Detection of the Pinewood Nematode, *Bursaphelenchus xylophilus*, Using a Real-Time Polymerase Chain Reaction Assay

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Accepted for publication 14 January 2005.

ABSTRACT

Cao, A. X., Liu, X. Z., Zhu, S. F., and Lu, B. S. 2005. Detection of pinewood nematode, *Bursaphelenchus xylophilus*, using a real-time polymerase chain reaction assay. *Phytopathology* 95:566-571.

The pinewood nematode, *Bursaphelenchus xylophilus*, has caused significant damage to pine plantations both in East Asia and North America and is an important quarantine organism. A real-time polymerase chain reaction (PCR) assay was developed to detect *B. xylophilus*. A set of primers and probe specific for *B. xylophilus* was designed to target the

ribosomal DNA internal transcribed spacer region. Optimal primer concentration, Mg²⁺ concentration, and extension temperature were 400 nM, 3.0 mM, and 60°C, respectively. The assay was highly specific and sensitive, detecting as little as 0.01 ng of *B. xylophilus* DNA. The real-time PCR assay also successfully detected *B. xylophilus* in field samples, and it should be very useful for quarantine purposes.

Additional keywords: cycle threshold, normalized fluorescence.

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle (= *B. lignicolus*), is the casual agent of pine wilt disease (18,19), an important disease to conifer trees. The nematode is native to North America and has been associated with pine wilt in Canada, Mexico, and the United States (5,7,14,23). *B. xylophilus* may have been introduced into Japan from North America in the early 1900s and subsequently introduced into China and Korea from Japan (8,30).

The European Plant Protection Organization placed the pinewood nematode on the A1 list of quarantine pests in July 1985 (25); the nematode, however, was reported in Portugal in 1999 (22). During the 1990s, this quarantine on green lumber exports to Europe caused an estimated annual loss to the American forest industry of US \$100 million (9).

In China, the pinewood nematode was first detected in Jiangsu in 1982 and was subsequently reported in Guangdong, Anhui, Shandong, Zhejiang, Hongkong, and Taiwan (33). From 1985 to 1995, *B. xylophilus* destroyed approximately one million pine trees in China (24). To prevent its further introduction and spread, *B. xylophilus* has been listed as a quarantine pest in China. Within the past decades, China has developed comprehensive regulations to eliminate significant pest risks associated with importing coniferous wood chips, unseasoned lumber, and logs from *B. xylophilus*-infested areas or countries.

Effective quarantine requires efficient detection. However, detection of *B. xylophilus* is complicated by *B. mucronatus* Mamiya & Enda. *B. mucronatus* is widely distributed in Asia and Europe, is not pathogenic to pines under field conditions, but is morphologically similar to *B. xylophilus* (8,20). The only major morphological difference between the species is the shape and size of a mucro on the tail of female *B. mucronatus*. The occurrence of interspecific forms also makes it difficult to distinguish between these two closely related species (28).

To distinguish *B. xylophilus* from *B. mucronatus*, researchers have developed DNA-based methods, including restriction fragment length polymorphism (15,34), random amplified polymorphism DNA (27,35), polymerase chain reaction (PCR) (11,21,32), as well as DNA fingerprinting probes derived from the repeated DNA sequence elements (11,28). Although these methods differentiate *B. xylophilus* from *B. mucronatus*, they are too time-consuming for quarantine purposes. A less time-consuming PCR-based assay, referred to as real-time PCR, has recently shown promise in the specific and rapid detection of bacteria (3,26,29), fungi (6), and other organisms (2,31). This technique relies on the combination of a primer set and an additional dual-labeled fluorogenic probe (14) to enable continuous monitoring of amplicon synthesis during thermocycling; the technique requires no post-PCR handling for target quantification (26).

In this paper, a *B. xylophilus*-specific real-time PCR assay was developed and optimized. The internal transcribed spacer (ITS) region was used because, although largely conserved, it differs between *B. xylophilus* and *B. mucronatus* (4,15–17,21,22,34). Furthermore, the ITS sequence occurs in multiple copies in the genome and the sequence information is available in GenBank. The specificity, sensitivity, and application of the assay are demonstrated.

MATERIALS AND METHODS

Sources of nematodes and DNA samples. Nine isolates of *Bursaphelenchus* spp. were obtained from laboratory stocks and cultured on potato dextrose agar plates (Difco, Detroit, MI) colonized with the fungus *Botrytis cinerea* (Table 1). One isolate of *Caenorhabditis elegans* was supplied by the Caenorhabditis Genetics Center (Minneapolis, MN) and was cultured on nematode growth medium. Three DNA samples of *Bursaphelenchus* spp. were provided by W. M. Ye (University of New Hampshire, Durham).

DNA extraction. Two methods were used to extract genomic DNA from nematodes. When the DNA was extracted from a large number of nematodes (including mixed life stages within a spe-

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DOI: 10.1094/PHYTO-95-0566

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cies or mixed species), the method of Webster et al. (28) was used. When the DNA was extracted from one to four nematodes, the method of Zheng et al. (34) was used with the following modifications. One to four nematodes were placed into 15 µl of double-distilled water on a glass slide and cut into small pieces with a sterile side-sharpened needle. The nematode fragments in 10 µl of suspension were transferred into a sterile 0.5-ml thin-walled PCR tube containing 8 µl of nematode lysis buffer (12.5 mM KCl, 2.5 mM Tris-HCl [pH 8.3], 0.03 mM Mg²⁺, 0.025 mM dithiothreitol, 0.12% Tween 20, and 1 µl of proteinase K). Tubes were incubated at 65°C for 60 min followed by 95°C for 3 min.

DNA concentration was determined by measuring the absorbance at 260 nm with a SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA). If necessary, DNA was diluted with ultra-purified water.

ITS primers and TaqMan probe design. Genus-specific primers F194 (5'-CGTAACAAGGTAGCTGTAG-3') and 5368 (5'-TTTCACTCGCCGTTACTAAGG-3') were used to amplify the ITS1, 5.8S, and ITS2 rDNA region of *Bursaphelenchus* spp. (15). PCR products from five isolates each of *B. xylophilus* and *B. mucronatus* were cloned and sequenced. The nucleotide sequence of ITS1-5.8S-ITS2 region was submitted to the GenBank/

EMBL database (accession no. U92464), and related sequences were aligned using the ClustalX 1.81 package (PE Applied Biosystems, Foster City, CA). The DNA regions demonstrating the greatest dissimilarities between species were selected for designing *B. xylophilus*-specific PCR primers and TaqMan probe. The primers and TaqMan probe were designed using the software program Primer Express version 2.0 (PE Applied Biosystems). Primers and probe were synthesized by PE Applied Biosystems.

Real-time PCR assay. Real-time PCR was performed in 50-µl reactions using MicroAmp Optical 96-well plates and MicroAmp Optical Caps (PE Applied Biosystems). The 50-µl PCR mixture consisted of 5 µl of 10× reaction buffer (1 mM Tris-HCl [pH 9.0], 5 mM KCl, and 0.01% Triton X-100), 4 mM Mg²⁺, 200 µM dNTP, 2 units of AmpliTaq DNA polymerase, 400 nM of each primer, 200 nM TaqMan probe, and 10 ng of template DNA. As a negative control, ultrapure water rather than template DNA was included in each PCR run. Chemicals were purchased from Promega (Madison, WI). The ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) was used for amplification and fluorescence measurement at each temperature step and cycle during the reaction. Thermal cycling conditions consisted of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and then

TABLE 1. Isolates and origins of *Bursaphelenchus* species and *Caenorhabditis elegans*

Nematode species	Isolate	Source of DNA template ^a	Geographical location
<i>B. xylophilus</i>	BX1	Juvenile	Guangdao, Shandong, China
<i>B. xylophilus</i>	BX2	Juvenile	Nanjing, Jiangsu, China
<i>B. xylophilus</i>	BX3	Juvenile	Nanjing, Jiangsu, China
<i>B. xylophilus</i>	BX4	Juvenile	Guangzhou, Guangdong, China
<i>B. xylophilus</i>	BX5	Juvenile	Langyanshan, Anhui, China
<i>B. xylophilus</i>	BX6	DNA	Mito, Ibaraki, Japan
<i>B. mucronatus</i>	BM1	Juvenile	Nanjing, Jiangsu, China
<i>B. mucronatus</i>	BM2	Juvenile	Japan
<i>B. mucronatus</i>	BM3	Juvenile	Grünberg, Germany
<i>B. mucronatus</i>	BM4	Juvenile	Zusmarshausen, Germany
<i>B. fraudulentus</i>	BF	DNA	Zusmarshausen, Germany
<i>B. tusciae</i>	BT	DNA	Scopeti, Florence, Italy
<i>C. elegans</i>	CE11	Juvenile	Caenorhabditis Genetics Center, Minneapolis, USA

^a DNA was obtained either from juvenile nematodes in culture or as DNA from other laboratories.

Fig. 1. Specificity of the real-time polymerase chain reaction assay for *Bursaphelenchus xylophilus*. The assays were conducted with 10 ng of DNA template from *B. xylophilus* (BX1-BX6), *B. mucronatus* (BM1-BM4), *B. fraudulentus* (BF), *B. tusciae* (BT), or *Caenorhabditis elegans* (CE11) and using the *B. xylophilus*-specific primer set BXF and BXR and TaqMan probe BXT under optimized conditions. CK = control without DNA template. Representative data from 1 of 10 experiments are shown. To avoid overlapping and confusing symbols, the same symbol (-o-) was used for treatments that produced no fluorescence.

45 s at 60°C. The ABI Prism Sequence Detector Software (version 1.6) was used to collect and analyze data of normalized fluorescence (ΔRn) and cycle threshold (Ct). The ΔRn value represents a difference in fluorescence intensity between two cycles; Ct refers to the specific cycle number at which the ΔRn value becomes greater than the base line. The Ct value is inversely proportional to the log of the initial template of DNA and has been shown to be the most reliable parameter to detect and quantify target DNAs with real-time PCR (13). Triplicates were performed in each experiment, and each experiment was repeated at least three times.

Optimal conditions were determined by performing the real-time PCR with concentration gradients of primers (50 to 800 nM) and Mg^{2+} (1.0 to 4.5 mM) at different annealing and extension temperature (55, 58, 60, 61, 62, and 63°C). Conditions were considered optimal if they produced the smallest Ct values and the

most fluorescence. Ten nanograms of genomic DNA of *B. xylophilus* isolate BX2 was used for optimization of conditions.

Specificity and sensitivities of *B. xylophilus* real-time PCR assay. DNA templates (10 ng each) extracted from isolates of *B. xylophilus*, other *Bursaphelenchus* spp., and *C. elegans* were used to examine the specificity of primers and probe using the optimized PCR parameters. To insure the integrity of DNA, the primers F194 and 5368 (15) were used to amplify all extracts except that from *C. elegans*; amplification was successful in each case (data not shown), indicating that the DNA from all *Bursaphelenchus* spp. was capable of amplification.

To determine the minimum DNA detection threshold, 100 ng of genomic DNA of *B. xylophilus* (isolate BX2) was diluted serially (10-fold) until the target DNA could not be detected by real-time PCR. Triplicates of each concentration were amplified according to the optimal conditions described previously.

Fig. 2. Sensitivity of the real-time polymerase chain reaction assays for *Bursaphelenchus xylophilus* using **A**, a 10-fold dilution series of *B. xylophilus* (isolate BX2) genomic DNA or **B**, genomic DNA extracted from single nematodes of *B. xylophilus* or *B. mucronatus*. **A** includes three replications of each dilution, and Ct refers to the cycle number at which fluorescence first increased. **B** includes five individual nematodes of *B. xylophilus* (BX1-BX5) and one nematode of *B. mucronatus* (BM1). Representative data from one of three experiments (**A**) or one of six experiments (**B**) are shown. CK = control without DNA template. To avoid overlapping and confusing symbols, the same symbol (○) was used for treatments that produced no fluorescence.

An experiment was conducted to determine whether the assay could detect genomic DNA extracted from single nematodes. DNA was extracted from individual nematodes of five *B. xylophilus* isolates and one isolate of *B. mucronatus*; a control without DNA was included. Real-time PCR was conducted as described previously, and the experiment was repeated six times.

Application of real-time PCR to field samples. Three pieces (≈ 5 by 8 by 1.5 cm) of pinewood were collected from the trunks of symptomatic trees in each of four pine forests. The forests were located in Aobaodi (Zhejiang Province), Langxi (Anhui Province), Liyang (Jiangsu Province), and Nanjing (Jiangsu Province), China. In the laboratory, the phloem was removed and the xylem was cut into small blocks (≈ 1 to 2 mm). One sample from each forest (approximately 20 g of xylem blocks) was placed on a Baermann funnel at room temperature. Nematodes were recovered after 2 h.

From each sample, 10 to 20 nematodes were examined individually and identified to species based on morphology. One to four individuals of the same nematodes were then subjected to real-time PCR using the *B. xylophilus*-specific primers and probe. The remaining nematodes in each sample were collectively subjected to real-time PCR using the *B. xylophilus*-specific primers and probe.

RESULTS

Primers and TaqMan probe design. PCR amplification of the rDNA-ITS region from *B. xylophilus* and *B. mucronatus* generated products 784 bp long. Nucleotide sequences of PCR products from five isolates of *B. xylophilus* were aligned; no substitutions were found (100% similarity). Nucleotide sequences of PCR products from five isolates of *B. mucronatus* also were aligned; 26 substitutions were detected (99.7% similarity). The pair-wise comparison of *B. xylophilus* and *B. mucronatus* sequences revealed 119-bp substitutions among 784-bp nucleotides (84.8% similarity). The region unique to *B. xylophilus* and conserved across the five isolates of *B. xylophilus* was selected for the development of species-specific primers and a TaqMan probe reporter. The forward primer (BXF) and reverse primer (BXR) were 5'-GATGATGCGATTGGTGACT-3' (35 to 53 bp) and 5'-AACGACGCGAATCGAACC-3' (75 to 92 bp), which amplify the sequence

from 54 to 74 bp in the ITS1 region. The TaqMan sequence-specific probe (BXT) was 5'-CGGTTGCCGCGCATGATGG-3' (55 to 73 bp) and labeled at the 5' end with fluorescent reporter dye 6-carboxy-fluorescein (FAM) and at the 3' end with quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) (TaKaRa Bio Inc., Shiga, Japan).

The normalized fluorescent intensity of the reporter dye (ΔRn) was plotted against cycle number to graph PCR amplification and to determine the Ct. The smallest Ct values (and maximum fluorescence) were obtained with 400 nM forward primer, 400 nM reverse primer, 3.0 mM Mg^{2+} , and an extension temperature of 60°C (data not shown).

Specificity and sensitivity of TaqMan PCR primers and probe. The specificity of the primers and dual-labeled fluorescent probe was examined with genomic DNA extracted from six isolates of *B. xylophilus*, four isolates of *B. mucronatus*, and one isolate each of *B. fraudulentus*, *B. tusciae*, and *C. elegans*. PCR was conducted with 40 thermal cycles and the optimized conditions. All DNA extracts from *B. xylophilus* produced detectable fluorescence, whereas the DNA extracts from other nematodes produced no detectable fluorescence (Fig. 1).

The sensitivity of the real-time PCR assay was examined with a 10-fold dilution series of 100 ng of *B. xylophilus* DNA and with DNA extracted from single nematodes. The PCR assay detected DNA template concentrations as low as 0.01 ng (Fig. 2A). The Ct values were correlated with the DNA template concentration ($R^2 = 0.996$), indicating the validity of the assay and its potential for quantification of target DNA. The PCR assay also detected DNA from single specimens of *B. xylophilus* (Fig. 2B).

Application of real-time PCR to field samples. Specimens of *Bursaphelenchus* spp. were recovered from all four samples of field-collected pinewood. Based on morphology, *B. xylophilus* but no other *Bursaphelenchus* sp. was present in two samples from Nanjing and Langxi, and both *B. xylophilus* and *B. mucronatus* were present in samples from Aobaodi (72% *B. xylophilus* and 23% *B. mucronatus*) and Liyang (64% *B. xylophilus* and 26% *B. mucronatus*). Bacterial-feeding nematodes were also found in all four samples. Genomic DNA from individual nematodes morphologically identified as *B. xylophilus* produced detectable fluorescence but genomic DNA from individual nematodes mor-

Fig. 3. Detection of *Bursaphelenchus xylophilus* in field samples using real-time polymerase chain reaction. Data are from individual nematodes of *B. xylophilus* (BX7-BX10) and *B. mucronatus* (BM5 and BM6), and from bulked nematode samples (MX1-MX4) recovered from infested pinewood from Aobaodi (MX1), Langxi (MX2), Liyang (MX3), and Nanjing (MX4). The bulk samples contained 600 to 1,000 nematodes with the following percentages of *B. xylophilus* and *B. mucronatus*: 72 and 23% (MX1); 96 and 0% (MX2); 94 and 0% (MX3); and 64 and 26% (MX4). *Caenorhabditis elegans* (CE11) was included as a non-*Bursaphelenchus* spp. control. CK = control without DNA template. To avoid overlapping and confusing symbols, the same symbol (-o-) was used for treatments that produced no fluorescence.

phologically identified as *B. mucronatus* did not (Fig. 3). The bulked nematode samples from Nanjing, Langxi, Aobaodi, and Liyang contained 600, 920, 1,001, and 780 nematodes, respectively. All of these bulked samples contained *B. xylophilus* and all produce detectable fluorescence (Fig. 3).

DISCUSSION

Long-distance transmission of *B. xylophilus* probably occurs with transportation of wood products including infested logs, packing boards, and wood chips (9,10). Rapid and accurate detection of the nematode in these products is critical to prevent nematode introduction and is the basis for quarantine regulation (1,16). Using morphology to detect *B. xylophilus* in pinewood is difficult because *B. xylophilus* is morphologically similar to related but nonpathogenic species. Molecular techniques such as DNA hybridization, restriction enzyme reactions, and PCR based on species-specific DNA fragments have been successfully used for detection and identification of *Bursaphelenchus* species, especially *B. xylophilus* and *B. mucronatus* (4,12,15–17,21,22,32,34). But a major limitation of these previously described methods is that they are time-consuming, requiring at least 2 h per determination.

The real-time PCR assay developed in this study saves time because it is largely automated, it enables simultaneous analysis of up to 96 samples, and it requires no post-PCR gel electrophoresis. The fluorescent PCR technology also yields more robust results than other PCR methods (29) when small amplicons of 20 to 100 bp in length are to be analyzed. Moreover, the assay was specific for *B. xylophilus* (it did not amplify DNA from other *Bursaphelenchus* species), was sensitive (it detected 0.01 ng of template DNA), and detected *B. xylophilus* obtained from field-collected pinewood.

Although other nematodes present in pinewood could conceivably interfere with detection of *B. xylophilus* by real-time PCR, the diversity of nematodes in pinewood is limited. Only a few nematode species (*B. xylophilus*, *B. mucronatus*, and bacterivores) were found in our field samples, and the real-time PCR assay detected *B. xylophilus* in these bulk samples. For quarantine determinations, the testing of bulk nematode samples will certainly be more efficient than testing of individual nematodes.

The efficiency of the real-time PCR assay could be increased if we could eliminate the need to recover nematodes from pinewood, i.e., if nematode DNA could be extracted directly from the wood rather than from recovered nematodes. We tried several times to do this but failed (data not shown). A new machine, however, will soon decrease the time needed to recover nematodes from pinewood (C. S. Pan, *personal communication*), and this will further increase the usefulness of the real-time PCR assay.

ACKNOWLEDGMENTS

We thank S. Y. Chen, S. D. Li, R. Ma, and three anonymous reviewers for useful suggestions; W. M. Ye for providing DNA samples of *Bursaphelenchus* spp.; M. S. Lin, H. Xie, X. Y. Cheng, and L. F. Wang for providing nematode cultures; J. W. Zheng for providing an important reference; and the Caenorhabditis Genetics Center at the University of Minnesota for providing *Caenorhabditis elegans*. This research was supported by Chinese Academy of Sciences (Project no. KSCX1-SW-13-02-04).

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