Satellite DNA as a target for TaqMan real-time PCR detection of the pinewood nematode, *Bursaphelenchus xylophilus*

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SUMMARY

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, is a major pathogen of conifers, which impacts on forest health, natural ecosystem stability and international trade. As a consequence, it has been listed as a quarantine organism in Europe. A real-time PCR approach based on TaqMan chemistry was developed to detect this organism. Specific probe and primers were designed based on the sequence of the *Msp*I satellite DNA family previously characterized in the genome of the nematode. The method proved to be specific in tests with target DNA from PWN isolates from worldwide origin. From a practical point of view, detection limit was 1 pg of target DNA or one individual nematode. In addition, PWN genomic DNA or single individuals were positively detected in mixed samples in which *B. xylophilus* was associated with the closely related non-pathogenic species *B. mucronatus*, up to the limit of 0.01% or 1% of the mixture, respectively. The real-time PCR assay was also used in conjunction with a simple DNA extraction method to detect PWN directly in artificially infested wood samples. These results demonstrate the potential of this assay to provide rapid, accurate and sensitive molecular identification of the PWN in relation to pest risk assessment in the field and quarantine regulation.

INTRODUCTION

Pine wilt is a major disease of pine (*Pinus* spp.) forests caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus.* The PWN is indigenous to North America where it is not considered as a pathogen on native pine species. However, the nematode constitutes a severe pest in countries where it has been introduced. For example, about 30% of the total area of pine forest is estimated to be infested by this species in Japan (Mamiya, 2004), following its introduction via the timber trade. Owing to the potential threat to forest ecosystems, regulations have been introduced in many countries to prevent introduction and/or further spread of *B. xylophilus.* In Europe, the recent discovery of the PWN in a restricted area in Portugal (Mota *et al.*, 1999) has led to its listing as a quarantine pest, and to the implementation of national large-scale surveys to monitor the pathogen and special phytosanitary measures for conifer wood packaging materials imported from areas where the nematode has been recorded (EU directive 2000/29/EC). To be effective, such regulation steps strongly depend on accurate detection of the pathogen.

More than 70 species of *Bursaphelenchus* are currently recognized, and their morphological identification requires a high level of expertise (Ryss *et al.*, 2005). In particular, species belonging to the 'pinewood nematode species complex', such as *B. xylophilus* and *B. mucronatus*, share several morphological characteristics, and may even be subject to reciprocal genetic exchange through hybridization (De Guiran and Bruguier, 1989). In recent years, conventional PCR based on direct visualization of amplicons (Castagnone *et al.*, 2005; Kang *et al.*, 2004; Matsunaga and Togashi, 2004; Takeuchi *et al.*, 2005) or RFLP analysis of amplicons (Burgermeister *et al.*, 2005) have provided useful methods for identification of the PWN. However, the multistep process of amplicon analysis, involving gel electrophoresis, and the risk of post-PCR contamination mean that conventional PCR protocols are not appropriate for high-throughput routine testing. To overcome such limitations, real-time PCR coupled to automated product analysis appears to be a promising alternative, as post-PCR handling for target detection is no longer required. Instead, fluorescent monitoring of amplicon accumulation allows target detection, based on the cycle threshold (Ct) value, i.e. the number of PCR cycles at which the amplicon product curve exceeds fluorescence background (Heid *et al.*, 1996). Applicability of this

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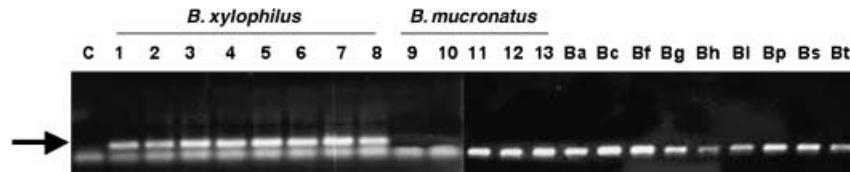E-mail: Philippe.Castagnone@sophia.inra.fr

Fig. 1 Specific amplification of *Bursaphelenchus xylophilus* satellite DNA using primers BsatF and BsatRV in conventional PCR. The arrow indicates the specific fragment amplified in *B. xylophilus* isolates only. Assays were performed using 10 ng of genomic DNA template. In the negative control, DNA was replaced by water. The order of the lanes in the gel is the same as for the *Bursaphelenchus* spp. isolates listed in Table 3.
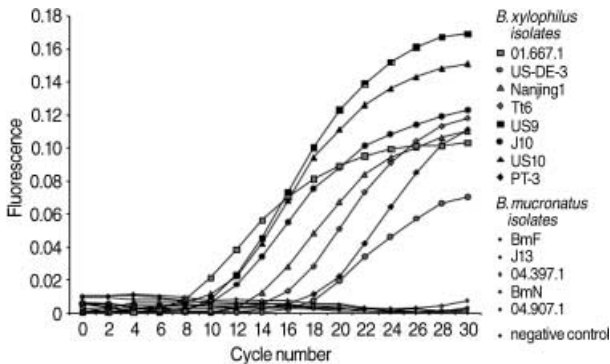

Fig. 2 Specificity of the TaqMan real-time PCR assay for *Bursaphelenchus xylophilus*. Assays were performed using 10 ng of genomic DNA template from the *B. xylophilus* and *B. mucronatus* isolates listed in Table 3. In the negative control, DNA was replaced by water. To avoid overlapping and confusing symbols, the same symbol (○) was used for all the *B. mucronatus* isolates.
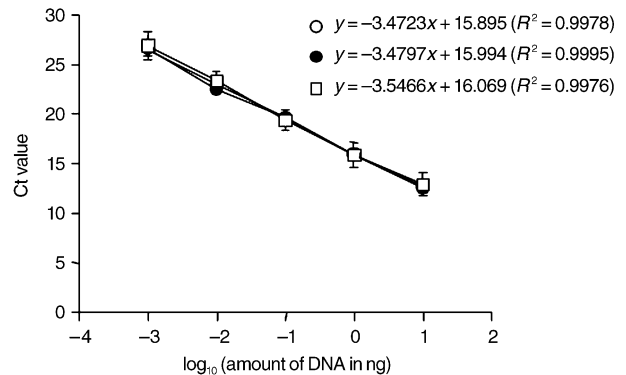

Fig. 3 Standard curves showing Ct values plotted against the $\log_{10}$ of known amounts of *Bursaphelenchus xylophilus* genomic DNA in three independant experiments (average of five replicates ± SD). Nematode genomic DNA was serially diluted to yield final amounts ranging from 10 ng to 1 pg. For each experiment, the regression line equation and its R^2 value are displayed.

new technology to the detection of plant-parasitic nematodes has very recently been tested (Holeva *et al.*, 2006; Madani *et al.*, 2005), including the PWN (Cao *et al.*, 2005), using ribosomal DNA as a target sequence.

The aim of this study was the development and evaluation of a real-time PCR assay for routine sample screening to detect the PWN, based on the specific *Msp*I satellite DNA family previously cloned in the genome of the parasite (Tarès *et al.*, 1993). To improve the accuracy of real-time PCR, TaqMan chemistry has been incorporated into our assay. This chemistry combines the use of a target-specific oligonucleotide probe dual-labelled with a donor fluorophore and an acceptor dye, and the 5′-exonuclease activity of Taq polymerase (Holland *et al.*, 1991), thus combining the sensitivity of PCR and the specificity of DNA hybridization.

RESULTS

Specificity and sensitivity of TaqMan real-time PCR assays

In a previous study, we showed that the *Msp*I satellite DNA repetitive unit was not detected by conventional PCR in the genome of three congeneric species, i.e. *B. mucronatus, B. tusciae*

and *B. leoni* (Castagnone *et al.*, 2005). Here, in conventional PCR, the BSatF and BSatRV primers amplified a fragment of the expected size (approximately 80 bp) from all the *B. xylophilus* isolates but did not amplify from isolates belonging to the ten other *Bursaphelenchus* species tested nor from the negative control (Fig. 1). In TaqMan real-time PCR optimized assays, genomic DNA from the *B. xylophilus* isolates was specifically amplified, while fluorescence remained below the threshold values for the negative control and the isolates of the non-target species *B. mucronatus* (Fig. 2). Some variation in Ct values was observed between *B. xylophilus* isolates, which suggested variation between isolates in the genomic content of the *Msp*I satDNA family.

The sensitivity of the nematode TaqMan PCR assays was measured with ten-fold dilution series of purified genomic DNA. *Bursaphelenchus xylophilus* target DNA was detectable in samples containing as little as 1 pg DNA (Fig. 3). Standard curves showed a negative linear correlation between Ct values and the $\log_{10}$ of the known amount of DNA over a range of 10 ng to 1 pg (Fig. 3). The same result was confirmed in three separate experiments using different dilution series, which illustrates the sensitivity and linearity of the detection within a wide range of input template DNA amount (Fig. 3).
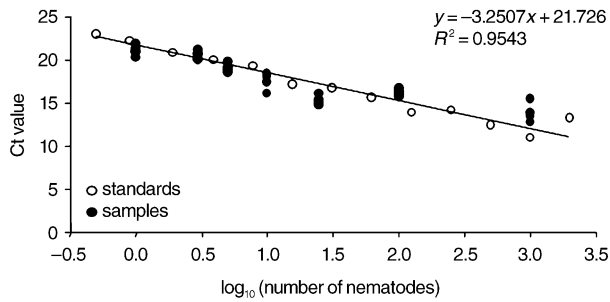
Fig. 4 Real-time PCR testing of batches of individual *Bursaphelenchus xylophilus*, showing the correlation between Ct values and numbers of nematodes, in comparison with a standard curve constructed using serial dilutions of a DNA extract prepared from 2000 nematodes. For the standard curve, the regression line and its R^2 value are displayed.

$$y = -3.2507x + 21.726$$
$$R^2 = 0.9543$$

Table 1 Real-time PCR (TaqMan) detection of *Bursaphelenchus xylophilus* in mixed samples. Ct values are means ± SD.

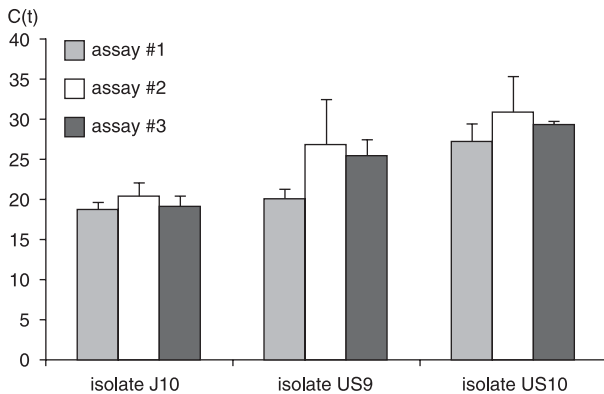| | Composition of the samples | | Percentage *B. xylophilus* | |
	B. xylophilus	*B. mucronatus*	in the samples	Ct
DNA samples	10 pg	1 ng	1%	24.85 ± 0.74
	10 pg	10 ng	0.1%	24.25 ± 0.61
	10 pg	100 ng	0.01%	24.91 ± 0.82
Nematode samples	1 nematode	1 nematode	50%	25.58 ± 2.35
	1 nematode	4 nematodes	20%	23.47 ± 1.84
	1 nematode	19 nematodes	5%	22.64 ± 0.66
	1 nematode	99 nematodes	1%	23.55 ± 1.96

Fig. 5 Comparison of real-time PCR testing of individual nematodes of three different *Bursaphelenchus xylophilus* isolates as performed in three independent assays (average of five replicates ± SD).

Application of TaqMan assays to individual nematodes

Detection of the target DNA from batches of individual *B. xylophilus* nematodes was performed by comparison of the Ct values of the samples to those obtained from the standard dilutions of DNA prepared from 2000 nematodes, using in both cases the simplified DNA extraction method. Samples consisted of DNA extracted from batches of 1–1000 individual nematodes, and the corresponding average Ct values ranged from approximately 22 to 15, in good correlation with the standard curve (Fig. 4). In particular, detection of single nematodes was always efficient, which confirms the sensitivity of the detection. In addition, if we compare the average Ct value for one nematode to the results of the dilution assays with genomic DNA (Fig. 3), we can extrapolate that the analytical sensitivity of the real-time PCR assay with genomic DNA is approximately equivalent to 1/25 nematode per reaction. Very similar results were obtained with

different *B. xylophilus* isolates, and showed reproducible results between independent assays (Fig. 5).

Application of TaqMan assays to mixed samples

In order to evaluate the TaqMan assay in conditions closer to real quarantine determination, we tested the specific detection of the target in mixed samples. In a first step, samples consisted of mixtures of genomic DNA from both *B. xylophilus* (10 pg) and *B. mucronatus* (varying amounts from 1 ng to 100 ng). *B. xylophilus* DNA was unambiguously detected when present from 1% to 0.01% in the mixed sample (Table 1). In our conditions, the dilution level did not affect the sensitivity of the detection (with Ct values ranging from 24.25 to 24.91; Table 1). In a second series of experiments, *B. xylophilus* and *B. mucronatus* individual nematodes were mixed in varying proportions, the target species representing from 50% to 1% of the sample. Again, one single *B. xylophilus* was always specifically detected in the different mixtures tested (Table 1).

Detection of PWN in artificially infested wood by real-time PCR

In order further to validate the *Msp*I satellite DNA family as a target of interest for PWN diagnostics, TaqMan assays were conducted with templates resulting from direct processing of 0.1-g fragments of wood artificially inoculated with the nematode. Indeed, PWN DNA was detected in a variety of PWN-infested wood samples, either trunk pieces or smaller branches (Table 2). In each sample, the number of nematodes was estimated after extraction in Baermann funnel, and ranged from ten to 138 nematodes (Table 2). For the trunk samples, PWN DNA was amplified in undiluted extracts, while it was necessary to dilute the small branch extracts 1:10 to get amplification, suggesting a certain amount of PCR inhibitors were carried over in these extracts. No fluorescence was detected for the negative controls tested (non-inoculated wood samples).

Table 2 Real-time PCR (TaqMan) detection of *Bursaphelenchus xylophilus* in wood, and nematode isolation results for replicate samples. Samples of 0.2 g of infested wood were cut into small pieces, then divided in half. DNA was extracted from one half of the sample (0.1 g), and nematodes were isolated from the other half, and the numbers of adults and juveniles were recorded. Ct values are means ± SD.

Sample	Nematodes isolated	Ct
Trunk 1.5–2 cm diameter	70 adults, 68 juveniles	16.82 ± 0.06*
Trunk 1.5–2 cm diameter	7 adults, 3 juveniles	22.49 ± 0.10*
Small branches < 5 mm diameter	10 adults, 8 juveniles	25.76 ± 0.08†

*Extracts tested without dilution.
†Extract diluted 1 : 10.

DISCUSSION

A real-time PCR assay, using TaqMan chemistry, has been developed for the specific detection of the PWN, a quarantine organism in Europe. Compared with conventional PCR assays, one major advantage of real-time PCR, in the particular context of diagnostics, is its wide detection range due to the determination of the Ct values at an early stage of the reaction. Furthermore, no time-consuming post-PCR detection of amplification products by gel electrophoresis is required. Within the real-time PCR assay, the use of the TaqMan chemistry gives a better specificity to the detection compared with non-specific DNA binding dyes (e.g. SYBR Green), for which detection may be disturbed by non-specific product formation (Mackay *et al.*, 2002; Schena *et al.*, 2006).

The specific distribution of satellite DNA observed here in *B. xylophilus* has been documented in many organisms, including entomopathogenic and plant parasitic nematodes (reviewed in Grenier *et al.*, 1997), and could be linked to some structural role of these sequences, i.e. chromosome folding. In particular, due to its close association with centromeric histones, it has been proposed that satellite DNA may be driving adaptive evolution of these proteins, leading in this way to the speciation process via a loss of compatibility between chromosomes in hybrids (Henikoff *et al.*, 2001). Moreover, such repetitive sequences evolve by means of concerted evolution, resulting ultimately in homogenization of changes among repeats within the genome and their subsequent fixation in members of reproductively isolated populations (i.e. biological species) in a process known as molecular drive (Dover, 1986).

In a recent report, Cao *et al.* (2005) described a TaqMan procedure that could be used to detect as little as 0.01 ng of *B. xylophilus* DNA, using the nematode ribosomal DNA internal transcribed region as a target. The assay described here appears ten-fold more sensitive, with a reproducible detection limit of 1 pg of genomic DNA. The higher sensitivity of the technique is due, at least in part, to the presence of a large number of monomers of the *Msp*I satellite in each cell of the PWN. Indeed, this repetitive family was estimated to constitute up to 30% of the genome of the nematode (Tarès *et al.*, 1993). Taking into account the genome size, estimated to be about 30×10^6 bp for the closely related species *B. mucronatus* (Leroy *et al.*, 2003), this indicates an approximate copy number of 56×10^3 per haploid genome. Comparatively, the number of copies of the rDNA cistron ranges between 50 and 100 in the PWN genome, as deduced from the number in the genome of the model nematode *Caenorhabditis elegans* (Sulston and Brenner, 1974). This magnitude clearly indicates that satellite DNA is a very suitable target for diagnostics, as recently exemplified with real-time PCR detection of the protozoan parasite *Trypanosoma brucei* (Becker *et al.*, 2004).

Our results showed some variability in Ct values among *B. xylophilus* isolates, which can be interpreted as a variation between isolates in the genomic content of the *Msp*I satellite DNA family. Although proportionality was achieved, for any tested isolate, between genomic DNA concentration or number of nematodes and fluorescence detection, this observation precludes the use of the *Msp*I repetitive family as a target region for quantitative analysis of unknown isolates. Nevertheless, because of the evolutionary patterns of satellite DNAs, such variation is not unexpected. Indeed, with respect to copy number, satellite DNAs can vary dramatically in their content among related organisms, and it has been proposed that extensive amplifications and deletions could act as a driving force in the evolution of these sequences (Ugarkovic and Plohl, 2002). Such variations in copy number have been exemplified previously in both plant- and animal-related genomes (e.g. Alix *et al.*, 1998; Pons *et al.*, 2002). However, in spite of such variation, the *Msp*I target sequence was always found as highly reiterated in all the *B. xylophilus* isolates tested, which constitutes, along with its species-specific distribution, a major advantage of satellite DNA as a target for detection purpose.

In wood samples, the PWN is often associated with the closely related non-pathogenic species *B. mucronatus*, which constitutes the most prevalent *Bursaphelenchus* species found in European coniferous forests (Braasch, 2001; L. Robertson, pers. com.). In this context, detection of the PWN in samples containing both species appears essential for diagnostic purposes. The procedure presented here meets such a requirement, as *B. xylophilus* genomic DNA or single individuals were positively detected in mixed samples, up to the limit of 0.01% or 1% of the mixture, respectively. From that point of view, it should be considered as a candidate methodology for diagnosing PWN infection in epidemiological surveys as well as in regulatory testing.

The presence in wood of substances that strongly inhibit PCR is a frequent problem for PCR-based systems used to detect plant pathogens in woody hosts (Langrell and Barbara, 2001). Despite the development of widely applicable plant DNA extraction

procedures designed to minimize co-extraction of inhibitory compounds, inhibition remains a major impediment to many routine applications, compromising both assay sensitivity and reliability (Bickley and Hopkins, 1999). In particular, polysaccharides and secondary plant metabolites (such as polyphenols and phenolic compounds) are common, PCR-inhibitory contaminants of DNA extracts made from wood samples. The method described here for DNA extraction from infested wood uses minimal disruption of the sample, such that PWN DNA can be detected but inhibitors in the wood, which could prevent amplification, are not released. However, dilution was needed to get positive amplification in the case of small branches, which indicated that substances inhibiting PCR were nevertheless present in such samples. Despite this, the ability of the real-time PCR assay to detect less than one genome equivalent per reaction allows extracts to be diluted without the risk of false negative results. Modifications to this method to standardize the protocol, increase throughput, increase the size of samples that can be processed and improve sampling could increase the advantages of routine direct testing of wood samples. Clearly, the need for such a test is especially pressing in light of the recent discovery of *B. xylophilus* in Europe (Mota *et al.*, 1999), and the increasing impact of this invasive pest on international trade regulation.

EXPERIMENTAL PROCEDURES

Biological materials

The *Bursaphelenchus* isolates used in this study, with their original host and geographical origin, when available, are listed in Table 3. Isolates were cultured monoxenically on the fungus *Botrytis cinerea* in a Petri dish on potato dextrose agar medium at 25 °C.

To test the detection of the nematode without prior isolation, wood samples were artificially inoculated as follows. Three-year seedlings of *Pinus pinaster* were inoculated at the base with *c.* 3000 nematodes of the *B. xylophilus* PT-3 isolate. Six weeks after inoculation (i.e. seedlings showing wilting symptoms extending over 50% of current-year needles), all the needles were removed, and the trunk was separated from the branches. From each edge of the trunk, several discs (3-mm thickness) were cut to constitute the tested wood samples. To check the success of infestation, the nematodes were extracted from 0.1-g wood samples using the Baermann funnel technique for 48 h (Mamiya, 1975).

DNA extraction from nematodes

Genomic DNA was purified from pooled nematodes of each isolate using the phenol/chloroform method (Sambrook *et al.*, 1989), quantified spectrophotometrically and aliquoted and

Table 3 Isolates of the *Bursaphelenchus* species tested.

Nematode species	Isolate	Host	Origin
B. xylophilus	J10	*Pinus densiflora*	Japan
B. xylophilus	US9	*P. halepensis*	USA
B. xylophilus	US10	*Abies balsamea*	USA
B. xylophilus	01.667.1	packaging wood	Canada
B. xylophilus	PT-3	*P. pinaster*	Portugal
B. xylophilus	Nanjing 1	*P. thumbergii*	China
B. xylophilus	Tt 6	?	Japan
B. xylophilus	US-DE-3	*P. taeda*	USA
B. xylophilus	J13	*P. thumbergii*	Japan
B. xylophilus	BmF	*P. pinaster*	France
B. xylophilus	BmN	*P. sylvestris*	Norway
B. xylophilus	03.397.1	*P. nigra*	France
B. xylophilus	04.907.1	*P. pinaster*	France
B. antoniae	Leiria	*Hylobius sp.*	Portugal
B. conicaudatus	BcoJ	*Ficus carica*	Japan
B. fraudulentus	BFHU	*Quercus robus*	Hungary
B. hofmanni	AT5w	*P. sylvestris*	Austria
B. glochis	03-814-1	*P. sylvestris*	France
B. luxuriosae	BluJ	*Acalolepta luxuriosa*	Japan
B. pinophilus	04-841-1	*P. sylvestris*	France
B. sexdentati	Guadalupe	*P. pinaster*	Portugal
B. tusciae	IT-14w	*P. pinea*	Italy

stored at −80 °C. Alternatively, DNA from a defined number of nematodes (1–2000 individuals) was extracted using a simplified procedure, as previously described (Castagnone *et al.*, 2005), with a slight modification. Unlike the original protocol, the volume of lysis buffer used was not constant but adapted to the number of nematodes, i.e. 3 μL for 1−4 nematodes and 20 μL for higher numbers of nematodes.

DNA extraction from PWN-infested wood samples

DNA extraction from infested wood was performed using a ChargeSwitch gDNA Plant kit from Invitrogen (Carlsbad, CA). Approximately 0.1 g of infested wood was cut into small pieces and placed into a plastic bag with 5 mL of CST lysis buffer containing 1% polyvinylpyrrolidone and 20 mM calcium chloride. The sample was lightly disrupted using a hammer, then 1 mL of lysate was removed and processed according to the manufacturer's instructions. Briefly, 100 μL sodium dodecyl sulphate was added to the lysate and incubated at room temperature for 5 min, then 400 μL precipitation buffer was added and centrifuged at maximum speed (approximately 18 000 *g*) for 5 min. Approximately 1 mL of supernatent was removed, and 100 μL of CST detergent and 40 μL CST beads were added. A PickPen 8-M from Bio-Nobile (Turku, Finland) was used to transfer the CST beads and bound DNA through two washing steps (1 mL CST Wash Buffer) and into 150 μL CST Elution Buffer in a 2.2-mL deep well plate, followed by removal of the Magnetic Particles. The DNA

was either tested immediately or stored at –20 °C for future analysis.

Primers and TaqMan probe design

Real-time PCR primers BSatF (5′-TGACGGAGTGAATTGACAA-GACA-3′) and BSatRV (5′-AAGCTGAAACTTGCCATGCTAAA-3′) were designed to amplify a 77-bp-long amplicon of the target sequence from *B. xylophilus Msp*I satDNA monomeric unit (accession number L09652). The fluorogenic TaqMan probe BSatS (5′-ACACCATTCGAAAGCTAATCGCCTGAGA-3′) was designed to anneal to a 30-bp region located between the primer pair. A BLAST search was performed on the amplicon sequence to ensure specificity. The probe was labelled at the 5′ end with 6-carboxyfluorescein (FAM) as reporter dye, and modified at the 3′ end with the quencher dye tetra-methylcarboxyrhodamine (TAMRA). Primers and probe were synthesized by Eurogentec (Belgium).

Amplification conditions

Optimal conditions were determined by performing real-time PCR assays with concentration gradients of primers (50–900 nM) and probe (50–250 nM) at different annealing and extension temperatures (58–65 °C). Conditions were considered as optimal if they produced the lowest Ct values and the highest fluorescence. By definition, Ct represents the number of amplification cycles at which the detected fluorescence exceeds the fluorescence background (Heid *et al.*, 1996). In our experimental conditions, the fluorescence of the negative controls used never exceeded such background, and thus no Ct values could be attributed to these negative controls.

Real-time PCR reactions were performed in a total volume of 25 μL in 0.2 mL Low Profile tube strips (ABgene, UK) containing 1 μL of genomic DNA. Each reaction contained 2.5 μL of 10× reaction buffer (qPCR Core Kit, Eurogentec, Belgium), 5 mM MgCl₂, 200 μM each dNTPs, 0.5 unit Taq polymerase (qPCR Core Kit) and 200 nM each primer and probe. Thermal cycling conditions consisted of 10 min at 95 °C, followed by 30 cycles of 15 s at 95 °C and 30 s at 59 °C.

Real-time PCR assays on nematode and nematode DNA samples were performed in a DNA Engine Opticon 2 (MJ Research). Data were analysed using the Opticon 2 Monitor software version 3.1 according to the manufacturer's instructions. The Ct values for each reaction were calculated automatically by the software by determining the PCR cycle number at which the reporter fluorescence exceeded background. Triplicate reactions were performed in each assay, and each assay was repeated at least three times.

Real-time PCR testing of wood extracts was performed on a SmartCycler II (Cepheid, Sunnyvale, CA). Each reaction consisted of 0.025 U/μL Hot Taq (Biogene, Kimbolton, UK), 1× PCR buffer, 0.2 mM each dNTP, 5.5 mM MgCl₂, 5% trehalose (w/v), 300 nM each primer and 100 nM probe. Cycling conditions were 10 min at 95 °C followed by 40 two-step cycles of 15 s at 95 °C and 1 min at 60 °C. Data were analysed using the default threshold setting of the SmartCycler software (30 fluorescence units). Extracts were tested undiluted and diluted 1 : 10 in nuclease-free water.

Conventional PCR reactions were performed as described above using Eurogentec qPCR Core Kit reagents, except that no probe was added to the reactions. Amplicons were analysed by electrophoresis in 2% agarose gels containing ethidium bromide in TBE buffer (Sambrook *et al.*, 1989), and visualized under UV light.

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