Genetic Structure of *Cronartium ribicola* Populations in Eastern Canada

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ABSTRACT

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The genetic structure of populations of *Cronartium ribicola* was studied by sampling nine populations from five provinces in eastern Canada and generating DNA profiles using nine random amplified polymorphic DNA markers. Most of the total gene diversity (H_t = 0.386) was present within populations (H_w = 0.370), resulting in a low level of genetic differentiation among populations in northeastern North America (F_{st} = 0.062). A hierarchical analysis of genetic structure using an analysis of molecular variance (AMOVA) revealed no statistically significant genetic differentiation among provinces or among regions. Yet, genetic differentiation among populations within regions or provinces was small (AMOVA ϕ_{st} = 0.078) but statistically significant ($P < 0.001$) and was several orders of magnitude larger than differentiation among provinces. This is consistent with a scenario of subpopulations within a metapopulation, in which random drift following migration and new colonization are major evolutionary forces. A phenetic analysis using genetic distances revealed no apparent correlation between genetic distance and the province of origin of the populations. The hypothesis of isolation-by-distance in the eastern populations of *C. ribicola* was rejected by computing Mantel correlation coefficients between genetic and geographic distance matrices ($P > 0.05$). These results show that eastern Canadian provinces are part of the same white pine blister rust epidemiological unit. Nursery distribution systems are controlled provincially, with virtually no seedling movement among provinces; therefore, infected nursery material may not play an important role in the dissemination of this disease. Long-distance spore dispersal across provincial boundaries appears to be an epidemiologically important factor for this pathogen.

Additional keywords: Mantel tests.

White pine blister rust, caused by the basidiomycete *Cronartium ribicola* J.C. Fischer, is a very severe disease of five-needle pines. The pathogen was introduced into North America at the beginning of the century and now covers most of the distribution range of white pines (20).

Management strategies to control tree diseases are often based on knowledge of the biology of the causal agent. For example, *C. ribicola* alternates between a *Ribes* spp. and a pine host; therefore, eradication of the *Ribes* host has been used as a control method (14,17). Information about the population biology and genetics of this pathogen is also important to tree improvement programs and the deployment of resistant planting material. Resistant *Pinus lambertiana* have been attacked by a virulent race of *C. ribicola* in California (9), and the spread of that race has been monitored (10, 11). Information about distance and rate of spread and the size of epidemiological units is important.

New knowledge about the population biology of this pathogen has started to accumulate in recent years, in particular with the use of molecular approaches. Genetic diversity in this fungus was shown to be distributed at a very fine scale. The largest proportion of genetic diversity was found among individual aecidia within cankers (4), a result consistent with the outcrossing nature of the pathogen (3).

Random amplified polymorphic DNA (RAPD) analysis of populations of *C. ribicola* from natural stands and plantations in Quebec indicated no genetic differentiation among regions separated by 1,000 km or between natural stands and plantations (5). Similarly, restriction fragment length polymorphism (RFLP) analysis of the rDNA gene revealed high levels of polymorphism but no geographic patterns of distribution in *C. ribicola* in western North America (26). These results suggested that the populations were panmictic and that gene flow was occurring among them. However, a test of the isolation-by-distance hypothesis (28), to determine whether or not the between-populations genetic diversity might be correlated to geographic distance, was not performed.

Also, in spite of this apparent absence of geographic-genetic correlation, differentiation among some populations of *C. ribicola* in Quebec was relatively large (e.g., 11%) and statistically significant (5). This genetic differentiation among populations could be attributable to a barrier to gene flow between some populations, to genetic drift following colonization events, to local selection, or to a combination of these factors. However, some populations sampled contained fewer than 10 individuals due to the limited number of sporulating cankers available (5). Therefore, it is possible that uneven sampling resulted in sampling error that may have inflated the level of genetic differentiation measured.

Although sampling sites covered over 1,000 km of longitudinal range and several hundred kilometers of latitudinal range, previous surveys of eastern *C. ribicola* populations were limited to natural stands and plantations in the province of Quebec (4,5). Despite the absence of genetic differentiation between rust populations in natural stands and plantations, it is still possible that nursery-produced seedlings are a source of inoculum for plantations as well as natural-stand epidemics. This could introduce a bias when attempting to calculate biological parameters such as gene flow. The seedling distribution systems in nurseries are controlled provincially, with little or no transfers among provinces; therefore, comparison of rust populations among provinces could reveal such a trend.

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The goal of this study was to determine the genetic structure of *C. ribicola* populations covering five eastern Canadian provinces with a hierarchical sampling scheme to determine whether (i) there is significant genetic differentiation associated with the province of origin and indirectly with the seedling distribution system; (ii) the isolation-by-distance hypothesis can be rejected, and (iii) the genetic differentiation observed among populations within regions (5) is also present when a larger and more evenly distributed sample size is analyzed.

MATERIALS AND METHODS

Sampling. Two hundred seventy-three single aecidia were collected in nine natural stands and plantations of *P. strobus* from eastern Canada. The nine sites were distributed over five provinces: three in Ontario, one in Quebec, one in New Brunswick, one in Nova Scotia, and three in Newfoundland (Fig. 1, Table 1). At least 10 trees were sampled in each pine stand during the month of May and the beginning of June 1995. To avoid airborne contamination or cross-contamination between aecidia, cankers were sampled prior to opening of the aecidia. Three single aecidia were collected individually on each canker (one canker per tree) 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 until DNA extraction.

DNA extraction. DNA was extracted from aeciospores by a modification of a protocol described elsewhere (4,5,12). The lyophilized spores (1 to 5 mg) were ground for 2 to 4 min with approximately 10 mg of diatomaceous earth (Sigma Chemical Co., St. Louis) and 50 µl of extraction buffer (700 mM NaCl; 50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 1% β-2-mercaptoethanol; and 1% cetyltrimethylammonium bromide) using disposable Kontes pestles (VWR-Canlab, Toronto, Canada). A volume of 350 µl of extraction buffer was added, and the samples were incubated at 65°C for 1 h.

The samples were then extracted with 400 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 500 µl of isopropanol and incubating at –20°C 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 agarose gel with a known amount of λ-*Hin*dIII fragments (Gibco BRL, Bethesda, MD). DNA was diluted to approximately 5 ng/µl), depending on DNA concentration, and stored in TE-8 buffer at –20°C.

RAPD amplification. Amplifications were carried out in volumes of 12.5 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 of each oligonucleotide, 2 µl of genomic DNA, and 0.5 unit of *Taq* DNA polymerase (Boehringer GmbH, Mannheim, Germany) (27). Amplifications were carried out in a thermal cycler (model PTC-60; MJ Research, Inc., Watertown, MA) programmed for a denaturation step at 94°C for 3 min, followed by one cycle at 35°C for 4 min for annealing and 72°C for 2 min for extension, 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 and OPC08 were selected based on previous results after a screening of 40 primers (4,5). Three additional primers, OPE15, OPK11, and OPK19, were retained for the current study after a screening

TABLE 1. Sample size and geographic location of populations of *Cronartium ribicola* in eastern Canada

Populations	Abbreviation	Province	Sample size
Ste-Camille-de-Bellechasse	SCB	Quebec	30
Temagomi	TON	Ontario	30
Minden	MIO	Ontario	30
Sault Ste. Marie	SON	Ontario	30
Trafalgar	TRA	Nova Scotia	30
Moncton	MON	New Brunswick	33
Gander River	GAR	Newfoundland	30
Little Grand Lake	LGL	Newfoundland	30
Sheffield Lake	STN	Newfoundland	30

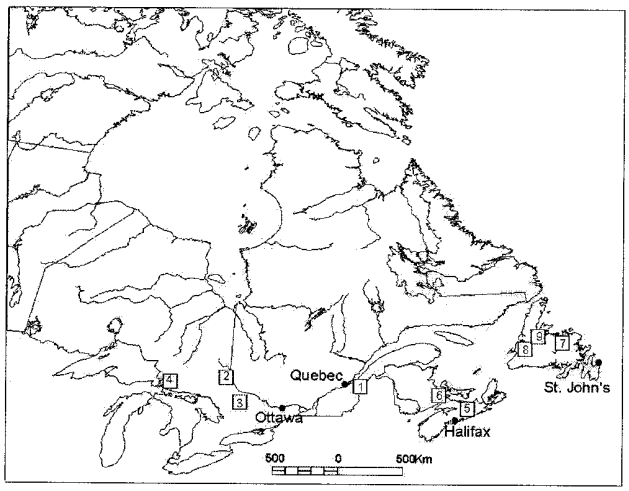

Fig. 1. Map of *Cronartium ribicola* populations sampled. 1, Ste-Camille-de-Bellechasse; 2, Temagomi; 3, Minden; 4, Sault Ste. Marie; 5, Trafalgar; 6, Moncton; 7, Gander River; 8, Little Grand Lake; and 9, Sheffield Lake.

TABLE 2. Frequency of random amplified polymorphic DNA (RAPD) markers, heterozygosity, and genetic differentiation for nine populations of aecidia of *Cronartium ribicola* in eastern Canada[a]

	Allele frequency (q)[c]									
Populations[b]	OPA01-700	OPA01-2000	OPC08-750	OPC08-900	OPE15-1600	OPK19-2000	OPK19-1700	OPK11-500	OPK11-900	Average heterozygosity
SCB (QC)	0.417	1.000	0.966	0.610	0.733	0.375	0.279	0.490	0.490	0.375
TON (ON)	0.417	1.000	1.000	0.455	0.455	0.388	0.388	0.733	0.798	0.359
MIO (ON)	0.522	1.000	0.983	0.522	0.582	0.455	0.455	0.553	0.417	0.398
SON (ON)	0.582	1.000	0.983	0.582	0.582	0.490	0.553	0.620	0.463	0.393
TRA (NS)	0.417	0.983	0.966	0.686	0.877	0.329	0.417	0.553	0.522	0.359
MON (NB)	0.591	0.969	0.985	0.641	0.655	0.440	0.201	0.358	0.555	0.368
GAR (NF)	0.455	1.000	1.000	0.777	0.490	0.490	0.755	0.329	0.610	0.359
LGL (NF)	0.455	1.000	1.000	0.610	0.832	0.329	0.208	0.417	0.375	0.340
STN (NF)	0.490	1.000	1.000	0.522	0.329	0.375	0.329	0.498	0.463	0.383

[a] Total expected heterozygosity (H_t) = 0.386, expected heterozygosity between populations (H_b) = 0.016, expected heterozygosity within populations (H_w) = 0.370, and inbreeding coefficient (F_{st}) = 0.062. Standard deviation of 0.023 was calculated by 1,000 bootstrap samplings over loci.
[b] NB = New Brunswick, NF = Newfoundland, NS = Nova Scotia, ON = Ontario, and QC = Quebec.
[c] Frequency of the null allele (q) for dominant RAPD markers was estimated by using Lynch and Milligan's (13) method.

of 60 primers with 10 samples of *C. ribicola* selected from different geographic regions. The selected primers yielded one or more repeatable polymorphisms. Discarded primers were monomorphic, yielded unrepeatable results, or did not yield DNA amplification products.

Amplification products were separated by electrophoresis on 1.5% agarose gels using 1× Tris-acetate-EDTA buffer (primers OPA01 and OPK19) or 1% agarose plus 0.5% synergel (Diversified Biotech, Newton Center, MA) in 0.5× Tris-phosphate-EDTA buffer (primers OPC08, OPE15, and OPK11). Polymerase chain reaction products were visualized by UV fluorescence after ethidium bromide staining.

Data analysis. Markers were scored and entered in a binary fashion in a matrix containing RAPD markers in columns (name of primer, followed by size of marker in base pairs following dash) and samples in rows. Considering that RAPD markers are dominant and the aecidiospores are presumably dikaryotic, data were interpreted as dominant markers in diploids. Usually, for diploid individuals, distinction between dominant homozygous and heterozygous is not possible. But statistical analyses enable interpretation of data using allelic frequencies. The frequency of null alleles was calculated by applying a correction for small sample size (13). Population genetic parameters such as expected heterozygosities (or gene diversity), genetic differentiation, and genetic distance were calculated by using corrections for bias in estimating the allele frequencies (13). Standard deviation of the F_{st} value was obtained by performing 1,000 bootstrap resamplings over loci (J. Beaulieu, *unpublished data*).

In addition, Euclidean distances between all pairs of RAPD multilocus phenotypes were subjected to an analysis of molecular variance (AMOVA) (2). This analysis partitioned the total variance into hierarchical components (among populations, among provinces, among regions, and within population). *F* statistics analogs were also derived to estimate population differentiation. The statistical significance of these population parameters was tested by performing 1,000 random permutations of the data set. For the AMOVA analysis, samples with missing markers were eliminated to avoid multilocus phenotype misidentification. Therefore, for this analysis, a total of 255 samples was analyzed.

Nei's (16) unbiased genetic distance was measured between all pairs of sites in order to determine whether or not populations of *C. ribicola* were structured geographically. To investigate relationships between populations, a phylogenetic tree was constructed using the phenetic approach of the unweighted pair group method with arithmetic averages (UPGMA) of the PHILIP software package (version 3.5; J. Felsentein, Department of Genetics, University of Washington, Seattle).

Statistical tests of population structure were performed by calculating Mantel's correlation coefficients between sets of matrices (19). To determine the relationship between genetic and geographic distances, linear, quadratic, and cubic models were tested by regression analysis using PROC REG (SAS Institute, Cary, NC). Both Nei's (16) unbiased distance measures and pairwise F_{st} calculated by AMOVA were tested in the model for correlation with geographic distance. A Mantel test was then performed between the geographic and genetic distance matrices. If isolation-by-distance takes place, a positive correlation would be expected between these two matrices. Standard product-moment correlation coefficients, *r*, were calculated between X_{ij} and Y_{ij}, the matrix elements at the intersection of row *i* and column *j* of the two matrices. 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. This analysis was performed using the software Progiciel R (Beta 3.0; Département des sciences biologiques, Université de Montréal, Montréal).

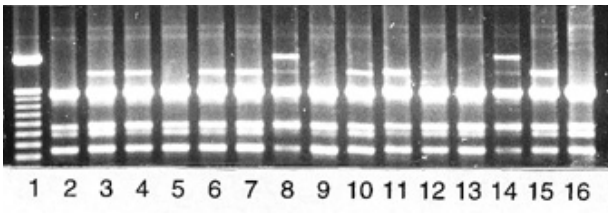

Fig. 2. Random amplified polymorphic DNA profiles for aecidiospore samples of *Cronartium ribicola* from five separate trees. Lanes 2 to 4, Quebec; lanes 5 to 7, Ontario; lanes 8 to 10, Nova Scotia; lanes 11 to 13, Newfoundland; and lanes 14 to 16, New Brunswick. DNA was amplified with primer OPA01. Lane 1 is a 100-bp DNA ladder.

RESULTS

The polymorphic markers revealed by the five primers in this study were present in every population sampled, and there was no instance of private alleles (i.e., an allele that was unique to one or a few populations) (Table 2). For example, marker OPA01-1700 was found in white pine blister rust populations from Quebec, Newfoundland, New Brunswick, Nova Scotia, and Ontario, representing a geographical range of 3,000 km (Fig. 2).

Marker frequency was estimated by using Lynch and Milligan's method (13) to account for the mostly dominant nature of RAPDs (Table 2). Estimated frequencies were relatively homogeneous among populations for some markers. Frequency of OPA01-1700 ranged from 0.417 to 0.591 (Table 2). However, other markers differed in estimated frequency among populations. For example, marker OPK19-1700 ranged in frequency from 0.208 to 0.755 (Table 2).

The expected heterozygosity (average gene diversity) for populations ranged from 0.340 to 0.398. Most of the total expected heterozygosity (H_t = 0.386) was present within populations (H_w = 0.370), resulting in a low level of genetic differentiation among populations in northeastern North America (F_{st} = 0.062) (Table 2).

The AMOVA also revealed a low but statistically significant level of genetic differentiation among populations (ϕ_{st} = 0.078; $P < 0.001$) (Table 3). Nevertheless, genetic differentiation among regions (Centre = Ontario and Quebec; and Maritimes = Newfoundland, New Brunswick, and Nova Scotia) was 300-fold lower than that among populations and not significantly different from zero (P = 0.386) (Table 3). Genetic differentiation was also not significantly different from zero (P = 0.90) among provinces (data not shown).

Nei's (16) unbiased genetic distance was measured between all pairs of populations. In general, genetic distance between populations was low (<8%) (Fig. 3). There was no apparent correlation between the region of origin and the genetic distance as viewed on the UPGMA dendrogram (Fig. 3). For example, populations MIO and SON from Ontario, STN from Newfoundland, and MON from New Brunswick were part of the same cluster, although they are separated by several thousand kilometers (Figs. 1 and 3). By con-

TABLE 3. Analysis of molecular variance of random amplified polymorphic DNA haplotypes for single aecidia samples for nine populations of *Cronartium ribicola* from two regions (Maritimes = Newfoundland, New Brunswick, and Nova Scotia; and Centre = Quebec and Ontario)

Source	df	Variance components	ϕ Statistics	Proportion of variance components (%)	*P* value[a]
Among regions	1	0.000	0.000	0.00	0.386
Among populations within regions	7	0.109	0.078	7.84	<0.001
Within populations	266	1.279	0.077	92.30	<0.001

[a] Probability of obtaining equal or larger value determined by 1,000 randomizations of the treatments.

trast, populations GAR and LGL, both from Newfoundland, were part of different clusters, although they were geographically separated by only a few hundred kilometers. Bootstrap analysis yielded no support for any of the nodes in the dendrogram (80% criterion).

Relationships between geographic distances and pairwise ϕ_{st} are shown in Figure 4. The data points are scattered throughout the plot, with no clear trend visible. No statistically significant correlation between geographic distances and pairwise ϕ_{st} was found by testing three different models (linear, quadratic, and cubic) by regression analyses. The analysis of genetic structure using Mantel statistics also confirmed the lack of correlation between the genetic and geographic distance matrices. The Mantel correlation coefficient between genetic and geographic distance matrices was slightly negative ($r = -0.029$) but not significantly different from zero ($P = 0.445$).

DISCUSSION

The results presented here showed low levels of population differentiation and no apparent geographic pattern in genetic diversity. Genetically closely related populations were often from different provinces, sometimes separated by several thousand kilometers, but genetically more distant populations were found within a province. Analysis of genetic structure using Mantel statistics allows a statistical test of population structure. These analyses revealed no statistical support to the hypothesis of isolation-by-distance (28). The results reported here are consistent with the hypothesis that long-distance migration and extensive gene flow take place among the *C. ribicola* populations studied. Aeciospores and uredospores are particularly well adapted to long-distance transport, and circumstantial evidence of migration over hundreds or thousands of kilometers has been reported (15,18).

Although there was no genetic differentiation between *C. ribicola* populations from plantations and natural stands of *P. strobus* (5), some populations had levels of gene diversity much lower than the average. It was hypothesized that introduction of the pathogen from nurseries might be responsible for population bottlenecks. If the nursery distribution system was an important factor in the epidemiology of this pathogen, it would be expected that some genetic differentiation would be observed among populations of rust originating from different provinces, because there is virtually no seedling movement among provinces. However, our results clearly show that all eastern Canadian provinces are part of the same white pine blister rust epidemiological unit, and long-distance migration must take place among provinces. This contrasts with the population structure of Scleroderris canker, another pine disease, caused by *Gremmeniella abietina* var. *abietina*, that had divergent populations in Quebec and Newfoundland, indicating limited natural spread but little within-province differentiation, suggesting extensive movement within provinces (7). Clearly, spore dispersal is responsible for the difference in population structure for these two pathogens. *G. abietina* var. *abietina* is presumably disseminated locally by rain-splashed asexual conidia, with possibly some long-distance spread on infected nursery seedlings at the time of outplanting.

Low levels of genetic differentiation also have been reported among geographically separated populations of other pine rusts using several types of genetic markers. RFLP patterns of the rDNA gene among western populations of *C. ribicola* (26), RAPD profiles among eastern *C. ribicola* populations (4,5) and *C. quercuum* f. sp. *fusiforme* (6), and isozyme profiles among *C. coleosporioides*, *C. comandrae*, and *C. arizonicum* populations (23) did not reveal apparent geographic patterns. However, this is the first study in which the isolation-by-distance hypothesis is tested.

One exception to these observations of low levels of genetic differentiation among distant populations of rusts was reported for a pine-to-pine rust, *Peridermium harknessii*, in which an F_{st} of 53% was reported among stands within North Dakota (22). However, genetically isolated zymodemes have been reported for this pine rust in western North America, with one zymodeme possessing mostly binucleate aeciospores and the second one possessing uninucleate aeciospores (24,25). Whether these two zymodemes can be considered part of the same biological species remains to be determined.

Genetic diversity among provinces or among regions was far smaller than among populations within provinces or regions. The analysis of genetic distance was also particularly informative. *C. ribicola* populations from Ontario (SON) and Newfoundland (STN), which are about 3,000 km apart, had almost identical genetic composition, as indicated by the low genetic distance. But some populations within regions clearly had distinct genetic composition. Populations GAR and STN from Newfoundland and TON and MIO from Ontario exhibited some of the largest genetic distances, even though these populations were situated within the same province.

The current study was designed to exclude possible sample effects, in particular due to the small sample size in some populations (5), that could artificially inflate the among-population genetic diversity. However, the current study yielded F_{st} and ϕ_{st} values that were of the same order of magnitude as those reported earlier (5), and the conclusions reached in that study are also supported by this article. Thus, our observations of greater genetic differentiation among local populations (within regions) reported here and by Hamelin et al. (5) are consistent with a scenario of subpopulations within a metapopulation, in which long-distance migration results in homogenization at the broader scale and extinction and recolonization results in genetic drift at the local scale (1,8,21). This scenario is particularly plausible for white pine blister rust. The fungus reproduces sexually annually, and primary inoculum for establishment of new infections consists of basidiospores from the telial host, a *Ribes* spp. However, repeated asexual cycles can potentially take place on the *Ribes* host. In cases in which long-distance propagation occurred by the dissemination of aeciospores or uredospores, followed by asexual cycles, founder effects are likely to occur.

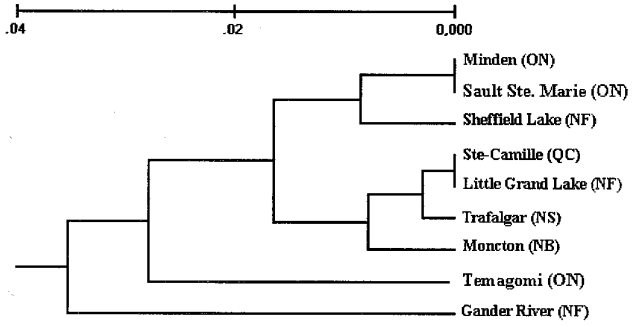

Fig. 3. Unweighted pair group method with arithmetic averages analysis of Nei's (16) unbiased genetic distance between nine populations of *Cronartium ribicola* from eastern Canada. No node was supported at the 80% bootstrap level.

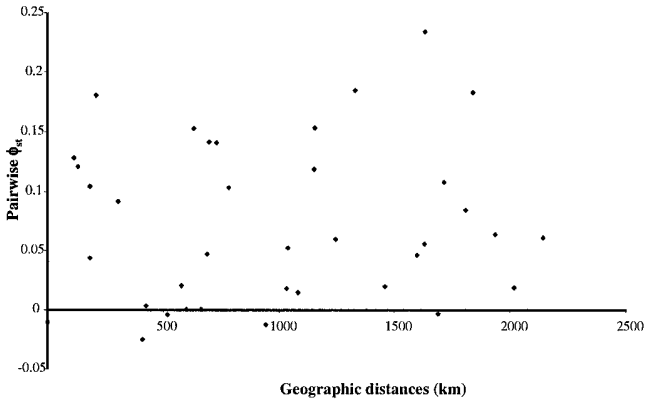

Fig. 4. Plot of pairwise ϕ_{st} and geographic distance among nine populations of *Cronartium ribicola* from eastern North America.

The current study, however, cannot establish which of these factors are important in shaping the population structure of *C. ribicola*. A comparison of the genetic composition of the different spore stages could answer these questions.

ACKNOWLEDGMENTS

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