Forest Pathology

For. Path.
doi: 10.1111/j.1439-0329.2010.00676.x

REVIEW

A rapid staining-assisted wood sampling method for PCR-based detection of pine wood nematode *Bursaphelenchus xylophilus* in *Pinus massoniana* wood tissue

By X. R. Wang[1,3], X. C. Kong[1], W. H. Jia[1], X. W. Zhu[1], L. L. Ren[1] and M. M. Mota[2]

[1]Laboratory of Plant Nematology, College of Natural Resources and Environment, South China Agricultural University, Guangzhou 510642, China; [2]NemaLab/ICAAM, Dept de Biologia, Universidade de Évora, Évora, Portugal; [3]E-mail: xinrongw@scau.edu.cn (for correspondence)

Summary

For reasons of unequal distribution of more than one nematode species in wood, and limited availability of wood samples required for the PCR-based method for detecting pinewood nematodes in wood tissue of *Pinus massoniana*, a rapid staining-assisted wood sampling method aiding PCR-based detection of the pine wood nematode *Bursaphelenchus xylophilus* (Bx) in small wood samples of *P. massoniana* was developed in this study. This comprised a series of new techniques: sampling, mass estimations of nematodes using staining techniques, and lowest limit Bx nematode mass determination for PCR detection. The procedure was undertaken on three adjoining 5-mg wood cross-sections, of 0.5 × 0.5 × 0.015 cm dimension, that were cut from a wood sample of 0.5 × 0.5 × 0.5 cm initially, then the larger wood sample was stained by acid fuchsin, from which two 5-mg wood cross-sections (that adjoined the three 5-mg wood cross-sections, mentioned above) were cut. Nematode-staining-spots (NSSs) in each of the two stained sections were counted under a microscope at 100× magnification. If there were eight or more NSSs present, the adjoining three sections were used for PCR assays. The *B. xylophilus* – specific amplicon of 403 bp (DQ855275) was generated by PCR assay from 100.00% of 5-mg wood cross-sections that contained more than eight Bx NSSs by the PCR assay. The entire sampling procedure took only 10 min indicating that it is suitable for the fast estimation of nematode numbers in the wood of *P. massonina* as the prelimary sample selections for other more expensive Bx-detection methods such as PCR assay.

1 Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* is the causal agent of pine wilt disease. *Pinus massoniana* is the dominant species of pine forestry in China. Since the 1980s, it has been attacked by PWN, which has spread to several provinces and caused extensive economic losses (Chen et al. 2005). *Bursaphelenchus xylophilus* is believed to be native to North America, where it has been reported (Dwinell 1993). It has now spread to Japan, China, Korea and Portugal (Mota et al. 1999; Suzuki 2002; Mota and Vieira 2008). Because of the obvious risk of further spread to other provinces in China and also to other European countries, surveys for *B. xylophilus* will be necessary throughout the country or continent to try detect this pest, or to delimit infested areas when positive results are obtained during the detection survey. Without efficient and consistent sampling methods, the results of detection and delimiting surveys will not be adequate. There are numerous scientific publications on *B. xylophilus*, which include sampling methods, but the methods are variable and there is no universally accepted methodology in this field (Schröder et al. 2009).

There are numerous scientific publications that deal with the detection of *B. xylophilus* from wood samples (Hoyer et al. 1998; Iwahori et al. 2000; Liao et al. 2001; Kang et al. 2004; Penas et al. 2004; Castagnone et al. 2005; Wu et al. 2005; François et al. 2007;

Received: 21.3.2010; accepted: 17. 6. 2010; editor: J. Hantula

Leal et al. 2007; Burgermeister et al. 2009; Zhuo et al. 2010). Most of the methods require the prior extraction of nematodes from wood. In some cases this method can be effective, but it is not always suitable for extracting nematodes from pine wood (Mamiya 1975), especially under low temperature conditions, when the nematodes are dead, or when they occur at low densities. To overcome this problem, Takeuchi et al. (2005) investigated a nested-PCR method developed in Japan to detect *B. xylophilus* from wood tissues, of three species of pine tree: *Pinus thunbergii*, *Pinus densiflora* and *Pinus taeda*. Wang et al. (2006) also developed a PCR-based method for detecting the *B. xylophilus* in 5-mg wood tissue of *P. massoniana* in China. Because of unequal distribution of more than one nematode species in wood (Ryss et al. 2005; Schröder et al. 2009; Zhao et al. 2009), and limited availability of wood samples required for the PCR-based method for detecting PWN in *P. massoniana*, it has become crucial to develop a fast and reliable wood sampling method for PCR-based detection in small wood samples of *P. massoniana*.

In this study, a staining method was developed for estimating the nematode mass in a 5-mg cross-section from a wood sample of *P. massoniana*. The limit of PWN mass required for successful PCR detection in a 5-mg sample was then proposed, based on statistical analysis. Thus, a rapid staining-assisted wood sampling for PCR-based detection of a PWN (*B. xylophilus*) in wood tissue of *P. massoniana* is herein presented, which consists of a series of new techniques and results including 'sampling, nematodes mass estimation by staining and lowest limit nematode mass determination for the PCR detection'.

2 Material and methods

2.1 Wood sample preparation

In October 2007, a $7 \times 7 \times 5$ cm wood piece, that was infected with PWN was cut from the southern part of the base crown of pine tree of *P. massoniana* died within in 1 month, since within 1 month after the death, as a result of pine wilt disease, of a *P. massoniana* tree, the nematode species composition on the tree consisted entirely of *B. xylophilus*. The wood was placed inside a plastic bag to maintain humidity and then transported back to our laboratory. A 10-g wood sample was used for nematode extraction at 25–30°C using a Baermann funnel (Mamiya 1975). The PWN and other nematodes were identified and counted under the microscope at 100× magnification (CH30; OLYMPUS). If pure PWN were present, the rest of the wood sample was cut into pieces of approximately $0.5 \times 0.5 \times 0.5$ cm and kept at 10°C for the following experiment. A total of 60 wood samples were collected from the 50-year-old pine forest in Guangdong Province, China.

2.2 Nematode population

Bursaphelenchus xylophilus was isolated from chips of infested *P. massoniana* wood using a Baermann funnel (Mamiya 1975). Identification of species was determined by means of microscopic examination and PCR assay (Wang et al. 2009). Isolates of *B. xylophilus* were reared on a lawn of *Pestalotiopsis* sp. cultured on PDA plates, at 25°C in the dark. Collected nematode species were stored in water at 4°C.

2.3 Staining nematodes in *Pinus massoniana* wood tissue

Staining nematodes in a longitudinal wood section of *P. massoniana* was done according to the method described by Byrd et al. (1983), with some modifications. A wood sample of $0.5 \times 0.5 \times 0.5$ cm was cut into longitudinal sections of about 0.015 cm in thickness. Sections were placed in 1 ml cold water and then heated to boiling point, after which 1 ml acid fuchsin was added. The mixture was then heated and stirring for 30 s, before another

1 ml acid fuchsin added, and stirred and heated again, as described above. The section was then transferred to cold water for rinsing (a few seconds), and photographed under a microscope of 100× magnification (CH30; OLYMPUS).

Staining nematodes in 5 mg wood cross-sections of *P. massoniana* was done by the same method as that described above, except that the wood sample of 0.5 × 0.5 × 0.5 cm was placed in 5 ml cold water and then heated to boiling point, in which 3 ml acid fuchsin was added. The mixture was then heated and stirred for 1 min before another 3 ml acid fuchsin was added, and stirred and heated for 30 s. The sample was then cooled to ambient environment. The stained sample was cut into many 5 mg wood cross-sections. For each section, its size was 0.5 cm (long) × 0.5 cm (wide) × 0.015 cm (thick) for photography under microscope (100×).

Twenty 0.5 × 0.5 × 0.5 cm wood samples were stained according to the method for staining nematodes in a wood cross-section of *P. massoniana* as described above. For each sample, five adjoining 5 mg wood cross-sections measuring 0.5 × 0.5 × 0.015 cm were selected (Fig. 1). Because nematodes were cross-cut and stained as nematode-staining-spots (NSSs) in a 5 mg wood cross-section, the nematode mass could be estimated by the mass of total NSSs in each 5 mg wood cross-section. The nematode mass of one NSS is equal to that of a nematode of 0.015 cm body length, so the total nematode mass in one 5 mg wood cross-section will be equivalent to the total body mass of NSSs which it contains. Therefore, the comparative nematode mass among five adjoining 5 mg wood cross-sections from one wood sample of *P. massoniana* would be equivalent to that of the total number of NSSs among each of the five adjoining 5 mg wood cross-sections. Nematode-staining-spots in each section were counted under a microscope. Multiple comparisons of a number of NSSs among each of five adjoining 5 mg wood cross-sections were made, SAS software 8.0 (SAS Institute Inc, Cary, NC, USA).

2.4 DNA extraction

DNA from a single *B. xylophilus* nematode was isolated based on the method of LIAO et al. (2001), which involves a simple procedure: a single adult or juvenile specimen was hand-picked and placed in 10 μl lysis buffer (2.5 mM DTT, 1.125% (w/v) Tween 20, 0.025% (w/v) Gelatin, 125 mM KCl, 25 mM Tris–HCl (pH 8.0), 3.75 mM $MgCl_2$), and placed in a container maintained at a temperature of −70°C for 30 min. Each tube was then incubated at 95°C for 15 min, then 65°C for 5 min, after which 1 μl of 20 mg/ml proteinase K was added, prior to incubation at 65°C for 1 h, followed by 95°C for 15 min. The next stage involved one of two options: either the PCR reaction was performed directly in the same tube, or the lysate was frozen at −20°C for subsequent procedures.

DNA extraction from infested wood samples of *P. massoniana* was carried out according to the methods described by WANG et al. (2009): a 5-mg wood cross-section containing nematodes was placed in 88 μl lysis buffer (2.5 mM DTT, 1.125% (w/v) Tween 20, 0.025% (w/v) Gelatin, 125 mM KCl, 25 mM Tris–HCl (pH 8.0), 3.75 mM $MgCl_2$). The sample was

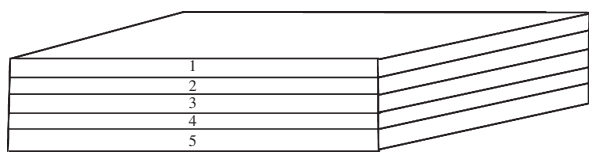

Fig. 1. Cross-section of a 0.5 × 0.5 × 0.5 cm piece of wood cut into five adjoining 5-mg sections of 0.5 × 0.5 × 0.015 cm.

then treated as follows: kept at −70°C for 30 min, incubated at 95°C for 15 min, and then at 65°C for 5 min, after which 10 μl of 20 mg/ml proteinase K was added to the solution, which was then vortexed prior to incubation: first at 65°C for 1 h, and then at 95°C for 15 min. After centrifugation at 15 300 *g* for 3 min at 4°C, the aqueous phase containing genomic DNA was transferred to a new tube and passed through 3S column kit (Shanghai Shenergy Biocolor Bioscience & Technology Company, China) according to the kit protocol. The final sample solution contained the genomic DNA.

2.5 PCR amplification

A pair of PCR primers specific to PWN was designed based on the nucleotide sequence data reported by Wang et al. (2006, 2009). It consisted of a forward primer P155 (sequence: 5′-CTACGTGCTGTTGTTGAGTTGGC-3′) and a reverse primer P538 (sequence: 5′-TGGTGCCTAACATTGCGCGA-3′), synthesized by Beijing SAIBAISHENG Biotic Company (Beijing, China). To detect PWN, PCR was performed on a 2 μl of the sample solution in a final volume of 25 μl, containing 2.5 μl of PCR buffer (10×), 2.5 μl of 25 mM $MgCl_2$, 2 μl of 2.5 mM dNTP mixture, 1.0 μl of each 10 μM primer, 0.1 μl of 0.5 U rTaq polymerase (TaKaRa Biotech, Japan) and 14.9 μl of double distilled water on a thermal cycler (TaKaRa, Japan) with the following cycling experimental conditions: 94°C for 3 min followed by 40 cycles at 94°C for 1 min, 50°C for 30 s, and 72°C for 45 s, ending with one cycle at 72°C for 10 min followed by storage at 4°C. Amplification products were separated on a 1.5% (w/v) agarose gel.

2.6 Determination of the lowest limit of *Bursaphelenchus xylophilus* nematode mass by staining 5-mg wood sample used for the PCR assay

Evaluation of nematode staining procedure for potential PCR inhibition was studied initially as follows: Nematodes were boiled and stained by acid fuchsin the same as that described above. A single boiled nematode and a single stained nematode were used in the PCR assay. Four replicate samples were used for each treatment. The above-mentioned method, using one single nematode, was employed for the DNA extraction procedures and the PCR amplification conditions.

Because acid fuchsin inhibited PCR amplification, this method was not successful for detecting a single stained PWN by the PCR assay. Statistic analysis indicated that there was no significant difference among the nematode mass of five adjoining 5 mg wood cross-sections. Then the relationship between number of NSSs and its PCR assay result in each 5 mg wood cross-section of *P. massonina* wood samples was studied. The PCR assay was undertaken on each of three adjoining 5 mg wood cross-sections, of 0.5 × 0.5 × 0.015 cm dimension, that was cut from a wood sample of 0.5 × 0.5 × 0.5 cm initially, then the larger wood sample was stained by acid fuchsin, from which two 5 mg wood cross-sections (that adjoined the three 5 mg wood cross-sections, mentioned above) were cut. Nematode-staining-spots in each of the two stained sections were counted under a microscope at 100× magnification (Fig. 2). The test was carried out on 20 replicates.

3 Results

3.1 Nematode staining

Fifty longitudinal wood sections and fifty 5 mg wood cross-sections with a thickness of 0.015 cm were stained, respectively. Thereafter, microscopic examination indicated that the body of nematode was stained purple-red, the longitudinal sections of wood were brown

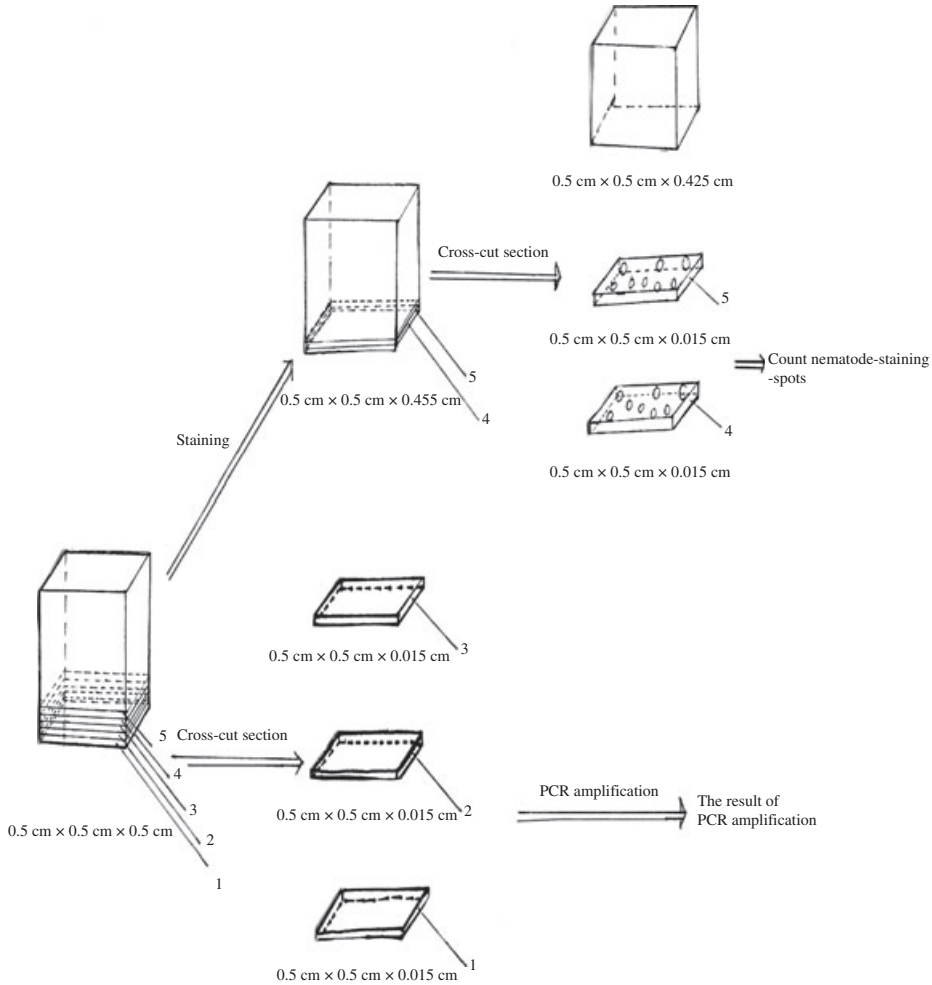

Fig. 2. Schematic diagram illustrating the method used for a rapid, staining-assistant wood sampling for PCR-based detection of pine wood nematode *Bursaphelenchus xylophilus* in *Pinus massoniana* wood tissue.

(Fig. 3a). It was noted that 90% of nematodes were straight and lay parallel to the xylem vessels of the pine wood (Fig. 3a). The bodies of few nematodes were bent in the mid-body region (Fig. 3b). While in wood cross-sections, cross-cut nematodes were easily seen under the microscope, appearing as a purple-red nematode-staining-spot (NSS) in the cross-section of xylem vessels in the wood of *P. massonina* (Fig. 4). Nematodes were successfully stained in 100% of the sections.

Nematode mass comparison among five adjoining 5 mg wood cross-sections was studied. The multiple-comparison result indicated no significant difference among the numbers of NSSs of five adjoining 5 mg wood cross-sections at the level of 0.05 ($p \geq 0.05$) (Table 1).

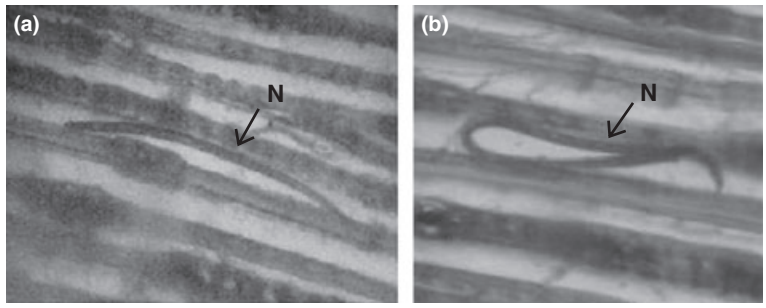

Fig. 3. Microscopic images (photographs) of stained nematodes in a longitudinal section of *Pinus massoniana* wood (at 100×).

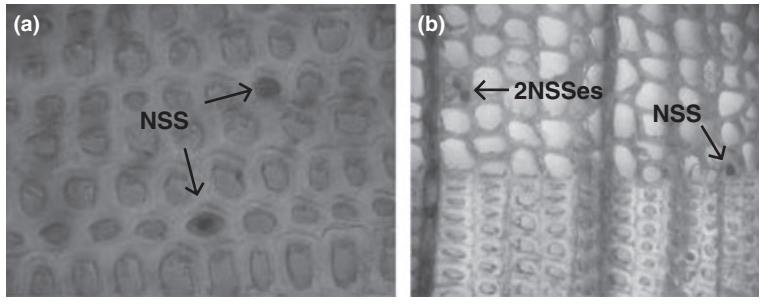

Fig. 4. Microscopic images (photographs) of nematodes in cross-section within *Pinus massoniana* wood tissue, (also cut in cross-section) at 100×.

Table 1. **Multiple comparisons of the number of nematode-staining-spots (NSSs) among five adjoining 5 mg cross-sections from a sample of *Pinus massoniana* wood (unit: number of NSSs/section).**

Cross-section number	1	2	3	4	5	F-value	p > F
Number of NSSs in one 5-mg wood cross-section	8.15 ± 1.64a	5.85 ± 0.93a	5.95 ± 0.99a	7.40 ± 2.12a	5.4 ± 0.82a	1.36	0.2574

The number of NSSs in one 5 mg wood cross-section is equal to the mean + standard error of 20 replicates.
For each row, the figure with the same letter is at the same level in terms of statistical analysis ($p > 0.05$).
Multiple comparison result was analyzed by the software SAS 8.0.

3.2 Determination of the lowest limit of *Bursaphelenchus xylophilus* nematode mass by staining the wood sample used for the PCR assay

Evaluation of nematode staining procedure for potential PCR inhibition was studied initially. Single boiled nematode or single stained nematode were used in PCR assays: 100% *B. xylophilus*-specific PCR products of 403 bp (DQ855275) were generated from the

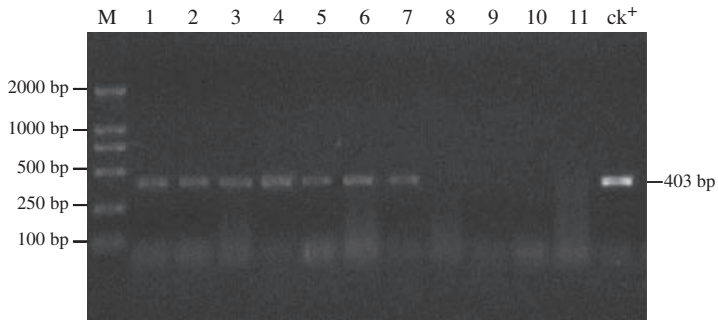

Fig. 5. Electrophoresis of the amplicons from a single specimen of pine wood nematode *Bursaphelenchus xylophilus* after boiling or staining by acid fuchsin. The forward primer was P155, the reverse primer was P538. Four replicates for each treatment were done. About 100% *B. xylophilus* specific PCR products were generated for single live *B. xylophilus* (lanes 1–3), and for the boiled nematode (lanes 4–7) and for a positive control (genomic DNA of *B. xylophilus*) (CK^+). No PCR products were generated for a single *B. xylophilus* stained by acid fuchsin (lanes 8–11). M, DL 2000 DNA ladder.

boiled nematode procedure (Fig. 5, lanes 4–7) and from a positive control (genomic DNA of *B. xylophilus*) (CK^+), no PCR products were generated from single individual of *B. xylophilus* that had been stained by acid fuchsin (Fig. 5, lanes 8–11), which indicates that the nematode staining procedure inhibits *B. xylophilus* PCR amplification.

Because there is no significant difference among the numbers of NSSs of five adjoining 5 mg-wood cross-sections, the relationship between number of NSSs and its corresponding PCR assay result in each set of five adjoining 5 mg wood cross-sections of *P. massonina* wood samples was studied. About 100% *B. xylophilus*-specific PCR products of 403 bp (DQ855275) were generated for the 5-mg wood cross-sections, with more than eight *B. xylophilus* NSSs (Fig. 6, lanes 1–3, 5, 6, 10, 19; Fig. 7, lanes 1–6).

4 Discussion

Although acid fuchsin has been widely used for staining nematodes in histopathological studies as well as nematode number counting (Byrd et al. 1983; Daykin and Hussey 1985; Dhandaydham et al. 2008), there has been no report on using acid fuchsin to stain PWN in wood cross-sections of pine wood tissue for nematode mass estimation. We have established the method to stain the PWN, both in longitudinal sections and in cross-sections of *P. massoniana* for nematode mass estimation in this study. For successful detection of stained nematodes under microscopic examination, the section thickness should be between 0.015 cm and 0.020 cm. If the section is thinner than 0.015 cm, the nematodes will be lost during the staining procedure; if it is thicker than 0.020 cm, the section will be too thick for stained nematodes to be seen under the microscope.

Because acid fuchsin inhibited PCR amplification, it was not successful in detecting a single stained PWN by means of PCR assays. In this research, the size for a 5-mg wood cross-section was $0.5 \times 0.5 \times 0.015$ cm (thickness). The total thickness of adjoining five 5-mg wood cross-sections will be 5×0.015 cm = 0.75 cm < 0.1 cm. The average length of *B. xylophilus* is around 0.0772.4 (0.0657.8–0.0809.6) cm in Guangzhou or 0.114 (0.096–0.131) cm in Nanjing, China (Cheng et al. 1983), or 0.081 (0.71–1.01) cm in Japan (Mamiya and Kiyohara 1972). Ninety percent of nematodes in *P. massonina* wood were straight and parallel to the xylem vessels of the pine wood (Fig. 3a), but few nematodes were bent in the mid region of the body (Fig. 3b). Nematode mass in each adjoining five 5-mg wood cross-section was supposed to be the same. A statistical comparison of the

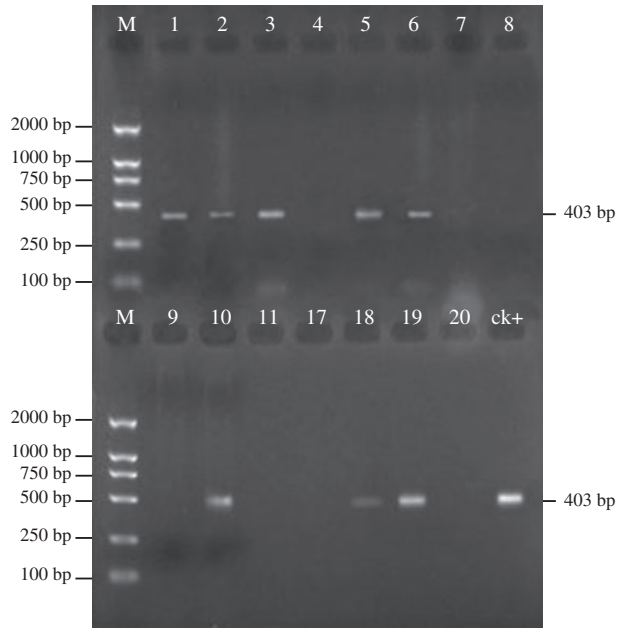

Fig. 6. Electrophoresis of the *Bursaphelenchus xylophilus*-specific amplicons derived from a 5 mg wood sample (cut in cross-section) with a number of stained- nematode-spots (NSSs). The forward primer was P155, the reverse primer was P538. Two replicates for each treatment were done. About 100% *B. xylophilus* specific PCR products were generated from a wood sample with more than eight *B. xylophilus* NSSs (lanes 1–3, 5, 6, 10, 19) and CK^+, a positive control (DNA of single individual *B. xylophilus*). About 50.0% *B. xylophilus*-specific PCR products were generated from a wood sample with six NSSs (lane 7, 18). No PCR products from a wood sample with <6 NSSs were generated (lanes 4, 7–9, 11, 17, 20). M, DL 2000 DNA ladder.

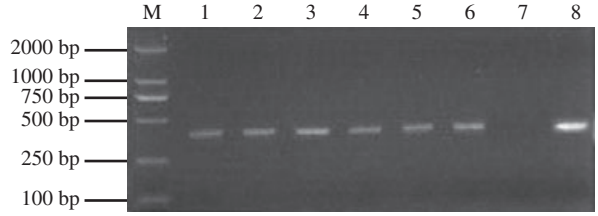

Fig. 7. Electrophoresis of *Bursaphelenchus xylophilus*-specific amplicon results derived from a 5 mg wood sample (cut in cross-section with more than eight *B. xylophilus* stained-nematode-staining-spots (NSSs). The forward primer was P155, the reverse primer was P538. Three replicates for each treatment were done. About 100% *B. xylophilus* specific PCR products were generated from wood sections with 11 *B. xylophilus* NSSs (lanes 1–3) and 12 *B. xylophilus* NSSs (lanes 4–6) and a positive control (lane 8, DNA of a single *B. xylophilus* specimen). No PCR products were generated for the negative control (lane 7). M, DL 2000 DNA ladder.

nematode mass among adjoining five 5 mg wood cross-sections indicated that no significant differences between each of the five adjoining 5 mg wood cross-sections ($p > 0.05$) (Table. 1). Among the adjoining five 5 mg wood cross-sections, two sections

could be used for staining and counting of nematode-staining spots, which were used for estimating nematode mass in the stained 5 mg wood cross-sections, the three remaining unstained pieces were used for PCR assays (Fig. 2). This study represents the first report of estimating nematode mass in wood samples by means of acid fuchsin staining before the wood sample was used for detecting nematodes by means of PCR assays.

Nematode mass determination in 5 mg wood cross-sections is an important factor for successful PCR assays. The nematode mass estimation was based on the NSSs number in one 5 mg wood cross-section. The nematode mass in one NSS is equal to that of a single nematode body of 0.015 cm in length. For each of the five adjoining 5-mg wood cross-sections, NSSs in two adjoining 5 mg wood cross-sections were counted and the remaining three 5 mg wood cross-sections were used for PCR assays. Results indicated that the lowest limit of nematode mass in a 5 mg wood cross-section for successful detecting of PWN by PCR assay was eight Bx NSSs (Table 2; Figs 6 and 7). Note that 8×0.015 cm = 0.12 cm, which is similar to the 1.6× the length of a nematode body (in Guangzhou). Therefore, the lowest nematode number in the wood sample should be 1.6×200 nematotodes/5 mg $\times$ 200 = 320 nematodes/g. There are about 70 species of *Bursaphelenchus* associated with pine trees (Ryss et al. 2005), and 12 nematodes species of *B. mucronatus*, *B. aberrans*, *B. corneolus*, *B. leoni*, *B. hunanensis*, *B. teratospicularis, Aphelenchoides resinosi*, *Seinura steineri*, *Ditylenchus Parvus*, *Odorhabdiplogaster xiphocaudatus, Rhabditida* sp. and *Parasitorhabditis* sp. in the wood of *P. massoniana* (Wang et al. 2009). All the nematodes were stained by acid fuchsin in the pine wood. Therefore, there was a need to establish that the eight nematode-staining-spots observed in a 5-mg wood cross-sections were all PWNs? This can be explained as follows: our another research on nematode species compositions in one wood sample based on nematode extraction

Table 2. **Number of stained nematode spots (NSSs) and its corresponding *Bursaphelenchus xylophilus*-specific PCR detection results in each set of five adjoining 5 mg wood cross-sections of *P. massonina* wood samples.**

Sample no.	Number of NSSs per 5 mg wood cross-section	*Bursaphelenchus xylophilus*-specific amplicons (see Fig. 6)
1	12	+
2	11	+
3	17	+
4	4	–
5	13	+
6	9	+
7	6	–
8	2	–
9	5	–
10	8	+
11	3	–
12	0	–
13	0	–
14	1	–
15	0	–
16	0	–
17	2	–
18	6	+
19	32	+
20	4	–

+, *B. xylophilus*-specific PCR products generated; –, No *B. xylophilus*-specific PCR products generated.

result from 236 wood samples of *P. massoniana* showed that there were either only one or maximum two nematode species in one wood sample of one individual *P. massoniana* tree. Within 1 month after the death, as a result of pine wilt disease, of a *P. massoniana* tree, the nematode species composition in the tree consisted entirely of *B. xylophilus.* After 3 months, however, there was a risk that other nematodes would be dominant in the wood sample, but the nematode density is <180 nematodes/g by Baermann funnel at 25–30°C (whose NSSs should be estimated as <8). As the present primers could differentiate *B. xylophilus* from 12 other nematodes (including *B. mucronatus*, *B. aberrans*, *B. corneolus*, *B. leoni*, *B. hunanensis*, *B. teratospicularis, Aphelenchoides resinosi*, *Seinura steineri*, *Ditylenchus parvus*, *Odorhabdiplogaster xiphocaudatus*, *Rhabditida* sp. and *Parasitorhabditis* sp.) (Wang et al. 2009), these present results were reliable.

Compared with traditional nematode extraction method by Baermann funnel, which extracts only active pine wilt disease nematodes from pine wood tissue under the proper temperature, and requires experts to identify nematodes, which would take longer than 24 h, this study describes a practical method to estimate nematode mass in wood sample before detecting nematodes by means of PCR assays to reduce PWN detecting cost by PCR assay, since all live PWN nematodes in the wood section can be stained by acid fuchsin and detected by the PCR assay. In our another study, it was found that in the case that the number of nematode-staining-spots were counted as 8, 16, 12 in a 5-mg wood cross-section and were successfully detected by the PCR assay, then according to our nematodes mass estimation method, the nematode extraction number using a Baermann funnel should be 320, 640, 480/g, respectively, but the real nematode extraction result was only 0.6, 1.6, 27.9 nematodes/g after 24 h, at 20°C, respectively.

Acknowledgements

The authors are grateful to Dr. P. Castagnone-Sereno (INRA, France) for his valuable advices on submitting the manuscript. This research is subsidized by the Natural Science Research Fund of Guangdong Province (04020597), National Technology Support Project of China (2006BAD08A19112), and National Natural Science Foundation of China (NSFC30771409).

References

Burgermeister, W.; Braasch, H.; Metge, K.; Gu, J. F.; Schröder, T.; Woldt, E., 2009: ITS-RFLP analysis, an efficient tool for differentiation of *Bursaphelenchus* species. Nematology **11,** 649–668.

Byrd, D. W. Jr; Kirkpatrick, T.; Barker, K. R., 1983: An improved technique for clearing and staining tissues for detection of nematodes. J. Nematol. **15,** 142–143.

Castagnone, C.; Abad, P.; Castagnone-sereno, P., 2005: Satellite DNA-based species-specific identification of single individuals of the pinewood nematode *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). Eur. J. Plant Pathol. **112,** 191–193.

Chen, F. M.; Tang, J.; Ye, J. Z., 2005: Pine wilt disease diagnostic method assessment. J. Anhui Agric. Univ. **32,** 22–25.

Cheng, H. R.; Lin, M. S.; Li, W. Q., 1983: Occurrence of pine wilt disease on *Pinus thunbergii* in Nanjing. For. Pest. **4,** 1–5. (In Chinese).

Daykin, M. E.; Hussey, R. S., 1985: Staining and histopathological techniques in nematology. In: An Advanced Treatise on Meloidogyne, Vol. **II**. Ed. by Barker, K. R.; Carter, C. C.; Sasser, J. N. Raleigh: North Carolina State University Graphics, pp. 39–48.

Dhandaydham, M.; Charles, L.; Zhu, H. Y.; Starr, J. L.; Huguet, T.; Cook, D. R.; Prosperi, J. M.; Opperman, C., 2008: Characterization of root-knot nematode resistance in *Medicago truncatula*. J. Nematol. **40,** 46–54.

Dwinell, L. D., 1993: First report of pinewood nematode (*Bursaphelenchus xylophilus*) in Mexico. Plant Dis. **77,** 846.

François, C.; Castagnone, C.; Boonham, N.; Tomlinsonr, R.; Lawson, C.; Hockland, S.; Quill, J.; Vieira, P.; Mota, M.; Castagnone-sereno, P., 2007: Satellite DNA as a target for TaqMan real-time PCR detection of the pine wood nematode, *Bursaphelenchus xylophilus*. Mol. Plant Pathol. **8,** 803–809.

Hoyer, U.; Burgermeister, W.; Braasch, H., 1998: Identification of *Bursaphelenchus* species (Nematode, Aphelenchoididae) on the basic of amplified ribosomal DNA (ITS-RFLP). Nachrichtenbl. des Dtsch. Pflanzenschutzdienst. **50,** 273–277.

Iwahori, H.; Kanzaki, N.; Futa, I. K., 2000: A simple polymerase chain reaction-restriction fragment length Polymorphism-aided diagnosis method for pine wilt disease. For. Pathol. **3,** 157–164.

Kang, J. S.; Choi, K. S.; Shin, S. C.; Moon, I. S.; Lee, S. G.; Lee, S. H., 2004: Development of an efficient PCR-based diagnosis protocol for identification of the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). Nematology **6,** 279–285.

Leal, I.; Margaret, G.; Eric, A.; Leland, H.; Michael, R., 2007: Application of a real-time PCR method for the detection of pine wood nematode, *Bursaphelenchus xylophilus*, in wood samples from lodgepole pine. Nematology **9,** 351–362.

Liao, J. L.; Zhang, L. H.; Feng, Z. X., 2001: A reliable identification of *Bursaphelenchus xylophilus* by rDNA amplification. Nematol. Mediterr. **29,** 131–135.

Mamiya, Y., 1975: Efficiency of Baermann funnel method for the extraction of pinewood nematodes. For. Pest. **24,** 115–119.

Mamiya, Y.; Kiyohara, T., 1972: Description of *Bursaphelenchus lignicolus* n. sp. (Nematoda: Aphelenchoididae) from pine wood and histopathology of nematode infested trees. Nematologica **18,** 120–124.

Mota, M.; Vieira, P., 2008: Pine Wilt Disease: A Worldwide Threat to Forest Ecosystems. Berlin (DE): Springer Science + Business Media B.V.

Mota, M. M.; Braasch, H.; Bravo, M. A.; Penas, A. C.; Burgermeister, W.; Metge, K.; Sousa, E., 1999: First record of *Bursaphelenchus xylophilus* in Portugal and in Europe. Nematology **1,** 727–734.

Penas, A. C.; Correia, P.; Brsvo, M. A.; Mota, M.; Tenreiro, R., 2004: Species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Parasitaphelenchidae) associated with maritime pine in Portugal. Nematology **6,** 437–453.

Ryss, A.; Vieira, P.; Mota, M.; Kulinich, O., 2005: A synopsis of the genus *Bursaphelenchus* Fuchs, 1937 (Aphelenchida: Parasitaphelenchidae) with keys to species. Nematology **7,** 393–458.

Schröder, T.; Mcnamara, D. G.; Gaar, V., 2009: Guidance on sampling to detect pine wood nematode *Bursaphelenchus xylophilus* in trees, wood and insects. Bull. OEPP **39,** 179–188.

Suzuki, K., 2002: Pine wilt disease – a threat to pine forest in Europe. Dendrobiology **48,** 71–74.

Takeuchi, Y.; Kanzakin, N.; Futai, K., 2005: A nested PCR-based method for detecting the pine wood nematode, *Bursaphelenchus xylophilus*, from pine wood. Nematology **7,** 775–782.

Wang, X. R.; Hu, Y. Q.; Zhu, X. W., 2006: Molecular detection of *Bursaphelenchus xylophilus* in the wood tissue of *Pinus massoniana* (Abstract). Chin. Edible Fungi (Suppl.) **25,** 163

Wang, X. R.; Zhu, X. W.; Hu, Y. Q.; Huang, H. H.; Kong, X. C.; Jia, W. H., 2009: A PCR-based method for detecting the pine wood nematodes – *Bursaphelenchus xylophilus* from *Monochamus alternatus*. Sci. Silvae Sin. **45,** 70–75.

Wu, X. Q.; Xiong, D.; An, Y., 2005: RAPD analysis of genetic relationships in inter and inner-species of *Bursaphelenchus xylophilus* and *B. mucronatus*. J. Nanjing For. Univ. (Natural Sci. Edn.) **29,** 1–4.

Zhao, L. L.; Jiang, P.; Humble, L. M.; Sun, J. H., 2009: Within-tree distribution and attractant sampling of propagative pinewood nematode, *Bursaphelenchus xylophilus*: an early diagnosis approach. For. Ecol. Manag. **258,** 1932–1937.

Zhuo, K.; Luo, M.; Cui, R. Q.; Liao, J. J., 2010: A multiplex one-step PCR method for the simultaneous identification of *Bursaphelenchus xylophilus*, *B. mucronatus* and *B. doui* – three species within the *xylophilus* group. For. Pathol. doi: 10.1111/j.1439-0329.2009.00638.x [Epub ahead of print.]