Genetic Diversity and Structure of the *Apiosporina morbosa* Populations on *Prunus* spp.

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ABSTRACT

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Populations of *Apiosporina morbosa* collected from 15 geographic locations in Canada and the United States and three host species, *Prunus virginiana*, *P. pensylvanica*, and *P. padus*, were evaluated using the sequence-related amplified polymorphism (SRAP) technique to determine their genetic diversity and population differentiation. Extensive diversity was detected in the *A. morbosa* populations, including 134 isolates from Canada and the United States, regardless of the origin of the population. The number of polymorphic loci varied from 6.9 to 82.8% in the geographic populations, and from 41.4 to 79.3% in the populations from four host genotypes based on 58 polymorphic fragments. In all, 44 to 100% of isolates in the geographic populations and 43.6 to 76.2% in populations from four host genotypes represented unique genotypes. Values of heterozygosity (*H*) varied from 2.8 to 28.3% in the geographic populations and 10.2 to 26.1% in the populations from four host genotypes. In general, the *A. morbosa* populations sampled from wild chokecherry showed a higher genetic diversity than those populations collected from other host species, whereas the populations isolated from cultivated chokecherry, *P. virginiana* 'Shubert Select', showed a reduction of genetic diversity compared with populations from wild *P. virginiana*. Significant population differentiation was found among both the geographic populations ($P < 0.05$) and populations from different host genotypes ($P < 0.02$). In the geographic populations, most of populations from cultivated and wild *P. virginiana* were closely clustered, and no population differentiation was detected except for the populations from Morris, Morden, and Winnipeg, Manitoba, Canada. Furthermore, the populations from *P. virginiana* in the same geographic locations had higher genetic identity and closer genetic distance to each other compared with those from different locations. Four populations from *P. virginiana*, *P. pensylvanica*, and *P. padus*, were significantly differentiated from each other ($P < 0.02$), except there was no differentiation between the Shubert Select and wild chokecherry populations ($P = 0.334$). Indirect estimation of gene flow showed that significant restricted gene flow existed between populations from different regions and host species. Gene flow rates (*Nm*) varied from <1 to 12.5, with higher gene flow rates among population pairs from the same host species ($P = 1.000$). The analysis of molecular variance revealed that a major genetic variance source came from the genetic variation among isolates within populations regardless of the origin and host genotype of the population. Although some locations had a limited number of isolates, the results of this study clearly showed that the genetic diversity and population differentiation of *A. morbosa* were closely associated with host genotypes and geographic locations, but mostly with the former.

Additional keywords: *Dibotryon morbosum*, genotypic diversity.

Black knot, caused by the fungus *Apiosporina morbosa* (Schwein.:Fr.) Arx (syn. *Dibotryon morbosum* (Schwein.:Fr.) Theiss. & Syd.), is a serious disease of *Prunus* spp. throughout North America (11,17,20,23,25,28). The pathogen primarily infects twigs or branches of *Prunus* spp. during spring (17,25) and overwinters in the form of mycelia in infected branches (13,25). It takes ≈2 years from the time of infection to form rough, greenish, brown-to-black, spindle-shaped galls (black knots) on twigs and branches on which pseudothecia develop during the winter (17,22,25).

In Canada, the pathogen infects a variety of *Prunus* spp., including chokecherry (*Prunus virginiana* L.), domestic plum (*P. domestica* L.), Japanese plum (*P. salicina* Lindl.), wild plum (*P. americana* Marsh), sour cherry (*P. cerasus*), and pin cherry (*P. pensylvanica* L.) (9,12,13,32,33). Significant production losses of sour cherry have occurred in the Niagara Peninsula in Ontario, Canada (17). Despite its wide distribution, pin cherry is affected mainly in eastern Canada (33). In western Canada, black knot disease has occurred mainly on wild and cultivated chokecherry (*P. virginiana*) and pin cherry (*P. pensylvanica*) (38). In recent years, disease incidence has increased in the ornamental chokecherry 'Shubert Select' in nurseries and cities in western Canada. Humidity and temperature are the most important factors governing the epidemiology of black knot disease in *Prunus* spp. (12). Release of ascospores depends on rainfall and temperature during early spring (11,17). Koch (11) demonstrated that removal of knots in late winter is effective for controlling black knot disease on plum trees. Excellent control also can be obtained by spraying lime sulfur on plum trees (11). Recent research demonstrated that fungicides, such as captan, were effective against black knot on plum (20).

Previous studies have shown that various strains of the pathogen may have some specificity to certain host plants (5,7,9,13,28). Cross-inoculations made among chokecherry, wild plum, cultivated plum, and cherry, using ascospores and conidia isolated from these hosts, did not successfully form any typical knots (7,13). However, the inoculations using spores from one chokecherry formed normal knots on another chokecherry tree (13). Gourley (9) reported that the ascospores from plum knots readily infected peach seedlings, but those from peach knots did not infect peach or plum seedlings. Similarly, Smith et al. (28) were unable to induce the black knot disease in *P. domestica* 'Stanley'

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using ascospores from *P. serotina* (black cherry). Early studies by Farlow (5) and Gilbert (7) also showed pathogen specificity to different hosts.

A number of molecular techniques have been used to investigate genetic diversity of pathogen populations (1,30,35,36,37) in plants. Among them, amplified fragment length polymorphism (AFLP) seems to be a more effective polymerase chain reaction (PCR)-based technique than others, such as random amplified polymorphic DNA (RAPD), because it produces much more polymorphic fragments. The sequence-related amplified polymorphism (SRAP) technique, which has been applied to gene tagging in *Brassica* plants, is a relatively new and highly efficient PCR-based technique (14). It is based on a PCR-based amplification using two primers and can produce highly reproducible polymorphic bands similar to the AFLP technique (14,31). However, the SRAP technique is simpler and less costly than AFLP because it omits the enzyme restriction, ligation of primer adapters, and preamplification done in the AFLP technique.

Although studies have been conducted on pathogen morphology (9,32), disease epidemiology (17,23,28), inoculation and host response (7,32,33), and disease control (9,20), no information has been reported on genetic variation and population differentiation of *A. morbosa*. Therefore, to obtain valuable information on genetic structure of the *A. morbosa* populations, we used the SRAP technique to develop molecular markers. The objectives of this study were to (i) characterize the population structure of *A. morbosa* from different geographic regions and host species by determining genetic diversity and population differentiation, (ii) analyze the natural selection pressure causing genetic diversity and restriction of gene flow among populations, and (iii) analyze the possible disease management strategies associated with the genetic structure of *A. morbosa*.

MATERIALS AND METHODS

Isolate collection. To analyze genetic diversity and differentiation of *A. morbosa* populations, 134 isolates were collected from 2- to 5-year-old *Prunus* spp., consisting of 15 geographic populations in Canada and the United States (Table 1). These geographic populations were isolated mostly from wild chokecherry and 'Shubert Select' (both *P. virginian*), and some from *P. pensylvanica*. In addition, four populations consisting of 96 isolates, collected from wild chokecherry, Shubert Select, *P. pensylvanica*, and *P. padus*, were used for investigating genetic diversity and population differentiation among populations defined by host genotypes (Table 2).

Black knots were excised from infected branches, transferred to the laboratory, and then washed and moistened under running tap water for 30 min. Knot samples were surface sterilized using 10% commercial bleach (0.5% NaHCl) for 2 min. After rinsing three times with sterile water, fresh epidermal tissues with fungal stroma were removed from the gall using a scalpel and macerated in a few drops of sterile water on a sterile slide using a round-ended glass rod. The macerate was streaked on water agar plates and incubated for 24 h at room temperature (20 to 24°C). Single germinating ascospores were marked on plates under the microscope, transferred to potato dextrose agar (PDA) (Difco Laboratories, MD) plates, and incubated at room temperature (20 to 24°C) to obtain pure single ascospore isolates. An isolate of *A. morbosa* (strain ATCC15085) was obtained from the American Type Culture Collection, Virginia, to compare morphological characteristics with *A. morbosa* isolates cultured from knots in this study.

DNA extraction. Conidia produced on PDA plates from 2-week-old single ascospore cultures were harvested by flooding the cultures with distilled water and scraping gently with a sterile glass slide to dislodge spores. The spore suspension was filtered through two layers of cheesecloth and adjusted to approximately 10^7 conidia ml^{-1} with a hemacytometer. Potato dextrose broth (75 ml) in a 250-ml flask was inoculated with 0.5 ml of conidial suspension of an isolate. The flasks were incubated at room temperature (20 to 24°C) for 24 h without agitation. A mycelium pad was formed and harvested by vacuum filtration through two layers of sterilized Miracloth (Calbiochem, CN Biosciences, Inc., La Jolla, CA). Mycelia were rinsed twice with sterile water and stored at –80°C until lyophilized. DNA of each isolate was extracted using the method described by Lodhi et al. (15). All DNA extracts were quantified using a spectrophotometer and adjusted to a final concentration of 5 ng μl^{-1} for PCR analysis.

Amplification of SRAPs. The PCR-based SRAP technique was used to analyze genetic diversity and population differentiation among both geographic and host-specific populations. PCR amplification reaction with the SRAP primers was performed in a 15-µl reaction volume containing 15 ng template DNA, 0.4 µM each of two primers, 0.75 unit of Taq polymerase (Invitrogen Life Technologies, ON, Canada), 100 mM Tris-HCl (pH 8.0), 500 mM KCl, 1.5 mM $MgCl_2$, and 0.1 mM each of dNTPs. The fragments were amplified in a programmable thermal controller (Genius, TECHNE Ltd., Cambridge, UK). The first five cycles were run at 94°C for 1 min, 35°C for 50 s, and 72°C for 1 min, for denaturing, annealing and extension, respectively. The remainder of the amplification consisted of 36 cycles at 94°C for 50 s, 50°C for

TABLE 1. Genetic diversity among 15 *Apiosporina morbosa* populations sampled from different regions based on sequence-related amplified polymorphism (SRAP) fingerprinting

Population	Location	SRAP data[a]				
		n	*g*	*s*	*r* (%)	*H*
JN03	Jeffries Nurseries, MB, Canada, 2003	17	10	0.273	42.0	0.208
WPG	Winnipeg, MB, Canada	11	7	0.249	44.8	0.169
REG	Regina, SK, Canada	11	6	0.358	55.5	0.211
JN02	Jeffries Nurseries, MB, Canada, 2002	6	4	0.290	51.7	0.197
NP	Neepawa, MB, Canada	5	5	0.376	61.4	0.240
MR	Morris, MB, Canada	9	4	0.267	48.3	0.182
WI	Wisconsin, United States	4	2	0.042	6.9	0.028
ON	Ontario, Canada	11	6	0.181	39.7	0.115
NF	Newfoundland, Canada	4	3	0.052	8.6	0.036
RMNP1	Riding Mountain National Park, MB, Canada	15	13	0.424	82.8	0.283
RMNP2	Riding Mountain National Park, MB, Canada	14	13	0.387	77.6	0.254
SA	Saskatoon, SK, Canada	8	8	0.350	68.4	0.239
WT	Waterton, AB, Canada	10	7	0.235	44.8	0.202
MI	Michigan, United States	4	2	0.115	18.9	0.079
MO	Morden, MB, Canada	5	3	0.180	39	0.113

[a] *n* = Population size, *g* = number of genotypes in populations, *s* = Shannon index, *r* = percentage of polymorphic loci (99% criterion), and *H* = average unbiased proportion heterozygosity.

50 s, and 72°C for 1 min. PCR repetitions using the same set of primers and isolates and different DNA preparations of the same isolates were conducted to check the reproducibility of results. To select effective primer combinations, 36 pairs of the SRAP primers were screened for the PCR amplification. The amplified PCR products were separated first by electrophoresis, using a denaturing 5% (wt/vol) polyacrylamide DNA sequencing gel containing 7.5 M urea. The silver-staining kit (Promega Corp., Madison, WI) was used for visualizing DNA bands. Manufacturer's instructions were followed for the fixation, staining, and development of polyacrylamide gel. Based on efficacy of primers producing polymorphic bands on polyacrylamide gels, out of 36 primer combinations, three pairs were chosen for generating the SRAP data: DC1 (5′-TAA ACA ATG GCT ACT CAA G-3′) and ODD30 (5′-GCG ATC ACA GAA GGA AGG T-3′), ODD14 (5′-TCG GTC TTT GTC GTT TCT A-3′) and ODD32 (5′-ACT GTG ATG TCG TTA CTG AT-3′), and ODD9 (5′-AGT TCC TCA GAC GCT ACC-3′) and ODD15 (5′-GCG AGG ATG CTA CTG GTT-3′). The PCR products produced with these selected primer combinations then were separated in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems and Hitachi, Ltd., Japan) to perform the fragment analysis. The protocols of the company were employed to fill polymer gel fluid, load PCR products, set the preferences of the data collection software, monitor the analysis run, and view and analyze raw data in the ABI Prism 3100 Genetic Analyzer. The fragment analysis data were collected by the data collection software based on sizes of PCR products. Raw data from a completed run first were viewed and analyzed using the GeneScan software. To visually view band patterns and screen the polymorphic bands, the fragment analysis data then were reconstructed into a gel image using the software Genographer (version 1.6), developed by Montana State University. The range of fragment sizes and visual intensity of bands for screening were able to be defined easily in the Genographer program, and the fragment analysis data were able to be viewed as a gel image, thumbnails, and graphs, which allowed for a fairly quick and easy way of scoring a gel. The presence and absence of all fragments between molecular sizes of 150 and 500 bp were scored visually for each isolate in the Genographer program. Bands representing molecular sizes >500 or <150 bp were not scored because the resolution was insufficient to discriminate between bands of various molecular sizes.

Statistical analysis. Populations were defined according to their geographic locations and host genotypes. Amplified fragments were scored manually as putative loci with two alleles, one allele indicating the presence of a fragment (designated as 1) and the other the absence (designated as 0) of homologous bands to create a binary matrix of isolates and molecular fragments of the different SRAP phenotypes. The softwares Population Genetic Analysis (POPGENE, version 1.32; Molecular Biology and Biotechnology Center, University of Alberta, Edmonton, Canada), and Tools for Population Genetic Analyses (TFPGA, version 1.3; Northern Arizona University) were used for statistical analysis of standard population genetics. The data were processed as a diploid model with two alleles per locus (16). Frequency of alleles was estimated by Taylor expansion estimator (16). Genetic diversity analysis was measured among 15 geographic populations (Table 1) and 4 populations from three different host species (Table 2). The mean genetic diversity, H, was calculated as $H = (1 - \Sigma P_i^2)$, where P_i is the frequency of allele i at the locus (19). Heterozygosity and percent polymorphic loci (99% criterion) were estimated for all populations. Genotypic diversity was calculated using Shannon's information index (24). Population structure was analyzed using F statistics (34) in order to test the significance for the null hypothesis of no differentiation at the corresponding hierarchical level. Differentiation among populations from geographical locations and host species also was estimated using an exact test (21) and by indirect estimation of gene flow using G_{st} with $Nm = 1/4(1 - G_{st})/G_{st}$ (19,26), where N is the effective population size, m is the immigration rate of gene flow, and Nm is the average number of migrants among populations per generation (26,27). Cluster analysis of multilocus SRAP genotypes was based on allele frequencies observed for each population. A phenogram was constructed using the unweighted pair-group method with arithmetic average (UPGMA) from a Rogers' modified genetic distance matrix (34,37) using the TFPGA 1.3 software package. Bootstrap sampling (1,000 replicates) was performed for statistical support of branches of the constructed phenogram (6). The analysis of molecular variance (AMOVA) was used to partition the total genetic variance within and among populations from different geographic regions and host species (4). The AMOVA was performed by treating a SRAP profile as a haplotype, using Arlequin software (version 2.0) provided by L. Excoffier (University of Geneva, Department of Anthropology and Ecology, Switzerland).

RESULTS

SRAP variation. In all, 58 polymorphic bands were generated from the 134 isolates of *A. morbosa* included in this research using three pairs of SRAP primers. Populations of *A. morbosa* collected from different regions in Canada and the United States showed various degrees of genetic variability (Table 1; Fig. 1). The number of polymorphic loci (based on the 99% criterion) varied from 6.9% of the population collected from Wisconsin (WI) to 82.8% of the population from Riding Mountain National Park (RMNP1) in Manitoba, Canada. Based on the 58 polymorphic bands, the percentage of unique genotypes (g/n) in geographic populations varied from 44 to 100% (Table 1). Almost every single isolate represented a unique genotype in the Neepawa (NP) (g/n = 5/5), RMNP1 (g/n = 13/15), RMNP2 (g/n = 13/14), and Saskatoon (SA) (g/n = 8/8) populations (Table 1). Similarly, the values of the Shannon index (s) of geographic populations also revealed variation in genotypic diversity with the higher diversity for the NP (s = 0.376), RMNP1 (s = 0.424), RMNP2 (s = 0.387), and SA (s = 0.350) populations, and lower in the WI (s = 0.042), Michigan (MI) (s = 0.115), and Newfoundland (NF) (s = 0.052) populations (Table 1). Heterozygosity (H) obtained by estimates of allele frequency based on a Taylor expansion (16) varied from 2.8 to 28.3% in the 15 geographic populations. Again, populations collected from NP, RMNP1, RMNP2, and SA showed a higher level of heterozygosity, whereas the NF, WI, and MI populations showed lower genetic diversity (Table 1).

Heterozygosity and genotypic diversity also were compared among four populations collected from different genotypic hosts: *P. virginiana* (wild chokecherry and 'Shubert Select'), *P. pensylvanica*, and *P. padus.* All parameters describing the genetic diversity among the populations sampled from four different host genotypes were variable (Table 2). The highest heterozygosity and genotypic diversity were observed in the population collected from wild chokecherry (s = 0.392, H = 0.261) and the lowest from *P. pensylvanica* (s = 0.166, H = 0.102) (Table 2). Polymorphic loci varied between the populations from different hosts, with the

TABLE 2. Genetic diversity of four *Apiosporina morbosa* populations sampled from three host species based on sequence-related amplified polymorphism (SRAP) fingerprinting[a]

Population	n	g	s	r (%)	H
Prunus virginiana 'Shubert Select'	55	24	0.247	48.3	0.182
P. padus	3	2	0.251	46.3	0.164
P. pensylvanica	15	9	0.166	41.4	0.102
P. virginiana (wild chokecherry)	21	16	0.392	79.3	0.261

[a] n = Population size, g = number of genotypes in populations, s = Shannon index, r = percentage of polymorphic loci (99% criterion), and H = average unbiased proportion heterozygosity.

highest number of polymorphic loci from wild chokecherry (79.3%). Approximately 76.2% (*g/n* = 16/21) of individual isolates from the wild chokecherry population and 43.6% (24/55) of isolates from Shubert Select represented unique genotypes.

Population structure. SRAP data were used to analyze population structure because the data proved to have a high level of discrimination between the populations based on either the geographic region or host genotype. Genetic identity and distances were different among 105 pair combinations of 15 geographic populations of *A. morbosa* (Table 3). Genetic identity, as presented by Nei's genetic identity index (19), ranged from the maximum values 0.996 (between the JN03 and JN02 populations from Jeffries Nurseries) and 0.992 (between the RMNP1 and RMNP2 populations from Riding Mountain National Park) to the minimum value 0.614 (between the WI and MI populations from the United States) (Table 3).

Clusters were generated for populations using the SRAP data by UPGMA dendrograms. UPGMA cluster analysis using modified Rogers' genetic distances between the *A. morbosa* geographic populations gave distances ranging from ≈10.1 to ≈55.0%, and the clustering obtained in the dendrogram was statistically well supported by 1,000 bootstrappings (Table 3; Fig. 2). The 15 populations were clustered into two distinct subgroups. The population pairs for JN03 and JN02, RMNP1 and RMNP2, Morris (MR) and Morden (MO), and Waterton (WT) and SA were more closely related to each other than to others within the larger subgroup. However, the Ontario (ON), NF, WI, and Winnipeg (WPG) populations, among which the ON and NF populations from pin cherry (*P. pensylvanica*) were closely clustered, formed a distinct subgroup with the larger genetic distance from other geographic populations. The two populations from the United States (WI and MI) fell into two different subgroups (Fig. 2).

The exact tests also indicated significant differentiation between *A. morbosa* populations from different regions (Table 4). The test of statistical probabilities for pairwise comparisons among 15 geographic populations led to rejection of the null hypothesis, stating an absence of differentiation, between most of 105 pair combinations of 15 geographic populations (Table 4). In general, population differentiation was not observed among the JN02, JN03, Regina (REG), RMNP1, RMNP2, WT, SA, and NP populations, which fell into a large lineage (Table 4; Fig. 2) ($P > 0.05$). However, they were significantly differentiated from the MI, ON, NF, WI, WPG, and MR populations ($P < 0.05$), except for MO (Table 4). The four populations ON, NF, WPG, and WI were clustered in the same subgroup (Fig. 1) and not significantly differentiated from each other (Table 4). Although the MR and MO populations were not differentiated from each other, they were significantly different from the rest of the geographic popu-

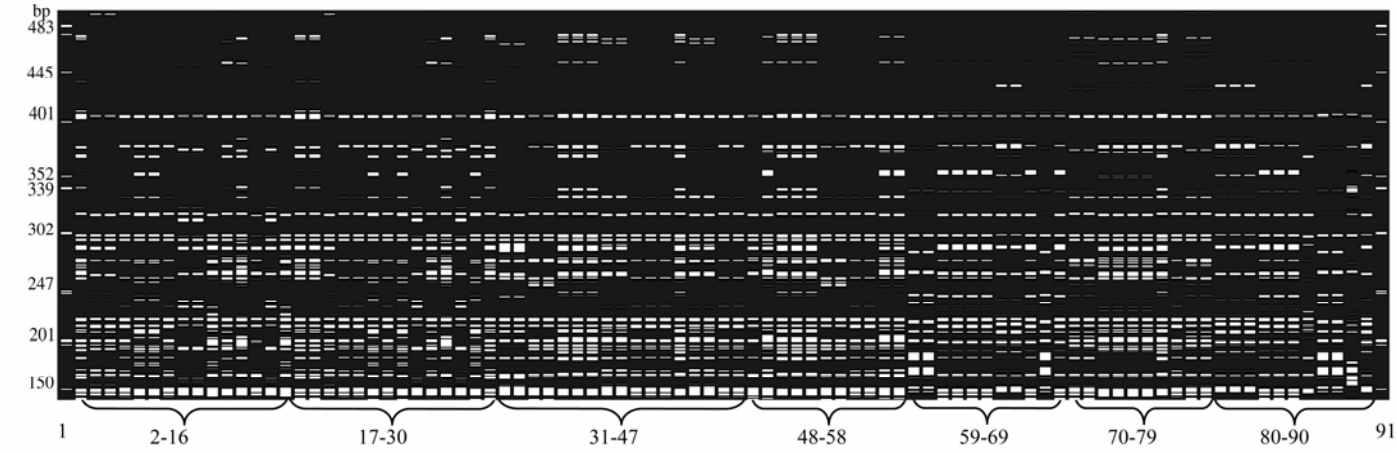

Fig. 1. Gel separating the polymerase chain reaction products amplified with the sequence-related amplified polymorphism (SRAP) primers DC1 and ODD30 against DNA samples of *Apiosporina morbosa* isolates. The gel was reconstructed by the Genographer program using the fragment analysis data generated by the ABI Prism 3100 Genetic Analyzer, and showed genetic diversity among the seven major populations: RMNP1 (lanes 2 to 16) and RMNP2 (lanes 17 to 30) collected from Riding Mountain National Park, Manitoba; JN03 (lanes 31 to 47) from Jeffries Nurseries, Manitoba; REG (lanes 48 to 58) from Regina, Saskatchewan; ON (lanes 59 to 69) from Ontario; WT (lanes 70 to 79) from Waterton, Alberta; and WPG (lanes 80 to 90) from Winnipeg, Manitoba. Lanes 1 and 91 were DNA ladders.

TABLE 3. Pairwise comparison of the genetic identity and distance among 15 geographic populations of *Apiosporina morbosa* in Canada[a]

Population	RMNP1	WPG	NP	RMNP2	JN03	MR	WI	JN02	REG	ON	NF	MO	WT	MI	SA
RMNP1	…	0.893	0.926	0.992	0.949	0.901	0.659	0.941	0.912	0.785	0.782	0.865	0.927	0.943	0.907
WPG	0.476	…	0.840	0.756	0.720	0.862	0.890	0.735	0.711	0.926	0.866	0.786	0.735	0.723	0.832
NP	0.315	0.367	…	0.898	0.903	0.924	0.830	0.896	0.856	0.894	0.862	0.852	0.958	0.891	0.951
RMNP2	0.156	0.462	0.278	…	0.968	0.901	0.646	0.969	0.918	0.777	0.783	0.827	0.932	0.943	0.921
JN03	0.223	0.493	0.299	0.191	…	0.850	0.651	0.996	0.976	0.747	0.757	0.761	0.949	0.910	0.925
MR	0.291	0.346	0.263	0.303	0.361	…	0.792	0.864	0.799	0.907	0.890	0.972	0.932	0.880	0.915
WI	0.568	0.334	0.406	0.533	0.528	0.447	…	0.674	0.655	0.867	0.933	0.703	0.820	0.614	0.819
JN02	0.235	0.476	0.303	0.184	0.101	0.342	0.536	…	0.978	0.763	0.781	0.767	0.943	0.943	0.925
REG	0.224	0.510	0.263	0.237	0.174	0.395	0.550	0.162	…	0.714	0.738	0.704	0.918	0.882	0.918
ON	0.429	0.257	0.369	0.440	0.465	0.348	0.361	0.448	0.502	…	0.966	0.832	0.912	0.736	0.902
NF	0.430	0.292	0.359	0.433	0.455	0.330	0.380	0.430	0.481	0.185	…	0.816	0.902	0.737	0.894
MO	0.357	0.437	0.367	0.406	0.369	0.191	0.527	0.422	0.450	0.390	0.408	…	0.848	0.838	0.829
WT	0.260	0.401	0.215	0.257	0.244	0.291	0.412	0.234	0.284	0.407	0.417	0.369	…	0.896	0.992
MI	0.250	0.505	0.301	0.355	0.307	0.345	0.561	0.300	0.353	0.491	0.410	0.393	0.326	…	0.916
SA	0.292	0.395	0.220	0.280	0.269	0.303	0.428	0.265	0.287	0.392	0.433	0.401	0.146	0.325	…

[a] Populations from RMNP = Riding Mountain National Park, WPG = Winnipeg, NP = Neepawa, JN = Jeffries Nurseries, MR = Morris, WI = Wisconsin, REG = Regina, ON = Ontario, NF = Newfoundland, MO = Morden, WT = Waterton, MI = Michigan, and SA = Saskatoon. Nei's genetic identity based on 58 sequence-related amplified polymorphism loci is above the diagonal, and Rogers' modified genetic distance coefficients are below the diagonal.

lations ($P < 0.05$). The two populations from the United States (MI and WI) were significantly differentiated from each other ($P < 0.0001$) (Table 4). Indirect estimation of gene flow between geographic populations using *Nm* as a measurement of population differentiation showed the rates of gene flow between populations ranged from <1 to 12.5 migrants per generation, meaning that gene migration was greatly restricted between geographic population pairs. Again, this indicated that differentiation existed among the geographic populations. In general, higher gene flow was observed among geographic population pairs that were not significantly differentiated from each other based on pairwise comparisons of possibility (Table 4). Lowest differentiation and greatest gene flow were measured between the JN02 and JN03 populations (*Nm* = 12.5, $P = 1.000$) collected from *P. virginiana* 'Shubert Select' in the same nursery, and between the RMNP1 and RMNP2 populations (*Nm* = 10.3, $P = 1.000$) collected from wild chokecherry in the same park. A relatively higher gene flow ($Nm > 4$, $P > 0.05$) also was observed between population pairs that were clustered in a lineage (Table 4; Fig. 2).

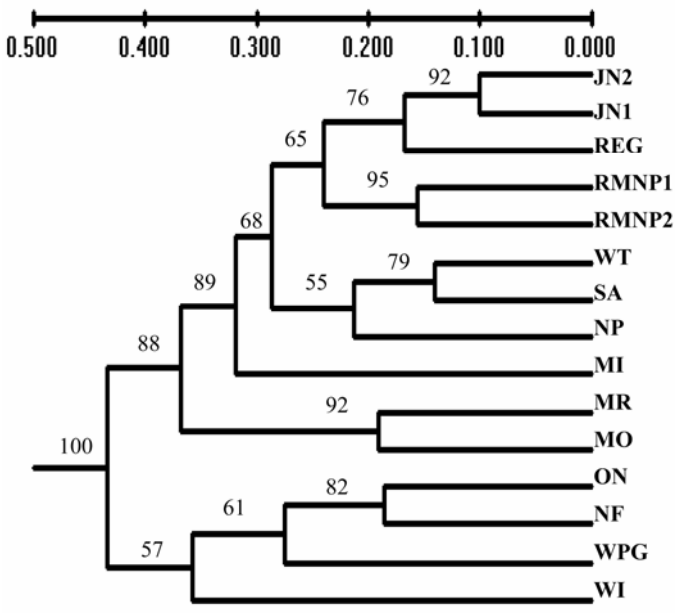

Fig. 2. Phenogram of Rogers' modified genetic distance between *Apiosporina morbosa* populations isolated from 15 geographic regions. Numbers at branches indicate the percentage of occurrence of the cluster in 1,000 bootstrapped phenograms.

Population structure also was analyzed for populations from different host species. Rogers' genetic distances among four *A. morbosa* populations isolated from different host genotypes ranged from ≈11.7 to ≈51.6%, (Table 5; Fig. 3). Based on the genetic identity and distances, the populations from Shubert Select and wild chokecherry were more closely related to each other than to other populations (Table 5; Fig. 3). Significant differentiation was detected between population pairs from different host species based on the exact test (Table 6). The populations from three host species were significantly differentiated from each other ($P < 0.05$), except for the Shubert Select and wild chokecherry populations ($P = 0.334$) (Table 6). The rate of gene flow for populations from different host species indicated that the gene flow was the greatest between populations from Shubert Select and wild chokecherry (*Nm* = 8.5) (Table 6). However, differentiation also was detected between the populations from the same host species located in different regions. For instance, three populations, WPG, JN03, and MR, all isolated from Shubert Select but from different geographic regions, were significantly differentiated from each other ($Nm < 2.0$, $P < 0.02$) (Table 4).

AMOVA also revealed a significant genetic differentiation among different geographic populations ($P < 0.001$), explaining 37% of total variance (Table 7). Furthermore, statistically significant genetic variation was observed within geographic populations, accounting for ≈63% of total genetic variation for *A. morbosa* (Table 7). AMOVA also was performed by partitioning the data among and within populations from host species. The results showed that 19.3% of total variance was due to differences among host genotypes and 80.7% of total genetic variance was caused by

TABLE 4. Pairwise comparison of gene flow and probability of population differentiation among 15 geographic populations of *Apiosporina morbosa* in Canada[a]

Population	RMNP1	WPG	NP	RMNP2	JN03	MR	WI	JN02	REG	ON	NF	MO	WT	MI	SA
RMNP1	…	2.461	2.862	10.307	5.221	1.110	0.931	5.507	4.117	1.062	1.296	1.339	3.440	1.721	3.733
WPG	0.026	…	1.505	2.166	1.956	1.434	2.802	1.611	0.386	2.992	2.211	0.641	0.626	0.484	0.648
NP	0.136	<0.0001	…	2.650	2.307	1.976	0.833	2.355	2.210	1.301	1.095	1.184	3.315	0.995	2.995
RMNP2	1.000	0.001	0.265	…	4.775	2.076	0.804	4.859	4.393	1.625	1.351	1.551	2.848	1.294	2.729
JN03	0.883	<0.0001	0.122	0.803	…	1.058	0.209	12.466	5.807	0.654	0.728	0.427	2.521	0.967	2.844
MR	0.017	0.015	0.027	0.012	<0.0001	…	0.533	1.199	0.578	2.168	1.964	3.959	1.754	1.071	1.551
WI	0.003	0.567	0.003	<0.0001	0.003	0.007	…	0.233	0.095	2.818	2.609	0.188	0.233	0.142	0.211
JN02	0.878	<0.0001	0.213	0.768	1.000	<0.0001	<0.0001	…	5.493	0.714	0.826	0.453	2.071	1.046	3.209
REG	0.703	<0.0001	0.367	0.734	0.988	<0.0001	<0.0001	0.996	…	0.415	0.491	0.203	2.273	0.455	2.417
ON	<0.0001	0.666	<0.0001	0.004	<0.0001	<0.0001	0.517	<0.0001	<0.0001	…	5.623	0.856	1.205	0.534	2.743
NF	<0.0001	0.779	<0.0001	0.001	<0.0001	<0.0001	0.421	<0.0001	<0.0001	0.967	…	0.840	0.948	0.577	2.716
MO	0.111	0.034	0.031	<0.0001	<0.0001	0.866	0.018	<0.0001	<0.0001	<0.0001	<0.0001	…	0.793	0.488	0.589
WT	0.082	<0.0001	0.268	0.112	0.158	0.002	0.021	0.108	0.180	<0.0001	<0.0001	0.009	…	1.508	4.625
MI	0.026	0.007	0.002	<0.0001	<0.0001	0.042	<0.0001	0.001	<0.0001	<0.0001	0.004	0.038	0.021	…	1.375
SA	0.101	0.013	0.304	0.096	0.097	0.017	<0.0001	0.078	0.156	<0.0001	<0.0001	0.013	<0.0001	0.002	…

[a] Populations from RMNP = Riding Mountain National Park, WPG = Winnipeg, NP = Neepawa, JN = Jeffries Nurseries, MR = Morris, WI = Wisconsin, REG = Regina, ON = Ontario, NF = Newfoundland, MO = Morden, WT = Waterton, MI = Michigan, and SA = Saskatoon. Estimates of the number of migrants (*Nm*) between populations are above the diagonal; probabilities of each pairwise comparison using the exact test are below the diagonal.

TABLE 5. Pairwise comparison of the genetic identity and distance among four populations of *Apiosporina morbosa* from three host species in Canada[a]

Population	'Shubert Select'	*Prunus padus*	*P. pensylvanica*	Wild chokecherry
P. virginiana 'Shubert Select'	…	0.892	0.942	0.975
P. padus	0.412	…	0.901	0.928
P. pensylvanica	0.357	0.516	…	0.914
P. virginiana (wild chokecherry)	0.117	0.370	0.371	…

[a] Nei's genetic identity based on 58 sequence-related amplified polymorphism loci is above the diagonal and Rogers' modified genetic distance coefficients are below the diagonal.

differences within host genotypes. The major source of the genetic variation came from differences within populations regardless of whether a population was based on geographic location or host genotype.

DISCUSSION

This study exhibited extensive genetic diversity in *A. morbosa* populations collected from different geographic regions of Canada and, to a lesser extent, the United States, and host genotype. The genetic differentiation in populations was associated with both host genotypes and geographic regions where the host was located. To our knowledge, this is the first report of genetic diversity and population structure in *A. morbosa* infecting wild and cultivated chokecherry and other *Prunus* host species.

The frequency of recombination through the sexual stage is an important genetic diversity source in fungal populations. Asexual reproduction by conidia results in clonal population structures that have distinctive features, such as widespread occurrence of identical genotypes and linkage between independent sets of genetic markers (18). However, the high level of diversity within a population, coupled with a lack of linkage among loci, is indicative of a genetically recombining population (10). In our study, we developed populations from single ascospores and found that a high level of genetic diversity existed in the *A. morbosa* population. In some populations, such as NP, RMNP1, RMNP2, and SA (Table 1), each isolate almost represented a new genotype, suggesting that a high level of recombination may occur in these *A. morbosa* populations, thus causing a high level of genetic diversity.

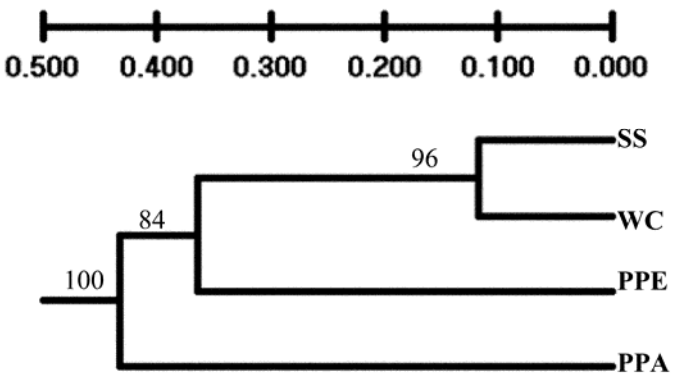

Fig. 3. Phenogram of Rogers' modified genetic distance between *Apiosporina morbosa* populations isolated from four host genotypes: wild chokecherry, *Prunus virginiana* (WC), *P. virginiana* 'Shubert Select'(SS), *P. pensylvanica* (PPE), and *P. padus* (PPA). Numbers at branches indicate the percentage of occurrence of the cluster in 1,000 bootstrapped phenograms.

In general, populations of *A. morbosa* from cultivated *P. virginiana* 'Shubert Select' had decreased genetic diversity compared with the populations from wild chokecherry (Tables 1 and 2). For example, genetic diversity among isolates of *A. morbosa* in the JN02, JN03, REG, MR, MO, and WPG populations from Shubert Select was notably lower than that observed among the NP, RMNP1, RMNP2, and SA populations collected from wild chokecherry. However, we did not find specific evidence to explain this reduction among *A. morbosa* isolates collected from Shubert Select. Diversity of host genotypes from which pathogens are isolated has been suggested as one of the factors responsible for the high value of genetic diversity of pathogens found in other host and pathogen systems (8,35). In our study, although Shubert Select and wild chokecherry are both *P. virginiana*, wild chokecherry has considerable genotypic diversity. The higher genotypic diversity of wild chokecherry compared with Shubert Select suggests that the existence of multiple genotypes in wild chokecherry also might create genetic diversity in the *A. morbosa* populations. Despite this diversity, compared with the populations from other host species, those from Shubert Select and wild chokecherry have the closest genetic relationship (Fig. 3). Similarly, the ON and NF populations isolated from pin cherry (*P. pensylvanica*) were closely clustered, and had no significant population differentiation (Nm = 5.6, P = 0.967), although the populations were collected from different regions. These findings again suggest that host genotype may play a role in population differentiation.

The age of populations in a sampling site also can influence genetic diversity. A recently colonized habitat will result in less diversity than an older population (3,29). Black knot disease has become more serious in recent years in Shubert Select, a dominant ornamental tree cultivar in cities and nurseries in western Canada. However, black knot is widespread in wild chokecherry and has a longer history than in Shubert Select. The higher genetic diversity in wild chokecherry reported in our study also might be related to a longer period of independent evolution of *A. morbosa* on wild chokecherry compared with the cultivated variety.

Our results showed that, among four populations based on host genotypes, there existed significant differences in gene flow. The populations from the same species of *P. virginiana* (Shubert Select and wild chokecherry) showed the highest gene flow value (Nm = 8.48, P = 0.334), suggesting that populations from wild

TABLE 6. Pairwise comparison of gene flow and probability of population differentiation among four populations of *Apiosporina morbosa* from three host species in Canada[a]

Population	'Shubert Select'	*Prunus padus*	*P. pensylvanica*	Wild chokecherry
P. virginiana 'Shubert Select'	…	1.877	3.396	8.483
P. padus	0.018	…	3.897	1.073
P. pensylvanica	<0.0001	0.019	…	2.471
P. virginiana (wild chokecherry)	0.334	0.010	<0.0001	…

[a] Estimates of the number of migrants (Nm) between the pair of populations are above the diagonal and probabilities of each pairwise comparison using the exact test are below the diagonal.

TABLE 7. Analysis of molecular variance for populations from 15 geographic populations and four *Apiosporina morbosa* populations from three host species in Canada using sequence-related amplified polymorphism markers[a]

Source of variation	df	Sum of squares	Variance components	P	Total variance (%)
Among regions	14	516.08	3.60	<0.001	37.16
Within regions	116	705.52	6.08	<0.001	62.84
Among host genotypes	4	83.30	1.93	<0.001	19.32
Within host genotypes	90	816.68	8.07	<0.001	80.68

[a] The total population of *A. morbosa* was partitioned into hierarchical components: among the 15 geographic regions (locations), within regions (isolates from a location), among host genotypes (isolates from three host species), and within a host genotype (isolates from a species).

chokecherry might be a source of inoculum for Shubert Select in nurseries and cities. However, further confirmation would be required through successful reciprocal inoculations using isolates from Shubert Select and wild chokecherry. Significant restriction of gene flow was found among populations from different host species, such as *P. virginiana* and *P. pensylvanica* ($Nm < 0.39$, $P < 0.02$). Even populations isolated from the same host species, but different genotypes, had some reduction in the rate of gene flow compared with populations from the host with the same genotype. For instance, the gene flow ($Nm = 10.31$, $P = 1.000$) between the JN02 and JN03 populations from Shubert Select was greater than that between the RMNP1 and JN03 populations (both *P. virginiana*) ($Nm = 5.22$, $P = 0.883$). It has been reported that *A. morbosa* exists as host-specialized strains (5,7,9,13,28). Previous studies revealed that isolates from black knots on chokecherry would not infect wild plum, and vice versa (38). Moreover, ascospores isolated from some peach knots did not infect other peach seedlings (9). The results of the present study supported these conclusions by the observation of high genetic diversity coupled with restricted gene flow between populations from different host genotypes.

Sexual reproductive isolation is another important assumption for molecular divergence among populations from different geographic regions (2). The longer the period of reproductive isolation, the greater is the divergence in genotypes because gene flow has been restricted between populations from isolated regions. This assumption might explain genetic variation and divergence between populations from the same host genotype, but in different regions, such as the relatively large differences observed in genetic diversity among the JN03 ($H = 0.208$), MO ($H = 0.113$), and WPG ($H = 0.169$) populations isolated from Shubert Select, and between the RMNP1 ($H = 0.283$) and WT ($H = 0.202$) populations isolated from wild chokecherry (Table 1). However, further experiments with more isolates will need to be conducted to confirm such an assumption.

Analysis of the genetic structure of *A. morbosa* showed that epidemics of black knot disease are caused by genetically diverse populations of the pathogen. Regardless of geographic location or host, the major source of genetic variation came from differences among isolates within populations. These findings have implications on quarantine and disease management. Resistant cultivars are one of the most effective means of disease control. Because of high levels of genetic diversity within populations, our results indicated that the expression of disease resistance to *A. morbosa* may be dependent on the strain of the pathogen. When screening breeding lines for resistance to black knot disease, it is advisable that several strains, or a broad selection of isolates, be used to effectively screen germ plasm for resistance in a region. Based on population differentiation, we also suggest that representative strains from each geographic region should be used for inoculating hybrids or lines obtained from breeding programs to find black knot resistance corresponding to different geographic regions.

When we surveyed black knot disease in western Canada in 2002 and 2003, we found numerous wild chokecherry patches fully covered by black knots. It is not known whether these disease patches are the inoculum sources for the recent increase of the disease in Shubert Select, a major ornamental chokecherry cultivar, in cities and nurseries in western Canada. Nevertheless, the closer genetic distance and relatively high rate of gene flow between populations from Shubert Select and wild chokecherry supports a hypothesis that wild chokecherry might be a prime inoculum source for Shubert Select. If this hypothesis is correct, eradicating these diseased wild chokecherry patches and locating new nurseries away from wild chokecherry may be helpful in management of black knot disease in the ornamental chokecherry industry. In addition, the variation of genetic diversity and restriction of gene flow between populations from different geographic regions suggests that quarantine for cuttings used for nurseries and young trees for planting in cities could break gene movement between populations from one region to another and reduce the risk for introduction of new strains between regions.

We found that there are many saprophytic fungi whose growth was associated with black knots caused by *A. morbosa*. Some of them, such as *Cladosporium* spp., were similar to *A. morbosa* in conidial morphology, causing confusion when conidia were used to develop pure *A. morbosa* cultures. To avoid the possibility that different species might be involved in population collections of *A. morbosa*, all isolates of *A. morbosa* in this study were developed by culturing single ascospores because of the distinctiveness of the ascospores of *A. morbosa*. However, ascospores of *A. morbosa* were observed mainly in a short period during early spring, after which it is difficult to find ascospores throughout rest of the year. In addition, we found that only a small proportion of knot samples could produce ascospores. For example, although we collected more than 600 black knot samples from different locations and host species in 2002 and 2003, only 134 isolates were recovered from single ascospores. Furthermore, at present there is no existing protocol for developing pseudothecia of *A. morbosa* on media. All these difficulties greatly restricted the expansion of isolate number of each population in this study. More isolates in each population would need to be examined to improve the robustness of the conclusions. Additional research is needed before stronger conclusions can be made with respect to selection pressures for genetic diversity and population differentiation of *A. morbosa.* As an initial study on the genetic diversity and population structure of *A. morbosa*, we believe that the results have provided some valuable information for future research on this disease and may serve as a baseline for monitoring population evolution of *A. morbosa*.

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