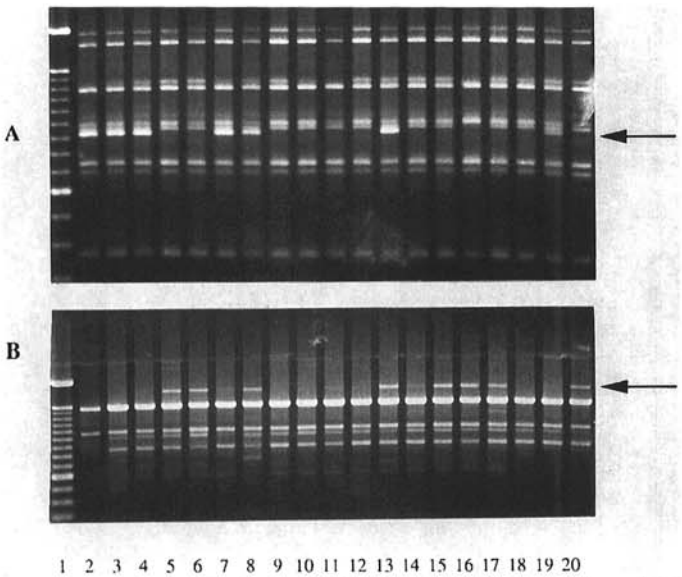

Fig. 1. Random amplified polymorphic DNA (RAPD) profiles for aecidial samples of *Cronartium ribicola* from a *Pinus strobus* plantation in St. Cyprien, Quebec. Lanes 2 to 4 and 5 to 7 contain RAPD profiles from three different aecidia from cankers CY21 and CY22, respectively; lanes 8 to 20 contain RAPD profiles from 13 different aecidia from canker CY23. Marker is 100-bp ladder (Gibco BRL). **A,** Primer OPC08; arrow indicates polymorphic marker OPC08-900. **B,** Primer OPA01; arrow indicates polymorphic marker OPA01-1700.

DNA extraction. DNA was extracted from aeciospores and spermatia by a modification of a protocol described elsewhere (8,18). The lyophilized spores were ground for 2 to 4 min with approximately 10 mg of diatomaceous earth (Sigma Chemical Co., St. Louis) and 100 µl of extraction buffer (700 mM NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% β-2-mercaptoethanol, and 1% cetyl-trimethyl-ammonium-bromide) using disposable Kontes pestles (VWR-Canlab, Toronto, Canada). Three hundred microliters of extraction buffer were added, and the samples were incubated at 65°C for 1 h.

The samples were then extracted with 600 µl of chloroform/isoamyl alcohol (24:1), finger-vortexed, and centrifuged at 10,000 × *g* for 5 min. The upper phase was pipetted into 1.5-ml Eppendorf microcentrifuge tubes, and the DNA was precipitated by adding 75 µl of 7.5 M ammonium acetate and 600 µl of isopropanol and placing it on ice for at least 30 min. The DNA was pelleted by centrifuging for 5 min at 10,000 × *g* and washed with 70% ethanol, after which the pellet was air-dried and resuspended in 20 µl of Tris-EDTA (TE)-8 buffer (10 mM Tris-HCl, pH 8, and 1 mM EDTA). DNA concentration was estimated by comparing the band intensity on an agarose gel with a known amount of λ-*Hin*dIII fragments (Gibco BRL, Bethesda, MD). DNA was diluted 1:2 to 1:25, depending on DNA concentration, and stored in TE-8 buffer at –20°C. Our results indicated that reproducible random amplified polymorphic DNA (RAPD) patterns were obtained with concentrations of 10 ng plus or minus a 10-fold dilution.

DNA amplifications. Amplifications were performed in volumes of 25 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.0001% gelatin, 100 µM of each dNTP (Pharmacia Biotechnology Inc., Uppsala, Sweden), 0.2 µM oligonucleotides, 2 µl (approximately 10 ng) of genomic DNA, and 0.5 unit of *Taq* DNA polymerase (Boehringer GmbH, Mannheim, Germany) (30). Amplifications were carried out in a thermal cycler (model PTC-60; MJ Research, Watertown, MA) programed for a denaturation step at 94°C for 3 min; followed by 1 cycle at 35°C for 4 min and 72°C for 2 min; and then 45 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min. The reactions ended with a 10-min extension at 72°C.

Primers (Operon Technologies Inc., Alameda, CA) OPA01 (5′-CAGGCCCTTC-3′), OPA09 (5′-GGGTAACGCC-3′), and OPC08 (5′-TGGACCGGTG-3′) were selected based on previous results (8). Amplification products were separated by electrophoresis on 1.5% agarose gels using 1× Tris-acetate-EDTA (TAE) buffer (primer OPA01) or 1% agarose plus 0.5% synergel (Diversified Biotech, Newton Center, MA) in 0.5× Tris-phosphate-EDTA (TPE) buffer (primers OPA09 and OPC08). Polymerase chain reaction (PCR) products were visualized by UV fluorescence after ethidium bromide staining.

The final numbers of samples for which reproducible RAPD profiles were obtained and analyzed were 209, 177, and 185 for

TABLE 1. Analysis of molecular variance (AMOVA) from a Euclidean distance matrix generated by scoring nine random amplified polymorphic DNA markers for aeciospore samples for three populations of *Cronartium ribicola* from plantations in Quebec

Comparisons[a]	Source	df	Variance	Percentage of total variance (%)	ϕ-statistics	Probability of a higher value
CY vs SA	Among sites	1	0.040	6.44	0.064	<0.001
	Among cankers within sites	93	0.138	22.28	0.238	<0.001
	Within canker	277	0.442	71.29	...	<0.001
CY vs PL	Among sites	1	0.017	2.67	0.027	0.027
	Among cankers within sites	93	0.143	22.86	0.235	<0.001
	Within canker	271	0.464	74.46	...	<0.001
SA vs PL	Among sites	1	0.039	6.69	0.067	<0.001
	Among cankers within sites	93	0.141	23.96	0.257	<0.001
	Within canker	263	0.408	69.36	...	<0.001

[a] Sites are white pine plantations: CY = St. Cyprien, SA = St. Alexis, and PL = Plessisville. Geographic locations are described by Hamelin et al. (8).

aecidia and 98, 98, and 106 for spermatia for sites CY, PL, and SA, respectively.

Analysis. All RAPD markers were treated as separate putative loci with two alleles, a marker allele, and a null allele. Since the aeciospores are presumably dikaryotic, markers were interpreted as dominant markers in diploids (20). An analysis of molecular variance (AMOVA version 1.55) was performed using a Euclidean distance matrix between all pairs of multilocus phenotypes (6,11). In this analysis, each canker was considered a population of aecidia. The AMOVA was used to partition the total variance into the following components: among sites, among cankers within sites, and within cankers. The variance components and F statistics analogs ϕ_{ct}, ϕ_{st}, and ϕ_{sc} were tested statistically by nonparametric randomization tests using 1,000 repetitions. Because of the limitation of the present version of the AMOVA software, the three sites were analyzed in a pairwise fashion for aecidia. This type of analysis gave the most accurate estimate of observed heterozygosities when both genotypic and phenotypic RAPD data were compared (13) and yielded results similar to those obtained with corrected measures of heterozygosities and genetic differentiation (8).

RESULTS

Nine of the 20 RAPD markers scored were polymorphic in aeciospores and were, therefore, informative for generating the Euclidean distance matrix. However, only six of these polymorphic markers could be scored reliably in spermatia. The three markers that were not scored could not be amplified consistently between repetitions of the same primer-template amplifications and were excluded from the analysis of DNA variability for spermatia. It was, therefore, not possible to directly compare diversity between the two types of spores, since the distance matrix was based on a different number of markers in aeciospores and spermatia.

A high level of genetic diversity was present between aecidia within cankers at all sites (Fig. 1A and B). Only 15 cankers (11%) surveyed at all three sites contained monomorphic aecidia, the remaining 120 cankers (89%) contained at least one aecidium with a different RAPD profile. Seventy-three cankers (54%) contained aecidia with more than two polymorphic RAPD markers.

Hierarchical partitioning of the genetic diversity by AMOVA revealed that 69 to 74% of the total diversity was attributable to the sampling of different aecidia within individual cankers ($P < 0.001$; Table 1), 22 to 24% was attributable to the sampling of different cankers within sites ($P < 0.001$), and 2.7% ($P = 0.027$) to 6.7% ($P < 0.001$) was attributable to the sampling of different sites (Table 1).

Surprisingly, different RAPD profiles were also found between spermatial samples within cankers (Fig. 2). To assess the contribution of the fertilizing spermatia to the aecidia, all cankers containing spermatia with more than one RAPD profile were excluded from the analysis. In the remaining 19 cankers, 50.2% of the RAPD loci had the null allele both in the spermatia and the aecidia, 30.3% of the loci had the marker allele both in the spermatia and the aecidia, and 19.4% of the RAPD loci had the null allele in the spermatia but the marker allele in the aecidia. When the multilocus genotypes (the combination of all RAPD markers for an individual sample) were considered, 75% of all aecidia were the result of outcrossing, i.e., at least one of the RAPD markers was absent in the spermatia but present in the aecidia. For example, marker C08-900 was absent in spermatial samples from canker CY21 (Fig. 2A), but present in all three aecidia of the same canker (Fig. 1A).

DISCUSSION

The presence of genetic diversity at a very fine scale with low levels of genetic differentiation between geographic locations reported here and elsewhere (8,9) is consistent with the hypothesis that homogenizing forces such as long distance migration of aeciospores and urediospores result in low levels of differentiation between geographic locations, but that sexual recombination and vector-driven dissemination of spermatia result in high levels of local genetic diversity. Alternatively, the low levels of genetic differentiation between geographic areas could be because of the sharing of a common recent ancestor.

The large proportion of the total diversity found between aecidia within cankers can be attributed, in part, to the biology of the fungus. Insects have been observed and trapped on cankers producing spermatia (7,12), and insect vectors are believed to be involved in spermatization. If an insect can visit several cankers and a canker can be visited by several insects, spermogonia on a canker could be spermatized by different spermatia. Our results show that genes are mixed at a very fine scale in the rust population and are consistent with the suggestion that insects are efficient vectors.

A practical implication of these findings is that the probability of finding two rust aecidia with different DNA profiles is high (7/10) when sampling within a single canker and that the probability is increased to approximately 9/10 by extending the sampling to different cankers within sites, but it is increased by less than 1/10 by sampling cankers on different sites.

The finding that most of the aecidia produced on cankers that had monomorphic spermatia were the result of outcrossing is consistent with the hypothesis of heterothallism in *C. ribicola* (12,23). However, selfing could not be excluded in 25% of the aecidia. Based on the marker frequencies (8), it can be expected that outcrossing occurred between fertilizing spermatia and spermogonia with identical RAPD profiles. Therefore, the proportion of outcrossing reported here should be considered an underestimate. An accurate estimate of outcrossing would require a larger number of polymorphic markers.

The presence of multiple spermatial genotypes within cankers might explain the mixed results obtained in studies of heterothallism in *C. ribicola* (10,12,23). When cankers were sealed to prevent external spermatization, a proportion of the cankers (up to

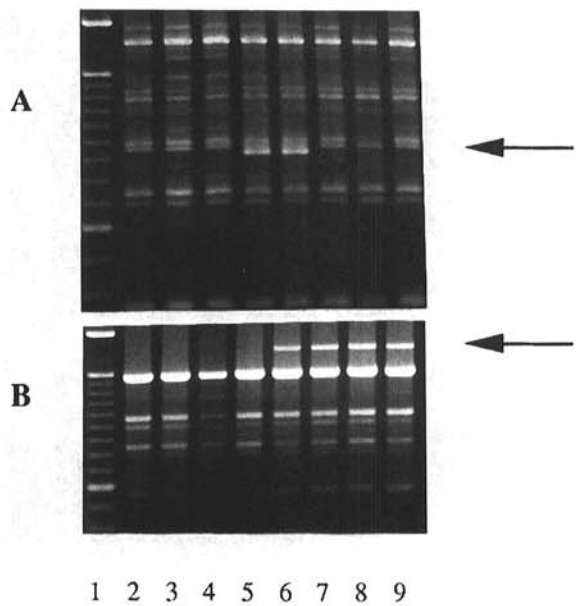

Fig. 2. Random amplified polymorphic DNA (RAPD) profiles for spermatial samples of *Cronartium ribicola* from a *Pinus strobus* plantation in St. Cyprien, Quebec. Lanes 2, 3, 4, and 5 contain RAPD profiles from two different spermatial samples on cankers CY21 and CY23, respectively; lanes 6, 7, 8, and 9 contain profiles for spermatia on cankers CY24 and CY25, respectively. Marker is 100-bp ladder (Gibco BRL). **A,** Primer OPC08; arrow indicates polymorphic marker OPC08-900. **B,** Primer OPA01; arrow indicates polymorphic marker OPA01-1700.

24%) still produced aeciospores the following spring (10,12). In addition, the pattern of forced spermatization was not clear, as there appeared to be more than one mating type involved (12). When monoaeciospore lines of *C. ribicola* derived from individual cankers were inoculated on pine seedlings, they segregated for lesion type, producing a pattern expected from a heterothallic rust (23). Nevertheless, a small but consistent proportion of the seedlings presumed homozygous for the lesion color produced mixed progeny.

If cankers studied by these workers also contained multiple monokaryotic haploid genotypes, as was the case in the populations of cankers sampled in our study, and if different mating types were present within cankers, heterothallic spermatization could have taken place within cankers without external sources of spermatia.

Two hypotheses can be proposed to explain the presence of monokaryotic haploid spermatia with different RAPD profiles on the same canker. The first hypothesis is that cankers were initiated from multiple infections. The possibility of confluent, mixed infection was raised to explain the production of aeciospores in apparently single infections in the heterothallic rust *Melampsora lini* (1). Experimental evidence of multiple infection was obtained for *C. quercuum* f. sp. *fusiforme* by analysis of inoculated seedlings (5). However, such experimental evidence is lacking for *C. ribicola*.

A second hypothesis is that spermatia could become established as haploid monokaryotic mycelium within cankers during the spermatization process. Germination and growth of spermatial mycelium under favorable conditions was observed in *Puccinia sorghi* (2). However, whether or not such mycelium can become established is uncertain. Most of the spermatial mycelia appear to be devoid of cytoplasm (2), and their growth between host cells might be an artifact of the inoculation conditions. There is no evidence that such spermatial growth occurs in *C. ribicola*, under either artificial or natural conditions.

The question of whether or not similar levels and distribution of genetic diversity should be expected for sampled DNA markers and virulence should be considered. Variability in lesion type was found between single-aeciospore isolates of *C. ribicola* within single galls (23). Greater DNA polymorphisms and pathogenic variability were reported between individual galls within a state than between states (9,25), and variability in virulence was found between single-aeciospore isolates within cankers of the fusiform rust (17). In the bean rust fungus *Uromyces appendiculatus*, congruence in phenetic groupings was found between virulence and isozymes (19) and between isozymes and RAPD markers (21).

If variability for virulence is also distributed at a very fine scale, single-gall inocula often used in inoculation studies were, in fact, composite inocula. This could explain why no difference in virulence was found when composite inoculum sources of fusiform rust were compared with single-gall inocula (22). If most of the genetic variability was present within cankers, with low levels of diversity between cankers and between sites, the bulking of inoculum within cankers would, in effect, homogenize the variability.

The results reported here clearly underline the importance of sampling aecidia and spermatia individually instead of collecting bulk samples for genetic studies and virulence surveys of *C. ribicola*. When we reconstructed some bulk isolates and scored RAPD markers, most of the variability was removed if samples containing a marker in approximately 50% of the samples were mixed (data not shown). Bulking samples within a canker would, therefore, mask much of the genetic variability present and result in an artificially homogenized population.

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