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Molecular Detection of *Apiosporina morbosa,* Causal Agent of Black Knot in *Prunus virginiana*

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ABSTRACT

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A specific and sensitive polymerase chain reaction (PCR) assay was developed to detect *Apiosporina morbosa*, the causal agent of black knot disease on chokecherry, *Prunus virginiana* (including the cultivar 'Shubert Select'). A pair of *A. morbosa*–specific forward and reverse primers (AMF and AMR) was designed from the internal transcribed spacer (ITS) regions of *A. morbosa*, preamplified by universal ITS primers ITS1 and ITS4, and compared with the ITS region sequences of *Fusarium*, *Alternaria*, *Phoma*, and *Cladosporium* species associated with black knots. The primers were tested for their specificity to *A. morbosa* detection in the PCR assays using DNA derived from 64 pure cultures, including 42 single-spore isolates of *A. morbosa* and 22 isolates of other fungi, as well as healthy and diseased plant branches collected from the field. A product of ~400 bp was amplified from DNA of all isolates belonging to *A. morbosa*. No product was amplified from DNA of other fungal species, confirming the specificity of the newly designed primers. Within plant tissues, the pathogen was detected at further distances from the edges of knots on thicker branches bearing larger knots compared with thinner branches bearing smaller knots. The PCR assay has shown high sensitivity, needing only 100 fg of the *A. morbosa* DNA for a reliable PCR amplification with the AMF and AMR primers.

Additional keywords: *Dibotryon morbosum,* 'Shubert Select'

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. (8,14,16,21). Fungal ascospores usually infect young branches during spring and cause rough greenish brown-to-black spindle-shaped galls (black knots) on twigs and branches (13,16). The pathogen overwinters in infected branches of *Prunus* spp. as mycelia on which pseudothecia develop (10). It has a long latent period on the plant. Generally, knots appear on infected twigs or branches 1 year after infection (13). A wide variety of both wild and cultivated species of *Prunus* can be infected by the fungus. In Ontario, the disease has occurred on many wild hosts such as *Prunus americana* Marsh., *P. virginiana* L., and *P. pensylvanica* L. (9). Plum trees such as the European plum (*P. domestica* L.) and the Japanese plum (*P. salicina* Lindl.) are also susceptible to black knot in eastern Canada (13). Significant production losses of sour cherry have been observed in the Niagara Peninsula in Ontario, Canada (13). In western Canada, black knot disease has occurred mainly on wild and cultivated chokecherry (*P. virginiana* L.) and pin cherry (*P. pensylvanica* L.) (21). Disease incidence has recently increased in nurseries and city plantings of the ornamental purple-leaved chokecherry cultivar *P. virginiana* 'Shubert Select' in the Prairie Provinces of Canada. Previous investigations showed that humidity and temperature are the most important factors governing the epidemiology of black knot disease in *Prunus* spp. (9). Release of ascospores depends especially on rainfall and temperature during early spring (8,13). Koch (8) demonstrated that removal of knots in late winter is effective for controlling black knot disease on plum trees. Excellent control can also be obtained by spray application of lime sulfur on plum trees (8), and recent research demonstrated that fungicides such as captan were also effective against black knot on plum and sour cherry (14). However, efficacy of captan seemed to depend on the application time. An application of captan on sour cherry in late May reduced black knot disease incidence to 0.4%, whereas applications of captan after early June were ineffective (14).

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Despite the effectiveness of fungicides, control is difficult once black knot disease becomes epidemic on trees. If the disease can be diagnosed at early development stages, management strategies such as chemical sprays can be implemented before epidemics develop. In addition, although *A. morbosa* is the causal agent of black knot, there are several other fungi from different fungal genera, such as *Fusarium*, *Penicillium*, *Phomopsis*, *Alternaria*, *Phoma*, and *Cladosporium,* that are associated with knots, primarily as saprophytes (9). This makes isolation and identification of the pathogen more difficult from infected plant tissues using traditional culture methods. Therefore, a rapid and accurate method for the specific detection of the causal agent of black knot in plant materials is needed for management of this disease. Traditional isolation and identification of *A. morbosa* can be time-consuming, thereby limiting management options in the nursery industry. A rapid diagnostic assay will aid in pathogen identification and lead to more effective management practices. The polymerase chain reaction (PCR)-based techniques for species-specific detection have become very popular (3). Compared with traditional diagnostic methods, the PCR-based techniques have certain advantages. For example, they do not always require culture of the target organisms prior to detection. Moreover, they are very sensitive, rapid, and specific (5,7,17). The objectives of this research were to (i) develop primers for specific detection of *A. morbosa*, and (ii) apply the primers in PCR reactions to rapidly detect the pathogen from infected plant tissues in field-grown plants.

MATERIALS AND METHODS

Source of isolates. Samples of black knots were collected from different geographic locations in Canada and in the United States, and from different host species, mostly wild chokecherry (*P. virginiana*) and its cultivar 'Shubert Select' (Table 1). Two methods were used to isolate *A. morbosa* from knots. First, fungi were isolated according to the methods of Koch (10). Knots were washed and moistened under running tap water for 30 min. Pieces (0.5 cm) from each knot sample were surface-sterilized using 10% commercial bleach (0.5% NaHCl) for 4 min. After rinsing with sterile water three times, five pieces were placed on petri plates

Table 1. *Apiosporina morbosa* isolates and other fungal genera from black knots on chokecherry and pin cherry trees in Canada

Isolate[b]	Species[c]	Host	Origin	Year of isolation	PCR product with[a]	
					ITS1/ITS4	AMF/AMR
3DF1	*Apiosporina morbosa*	'Shubert Select'	Jeffries Nurseries, MB[d]	2003	+	+
3DF2	*A. morbosa*	'Shubert Select'	Jeffries Nurseries, MB	2003	+	+
3DF5	*A. morbosa*	'Shubert Select'	Jeffries Nurseries, MB	2003	+	+
3DF7	*A. morbosa*	'Shubert Select'	Jeffries Nurseries, MB	2003	+	+
3DF8	*A. morbosa*	'Shubert Select'	Jeffries Nurseries, MB	2003	+	+
3DF9	*A. morbosa*	'Shubert Select'	Jeffries Nurseries, MB	2003	+	+
3DF10	*A. morbosa*	'Shubert Select'	Jeffries Nurseries, MB	2003	+	+
3DF 23	*A. morbosa*	'Shubert Select'	John Forsyth, Winnipeg, MB	2003	+	+
3DF 25	*A. morbosa*	'Shubert Select'	John Forsyth, Winnipeg, MB	2003	+	+
3DF 50	*A. morbosa*	'Shubert Select'	Abbotsfield, Winnipeg, MB	2003	+	+
3DF 53	*A. morbosa*	'Shubert Select'	Abbotsfield, Winnipeg, MB	2003	+	+
3DF 58	*A. morbosa*	'Shubert Select'	Hochman, Winnipeg, MB	2003	+	+
3DF 59	*A. morbosa*	'Shubert Select'	Lanyon, Winnipeg, MB	2003	+	+
3DF 60	*A. morbosa*	'Shubert Select'	Lanyon, Winnipeg, MB	2003	+	+
3DF 63	*A. morbosa*	'Shubert Select'	Wales Ave. Regina, SK	2003	+	+
3DF 66	*A. morbosa*	'Shubert Select'	Wales Ave. Regina, SK	2003	+	+
3DF 67	*A. morbosa*	'Shubert Select'	Wales Ave. Regina, SK	2003	+	+
3DF 68	*A. morbosa*	'Shubert Select'	Wales Ave. Regina, SK	2003	+	+
3DF135	*A. morbosa*	Wild chokecherry	Riding Mountain National Park-1, MB	2003	+	+
3DF136	*A. morbosa*	Wild chokecherry	Riding Mountain National Park-1, MB	2003	+	+
3DF143	*A. morbosa*	Wild chokecherry	Riding Mountain National Park-2, MB	2003	+	+
3DF148	*A. morbosa*	Wild chokecherry	Riding Mountain National Park-2, MB	2003	+	+
3DF152	*A. morbosa*	Wild chokecherry	Riding Mountain National Park-2, MB	2003	+	+
3DF154	*A. morbosa*	Wild chokecherry	Riding Mountain National Park-3, MB	2003	+	+
3DF156	*A. morbosa*	Wild chokecherry	Riding Mountain National Park-3, MB	2003	+	+
2DF3	*A. morbosa*	Wild chokecherry	Waterton, AB	2002	+	+
2DF7	*A. morbosa*	'Shubert Select'	University of Manitoba, MB	2002	+	+
2DF22	*A. morbosa*	Wild chokecherry	Bird's Hill, Winnipeg, MB	2002	+	+
2DF48	*A. morbosa*	'Shubert Select'	Superior, Wisconsin, USA	2002	+	+
2DF52	*A. morbosa*	*P. pensylvanica*	Lac Seul, ON	2002	+	+
2DF55	*A. morbosa*	*P. pensylvanica*	Lac Seul, ON	2002	+	+
2DF57	*A. morbosa*	'Shubert Select'	Morden, MB	2002	+	+
2DF59	*A. morbosa*	'Shubert Select'	Morden, MB	2002	+	+
2DF60	*A. morbosa*	Wild chokecherry	Waterton, AB	2002	+	+
2DF62	*A. morbosa*	Wild chokecherry	Morris, MB	2002	+	+
2DF64	*A. morbosa*	'Shubert Select'	Morden, MB	2002	+	+
2DF65	*A. morbosa*	Wild chokecherry	Waterton, AB	2002	+	+
2DF67	*A. morbosa*	Wild chokecherry	Morris, MB	2002	+	+
2DF68	*A. morbosa*	'Shubert Select'	Superior, Wisconsin, USA	2002	+	+
2DF77	*A. morbosa*	*P. pensylvanica*	Lac Seul, ON	2002	+	+
2DF78	*A. morbosa*	*P. pensylvanica*	Lac Seul, ON	2002	+	+
ATCC15085	*A. morbosa*	–	American Type Culture Collection, USA	2002	+	+
S1	*Fusarium*, sp-1	'Shubert Select'	Jeffries Nurseries, MB	2003	+	–
S2	*F.* sp-1	'Shubert Select'	John Forsyth, Winnipeg, MB	2003	+	–
S3	*F.* sp-1	'Shubert Select'	Abbotsfield, Winnipeg, MB	2003	+	–
S4	*F.* sp-2	Wild chokecherry	Riding Mountain National Park-1, MB	2003	+	–
S5	*F.* sp-2	'Shubert Select'	Jeffries Nurseries, MB	2003	+	–
S6	*F.* sp-3	Wild chokecherry	Riding Mountain National Park-1, MB	2003	+	–
S7	*F.* sp-3	Wild chokecherry	Riding Mountain National Park-2, MB	2003	+	–
S8	*F.* sp-4	Wild chokecherry	Riding Mountain National Park-3, MB	2003	+	–
S9	*Alternaria* sp-1	Wild chokecherry	Riding Mountain National Park-1, MB	2003	+	–
S10	*A.* sp-2	Wild chokecherry	Riding Mountain National Park-1, MB	2003	+	–
S11	*Phoma* sp-1	'Shubert Select'	John Forsyth, Winnipeg, MB	2003	+	–
S12	*P.* sp-1	'Shubert Select'	Abbotsfield, Winnipeg, MB	2003	+	–
S13	*P.* sp-1	'Shubert Select'	Jeffries Nurseries, MB	2003	+	–
S14	*P.* sp-1	Wild chokecherry	Morris, MB	2003	+	–
S15	*P.* sp-1	Wild chokecherry	Bird's Hill, Winnipeg, MB	2003	+	–
S16	*P.* sp-2	'Shubert Select'	Jeffries Nurseries, MB	2003	+	–
S17	*P.* sp-2	Wild chokecherry	Riding Mountain National Park-1, MB	2003	+	–
S18	*P.* sp-2	Wild chokecherry	Riding Mountain National Park-2, MB	2003	+	–
S19	*P.* sp-3	'Shubert Select'	Morden, MB	2003	+	–
S20	*Cladosporium* sp-1	Wild chokecherry	Riding Mountain National Park-3, MB	2003	+	–
S21	*C.* sp-2	'Shubert Select'	Jeffries Nurseries, MB	2003	+	–
S22	*C.* sp-2	'Shubert Select'	Jeffries Nurseries, MB	2003	+	–

[a] + and – indicate presence and absence of the approximately 550- and 400-bp DNA fragments amplified by the universal primer pair ITS1 + ITS4 and the *Apiosporina morbosa*–specific primer pair AMF + AMR, respectively.

[b] Representative isolates collected from different geographic locations and two host species, *Prunus virginiana* (wild chokecherry and 'Shubert Select' chokecherry) and *P. pensylvanica*. Their internal transcribed spacer (ITS) region amplifications with the ITS1 and ITS4 primers and the amplified products with the AMF and AMR primers are shown on the agarose gels in Figures 1 and 3, respectively.

[c] Isolates with the same number in the same genus share the identical ITS sequence.

[d] MB = Manitoba; SK = Saskatchewan; AB = Alberta; ON = Ontario.

containing 1.5% water agar. Fungal colonies formed around diseased tissues on the water agar plate in 2 to 3 days at room temperature (22°C). From the edge of each colony, a piece of mycelium was transferred onto potato dextrose agar plates (PDA) (Difco Laboratories, Detroit, MI) containing 100 ppm streptomycin sulfate (Sigma, Oakville, ON) to reduce bacterial contamination. The PDA plates were incubated for a week, and single conidial spores were transferred onto new PDA plates under a microscope with ×400 magnification to obtain single conidial spore isolates. In the second method, galls were surface-sterilized with 10% commercial bleach and washed 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 slide using a round-ended glass rod. The macerate was streaked on water agar plates and incubated for 24 h at room temperature. Single germinating ascospores were marked on plates under the microscope and were transferred to PDA plates to obtain pure single ascospore isolates. In addition, all other fungi associated with knots were isolated, purified, and stored for DNA analysis together with *A. morbosa* isolates. A list of the *A. morbosa* isolates, other black knot–associated fungal isolates from *Fusarium, Phoma, Alternaria,* and *Cladosporium*, and their sources is shown in Table 1. An isolate of *A. morbosa* (strain ATCC15085) was obtained from the American Type Culture Collection to compare morphological characteristics and sequences of the internal transcribed spacer (ITS) region with *A. morbosa* strains isolated from knots in Canada.

DNA extraction. Mycelia of each *A. morbosa* isolate were grown in a flask with 75 ml of potato dextrose broth (PDB) (Difco Laboratories). The PDB in the flask was inoculated with a 0.5-ml suspension of approximately 10^6 spores of an isolate per ml. The flasks were incubated at room temperature for 24 h without agitation. Mycelia of other gall-associated fungi were produced using a similar culture method, except that the flasks were incubated at room temperature for 3 to 4 days with agitation. Mycelia were harvested by vacuum filtration through two layers of sterilized Miracloth (Calbiochem, CN Biosciences, La Jolla, CA) and rinsed twice with sterile water, then stored at –80°C until lyophilized. DNA of isolates was extracted essentially by the method described by Lodhi et al. (12), except that polyvinylpyrrolidone was not used in the extraction procedure. All DNA extracts were quantified using a spectrophotometer and adjusted to a final concentration of 5 ng μl^{-1} for PCR analysis.

Amplification of ITS region and primer design. The 42 *A. morbosa* isolates and 22 isolates of *Fusarium, Phoma, Alternaria,* and *Cladosporium*, collected from different geographic locations and host species in Canada and the United States, were used in PCR amplification of the ITS regions (Table 1). The universal primers ITS1 (5′-TCCGTAGGTGAACCT-GCGG-3′) and ITS4 (5′-TCCTCCGCT-TATTGATATGC-3′) (20) were used to amplify the region spanning the 3′ end of the 18S rRNA gene, internal transcribed spacer 1 (ITS1), the 5.8 rRNA gene, ITS2, and the 5′ end of the 28 rRNA gene. PCR was conducted in a 25-µl reaction volume. Each reaction contained approximately 25 ng of template DNA, 2.5 µl of 10× PCR buffer, 1 unit of *Taq* DNA polymerase (recombinant) (Invitrogen Life Technologies, Burlington, ON, Canada), 100 mM Tris-HCl (pH 8.0), 500 mM KCl, 0.5 µM each of the ITS1 and ITS4 primers, 1.5 mM $MgCl_2$, and 0.2 mM each of dNTPs (Invitrogen Life Technologies). The temperature profile of the PCR was 40 cycles consisting of denaturing at 94°C for 2 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. A final extension at 72°C for 10 min followed. A negative control (no template DNA) was used in every experiment to test for presence of contamination in reagents. Amplified products were electrophoresed on 1.4% agarose gels containing ethidium bromide at 0.5 µg ml^{-1} with 1× TBE running buffer. A 100-bp DNA ladder (Invitrogen Life Technologies) was included on each gel as a molecular size standard. Since fragments amplified with the ITS1 and ITS4 primers from *A. morbosa* isolates and other fungal isolates were similar in size, specific primers needed to be designed for the detection of *A. morbosa*. To achieve this goal, all PCR products amplified with the ITS1 and ITS4 primers were purified using the High Pure PCR Product Purification Kit (Roche Applied Science, Penzberg, Germany) and were sent to the University Core DNA and Protein Services, University of Calgary, Alberta, Canada, for sequencing. The ITS sequences of *A. morbosa* isolates were aligned with those of all other fungi associated with black knots using the multiple sequence alignment software, CLUSTAL W (1.82) (EMBL-EBL, European Bioinformatics Institute). Two regions specific to *A. morbosa* were identified and used to design an *A. morbosa*–specific forward primer (AMF) and a reverse primer (AMR). The two primers were synthesized by Invitrogen Life Technologies.

Specificity and sensitivity of the *A. morbosa*–specific primers. All DNA samples of 42 *A. morbosa* isolates and 22 isolates of other fungal genera were used in PCR amplification for the specificity test of the AMF and AMR primers (Table 1). To test the sensitivity of the specific primers for the *A. morbosa* detection, 1 fg to 10 ng of serial dilutions of DNA of the *A. morbosa* isolate 3DF1 were used as templates for PCR amplification. The PCR conditions with the AMF and AMR primers were identical to those described above in the PCR reactions with the ITS1 and ITS4 primers, except that the annealing temperature was 58°C rather than 55°C. The PCR reaction with the ITS1 and ITS4 primers was performed as the positive control to compare the size of the product generated from the AMF and AMR primers and ensure that the template DNA was amplifiable.

PCR amplification from diseased plants. To compare spread of pathogen in diseased branches of different size, the *A. morbosa* primers AMF and AMR were tested against DNA samples extracted from diseased branches of ‘Shubert Select’ chokecherry within two diameter categories (Table 2). Diameters of the first set of 10 diseased branches ranged from 0.4 to 0.7 cm, on which black knots were 0.5-1 × 1-3 cm in size, whereas diameters of another set of 10 branches ranged from 2 to 2.5 cm, on which knots were 3-4 × 4-7 cm in size. The branches that produced only one knot on each were selected for tissue sampling, and branches from healthy trees served as the controls. The tissue samples on diseased branches were obtained from black knots, as well as 1, 3, 5, 7, 9, and 11 cm from the edges of knots (Table 2). DNA was extracted from the plant tissues using the method described by Lodhi et al. (12). The PCR was conducted with the AMF and AMR primers using the conditions described in the above reactions.

RESULTS

Isolation of *A. morbosa.* In 2002 and 2003, a total of 41 single-spore isolates of

Table 2. Detection of *Apiosporina morbosa* from diseased branches of *Prunus virginiana* ‘Shubert Select’

Branch diam. (cm)	Knot size (cm)	PCR assay with DNA extracted at different distances (cm) from edges of knots							
		Knot tissues	1[a]	3	5	7	9	11	Healthy branch
0.4-0.7	0.5-1 × 1-3	10/10[b]	9/10	1/10	0/10	0/10	0/10	0/10	0/10
2-2.5	3-4 × 4 -7	10/10	10/10	10/10	6/10	0/10	0/10	0/10	0/10

[a] Distance from edge of knot where plant tissues were removed.

[b] Number of branches in which 400-bp polymerase chain reaction (PCR) fragment was detected / total number of branches assayed.

A. morbosa and 22 isolates from the *Fusarium, Phoma, Alternaria,* and *Cladosporium* species associated with black knots were isolated and placed in our collection (Table 1). All isolates were used for DNA extraction, PCR reactions with the ITS1 and ITS4 primers, and the specificity test of the AMF and AMR primers.

Design of PCR primers. An approximately 550-bp DNA fragment of ITS region was PCR amplified from all *A. morbosa* isolates and other fungal isolates using the ITS1 and ITS4 primers (Table 1 and Fig. 1). The sequenced ITS region data showed that all isolates of *A. morbosa* including the ATCC15085 isolate had the identical ITS sequence, but a few isolates (2DF3, 2DF64, and 3DF54) had mutations in one to two sites (bases) (data not shown). ITS sequences of five *A. morbosa* representative isolates, including type isolate ATCC15085, have been submitted to GenBank. The accession numbers are AF493982, AF493983, AF493984, AY165751, and AY166451 (ATCC15085), respectively. For other gall-associated fungi, some isolates from the same genus had identical sequences, and thus sequences of 22 isolates of other fungi were classified into 11 different sequence categories. To design *A. morbosa*–specific primers, the sequence of the ITS region of *A. morbosa* isolate 3DF1 (Table 1), which represented sequences of *A. morbosa* and had an identical sequence to ATCC15085, was aligned with the 11 different ITS sequences of other fungal species. Sequencing data showed that isolates of *A. morbosa* had significantly different sequences from those of *Fusarium* spp., *Phoma* spp., and *Alternaria* spp. at the region used for designing the specific primers (data not shown). However, two isolates of *Cladosporium* spp. (*C.* sp-1 and *C.* sp-2) had high sequence similarity to *A. morbosa* at the region used for designing the primers (Fig. 2). The *A. morbosa*–specific primers were designed to specifically dis-

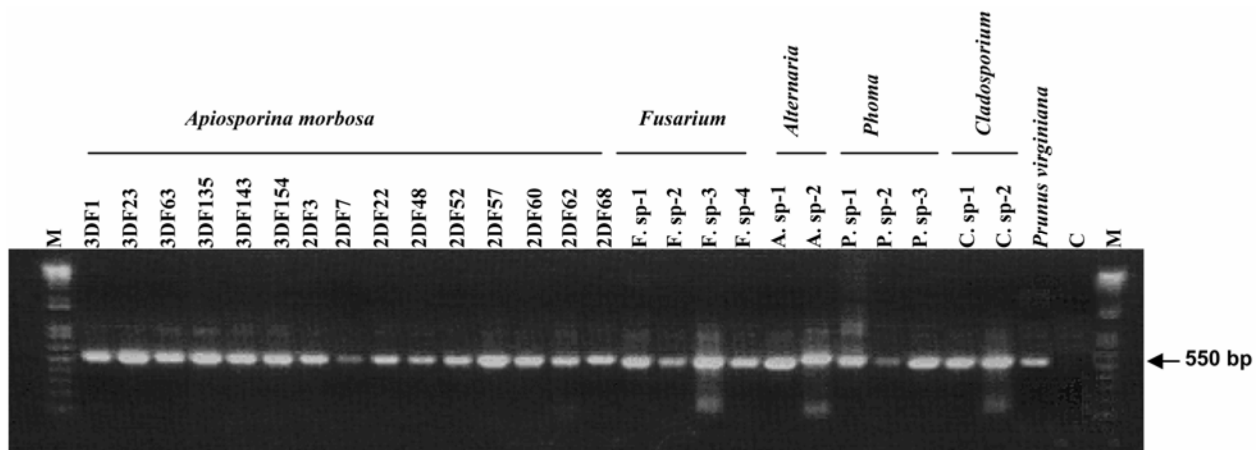

Fig. 1. An agarose gel showing bands amplified from the of rDNA with the primer pair ITS1 and ITS4 from 15 representative isolates of *Apiosporina morbosa*, 11 isolates of *Fusarium, Alternaria, Phoma,* and *Cladosporium* species associated with black knots, and healthy plants of *Prunus virginiana* 'Shubert Select'. M = 100-bp DNA ladder. C = negative control.

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A. morbosa     CGACTCTGTT GCCTCCGGGG CGACCCTGCC TT----CGGG CGGGGGCTCC GGGTGGACAC 60
C. sp-1        .......... .......... .......... ..TTCA.... .......C.. ..........
C. sp-2        ....G.A.G. .AA..T.C.. A.----.... ..----.TAA ......GA.. ..........

A. morbosa     TTCAAA-CTC TTGCGTAACT TTGCAGTCTG AGTAAACTTA ATTAATAAAT TAAAACTTTT 120
C. sp-1        A.....A... .......... .......... ......T... .......... .........C
C. sp-2        ......-... .......... .......... .......... .......... ..........

A. morbosa     AACAACGGAT CTCTTGGTTC TGGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG 180
C. sp-1        .......... .......... .......... .......... .......... ..........
C. sp-2        .......... .......... .......... .......... .......... ..........

A. morbosa     TGAATTGCAG AATTCAGTGA ATCATCGAAT CTTTGAACGC ACATTGCGCC CCCTGGTATT 240
C. sp-1        .......... .......... .......... .......... .......... ..........
C. sp-2        .......... .......... .......... .......... .......... ..........

A. morbosa     CCGGGGGGCA TGCCTGTTCG AGCGTCATTT CACCACTCAA GCCTCGCTTG GTATTGGGCA 300
C. sp-1        .......... .......... .A..GT.... .......... .......... .........G
C. sp-2        .......... .......... .......... .......... .......... ..........

A. morbosa     ACGCGGTCCG CCGCGTGCCT CAAATCGACC GGCTGGGTCT TCTGTCCCCT AAGCGTTGTG 360
C. sp-1        .......... .....C.... .......... .......... .......... C.........
C. sp-2        .......... .......... .......T.. .......... .......... ..........

A. morbosa     GAAACTATTC GCTAAAGGGT GTT-CGGGAG GCTACGCCGT AAAACAACCC CATTTCTAAG 420
C. sp-1        .......... .......... .CCA...... ..C......A .......A.. ..........
C. sp-2        .......... .......... ...-...... .......... .......... ..........
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Fig. 2. Sequence alignment of the partial rDNA internal transcribed spacer (ITS) sequences of *Apiosporina morbosa* (isolate 3DF1) and two *Cladosporium* isolates (*C.* sp-1 and *C.* sp-2) that have high similarity in sequence to *A. morbosa.* The *A. morbosa*–specific primers (AMF and AMR) were designed according to the two underlined bold regions. The ITS sequences of the *Fusarium, Phoma*, and *Alternaria* species were not aligned because they are significantly different in their ITS sequences from *A. morbosa.*

criminate against these two isolates. Therefore, the newly designed primers would differentiate *A. morbosa* from all other fungi, including *Cladosporium* spp. Two 19-bp sequences, 5′-GGGCGACCCTGCCTTCGGG-3′ and 5′-TACGGCGTAGCCTCCCGAA-3′, were identified in the ITS sequence of *A. morbosa* based on which pair of potential *A. morbosa*–specific primers, 5′-GGGCGACCCTGCCTTCGGG-3′ and 5′-ATGCCGCATCGGAGGGCTT-3′, were synthesized to serve as the *A. morbosa* forward (AMF) and reverse (AMR) primers, respectively (Fig. 2).

Specificity and sensitivity of the AMF and AMR primers. In specificity tests, 42 isolates of *A. morbosa* and 22 isolates from other fungal genera were examined in PCR reactions with the specific primers AMF and AMR. Only isolates of *A. morbosa* yielded a single PCR product approximately 400 bp in size when amplified with the AMF and AMR primers (Table 1 and Fig. 3). Isolates of *Fusarium*, *Phoma*, *Alternaria*, and *Cladosporium* associated with black knots as well as DNA of healthy plants, did not yield this product with the AMF and AMR primers (Table 1 and Fig. 3). A range of DNA concentrations of the *A. morbosa* isolate 3DF1 was tested to determine the sensitivity of the PCR assay with the AMF and AMR primers. For the reliable PCR amplification with the AMF and AMR primers, 100 fg to 10 ng of pure DNA from *A. morbosa* was sufficient (Fig. 4). No products were produced from the negative control.

Detection of *A. morbosa* from diseased plant tissues. In order to test the viability of the AMF and AMR primers when applied to clinical samples, the PCR assays were conducted with the specific primer pairs for detecting the pathogen from diseased tissues of black knots and plant tissues at different distances from the edge of knots. A single PCR product approximately 400 bp in size was detected from diseased tissues of knots and infected tissues up to 3 cm from edges of black knots on branches 0.4 to 0.7 cm in diameter (Table 2 and Fig. 5). Although most of the PCR product was detected in plant tissues within 1 cm from the edges of black knots, one of the 10 branches had the PCR product 3 cm from the edge of the knot. No amplified products were detected in plant tissues beyond 3 cm from the edge of knots in the 0.4 to 0.7 cm diameter branches. In contrast, in the branches 2 to 2.5 cm in diameter, all samples had the single PCR product detected within 3 cm and over half within 5 cm from edge of knots (Table 2 and Fig. 5). No product was detected in the tissues of healthy branches of any diameter.

DISCUSSION

In this study, we developed a pair of the *A. morbosa*–specific primers, AMF and AMR, which were used in PCR reactions to detect *A. morbosa*, the pathogen of black knot in *Prunus* spp. These PCR assays had very high specificity. They detected the specific fragment from all *A. morbosa* isolates tested, and no specific product was detected with this pair of primers from other fungi associated with black knots, nor from healthy plants. Practical tests done on diseased plants with the primers showed that the PCR assays detected the pathogen in infected plant tissues in the presence of other black knot–

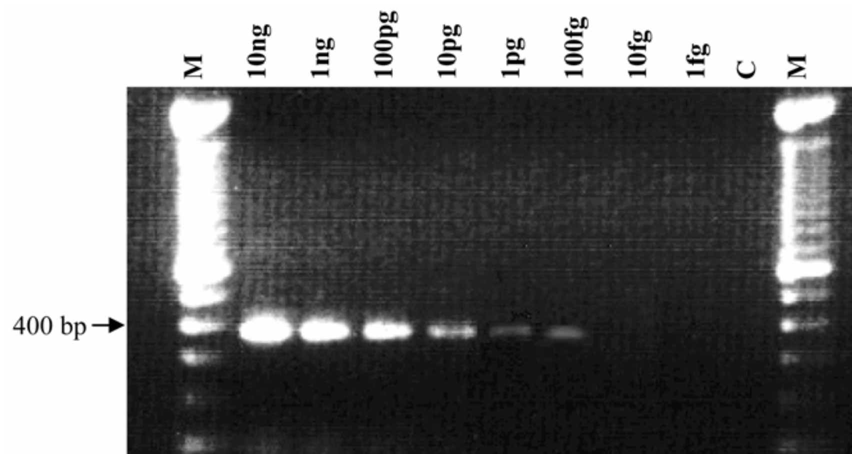

Fig. 4. An agarose gel showing sensitivity of the *Apiosporina morbosa*–specific primer pair AMF and AMR using a series of pure DNA dilutions from isolate 3DF1 as templates. M = 100-bp ladder. C = negative control.

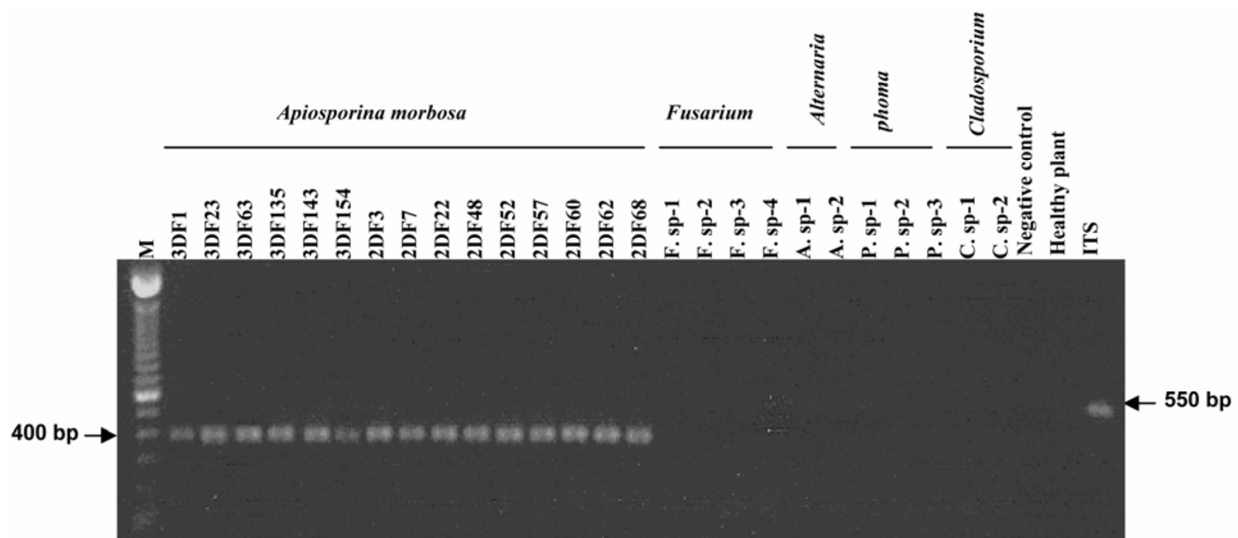

Fig. 3. An agarose gel showing a specific band amplified by polymerase chain reaction (PCR) amplification with the AMF and AMR primers from the *Apiosporina morbosa* isolates. DNA templates were extracted from 15 representative isolates of *A. morbosa*, 11 isolates of other fungal species with different sequences of internal transcribed spacer (ITS) of rDNA (*Fusarium, Alternaria, Phoma,* and *Cladosporium* associated with black knots) (Table 1), and a healthy plant of *Prunus virginiana* 'Shubert Select'. M is a 100-bp DNA ladder. The ~550-bp ITS product was amplified with primers ITS1 and ITS4 from *A. morbosa* isolate 3DF1 and served as a control of size compared with the ~400-bp product amplified with primers AMF and AMR from the *Apiosporina morbosa* isolates.

associated fungi. The PCR assay had high sensitivity, and it detected the specific fragment with 100 fg of DNA. To our knowledge, this is the first report of development of specific PCR primers for detection of *A. morbosa*.

The reliability of a PCR assay for detection of pathogens relies on the sensitivity of PCR primers specific to the target pathogen. Several strategies have been used to develop specific PCR primers for identification of plant pathogens. First, randomly amplified polymorphic DNA (RAPD) markers were commonly used to develop specific primers. Recently, RAPD markers have been used successfully to design specific primers for detection of *Stachybotrys elegans* from soil (17) and *Monilinia fructicola* from sweet cherry fruits (4). Second, specific genes also can be used to develop specific primers for identification of target pathogens. For example, specific nested primers for detection of the aflatoxin-producing fungi *Aspergillus flavus* and *A. parasiticus* from grains and food were successfully developed by the *omt-1* gene involved in aflatoxin biosynthesis (15). Third, ITS regions of rDNA have been widely used to design specific primers for identification of plant pathogens (1,6,7,21). The ITS regions are less conserved than RNA gene subunits, perhaps because they have no apparent functions (2). Therefore, there is relatively a rapid evolution in the ITS regions of rDNA, and thus high variation. It is reported that differences in ITS regions exist between closely related species and sometimes within species (18,19). This makes the ITS region a good candidate for developing specific PCR primers. In addition, rDNA is present in multiple copies in most fungal genomes. Therefore, PCR assays that amplify ITS regions of rDNA should be more sensitive than those that amplify single-copy genes. Because of these features of the ITS regions, the *A. morbosa*–specific primers were designed using the ITS regions of rDNA in the present research.

Unlike with other fungi, it is difficult to obtain pigment-less DNA samples from *A. morbosa* isolates. At the beginning of this study, we cultured the isolates of *A. morbosa* in PDB with shaking for 3 to 4 days. Although a sufficient amount of mycelia could be harvested by this method, all DNA samples extracted from these mycelium samples were brownish, probably because mycelia were too old and too many brown conidial spores were produced. PCR reactions using this type of DNA could be affected by pigment in the pathogen DNA. To eliminate pigment in DNA, we harvested only young mycelia for DNA extraction. To accomplish this, conidial spores were spread on the surface of PDB in a flask. The flask was held at room temperature for 24 h without any agitation. A young mycelium mat was formed after 24-h incubation. The DNA extracted from these mycelia was colorless and of higher quality.

The positive results obtained from asymptomatic tissues proximal to *A. morbosa* galls suggest that the PCR test is a sensitive diagnostic tool for early fungal pathogen detection in trees. PCR tests from tissues distal to galls and from noninfected stems were negative for *A. morbosa*. In addition, we found that the pathogen was detected at further distances from the edges of knots in thicker branches bearing larger knots compared with thinner branches bearing smaller knots. This may be related to time of pathogen infection. Thicker branches with larger knots may have been infected by the pathogen several years earlier, resulting in mycelia spreading further from infection sites. This result supports the conclusion drawn from a previous histological analysis that mycelia spread further from larger galls than from smaller ones (10).

This study was initiated to develop a pair of primers based on the ITS regions for rapid and specific detection of *A. morbosa*. We successfully tested young twigs with small black knots using the designed primers. Although the primers had good specificity and sensitivity for detecting the pathogen, more testing needs to be done in cities and nurseries where chokecherry is produced and grown in the landscape. In particular, if the pathogen can be detected early, increased efficacy of the chemical sprays may be achieved. Therefore in future work, it would be beneficial to widen the scope of plant tissue sampling to include tissues from different infection stages, tissues carrying spores, tissues at initial infection sites with no visible symptoms, and slightly swelled tissues and knots prior to production of fruiting bodies. Successful tests from these stages will greatly benefit the making of sound, economical management strategies for this disease. For example, transplanting rooted 'Shubert Select' chokecherry cuttings infected by the black knot pathogen is a likely source of disease spread in nurseries. If the cuttings are at the latent infection stage, the pathogen can be found through molecular detection before commercial sales are made from the nursery. The cuttings can be treated with fungicides or discarded at the nursery so that introduction of disease into new areas may be reduced. Similarly, if ornamental chokecherry trees infected by the fungus in towns, cities, or nurseries can be diagnosed through PCR with the specific primers at the initial disease development stage, an appropriate management practice such as chemical spray and removal of diseased branches can be done before black knot disease becomes epidemic, and thus risk of disease epidemics will be reduced greatly.

In the present study, the PCR assays were not quantitative and only detected the presence or absence of the pathogen. However, the recently developed real-time PCR assay can be used to determine the amount of target DNA in a sample (17). We believe that the highly sensitive PCR assays and primers developed in this research will be a basis for future studies using real-time PCR to determine the levels of *A. morbosa* in plant tissues. Such future work will involve applying the specific primers AMF and AMR to detect the concentrations of pathogen DNA at different distances from black knots using the real-time PCR technique. Furthermore, the spore density carried on plant surfaces may be determined by the real-time PCR technique. The information obtained from real-time PCR has the potential to enhance the accuracy of black knot disease surveillance and increase our understanding of the epidemiology of the host–pathogen interaction in the field.

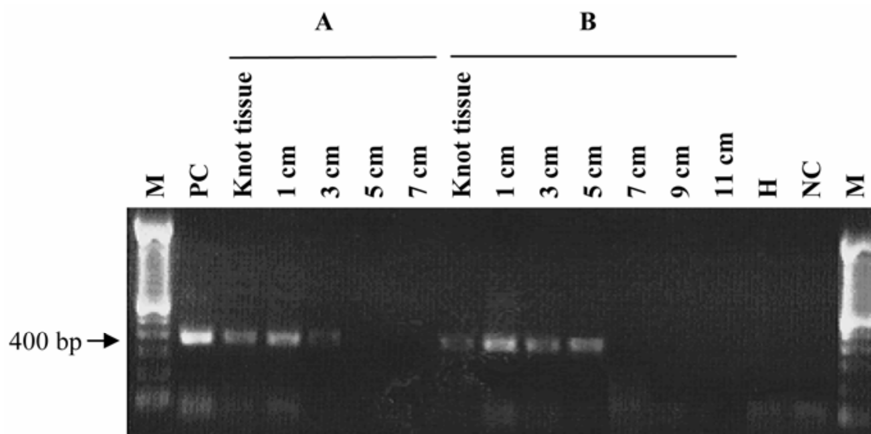

Fig. 5. An agarose gel showing the specific band amplified from *Apiosporina morbosa* with the AMF and AMR primers from diseased branches. DNA samples were isolated from black knots and infected plant tissues at different distances from edges of black knots on branches 0.4 to 0.7 cm in diameter (A), and branches 2 to 2.5 cm in diameter (B). M = 100-bp ladder. PC = positive control with pure DNA from isolate 3DF1. H = DNA from healthy plant. NC = negative control.

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