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Application of a real-time PCR method for the detection of pine wood nematode, *Bursaphelenchus xylophilus*, in wood samples from lodgepole pine

Isabel LEAL [1,*], Margaret GREEN [2], Eric ALLEN [1], Leland HUMBLE [1] and Michael ROTT [2]

[1] *Natural Resources Canada, Canadian Forest Service, Victoria, British Columbia, Canada, V8Z 1M5*
[2] *Centre for Plant Health, Canadian Food and Inspection Agency, Sidney, British Columbia, Canada, V8L 1H3*

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Summary – A real-time PCR polymerase chain reaction (real-time PCR) method was developed to detect and differentiate *Bursaphelenchus xylophilus* (pine wood nematode, PWN) from other wood-inhabiting nematode species. A primer set and a specific Taqman® fluorescent probe were designed to amplify target *B. xylophilus* heat shock protein 70 sequences. After optimization, this real-time PCR assay was shown to be highly specific and sensitive, detecting at least 0.005 ng of *B. xylophilus* genomic DNA, as well as DNA extracted from single nematodes. The practical application of this real-time PCR diagnostic method for the detection of *B. xylophilus* from actual wood samples of lodgepole pine (*Pinus contorta*, Dougl. var. *latifolia*) trees containing a heterogeneous population of nematodes, rather than pure cultures or individual nematodes, is demonstrated. This method works well in the presence of potential inhibitors associated with wood after Baermann extraction and thus eliminates the need to produce pure nematode samples through further culturing and/or isolation of nematodes with a high-power microscope.

Keywords – Baermann funnel, *Bursaphelenchus mucronatus*, Hsp70, locked nucleic acid, Taqman® probe.

Pine wilt disease was first noticed in Japan in the early 1900s but the pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, was not described as the causal agent until 1972 (Mamiya & Kiyohara, 1972). The nematode is vectored from an infested tree to a new host tree by longhorned beetles of the genus *Monochamus* (Coleoptera: Cerambycidae) (Mamiya & Enda, 1979; Kobayashi *et al.*, 1984; Lee *et al.*, 1990; Chen & Chao, 1998; Sousa *et al.*, 2001; Yang, 2004). Pine wilt disease has devastated pines and pine forests in Japan over the last century and has since spread to China, Korea and Taiwan (Kiyohara & Tokushige, 1971; Enda, 1988; Yang & Wang, 1989; Kang *et al.*, 2004). The first record of PWN in Europe was reported in Portugal (Mota *et al.*, 1999). Pine wood nematode is native to North America (Knowles *et al.*, 1983; Blakeslee *et al.*, 1987; Bowers *et al.*, 1992) with a single report of its presence in Mexico (Dwinell, 1993) but, in contrast to the Asian countries and Portugal, mortality due to pine wilt disease in North America is limited to exotic pine species (most notably European *Pinus sylvestris* L.) and artificial plantings such as Christmas tree farms and ornamental conifer plantings (Wingfield, 1983; Wingfield *et al.*, 1984; Bergdahl, 1988).

The international spread of PWN and its vectors occurs mainly through the movement of beetle-infested logs, untreated wood products and wood packaging material. Consequently, to prevent further spread and new introductions, PWN has been placed on the A1 list of the European Plant Protection Organization (EPPO) (Liebhold *et al.*, 1995; Evans *et al.*, 1996; Dwinell, 1997; Mireku & Simpson, 2002; http://www.eppo.org/QUARANTINE/listA1.htm). Thus, accurate techniques for the detection and identification of PWN may be required in order to comply with quarantine regulations and to prevent the movement of PWN between countries.

Traditionally, diagnosis of *B. xylophilus* has been based on morphological characters. However, the morphological similarity of adult PWN to *B. mucronatus* (Mamiya & Enda, 1979) and the inability to distinguish the juvenile stages of *B. xylophilus* make accurate morphological identifications difficult. The presence of a mucron on the tails of mature female *B. mucronatus* is the primary

* Corresponding author, e-mail: ileal@pfc.cfs.nrcan.gc.ca

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character used in distinguishing this nematode from *B. xylophilus.* However, specimens from a less pathogenic form of *B. xylophilus* have a mucronate tail (Wingfield *et al*., 1983), and a structure similar to a mucron has also been observed on adult females of *B. xylophilus* (Iwahori *et al*., 2000). Thus, based on morphology alone, it is difficult to differentiate between these two species. Molecular detection tools, which are simple, rapid, and reliable, can be used to determine with certainty the presence of this nematode in wood.

To distinguish between *B. xylophilus* and *B. mucronatus*, multiple molecular techniques have been developed: PCR-RFLP (Hoyer *et al*., 1998; Braasch *et al*., 1999; Iwahori *et al*., 2000; Burgermeister *et al*., 2005); RAPD techniques (Braasch *et al*., 1995; Irdani *et al*., 1995, Wang *et al.*, 1999), PCR-based diagnostics with species-specific primers based on: intergenic (IGS) sequences (Kang *et al*., 2004), internal transcribed spacer (ITS) regions (Matsunaga & Togashi, 2004), satellite DNA (Castagnone *et al*., 2005), heat shock protein 70 (Hsp70) (Leal *et al*., 2005), ITS nested PCR (Takeuchi *et al*., 2005); and a real-time PCR assay based on ITS sequences (Cao *et al*., 2005). Real-time PCR offers an advantage over conventional PCR in that it is generally more sensitive and it is less time-consuming (no post-PCR processing, such as electrophoretic separation and staining, which helps to reduce potential contamination issues).

In this paper we report a real-time PCR assay with a primer set and a Taqman® fluorescent probe designed to amplify and detect a region of the Hsp70 gene sequence specific to *B. xylophilus*. This region was used previously for the development of a conventional PCR method to detect and differentiate PWN (Leal *et al*., 2005).

Materials and methods

Nematode isolates

Pure cultures of five *B. xylophilus* isolates (US-DE-2, USA; Ne15, Canada; Q52-A, Canada; Ne12, China; PT-3 w, Portugal) were used in this study. In addition, two isolates of *B. mucronatus* (DE-30w and DE-18w, Germany), and one isolate of *B. conicaudatus* (Ne5b), *B. doui* (Ne26), *B. fraudulentus* (DE-10w), *B. singaporensis* (Ne7), *B. hofmanni* (DE-6w) and *B. thailandae* (Kr-2w) were used as controls in this study. The first five of these seven species belong to the *xylophilus*-group (Braasch, 2001; Metge *et al*., 2006). Isolates were grown on a lawn of *Botrytis cinerea* cultured on 2% MEA (malt extract agar) plates at room temperature for 1 month, and subsequently at 10°C for another 2 months in the dark before sub-culturing.

DNA extraction

The method of Burgermeister *et al.* (2005) was used with the following changes: incubation of sample homogenate was performed at 56°C overnight, instead of 3 h, and carrier RNA was only used when DNA was extracted from single nematodes. Elution buffer (10 mM Tris/HCl, pH 8.0) was applied to the membrane of the dried mini-column and incubated for 5 min prior to centrifugation of the sample to elute the DNA. Samples were eluted in 30 μl (for single nematodes), and 50 μl (for the rest of samples). Nucleic acid extracts were heated at 55°C for 5 min to remove any residual ethanol that could later affect the measurement of DNA quantity and quality as well as PCR amplification. The purity, quality and concentration of nucleic acids were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Design of species-specific primers

Hsp70 primers and Taqman® probe design

Specific primers and Taqman® probe were designed to the *B. xylophilus* and *B. mucronatus* Hsp70 sequence alignment, showing nucleotide differences between these two species (Leal *et al*., 2005), by using Beacon Designer 4 (Premier BioSoft International, Palo Alto, CA, USA). The addition of locked nucleic acids (LNAs) in all three sequences was performed using the LNA design tool available from Integrated DNA Technologies, IDT (http://www.idtdna.com/analyzer/Applications/lna/). Primes and probes were obtained from IDT (Integrated DNA Technologies, Coralville, IA, USA). The Taqman® probe was dual-labelled: at the 5′ end with fluorescent reporter dye (6-carboxy-fluorescein, FAM) and at the 3′ end with a dark quencher dye (Iowa Black, FQ). The sequences of the forward and reverse primers are: 5′-TAAGATGTC+-TTTT+AC+AGATGC+CAAG-3′ and 5′-GCC+TGGACGAC+CTTGAAT-3′, respectively. The probe sequence is 5′-FAMAT+TGG+CCGCAAATT+CGA+TGAA+CC IAblkFQ-3′. The + sign indicates and follows the nucleotide that is modified by a LNA.

REAL-TIME PCR AMPLIFICATION

Real-time PCR conditions for Hsp70 target

Real-time PCR was conducted in a 20 μl reaction volume containing 5 μl of template, 50 mM Tris (pH 8.3), 0.25 mg ml^{-1} non-acetylated BSA (Sigma), 0.1 μM probe, 0.7 μM forward primer, 0.5 μM reverse primer, 0.4 mM dNTPs (Roche Diagnostics Canada, Québec, Canada), 5.0 mM $MgCl_2$ and 1.0 unit FastStart *Taq* DNA polymerase (Roche Diagnostics Canada). PCR amplification was carried out using the following parameters: an initial denaturation and activation of the FastStart *Taq* polymerase at 95°C for 10 min followed by 45 cycles of amplification, with each cycle consisting of denaturation at 94°C for 5 s, annealing at 62°C for 20 s, and extension at 72°C for 10 s. The Roche LightCycler 1.5 version 3.5 software was used for all real-time PCR amplifications.

Real-time amplification using dUTP and degradation by UNG

Real-time PCR incorporating dUTP preceded by treatment with a heat-labile uracil-N-glycosylase (UNG) was conducted in a 20 μl reaction volume containing 5 μl template, 50 mM Tris (pH 8.3), 0.25 mg ml^{-1} BSA, 0.1 μM probe, 0.7 μM forward primer, 0.5 μM reverse primer, 5.0 mM $MgCl_2$, 0.8 mM dUTP, 0.4 mM dATP, 0.4 mM dGTP, 0.4 mM dCTP (Roche Diagnostics Canada), 0.25 U UNG (Roche Diagnostics Canada) and 1.0 unit FastStart *Taq* DNA polymerase (Roche Diagnostics Canada). PCR amplification was carried out as described above with the addition of an initial 30°C incubation step required for the degradation of any uridine-containing carryover products from previous PCR amplifications. The following parameters were used: 30°C for 30 min, 95°C for 10 min, followed by 45 cycles of 94°C for 5 s, 62°C for 20 s and 72°C for 10 s.

Specificity and sensitivity of B. xylophilus*-specific real-time PCR assay*

To examine the specificity of the real-time PCR assay, DNA samples (5 ng) of *B. xylophilus* and seven other *Bursaphelenchus* species were subjected to the optimised PCR parameters. To determine the sensitivity of the assay, we carried out a ten-fold serial dilution of *B. xylophilus* genomic DNA over the range of 50-0.005 ng. Six replicates of each concentration were amplified using the optimal parameters of the real-time PCR assay. To determine if the total amount of DNA extracted from a single *B. xylophilus* nematode was sufficient for amplification, and detectable within the linear range of this assay, DNA was extracted from eight individual nematodes that had been isolated previously from pure cultures. DNA was extracted from the single nematodes as described above eluting in 30 μl ultra-pure water instead of Tris buffer. This final extract was dried down using a speed vac (vacuum concentrator) and the final pellet resuspended in 5 μl 10 mM Tris (pH 8.0). The entire 5 μl volume was used as template in subsequent real-time PCR amplification according to the conditions and parameters described above. In addition, DNA was also extracted from samples of cultured *B. xylophilus* containing ten or 100 counted and pooled nematodes. Ten-fold dilutions of these extracts were then used as template and amplified to determine if a diluted DNA template, theoretically equivalent to a single nematode or ten nematodes, would amplify similarly to a sample template containing the total amount of DNA extracted from a single nematode.

Hsp70 real-time PCR assay application to field samples

The Baermann extraction method was used to recover nematodes from wood samples of lodgepole pine trees as described previously (Leal *et al*., 2005). Prior to DNA extraction, the sample vial consisting of nematodes in 20 ml of water was concentrated by centrifugation at 17 000 *g* for 5 min. The bulk of the supernatant was removed and the samples spun again at 17 000 *g* for 5 min to further concentrate the nematodes by removing all but the last 1-1.5 ml of fluid. The pellet containing the nematodes was re-suspended in the remaining supernatant and transferred to a 1.5 ml microcentrifuge tube, and was pelleted again at 12 000 *g* for 5 min. The resulting supernatant was removed by careful pipetting, leaving the pellet containing the nematodes with no more than 100 μl of solution remaining. This solution was used for DNA extraction and real-time PCR analysis.

Results

PRIMERS AND TAQMAN® PROBE DESIGN

The *B. xylophilus* Hsp70 gene was selected as the target sequence for the design of a specific set of real-time PCR primers and Taqman® probe which amplify and detect a 102-bp fragment from this gene sequence. Based on previous sequence alignments (Leal *et al*.,

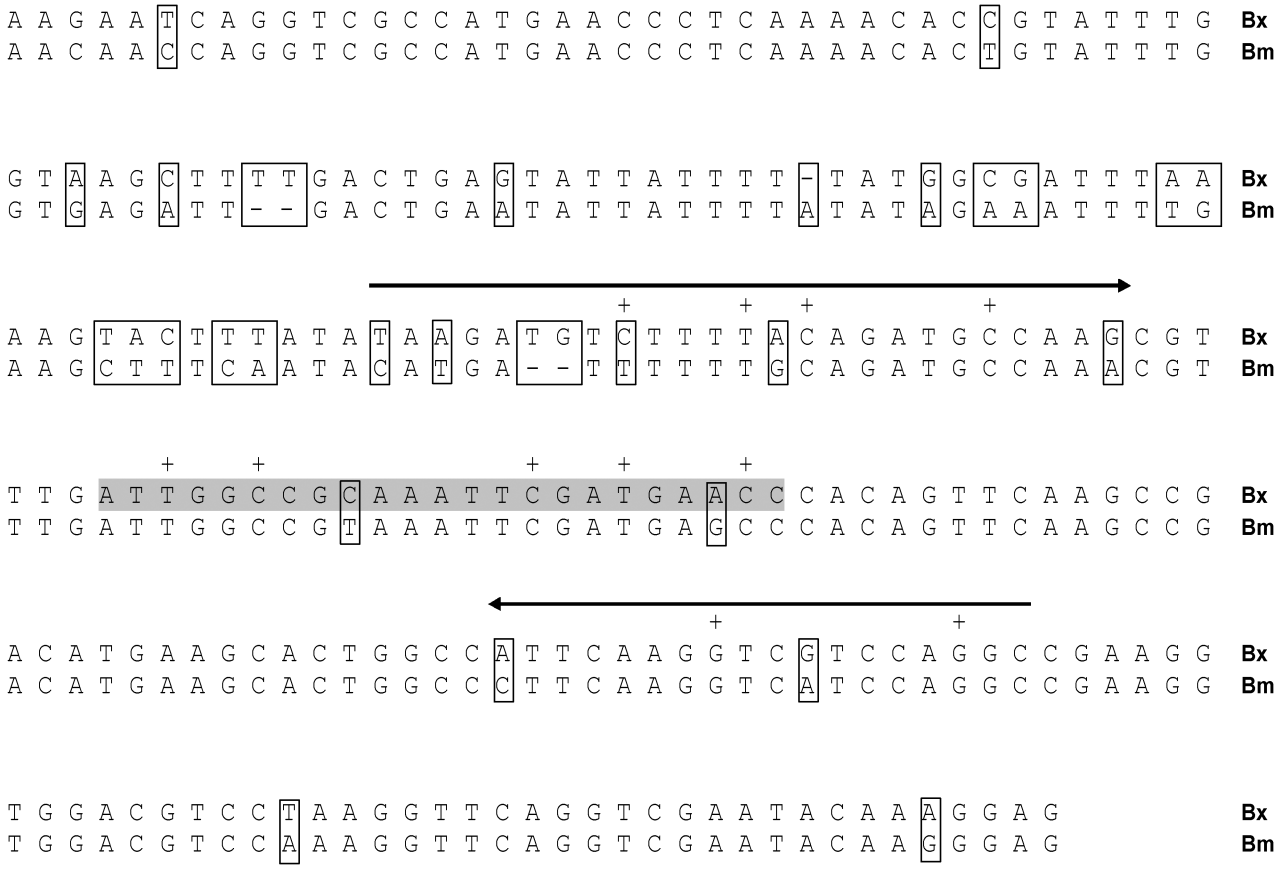

Fig. 1. *Alignment of Hsp70 consensus sequences from* Bursaphelenchus xylophilus *(Bx) and* B. mucronatus *(Bm) isolates. The primer set annealing sites are represented by arrows above the alignment (forward: Bx F and reverse: Bx R). The shaded portion of the Hsp70 sequence represents the annealing site of the Taqman® probe. The symbol '+' indicates the position of incorporated locked nucleic acids. Nucleotide sequence differences between* B. xylophilus *and* B. mucronatus *are boxed. Dashes indicate gaps in the corresponding sequence and were added in for the alignment.*

2005) this region was chosen since it was both readily available and showed a significant amount of sequence diversity. However, regions that were most divergent between these two species were also more AT rich and, as a result, the design of conventional primers and probes to these sequences with acceptable melting temperatures (T_m) resulted in oligonucleotides of excessive length and poor specificity (unpubl.). To rectify this situation, locked nucleic acid nucleotides were incorporated into the design of the primers and probe to decrease length, increase T_m and, as a result, increase specificity (see Fig. 1). The LNA base is a bicyclic RNA analogue that increases the thermal duplex stability, and improves the specificity of probe hybridisation to its target sequence with higher T_m (Demidov, 2003).

Specificity and sensitivity of real-time PCR assay, and amplification of single PWN

Potential problems encountered with PCR inhibitors present in wood were reduced by extracting DNA using a micro version of the silica-based membrane adsorption method (Burgermeister *et al.*, 2005). DNA extraction using this method was found to be an improvement in terms of eliminating PCR inhibitors present in wood to the one used previously with magnetic beads (Leal *et al.*, 2005; data not shown). The specificity of the assay was confirmed using DNA extracted from five isolates of *B. xylophilus* (Bx), two isolates of *B. mucronatus* (Bm), and one isolate of each of the following species: *B. doui* (Bd), *B. fraudulentus* (Bf), and *B. singaporensis* (Bs), *B. hofmanni* (Bh), *B. thailandae* (Bt) and *B. conicaudatus*

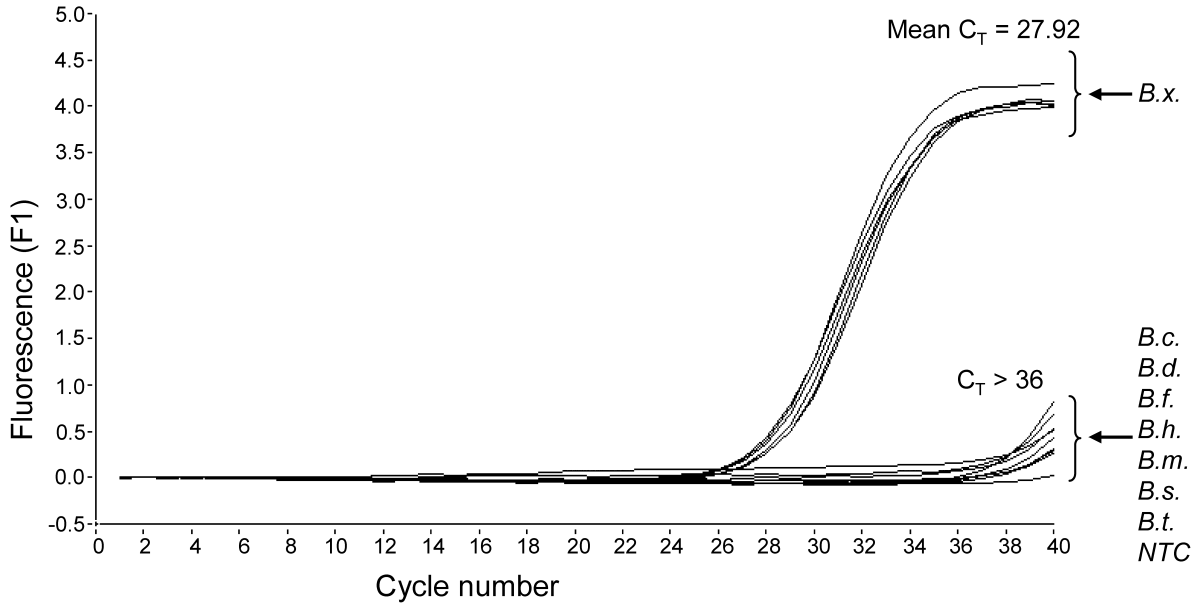

Fig. 2. *Amplification plot of real-time PCR assay showing specificity for detection of* Bursaphelenchus xylophilus*. The amplifications were carried out using 5.0 ng of DNA template from five isolates of* B. xylophilus *(Bx): Bx(2), PT3, Q52, Ne 15, and Ne 12, two isolates of* B. mucronatus *(Bm): DE 30, and DE 18, and one of* B. conicaudatus *(Bc): Ne5b;* B. doui *(Bd): Ne26;* B. fraudulentus *(Bf): DE10;* B. singaporensis *(Bs): Ne7;* B. hofmanni *(Bh); DE6w;* B. thailandae *(Bt): Kr2w, and NTC (no template control).*

(Bc). DNA from all *B. xylophilus* isolates could be amplified, whereas DNA extracted from all other seven species tested failed to give an amplification signal (cycle threshold value (C_T) $>$ 36), as shown in Figure 2. This figure shows single replicates, but the assay was performed in duplicate for all samples. As an amplification control, the quality of the purified nematode genomic DNA used in this test was confirmed using non-species-specific ITS primers (Burgermeister *et al.*, 2005), which resulted in the positive amplification from all *Bursaphelenchus* species (data unpubl.).

Sensitivity of the real-time PCR assay was evaluated using a ten-fold dilution series of *B. xylophilus* genomic DNA, starting with a final template amount of 50 ng down to a lower limit of 0.005 ng. A standard curve was plotted from the linear regression of logarithmic amount *B. xylophilus* DNA *vs* the C_T values as shown in Figure 3. The reaction is linear over a dynamic range of 50-0.005 ng with a correlation coefficient, $r = -1.00$, which represents six replicates of each dilution. The PCR efficiency of the assay, calculated from the slope of the linear regression curve using the equation $E = 10^{-1/\text{slope}}$, was 1.92 or 92%. The lowest amount of DNA analysed, 0.005 ng, resulted in amplification in all six replicates and was therefore defined as the limit of detection (LOD) for this assay. Data shown in Table 1 represents three independent experiments and demonstrate the excellent repeatability and reproducibility of this assay. All reactions containing *B. xylophilus* template DNA amplified a fragment of the correct size (102 bp), determined by agarose gel electrophoresis (unpubl.).

As this assay may be used as a diagnostic tool, it is important to prevent potential contamination in the form of carryover from previously PCR-amplified material. We included an enzymatic decontamination procedure based upon dUTP and uracil N glycosylase (UNG) to the Hsp70 real-time PCR assay. During real-time PCR, dUTP is incorporated into the PCR product and, before a subsequent amplification reaction, contaminating PCR products are degraded using UNG which is heat-labile and inactivated at the beginning of the amplification reaction (by heating at 95°C). Based on the construction of a standard curve, using six replicates of the same genomic DNA and concentrations as performed in Figure 3, we observed that with the inclusion of this assay modification there was a decrease in PCR efficiency ($E = 1.82$) and a 10-fold decrease in the sensitivity.

We have also shown that this real-time PCR assay is sensitive enough to detect a single nematode. This result

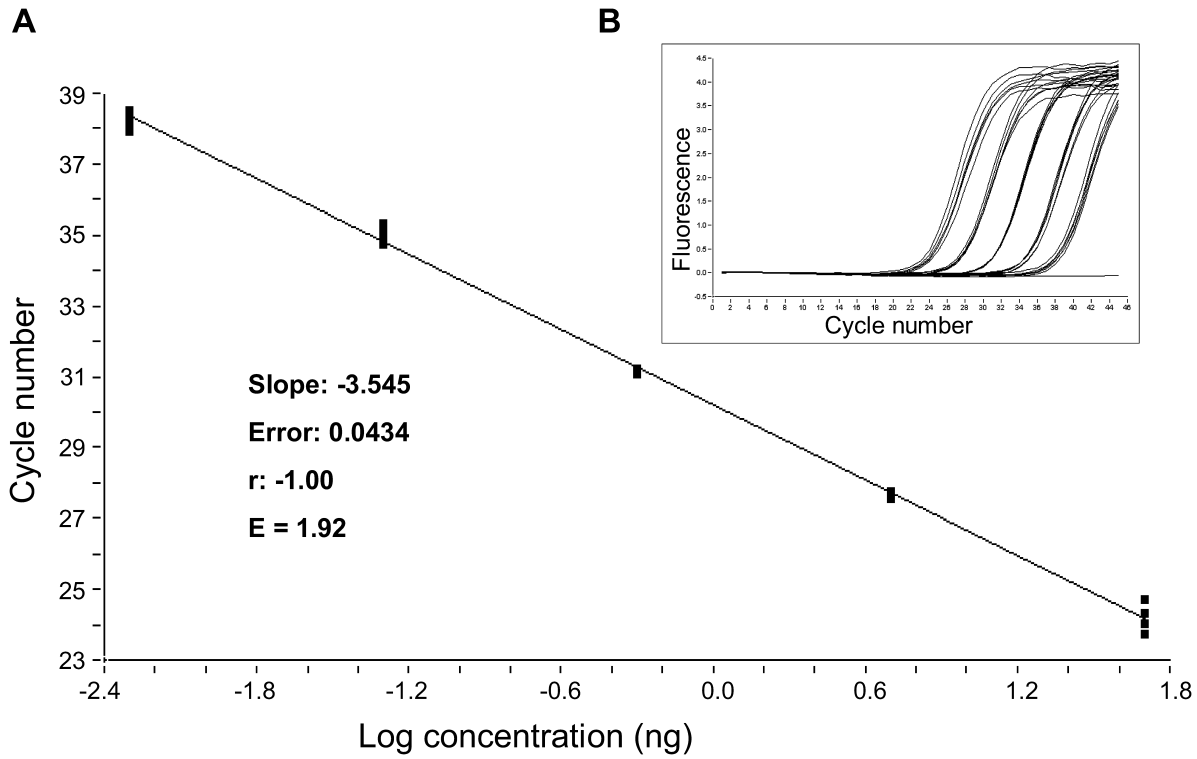

Fig. 3. *Sensitivity and linearity of the real-time PCR assay. A: Standard curve of threshold cycle number plotted against the log of the initial template concentration in nanograms. The ten-fold serial dilutions are linear over five logs. Data represent six replicates of each dilution. Efficiency (E)* $= 10^{-1/slope}$*; B: Amplification plot of same experiment showing the ten-fold dilution series of DNA concentrations (50.0-0.005 ng) replicated six times. No amplification was observed in control reactions that did not contain* Bursaphelenchus xylophilus *genomic DNA.*

Table 1. *Reproducibility of real-time PCR with Hsp70 primers and Taqman® fluorescent probe using* Bursaphelenchus xylophilus *genomic DNA in ten-fold dilution series (three independent experiments with replicates within experiments).*

B. xylophilus DNA (ng)	Mean C_T (±SD)			Overall mean C_T	SE	CV (%)
	Exp 1	Exp 2	Exp 3			
5	27.66 (0.07)	27.92 (0.06)	27.82 (0.10)	27.80	0.13	0.47
0.5	31.14 (0.05)	31.64 (0.01)	31.53 (0.09)	31.53	0.26	0.82
0.05	35.07 (0.22)	35.37 (0.12)	35.26 (0.06)	35.26	0.15	0.42
0.005	38.26 (0.23)	38.10 (0.01)	38.55 (0.07)	38.55	0.23	0.61

C_T, cycle threshold; SD, standard deviation; CV, coefficient of variation; SE, standard error.
Numbers in parentheses are SDs of replicates within experiments.
Six replicates of each DNA amount were used in Experiment 1, duplicates in Experiment 2 and triplicates in Experiment 3.
SE was calculated as the SD of the means from the three experiments.

was obtained by amplifying the total amount of DNA extracted from single *B. xylophilus* nematodes. The mean C_T value of individual single nematodes was 33.7 with a standard deviation of 0.77 (Fig. 4). As a comparison, we carried out ten-fold serial dilutions of DNA extracted from 100 and ten isolated *B. xylophilus* nematodes from pure cultures. The serial dilutions corresponding to ten and one *B. xylophilus* nematodes were amplified in four

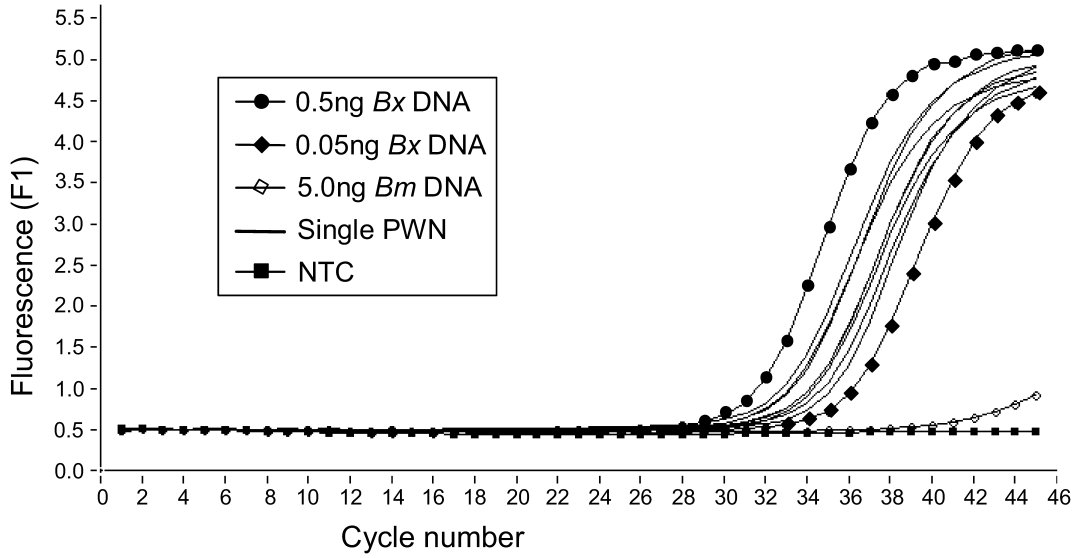

Fig. 4. *Real-time PCR amplification plot showing amplification curves of eight different reactions using DNA from single* Bursaphelenchus xylophilus *(Bx) nematodes (solid lines) as template flanked by 0.5 ng and 0.05 ng of purified* B. xylophilus *DNA as template (circle and square line points), respectively. A single reaction using 5.0 ng of purified* B. mucronatus *(Bm) DNA (open diamond line points) was also performed. One microgram of carrier RNA in a NTC (squared line points) was included as a negative control.*

replicates (for each dilution). Mean C_T values for the ten and one nematodes, prepared by dilution, were 30.48 and 33.46, respectively. These results demonstrated that diluted templates prepared from DNA extractions of larger numbers of nematodes amplified similarly as those templates prepared from single nematodes.

Evaluation of field sample extracts for potential PCR inhibition

DNA extracted from several field samples, which tested negative using the Hsp70 real-time PCR assay, were subsequently used as PWN-free field sample negative PCR controls. Twenty ml of water collected using the Baermann funnel from this wood was concentrated, DNA extracted, and eluted into a final volume of 50 μl. To determine whether this extract contained PCR inhibitors, 5 μl from the 50 μl were spiked into samples containing 5 ng of purified *B. xylophilus* DNA. The spiked samples were amplified, and compared to the amplification of the non-spiked negative control extracts as well as to the amplification of 5 ng purified control *B. xylophilus* DNA only. All reactions were performed in duplicate. Amplification of both spiked and purified *B. xylophilus* DNA control reactions, shown in Figure 5, resulted in comparable C_T values (27.81 and 27.81 for duplicate spiked reactions and 27.77 for purified *B. xylophilus* DNA control reaction). Based on the similar C_T values and shapes of the individual amplification curves from both spiked and non-spiked reactions, no PCR inhibition due to wood extracts was observed. No amplification was observed for those reactions corresponding to the negative field sample extracts alone.

Real-time PCR assay using lodgepole pine wood samples

The sensitivity of the real-time PCR assay was evaluated using wood sampled from dead and dying lodgepole pine trees attacked by mountain pine beetle, *Dendroctonus ponderosae* Hopkins. Four trees were selected for testing, and three out of the four samples produced detectable fluorescence and amplification (Fig. 6). The identities of the amplified fragments were confirmed to be the expected *B. xylophilus* Hsp70 gene sequences by nucleotide sequence analysis (unpubl.).

Discussion

Real-time PCR is a powerful analytical tool that can be used to detect the presence of an invasive species in

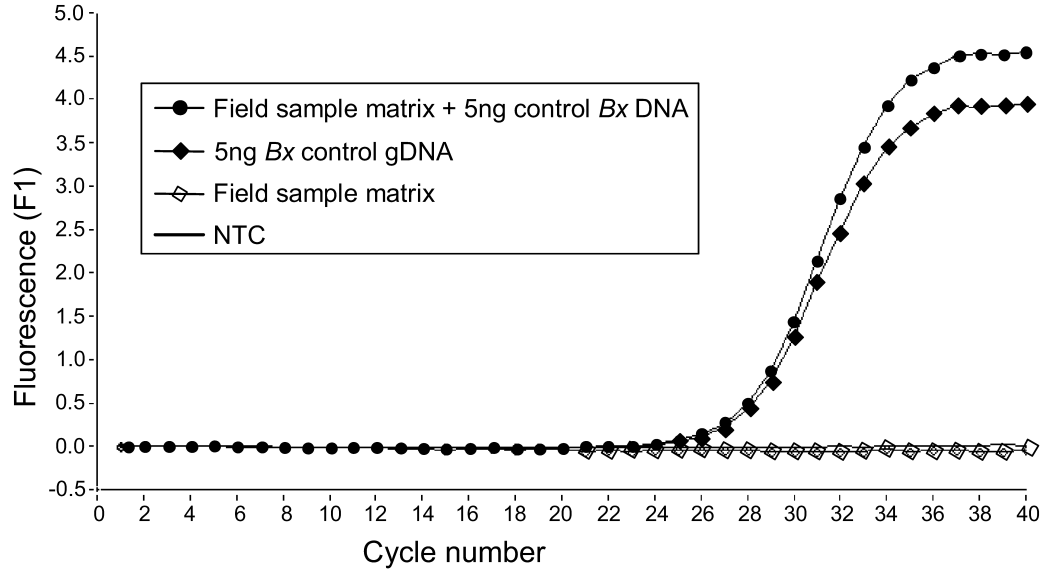

Fig. 5. *Evaluation of field sample extracts for potential PCR inhibition. Five microlitres of DNA extracts purified from Baermann funnel extracts of wood known to be free of* Bursaphelenchus xylophilus *(Bx) (representing the sample matrix) were PCR amplified in a reaction containing 5.0 ng of* B. xylophilus *DNA. PCR amplifications were compared to control reactions containing 5.0 ng of purified* B. xylophilus *DNA only. Additional controls were field sample matrix only and NTC (no template control).*

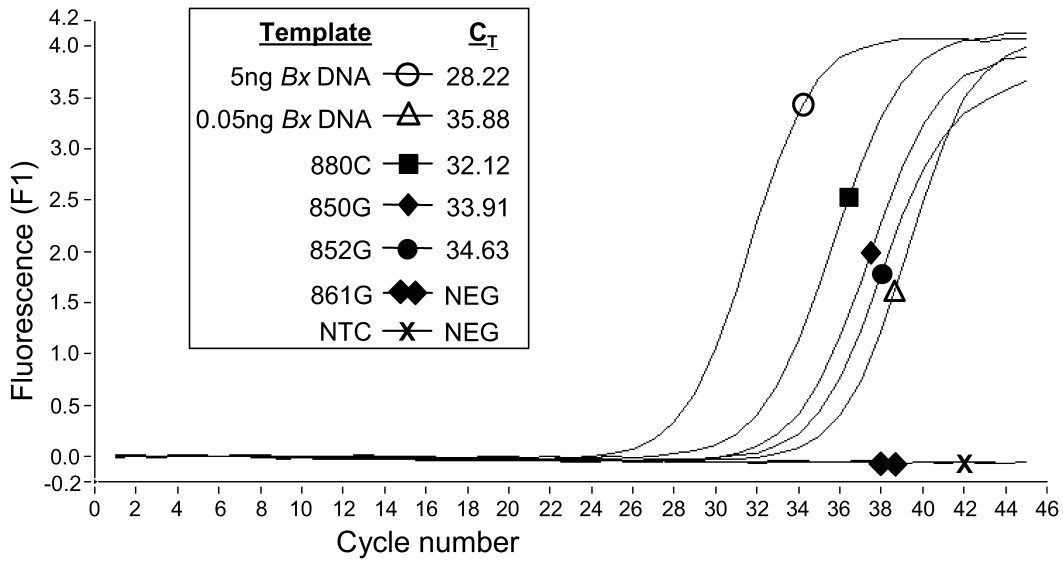

Fig. 6. *Real-time PCR amplification of PWN nematodes extracted from wood samples flanked by* Bursaphelenchus xylophilus *(Bx) genomic DNA standards (5.0 ng and 0.05 ng) using Hsp70 primers and Taqman® fluorescent probe. Four trees were selected for testing, and three (880C, 850G and 852G) produced detectable fluorescence while the fourth (861G) gave no detectable fluorescence (NEG). Lines are identified by symbols given in key. Five microlitres of undiluted field sample DNA extract was used as template. (C_T = cycle threshold value; NTC = no template control.)*

complex biological environments such as wood. Detection of a single PWN from among a spectra of other nematode species in wood requires an assay that is sensitive, robust in the presence of potential PCR inhibitors, and that can discriminate *B. xylophilus* from closely related species. Real-time PCR performed with hybridisation probes introduces an additional level of specificity over conventional PCR. With real-time PCR, the risk of contamination is reduced because it is a closed tube system and does not require post-PCR manipulation (agarose gel electrophoresis) for routine analysis, making it less time consuming than conventional PCR. In addition, real-time PCR is more suitable for automation, allowing for high-throughput analyses.

The introduction of *B. xylophilus* most likely occurs *via* forest products, including logs (barked and debarked), and packaging wood material carrying the *Monochamus* vector (Liebhold *et al*., 1995; Evans *et al*., 1996; Dwinell, 1997). To prevent the introduction of PWN into new host ranges, and to abide by quarantine regulations, it is critical to detect PWN efficiently and accurately. Both *B. xylophilus* and *B. mucronatus* are found in pine wood and are morphologically very similar. As a result detection of *B. xylophilus* using morphology alone is difficult and requires highly trained personnel for accurate identification. Molecular techniques using conventional PCR to detect and differentiate *B. xylophilus* with species-specific primers targeting ribosomal RNA regions (Kang *et al.*, 2004; Matsunaga & Togashi, 2004; Takeuchi *et al*., 2005), satellite DNA (Castagnone *et al.*, 2005) and the Hsp70 gene (Leal *et al*., 2005) have been developed.

The Hsp70 gene has been used previously as a target sequence to distinguish nematode species (Nikolaidis & Scouras, 2002). Although the Hsp70 gene is present in fewer copies compared to ribosomal RNA genes and satellite DNA in the *Bursaphelenchus* genome, the Hsp70 real-time PCR assay reported here is a very robust, sensitive and species-specific assay. In order to accomplish this, it was necessary to modify the real-time PCR primers and probe by incorporating LNAs to ensure that these oligonucleotides meet acceptable criteria for melting temperatures, length, and specificity.

The Hsp70 real-time PCR assay was shown to be specific for *B. xylophilus* with no amplification observed for the seven other non-target *Bursaphelenchus* species, including five species of the *xylophilus*-group (Braasch, 2001; Metge *et al*., 2006). The sensitivity of the assay was very good, with a LOD of 0.005 ng of genomic DNA, easily sensitive enough to detect DNA from individual nematodes. The enzymatic decontamination procedure using dUTP and UNG can have a decreasing affect on PCR efficiency and sensitivity (Pang *et al.*, 1992). However, in this study, even though the sensitivity of the overall assay was reduced ten-fold by using this approach, the limit of detection and the linear range of the assay (50-0.05 ng) would still be low enough to detect a single nematode and, therefore, it would be suitable if required for diagnostic purposes. A real-time PCR assay targeting the ITS sequence of PWN developed by Cao *et al.* (2005), was also sensitive enough to detect single nematodes. In the present work, the Hsp70 real-time PCR assay detected *B. xylophilus* extracted from field-collected lodgepole pine by the Baermann extraction method. Since wood is known to contain PCR inhibitors (Lee & Cooper, 1995; Langrell & Barbara, 2001), it is critical for the molecular detection of PWN from field samples to use a DNA extraction protocol that removes potential PCR inhibitors co-extracted with wood, as these are unavoidably present when using the Baermann extraction procedure. There was no indication of PCR inhibition due to wood sample extracts. Cao *et al.* (2005) also detected PWN obtained from wood samples. However, in their assay nematodes were first isolated and separated under a microscope before DNA extraction. This increases the number of handling steps required, as well as reducing the demands on the PCR assay with respect to the complexity of the matrix being assayed. In particular, there is less potential influence on sensitivity due to PCR inhibitors or contaminating DNA from closely related nematode species. The fact that the Hsp70 real-time PCR was not influenced by potential inhibitors carried through the nucleic acid extraction procedure of the wood is demonstrated by the high PCR efficiency (92%) and good linearity ($r = 1.00$) reported here for the assay. Unfortunately, this linearity and efficiency could not be compared to the real-time PCR developed by Cao *et al.* (2005) because these authors did not report a standard curve or the PCR efficiency of their assay. A nested PCR-based method for detecting PWN from Japanese black pine, *Pinus thunbergii* Parl., was developed by Takeuchi *et al*. (2005). However, these authors detected PWN by adding single nematodes to very small samples of pine wood (80 mg) prior to DNA extraction. They did not use the Baermann funnel technique, which usually recovers nematodes from 30 g of wood, and thus their sampling method was limited.

Based on the results reported in this paper, a detection threshold for a presence/absence diagnostic test method

for the detection of *B. xylophilus* in wood samples was set. In ongoing studies to determine the pests and pathogens associated with lodgepole pines killed by the mountain pine beetle in British Columbia, Canada, a C_T value of 35 will be used as a conservative detection threshold for the positive identification of *B. xylophilus* in wood samples. This threshold is based on the mean cycle threshold value, $C_T = 33.75$ (± 0.77), determined from the amplification of single nematodes. However, when detecting PWN from field samples, it should be noted that if only one-tenth of the total DNA extract volume is tested (*i.e.*, 5 μl), a detection threshold of C_T 35 would indicate the presence of less than ten nematodes in the entire sample extract. This detection threshold is conservative as the likelihood of finding only ten nematodes in wood sampled from pine wilt diseased trees is very low. According to Mamiya (1983), the nematode population in the wood of mature trees is more than a 1000 nematodes/g of dry wood from 40-60 days after inoculation.

The Hsp70 real-time PCR assay works well with DNA extracted from nematodes recovered by Baermann extraction, bypassing the need to produce pure nematode samples through further isolation and/or culturing. In addition, this assay also has the potential for quantifying *B. xylophilus* DNA from wood samples and, thus, it can represent a very useful and practical method to detect the amount of *B. xylophilus* present.

For quarantine regulations, the testing of a mixed heterogeneous population of nematodes from wood samples after the Baermann extraction will certainly be more efficient than testing individual nematodes after culturing, and/or separating the nematodes. The Baermann funnel extraction method was used to recover nematodes from wood tissue and, by using this method, positive PCR results should indicate the presence of live nematodes, although it is possible that small amounts of DNA from dead nematodes could be eluted thorough the Baermann process. This possibility is currently under investigation. When molecular techniques are used for quarantine purposes to detect PWN in wood products, it is critical to make a distinction between live and dead nematodes. Other strategies to differentiate between live and dead nematodes could be investigated, such as the use of reverse transcription-PCR (RT-PCR). Detection of mRNA (messenger RNA) in Baermann funnel extracted field samples by RT-PCR would indicate the presence of live cells since mRNA is degraded quickly in dead cells.

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