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Detection of the pine wood nematode using a real-time PCR assay to target the DNA topoisomerase I gene

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Abstract A species-specific real-time PCR assay targeting the DNA topoisomerase I gene has been developed to detect *Bursaphelenchus xylophilus*. The specificity of the assay was confirmed by the lack of amplification of genomic DNA from other *Bursaphelenchus* or *Seinura* species. The sensitivity test showed that the limit of the reaction was 0.01 ng of genomic DNA or one individual nematode, as small as an egg. The validity of the real-time PCR assay was evaluated by analyzing mixed nematode samples extracted from diseased pine trees in which *B. xylophilus* was associated with several closely related species, *B. mucronatus*, *B. hofmanni*, *Aphelenchoides macronucleatus*, *S. lii* and *S. wuae*. These results demonstrate the potential of the assay to provide rapid, specific and sensitive molecular identification of *B. xylophilus* for use in pest risk assessment and quarantine regulations.

Keywords *Bursaphelenchus xylophilus* · Identification · Taqman probe

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Introduction

The pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is distributed in Canada, China, Japan, Korea, Mexico, Portugal and USA (Mamiya 1983; Mota et al. 1999). It causes rapid wilting of *Pinus thunbergii* and *P. densiflora* in East Asia and *P. pinaster* in Portugal Europe, which results in millions of dead pine trees every year (Mamiya 1983; Mota et al. 1999; Penas et al. 2004). Despite enormous impact on the pine forests, there is no efficient and environmentally friendly method available to prevent infestation. Rapid and accurate detection is critical to prevent the introduction of *B. xylophilus*, especially in pest free areas.

More than 70 *Bursaphelenchus* species are described worldwide, and their morphological identification requires a high level of expertise (Ryss et al. 2005). In order to effectively distinguish *B. xylophilus* from other species, such as *B. mucronatus* and *B. fraudulentus*, which share some important morphological features, molecular technologies based on conventional PCR (Harmey and Harmey 1993; Matsunaga and Togashi 2004; Castagnone et al. 2005) and RFLP analysis (Zheng et al. 2003; Burgermeister et al. 2005) have been used. However, restriction digestion of the DNA or time-consuming post-PCR detection of amplification products by gel electrophoresis is needed. Those disadvantages may limit high-throughput routine

testing. Instead, real-time PCR permits the monitoring of the reaction during the amplification process, eliminating the need for additional steps and thus reducing the risk of contamination. Coupled to automated product analysis software, the real-time PCR technique allows continuous monitoring of the sample during PCR using hybridization probes or dyes (Kubista et al. 2006). Real-time PCR techniques have been developed to detect plant-parasitic nematodes, including PWN (Cao et al. 2005; Holeva et al. 2006; François et al. 2007; Leal et al. 2007).

When real-time PCR assays were used to detect organisms including the PWN, primers were often derived from rDNA genes or their internal transcribed spacer (ITS) regions (Toyota et al. 2008). These genes are popular because of little intraspecific DNA sequence variability and multiple copies in a single genome (Borneman and Hartin 2000). However, rDNA is a highly conserved region and a risk of nonspecific amplifications may be involved (Bialek et al. 2000). Because of slightly less conservatism, ITS regions have been used as the most popular targets to develop the molecular techniques to identify organisms. However, this method also has limitations, due to the possible presence of different copies of the ITS within a single genome coupled with difficulties in the application of the ITS as a molecular marker. For example, Mulet et al. (2009) discovered that, in 29 out of 44 *Pseudomonas* type strains, two DNA bands were obtained using ITS1 primer. Similar, Fatehi and Bridge (1998) received more than one PCR product following analysis of the ITS region of *Ascochyta* spp.

Although not yet shown, the occurrence of several copies of ITS in a single PWN genome cannot be ruled out. Therefore, molecular techniques which target other functional genes than the rDNA genes and their ITS regions might be preferable especially for routine testing. The primary goal of the present study was to design and test primer pairs and a Taqman probe for real-time PCR amplification to identify *B. xylophilus* targeting the DNA topoisomerase I gene and also to evaluate the potential of the real-time PCR in diagnosis of plant quarantine nematodes.

Materials and methods

Nematodes Nematodes were cultured on *Botrytis cinerea* for 1 week at 25°C grown on autoclaved barley grains and extracted by Baermann funnels for 2 h at 25°C and washed three times with M9 buffer (42.3 mM Na_2HPO_4, 22 mM KH_2PO_4, 85.6 mM NaCl and 1 mM $MgSO_4$, pH 7.0) to remove any remaining *B. cinerea* mycelium (Kikuchi et al. 2004). Nematode isolates used in the paper are listed in Table 1.

DNA extraction DNA extraction from a large number of nematodes was performed as described in Smits et al. (1991) with the following changes: Nematodes were extracted by Baermann funnels, collected in 10 ml tubes and precipitated by centrifuging at

Table 1 Isolates and origins of *Bursaphelenchus* and other nematode species for real-time PCR

Species	Origin	Host
B. xylophilus		
	AN19# Unknown	China
JL1	Nanjing, Jiangsu, China	*Pinus massoniana*
ZF4	Fuyang, Zhejiang, China	*P. massoniana*
GD1	Dongguan, Guangdong, China	*P. massoniana*
GX3	Guilin, Guangxi, China	*P. massoniana*
AA1	Anqing, Anhui, China	*P. massoniana*
JG4	Ganzhou, Jiangxi, China	*P. massoniana*
GZZ2	Zunyi, Guizhou, China	*P. massoniana*
SG3	Guangan, Sichuan, China	*P. massoniana*
CZ1	Chongqi, China	Unknown
SC2	Changdao, Shangdong, China	*P. thunbergii*
TANG	Karatu, Japan	*P. thunbergii*
WYS	Fukuoka, Japan	*P. thunbergii*
ZHUI	Miyazaki, Japan	*P. densiflora*
GONG	Miyazaki, Japan	*P. thunbergii*
TIAN	Kumamoto, Japan	*P. thunbergii*
C14-5	Japan	Unknown
US7#	USA	Wood chips
B. mucronatus		
AN5#	Anhui, China	Unknown
12#	Japan	Wood chips
13#	Taiwan, China	Wood chips
14#	Korea	Wood chips
B. hofmanni		
SF2	Sichuan, China	Unknown
Seinura wuae		
Sw1	China	Unknown

1,500 g for 5 min. The supernatant was removed and the nematode pellet was transferred with water into an empty 1.5 ml tube and centrifuged again at 1,500 g at room temperature for 5 min. Excessive water was removed with a pipette. Nematode biomass was calculated by weighing the empty tube and the tube plus nematodes after centrifugation and removal of the supernatant. The nematode precipitate of about 100 mg was ground in a mortar with liquid nitrogen until a fine white powder was produced. The powder was collected and 1 ml of extraction buffer (10 mM Tris-Cl, pH=8.0, 1% SDS, 0.4 M NaCl, 5 mM EDTA) per 100 mg of initial nematodes was added. Then 50 μl of proteinase K (20 μg l^{-1}) per 1 ml of extraction buffer was added and the mixture was incubated for 30 min at 37°C, and then for 60 min at 65°C. The solution was twice extracted with equal volumes of phenol–chloroform–isoamylalcohol (25:24:1) and chloroform–isoamylalcohol (24:1). The DNA was precipitated from the final aqueous phase by adding two volumes of 96% ethanol and 0.1 vol of 3 M sodium acetate (pH 5.2). The purified pellet was resuspended in 50 μl of TE buffer (10 mM Tris-Cl, pH=8.0, 1 mM EDTA) and the nucleic acid concentration was measured in a spectrophotometer at 260 nm.

DNA was extracted from single nematodes at different development stages, including eggs, second-stage, third-stage and fourth-stage juveniles, females and males. Individuals were selected using a hollow glass capillary and transferred into 20 μl lysis buffer (50 mM KCl, 10 mM Tris, pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP 40, 0.45% Tween 20, and 1.6 mg ml^{-1} Proteinase K) in 1.5 ml Eppendorf tubes. Tubes were checked under the microscope to verify that each tube contained only one specimen. Tubes containing nematodes were placed at −80°C for 30 min, at 65°C for 1 h and at 95°C for 20 min, consecutively. Afterwards, the tubes were centrifuged at 16,000 g for 5 min. Finally, the DNA suspension was used for real-time PCR amplification.

DNA topoisomerase I gene and the target sequence of PCR primers A suppression subtractive hybridization (SSH) cDNA library had been constructed from cDNA of *B. xylophilus* isolate AN19#F1-1, which was driven by cDNA of *B. mucronatus* isolate AN5#F7-1 according to the manual of the PCR-select cDNA subtraction kit (Clontech, USA). The cDNA XTOP (189 bp) of the topoisomerase I gene (accession number FG589696) was generated from the cDNA library (data unpublished). 3′ RACE (rapid amplification of cDNA ends) of XTOP was synthesized using the First-Choice RLM-RACE Kit (Ambion, USA). The PCR amplification was primed by a gene-specific primer (3′TOPP: CAGTC CCAAT ATCTC GTGGT TTT) and the 3′RACE adaptor outer primer provided in the kit. The PCR product was sequenced and the sequence was submitted to GenBank database (accession number FJ238051). Primers TOPCF (5′TGGCC CTATA TTTCA TCGAC AA3′) and TOPCR (5′TCCTT GAGCC TGTCC ACTTT T3′) designed from 3′ RACE product sequence of XTOP were used to amplify the genomic DNA (gDNA) of *B. xylophilus* and a 1,078 bp DNA fragment was obtained. The amplification fragment contained an intron sequence of 1,008 bp (accession no. FJ238052). The intron region of the topoisomerase I gene was used as the target to develop the primers and Taqman probe.

PCR primers and Taqman probe set Based on the sequence of the intron region of the topoisomerase I gene, the primer pairs TOPO-F (5′ACCAT TCGGT TGGCT CTGTT3′)/TOPO-R (5′CCCTA AGGCG TCGGT GAAC3′) defined a 57-bp-long amplicon of *B. xylophilus* using the Primer Express software version 3.0 (Applied Biosystems, USA). The fluorogenic Taqman probe TOPO-P (5′ TAGCT GAGCA TCTTT T 3′) was designed to anneal to the 16 bp region between the primer pairs. The probe was labeled at the 5′ end with 6-carboxyfluorescein (FAM) as a reporter dye, and modified at the 3′ end with the quencher dye tetra-methylcarboxyrhodamine (TAMRA). The primers and Taqman probe were synthesized by Invitrogen Corporation (Shanghai, China). The primers and Taqman probe were used to detect gDNA of *B. xylophilus* by real-time PCR.

PCR assay Real-time PCR was performed as specified in the Taqman Universal PCR Master Mix (Applied Biosystems, USA) manual, with 1 μl of gDNA template, 0.5 μM forward and reverse primers and 0.5 μM Taqman probe in a total volume of 10 μl. The ABI Prism 7500 Real Time PCR System (Applied Biosystems, USA) was used for amplification and fluorescence measurement. Thermal cycling conditions consisted of 2 min at 50°C, 10 min at 95°C, then 40

cycles of 15 s at 95°C, 33 s at 55°C, followed by 33 s at 72°C. Blank lysis buffer or ddH_2O were included in each PCR run as negative controls. Triplicate reactions were performed in each assay, and each assay was repeated at least three times. Data were analyzed using the 7500 system software version 1.3.0 (Applied Biosystems, USA) according to the manufacture's instructions. The Ct values were calculated by using a threshold value of 0.2 and the automatic baseline setting. After the real-time PCR, the products were analyzed by electrophoresis on 4% agarose gels at 100 V for 30 min, stained with ethidium bromide (0.5 μg ml^{-1}) for 20 min, and visualized with a UV transilluminator and photographed.

In order to assess the interference of wood extract to the real-time PCR, 5 g of fresh nematode-free pine wood sample of *P. massoniana* and *P. thunbergii* was cut into small pieces, placed in a Baermann funnel, and loaded with 100 ml (20 fold volume) of distilled ddH_2O to submerge the wood piece at room temperature over night. 10 ml of the wood extract (Hws presents the wood extract of *P. thunbergii* and Mws presents the wood extract of *P. massoniana*) was collected using the Baermann funnel. A series of pine wood extracts (0.5 μl, 1 μl, 1.5 μl, 2 μl, 2.5 μl) was added into a total PCR reaction volume of 10 μl, in which 1 ng of gDNA of *B. xylophilus* was added as described above. As a negative control, 100 μl of wood extract Hws and Mws was used to extract DNA with the DNA extraction method from a large number of nematodes. The DNA extraction product of wood extract was re-suspended by TE and used as negative template in the real-time PCR. The wood extract and ddH_2O were included in each PCR run as negative controls. Positive controls were included in each PCR run. Triplicate reactions were performed in each assay and each assay was repeated at least three times. A univariate Analysis of Variance (ANOVA), utilizing a general linear model, was conducted with Ct as the dependent variable and the Hws or Mws groups (different volume of wood extract) as the independent variable. Statistical significance was defined as $P<0.05$. Statistical analysis was performed with the SPSS 13.0 software program (SPSS Inc., Chicago, USA).

In order to determine the effect of the wood extract on the detection of single nematodes, single nematodes were lyzed in 20 μl lysis buffer, as described above, and 1.25 μl of the gDNA suspension was used for real-time PCR amplification. In the reaction mixture, 1.25 μl of the wood extract was used. Blank lysis buffer, ddH_2O and the DNA extraction product of wood extract were included in each PCR run as negative controls. Triplicate reactions were performed in each assay, and each assay was repeated at least eight times. The statistical data analysis for difference between the Ct value of real-time PCR containing 1.25 μl of the DNA suspension within the addition of the equal volume of wood extract Hws or Mws and those without was performed by means of an independent samples t-test with a significance level of 0.05. Statistical analysis was performed with the SPSS 13.0 software program (SPSS Inc., Chicago, USA)

Detection of Bursaphelenchus in naturally infested wood using real-time PCR Six samples of pine wilt disease specific to pine wood were collected from six pine forests in an epidemic area in China. These forests were located in Liyang (S1, Jiangsu Province), Yizheng (S2, Jiangsu Province), Xiang Mountain (S3, Zhejiang Province), Chuzhou (S4, Anhui Province), Changdao (S5, Shangdong Province) and Xiamen (S6, Fujian Province). Approximately 5 g of each pine wood sample was cut into small pieces, placed in a Baermann funnel (with approximate 10-cm diameter and 150 ml capacity), loaded with approximately 100 ml of distilled water to submerge the wood piece at room temperature overnight, and 10 ml of nematode suspension was collected. Part of the sample was used for morphological identification. 10 μl of the nematode suspension was added into equal volumes of nematode lysis buffer. Nematode DNA was extracted from small amounts of nematode as described above. 2.5 μl of supernatant was used as a template to execute real-time PCR. Purified DNA of *B. xylophilus* isolate AN19# was used as a positive control. The purified DNA of *B. mucronatus* AN5#, the extract of the stem of a healthy pine seedling cultured in the lab and the blank lysis buffer were used as negative controls. Triplicate reactions were performed in each real-time PCR assay and each assay was repeated three times.

Results

Specificity of the real-time PCR assay Real-time PCR primers and the Taqman probe were tested on DNA

from 18 isolates of *B. xylophilus*, 4 isolates of *B. mucronatus*, 1 isolate of *B. hofmanni* and 1 isolate of *Seinura wuae,* taken from different geographical locations and *Pinus* species (Table 1). Of these nematode samples, eighteen positive signals were obtained only when the template was DNA from *B. xylophilus* (Fig. 1). In order to confirm the results of real-time PCR reaction, the agarose gel electrophoresis was performed and the result indicated that all reactions containing *B. xylophilus* template DNA amplified a fragment of the correct size, 57 bp (Fig. 2). The results showed the specificity test is repeatable, reliable and the primers and Taqman probe are specific to *B. xylophilus*.

Sensitivity of the real-time PCR assay The sensitivity of the real-time PCR assay was examined using 10-fold dilutions of *B. xylophilus* genomic DNA between 100 ng and 0.01 ng. A standard curve was plotted from the linear regression of the logarithmic amount of *B. xylophilus* gDNA versus the Ct values. The regression line (plot of Ct against −log (dilution factor)) was linear and the regression coefficient was −1.00. The slope of the regression line was 3.7258±0.051. The PCR efficiency (E) of the assay calculated with the equation $E = 10^{-1/\text{slope}}$ was 1.86± 0.016 (Fig. 3). This was true for replicates and was therefore defined as the limits of detection (LOD) for this assay. For assessing the potential effect of wood extract on the Ct value of the real-time PCR assay, 0.5 µl, 1 µl, 1.5 µl, 2 µl, 2.5 µl of wood extract solution was added to the real-time PCR reaction mixture which contained 1 ng of purified gDNA of *B. xylophilus*. All positive controls gave positive results and all negative controls were negative in each PCR run. The univariate analysis of variance showed no significant difference between the Ct value of the real-time PCR reaction containing 1 ng of purified gDNA of *B. xylophilus* within or without the addition of different volume of Hws (P=0.389) and Mws (P=0.46) (Table 2).

Detection of single B. xylophilus Single nematodes at the development stages of egg, 2nd through 4th stage juvenile, males and females were used to assess the accuracy and repeatability of the PCR assay. The Ct

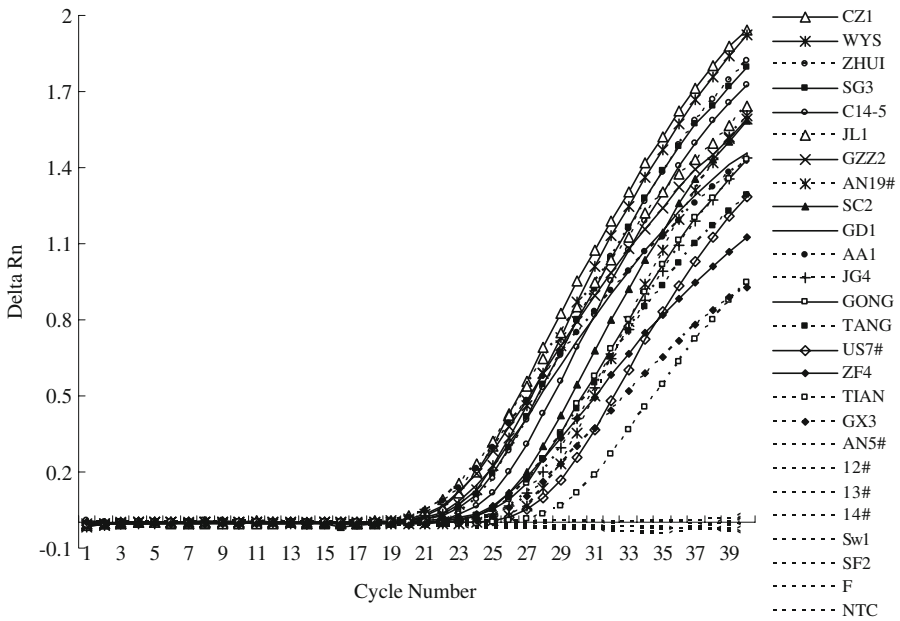

Fig. 1 Specificity of the real-time PCR assay for *Bursaphelenchus xylophilus*. The assays were conducted with genomic DNA from *B. xylophilus*, *B. mucronatus*, *B. hofmanni* and *S. wuae* isolates listed in Table 1. gDNA of *Botrytis cinerea* (F) and ddH_2O (NTC) were used as negative controls. To avoid overlapping and confusing symbols, the same symbol (----) was used for all *B. mucronatus*, *B. hofmanni*, *S. wuae* isolates, *B. cinerea* and the negative control which all produced no detectable fluorescence. The Ct values were calculated by using a threshold value of 0.2 and the automatic baseline setting. Each reaction was repeated at least three times

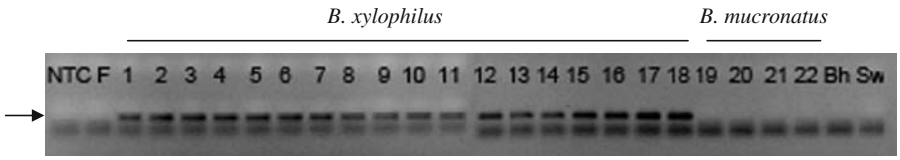

Fig. 2 Agarose gel electrophoresis of the real-time PCR products. The arrow indicates the specific 57 bp fragment amplified in *B. xylophils* isolates only. Assays were performed using 10 ng of gDNA template. NTC represents the negative control in which DNA was replaced by ddH_2O. F represents the g DNA template of the fungus *Botrytis cinerea*. Lane 1–18 represent isolates of *B. xylophilus*. Lane 19–22 represent isolates of *B. mucronatus*. Bh and Sw represent isolates of *B. hofmanni* and *Seinura wuae* respectively

value for egg, 2nd through 4th stage juvenile, males and females were 32.55±0.724, 32.71±0.596, 32.37±0.744, 32.30±0.791, 31.28±0.748 and 32.21±0.757, respectively. The average Ct value of a single nematode was 32.22±0.849. 100 ng gDNA template of *B. xylophilus* (B.x100) was used as a positive control with the Ct value of 21.28±0.503. 100 ng gDNA template of *B. mucronatus* (B.m100) and blank lysis buffer (NTC) were negative (Fig. 4). These results showed that the PCR assay can successfully detect nematodes at any life stage, including eggs. The t-test for independent samples showed no significant differences between the Ct value of the real-time PCR reaction containing the single nematode DNA suspension of *B. xylophilus* within or without the addition of the wood extract of *P. thunbergii* (P=0.188) and *P. massoniana* (P=0.167) (Table 2).

Detection of Bursaphelenchus in naturally infested wood using real-time PCR Morphological identification confirmed that all six pine wood samples from epidemic areas contained *B. xylophilus*. In addition, those samples contained other *Bursaphelenchus* species such as *B. mucronatus*, *B. hofmanni* and some other nematode species including *Aphelenchoides macronucleatus*, *Seinura lii, S. wuae*. The results of real-time PCR correspond to those from morphological detection (Fig. 5). It indicates that the real-time PCR assay is specific and reliable to detect *B. xylophilus*.

Discussion

Bursaphelenchus xylophilus is an important quarantine pine pathogen which has impact on forest health, natural ecosystem stability and international trade. In this paper we demonstrate that real-time PCR assay targeting the DNA topoisomerase I gene is a simple, rapid and accurate method for identifying *B. xylophi-*

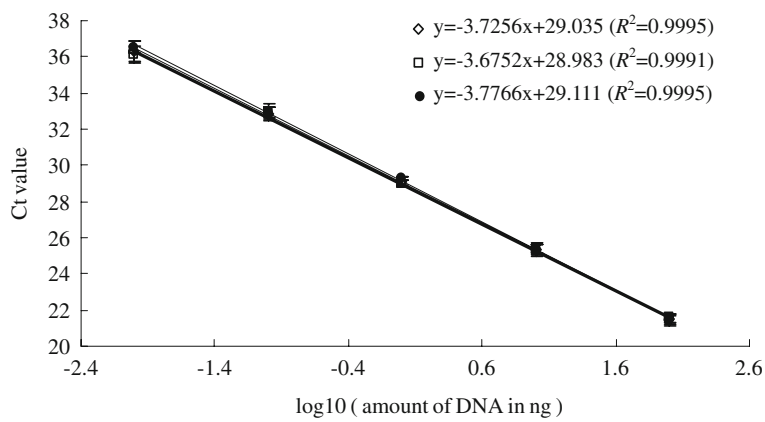

Fig. 3 Standard curve showing Ct values plotted against the $\log_{10}$ of serially diluted genomic DNA of *Bursaphelenchus xylophilus,* ranging from 100 ng to 0.01 ng in three independent experiments (average of five replicates ± SD). For each experiment, the regression line equation and its R^2 value are displayed. 100 ng gDNA of *B. mucronatus* isolate AN5# and ddH_2O were used as negative controls and produced no detectable fluorescence

Table 2 The effect of pine wood extract on the Ct value of the real-time PCR

Template	Wood sample extract [a]		Ct (mean ± SD)
1 ng purified gDNA	0.5 μl	Hws	32.15±0.96
		Mws	32.05±0.51
	1 μl	Hws	32.61±0.41
		Mws	31.61±0.55
	1.5 μl	Hws	32.52±1.01
		Mws	32.07±0.53
	2 μl	Hws	32.85±0.53
		Mws	31.80±0.36
	2.5 μl	Hws	32.55±0.79
		Mws	32.12±0.37
	Pc		31.93±0.21
gDNA supernatant of single nematode	1.25 μl	Hws	33.36±0.61
		Mws	33.33±0.62
	Pc		33.82±0.71

[a] Hws, Mws represent the pine wood extract of *P. thunbergii* and *P. massoniana*, respectively. Pc represents the positive controls which contain gDNA template of *B. xylophilus* without the pine wood extract

lus. Previous real-time PCR or conventional PCR reactions for detecting *B. xylophilus* generally used primers and probes that were designed from rDNA and their ITS regions or satellite DNA because multiple gene copies are usually present within a single genome (Matsunaga and Togashi 2004; Cao et al. 2005; François et al. 2007). The rDNA is the most frequently sequenced portion of the genomes. Therefore, the nucleotide diversity in this zone within and between species is much better known than for any other locus, which provides more confidence on the specificity of the corresponding PCR primers. rDNA is, however, a highly conserved region and therefore bears the risk of nonspecific amplifications (Bialek et al. 2000). ITS regions have been used as the most popular targets to develop molecular techniques to identify organism to the genus and/or species level. However, this method can be limited by the presence of several different copies of the ITS within a single genome (Mulet et al. 2009; Fatehi and Bridge 1998; Locatelli et al. 2002; Fenton et al. 1998).

The primers and Taqman probe used in the presented real-time PCR assay were designed based on an intron sequence of the DNA topoisomerase I gene of *B. xylophilus*, which is present in all eukaryotes and its sequence is conserved. It is the first report on the detection of *B. xylophilus* by targeting the DNA topoisomerase I gene, even though

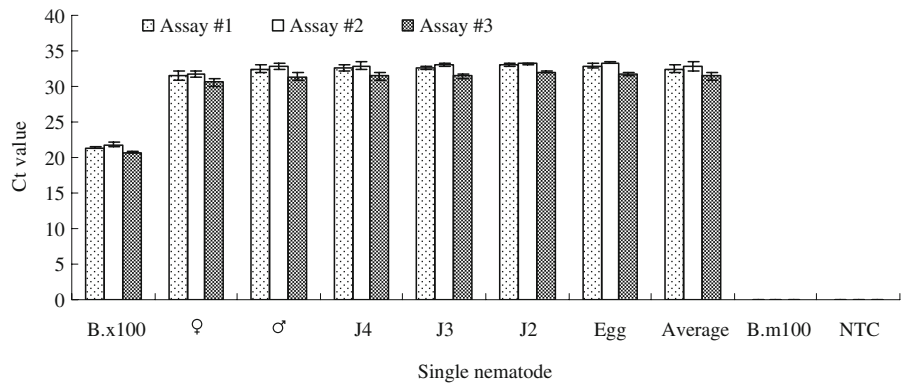

Fig. 4 Real-time PCR detection of single *Bursaphelenchus xylophilus* at different development stages. Egg, L2, L3, L4, ♂, ♀ represent egg, 2nd, 3rd, 4th stage juvenile, female and male nematodes respectively (average of three replicates ± SD). B. x100 represents 100 ng gDNA template of *B. xylophilus* used as a positive control with the Ct value of 21.28±0.503. B.m100 and NTC representing 100 ng DNA template of *B. mucronatus* and a blank lysis buffer as negative controls

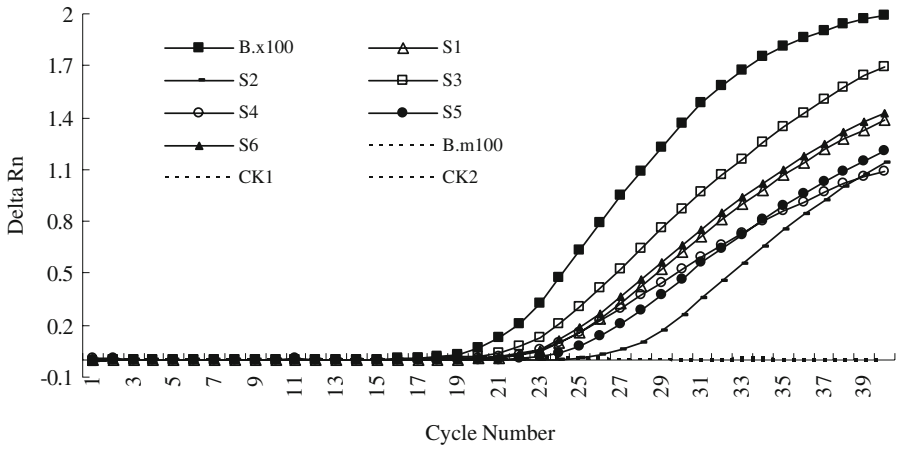

Fig. 5 Real-time PCR assay from naturally infested wood. 100 ng genomic DNA of *B. xylophilus* AN19# (B.x 100) was used as a positive control with a Ct value of 21.56±0.219. S1, S2, S3, S4, S5, S6 represent the six field samples described. The Ct value for each sample were 25.57±0.239, 29.39±0.17, 23.90±0.268, 25.66±0.141, 26.89±0.144, 25.21±0.135, respectively.100 ng genomic DNA from *B. mucronatus* AN5# (B.m 100), healthy pine wood (CK1) and blank lysis buffer without nematodes (CK2) were used as negative controls which all produced no detectable fluorescence

there were some successful applications to fungal species using the DNA topoisomerase II gene (Kanbe et al. 2002, 2003). Our work shows that real-time PCR targeting the purified gDNA topoisomerase I gene can detect the gDNA template as small as 0.01 ng, and its sensitivity is similar to that reported by Cao et al. (2005) using primers and a Taqman probe derived from ITS region. François et al. (2007) reported a real-time PCR assay which can detect the limit of 1 pg of nematode DNA because of multiple gene copies of satellite DNA present within a single genome. As well as the topoisomerase I gene, HSP70 also has been used as a target of RFLP analysis and PCR detection of *B. xylophilus* (Takemoto et al. 2005; Leal et al. 2005, 2007; Takemoto and Futai 2007). It is evident that recent progress in nematode genome sequencing and expressed sequence tag (EST) projects (Kikuchi et al. 2007) will give more promising and reliable gene candidates for diagnostic developments.

The focus of this paper is on the real-time PCR. Compared with conventional PCR, the real-time PCR provides a rapid, simple, sensitive, and quantitative option. The real-time PCR technology yields more robust results than conventional PCR, especially when the pine wood nematode sample size is very small (Cao et al. 2005; Wang et al. 2005; Ge et al. 2005). Furthermore, no time-consuming post-PCR detection of amplification products by gel electrophoresis is required. Finally, a Taqman probe of real-time PCR increases detection specificity compared to other non-specific DNA dyes like SYBR Green (François et al. 2007).

One limiting factor in PCR-based detection assays is the presence of PCR-inhibitors in the DNA extract, such as wood substances (Malvick and Grunden 2005; Wilson 1997; Langrell and Barbara 2001). However, Leal et al. (2007) discovered that real-time PCR assays produced equal PCR efficiency and detectable fluorescence regardless of the presence or absence of wood extract. In our work, the DNA templates were extracted from nematodes cultured on *B. cinerea* or recovered from pine wood by Baermann funnel extraction. *B. xylophilus* can be easily extracted. This facilitates both the collection of nematodes and the extraction of their DNA. In the experiment, 20 fold volume of ddH_2O was used to submerge the fresh nematode-free wood and obtain the wood extract. We did not discover any inhibition of the real-time PCR by wood extracts irrespective of whether the template of PCR was purified gDNA or the DNA suspension of a single nematode. Therefore, it might be possible, that *B. xylophilus* could be detected in ground wood tissue without previous extraction. However, this needs to be tested in further experiments. In conclusion, we have developed an efficient real-time PCR assay that specifically and accurately detects and identifies *B. xylophilus* by targeting the DNA topoisomerase I gene.

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