

Direct molecular detection of the pinewood nematode, *Bursaphelenchus xylophilus*, from pine wood, bark and insect vector

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Abstract The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, is the causal agent of pine wilt disease. The international economic impact of the introduction of the PWN into new areas has highlighted the need for the development of accurate and reliable detection methods of *B. xylophilus*, which are essential to define aspects of its control and management. In the present study, a methodology was developed for the direct detection of PWN by conventional PCR assay, with a species specific set of primers based on PWN satellite DNA, using total DNA extracted directly from maritime pine, *Pinus pinaster*, wood and bark samples, and from the insect vector, *Monochamus galloprovincialis*. This methodology involves homogenisation of wood, bark and insects using liquid nitrogen, DNA extraction and one or two PCR amplification steps, which permit the rapid and direct detection of one single nematode present in 100 mg of wood and bark and in one entire insect without the preliminary steps of nematode extraction.

Keywords *Monochamus galloprovincialis* · PCR · *Pinus pinaster* · Pine wilt disease · PWN

Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, is the causal agent of pine wilt disease (PWD). This nematode causes cell destruction leading to death of the host tree in a few months. Transmission of PWN to new trees is carried out by insects, belonging mainly to the genus *Monochamus*. Long-range spread of PWN occurs as a result of human activity in wood transport (Jones et al. 2008). In Europe, *B. xylophilus* has been reported in Portugal (Mota et al. 1999) and in Spain (Robertson et al. 2011) in maritime pine, *Pinus pinaster*. Its presence, in Portugal, was associated with the insect vector *Monochamus galloprovincialis* (Sousa et al. 2001). The economic importance of the introduction of PWN into new areas has highlighted the need for accurate detection of this species which is fundamental to define guidelines for control and management, to improve quarantine regulations and to prevent further spread of the disease. Traditionally, detection of PWN has been based on morphological characters after their extraction from wood samples. However, this methodology involves several time consuming steps and requires a high level of taxonomical expertise. Besides, PWN morphological identification can be sometimes difficult or impossible when only juvenile stages are detected or due to

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the variation found in the female tail (round, digitate or mucronate) of some isolates (Fonseca et al. 2008). In practical, the female tail terminus is the only morphological character used to distinguish *B. xylophilus* from *B. mucronatus*, a closely related non-pathogenic species with mucronate female tails (Mamiya and Enda 1979). Therefore, the identification by morphological characters can lead to a mistaken diagnosis. Molecular detection methods are simpler, faster and reliable and thus several PCR based methods to detect *B. xylophilus* with species-specific primers have been developed by targeting either rDNA, ITS regions, satDNA or Hsp70 and DNA topoisomerase I genes (Iwahori et al. 1998, 2000; Kang et al. 2004; Matsunaga and Togashi 2004; Cao et al. 2005; Castagnone et al. 2005; Leal et al. 2005, 2007; Kang et al. 2009; Huang et al. 2010; Zhuo et al. 2011). However, few studies on the use of these methodologies for direct detection of PWN in pine wood and insect vector, without the preliminary steps of nematode extraction, have been conducted (François et al. 2007; Takeuchi and Futai 2007; Kikuchi et al. 2009; Takeuchi and Futai 2009; Kanetani et al. 2011; Wang et al. 2010; Hu et al. 2011; Wang et al. 2011). In the present study, a new methodology was developed for the direct detection of PWN, performed by PCR amplification of the species specific *MspI* satDNA, leading to a pattern of monomer and multimers of the 160 bp monomer (Tarés et al. 1994; Castagnone et al. 2005), as recommended by the European and Mediterranean Plant Protection Organization (EPPO) (2009), using total DNA extracted from *P. pinaster* wood and bark samples and from the insect vector, *M. galloprovincialis*.

Materials and methods

Nematode isolate

Nematodes from a Portuguese *B. xylophilus* isolate (BxPt12G), maintained in cultures of *Botrytis cinerea* grown on Malt Extract Agar (MEA) medium at 25°C, were used for DNA extraction (positive controls) and to infest bark samples and insects. For DNA extraction, nematodes were first concentrated by centrifugation and then homogenised using a plastic homogeniser.

Wood samples

Eight maritime pine (*P. pinaster*) wood samples, seven collected from trees, in Oliveira do Hospital, Coimbra District, Portugal, and one from a heat treated pallet, as a negative control, were used for DNA extraction (Table 1).

In order to estimate the number of PWN and other nematodes present in 100 mg of wood (used for DNA extraction), nematodes were extracted from 100 g of wood, using the Whitehead and Hemming tray method. After 48 h, the resulting nematode suspensions were passed through a 20 µm sieve and the nematodes counted and identified on the basis of the species specific morphological characters.

Bark and insect samples

Non-infested bark samples, collected from *P. pinaster* trees in Oliveira do Hospital, were artificially infested with 1,000, 10 or 1 PWN. Non-infested dead adult

Table 1 Number of nematodes present in 100 mg of *Pinus pinaster* wood samples

Code	<i>Pinus pinaster</i>	Nematodes/100 mg (n°)	
		<i>Bursaphelenchus xylophilus</i>	Other nematodes (Rhabditidae and Aphelenchoididae)
Pp1	Tree	34	0
Pp2	Tree	5	0
Pp3	Tree	2	1
Pp4	Tree	1	0
Pp5	Tree	1	1
Pp6	Tree	0	0
Pp7	Tree	0	13
Ht1	Heat treated pallet	0	0

insects, *M. galloprovincialis*, were artificially infested with 1,000, 100, 10, 5 or 1 PWN. The nematodes were pipetted or handpicked into a small hole made on bark and on insects. Non-infested bark and insects were used as negative controls.

DNA extraction

Total DNA was extracted from 100 mg of pine wood and bark samples using the commercial kit DNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions. Samples were first ground to powder using liquid nitrogen and elution of total DNA from the dried membrane of the mini-columns was performed with 100 µl of supplied elution buffer. Furthermore, in order to evaluate the applicability of this DNA extraction method, to nematode suspensions obtained from infected *P. pinaster* wood samples after the tray method extraction, a 20 ml nematode suspension free of PWN and another with a single PWN, were used for DNA extraction. Nematodes were first concentrated by centrifugation and then homogenised using a plastic homogeniser. The same kit was also used to extract nematode DNA from the BxPt12G isolate (positive control).

Total DNA from *M. galloprovincialis* insects was extracted with the commercial kit DNeasy Blood and Tissue kit (Qiagen), according to the manufacturer's instructions, using one insect for each extraction. Insects were first ground to powder using liquid nitrogen and the elution of DNA from the dried membrane of the mini-columns was performed with 100 µl of supplied elution buffer. The same kit was used to extract nematode DNA from the BxPt12G isolate (positive control).

Three biological replicates were performed for each sample.

PCR amplification

PCR amplifications were performed using 5 µl of extracted DNA (1 µl for positive control) and 1 U of Dream Taq DNA polymerase (Fermentas) in 1× Dream Taq buffer, 0.2 mM of each dNTPs and 0.4 µM of J10-1 and J10-2Rc primers, specific for PWN satDNA (Castagnone et al. 2005). All reactions were carried out in a thermal cycler (Bio-Rad) with an initial denaturation step of 94°C for 5 min followed by 15 reaction cycles of 94°C for 30 s, annealing at 49°C

for 1 min and extension at 72°C for 2 min and then a final extension at 72°C for 5 min. The number of cycles and the annealing temperature of PCR conditions were modified compared to those described by Castagnone et al. (2005) for individual nematodes. Second PCR amplifications were performed to increase the sensitivity of the first test. For those 1st PCR products not visible or slightly visible by agarose gel electrophoresis, 1 µl of 1:10 dilutions were used as template in the 2nd PCR. The re-amplification PCR conditions were the same as described above for the 1st PCR. The amplification products were separated and visualized by agarose gel electrophoresis according to standard procedures (Sambrook et al. 1989).

Results

PWN detection in wood samples

The number of PWN and other nematodes estimated for 100 mg of the pine wood samples is presented in Table 1. Using total DNA extracted from these samples as PCR template, the specific pattern of monomer and multimers of the 160 bp monomer, specific of PWN, was detected after the 1st PCR in samples Pp1, Pp2 and Pp3 (Fig. 1a). The positive signs of Pp3 sample increased after re-amplification of the 1st PCR product, and positive signs for wood samples Pp4 and Pp5 were detected (Fig. 1b). No PCR amplification was detected in sample Pp7, with only other nematodes, and in samples Pp6 and Ht1, with no nematodes (Fig. 1a and b). Using total DNA extracted from nematode suspensions obtained from wood samples after nematodes extraction, PWN was effectively detected in the suspension containing 1 PWN, after re-amplification of the 1st PCR product (data not shown).

PWN detection in bark samples

After the 1st PCR, PWN was effectively detected in bark samples with 1,000 nematodes. A slight sign was visible for samples with 10 PWN and no sign was detected in samples with 1 PWN (Fig. 2a). After re-amplification of the 1st PCR products, the positive signs of these samples increased and a positive sign for the bark sample infested with 1 PWN was obtained (Fig. 2b).

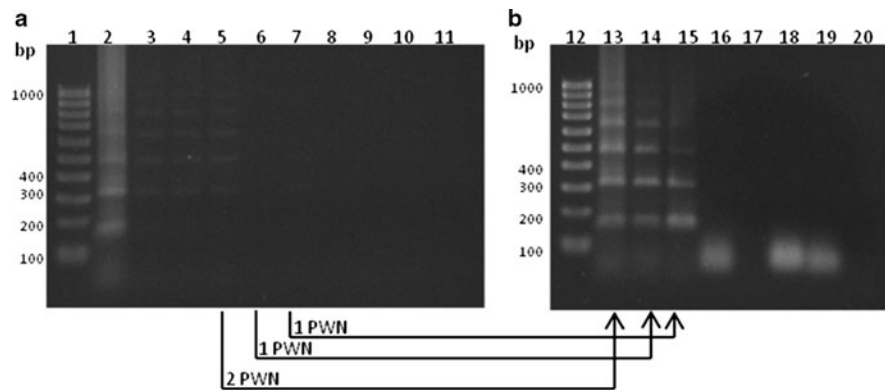


Fig. 1 Pinewood nematode molecular detection in wood samples. 1st PCR (a) using DNA from: *Bursaphelenchus xylophilus* (positive control) (2); Pp1 (34 PWN) (3); Pp2 (5 PWN) (4); Pp3 (2 PWN) (5); Pp4 (1 PWN) (6); Pp5 (1 PWN) (7); Pp6 (0 PWN) (8); Pp7 (0 PWN) (9); Ht1 (0 PWN) (10). Re-amplification of

1st PCR products (b) using a 1:10 dilution of product in lane 5 (13); 1:10 in lane 6 (14); 1:10 in lane 7 (15); 1:10 in lane 8 (16); 1:10 in lane 9 (17); 1:10 in lane 10 (18); 1:10 in lane 11 (19). DNA HyperLadderIV (Bioline) (1 and 12); no template control (NTC) (11 and 20)

PWN detection in the insect vector, *M. galloprovincialis*

After the 1st PCR, PWN was effectively detected in insects infested with 1,000, 100 and 10 PWN and no visible signs were obtained for insects infested with 5 and 1 PWN (Fig. 3a). After re-amplification of the 1st PCR products, positive signs for the insect samples infested with 5 and 1 PWN were detected (Fig. 3b).

Discussion

Total DNA, extracted from wood, bark and nematode suspensions of pine wood samples and from *M. galloprovincialis*, was successfully used as template for PCR detection of PWN by amplification of the species specific satDNA, generating a PCR specific pattern of monomer and multimers of the 160 monomer unit, as previously described (Tarés et al. 1994; Castagnone et al. 2005). The results obtained show that these DNA extraction methods are suitable for obtaining DNA free from PCR inhibitors, which are commonly present in wood and insects (Wilson 1997). The re-amplification of the 1st PCR product increased the sensitivity of the test to 1 PWN in naturally infected wood samples and artificially infested bark and insect samples. Furthermore, the reproducibility and specificity of the test were ascertained repeating all the experiments three times and using DNA from the

closely related species, *B. mucronatus*, as a negative control (data not shown) and also total DNA extracted from wood with other nematodes (families Rhabditidae and Aphelenchoididae).

This methodology is easy to perform, not very expensive and not so time consuming. When the results are negative it is necessary to use a larger amount of screening material, using more replicates or applying this methodology to a nematode suspension obtained from 100 g of wood/bark sample by using the tray extraction method. The application to nematode suspensions from *P. pinaster* wood samples, was also shown to be effective in the detection of PWN.

To date, several DNA extraction methods have been used for PCR based detection of PWN directly from wood samples. One previously described method, an adaptation of the cetyltrimethylammonium bromide (CTAB) method, was efficient, but laborious and time consuming (Takeuchi et al. 2005; Takeuchi and Futai 2007, 2009). Another method described by Kikuchi et al. (2009) and Kanetani et al. (2011), cannot be reproduced as there is no description of the buffers and the mentioned kit is not commercially available. The DNA extraction methods, directly from wood samples, described by Wang et al. (2010) and Hu et al. (2011) are time consuming and use only 5 mg of wood sample as starter material, which is a much smaller sample than the 100 mg used in this study. Furthermore, another DNA extraction method from

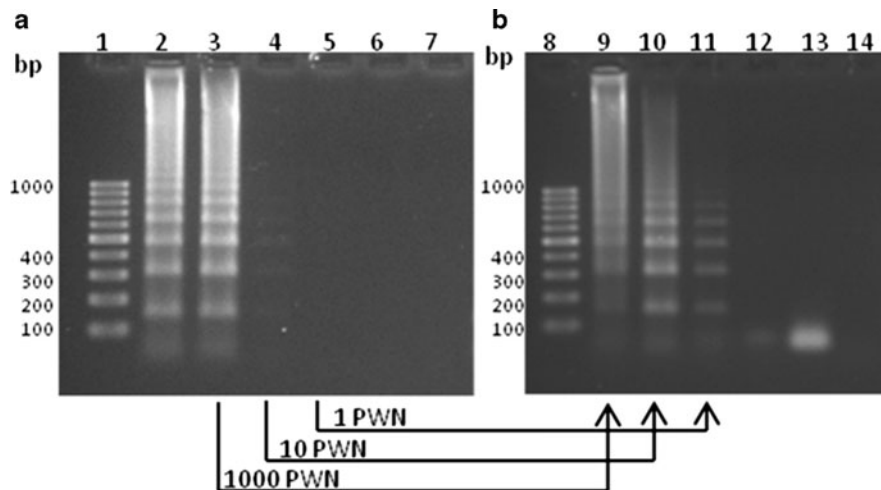


Fig. 2 Pinewood nematode molecular detection in *Pinus pinaster* bark samples. 1st PCR (a) using DNA from: *Bursaphelenchus xylophilus* (positive control) (2); bark with 1,000 PWN (3); bark with 10 PWN (4); bark with 1 PWN (5); non-infested bark (6). Re-amplification of 1st PCR products (b)

using a 1:100 dilution of product in lane 3 (9); 1:10 in lane 4 (10); 1:10 in lane 5 (11); 1:10 in lane 6 (12) and 1:10 in lane 7 (13). GeneRuler 100 bp DNA Ladder (Fermentas) (1 and 8); NTC (7 and 14)

100 mg of wood was successfully used for PWN direct detection by real-time PCR (François et al. 2007), however this method is more laborious and time consuming than the one described here. None of these methods have been tested for bark samples.

Regarding the insect vector, direct molecular detection of PWN using DNA extracted from 2 mg of stored tracheal tissue of *M. alternatus*, was recently

reported (Wang et al. 2011). In the present study, the molecular detection of PWN was performed using one entire insect *M. galloprovincialis* for the DNA extraction, without the need of dissecting the insect. This technique is an useful tool for the detection of the PWN from its insects vector as soon as they are caught from pine trees and could be used as a routine procedure.

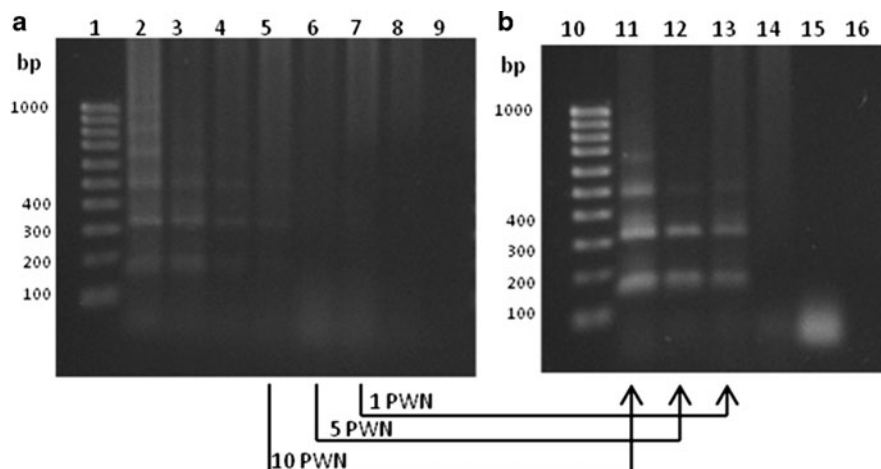


Fig. 3 Pinewood nematode molecular detection in the insect vector *Monochamus galloprovincialis*. 1st PCR (a) using DNA from: *Bursaphelenchus xylophilus* (positive control) (2); insect with 1,000 PWN (3); insect with 100 PWN (4); insect with 10 PWN (5); insect with 5 PWN (6); insect with 1 PWN (7); non-

infested insect (8). Re-amplification of 1st PCR products (b) using a 1:10 dilution of product in lane 5 (11); 1:10 in lane 6 (12); 1:10 in lane 7 (13); 1:10 in lane 8 (14); 1:10 in lane 9 (15). DNA HyperLadderIV (Bioline) (1 and 10); NTC (9 and 16)

The methodology described herein is very sensitive allowing the detection of 1 PWN/100 mg of wood and bark samples and 1 PWN/insect, without the preliminary steps of nematode extraction, constituting a new tool for the detection and identification of PWN, which is fundamental to define control and management strategies.

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