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RESEARCH ARTICLE

CHEN Fengmao, YE Jianren, TANG Jian, WU Xiaoqin

Discrimination of *Bursaphelenchus xylophilus* and *Bursaphelencus mucronatus* by PCR-RFLP technique

Abstract A polymerase chain reaction–restriction fragment length polymorphism analysis was used to discriminate isolates of *Bursaphelenchus xylophilus* and *B. mucronatus*. The amplifications of *B. xylophilus* isolates yielded one fragment of approximately 890 bp and that of *B. mucronatus* was about 930 bp. Digestion of amplified products of each nematode isolate with five restriction endonucleases revealed the following results: 1) *Dra* I digestion of the internal transcribed spacer (ITS) products of *B. xylophilus* populations yielded two fragments of 510 and 380 bp. *Dra* I could not digest the ITS products of *B. mucronatus* populations; 2) *Sal* I could not digest the ITS products of all *B. xylophilus* populations, but it could digest those of *B. mucronatus* populations into two fragments, which were 720 and 220 bp; 3) digested products of four *B. xylophilus* populations by *Msp* I yielded two fragments of 530 and 360 bp, except GZ02, which could not be digested. *B. mucronatus* populations yielded three fragments: 340, 290, and 180 bp; 4) all populations of *B. xylophilus* and *B. mucronatus* could not be digested by *Apa* I; 5) digestion of the ITS products of *B. xylophilus* and *B. mucronatus* yielded two fragments of 520 and 370 bp, and 530 and 400 bp respectively. The restriction endonucleases *Dra* I and *Sal* I could be used to identify *B. xylophilus* and *B. mucronatus*. Because the results of digestion of *B. xylophilus* and *B. mucronatus* were markedly different, they were very easy to be identified and applied; *Msp* I and *Xho* I were not suitable for identification of *B. xylophilus* and *B. mucronatus* and *Apa* I could not identify and distinguish between *B. xylophilus* and *B. mucronatus*.

Keywords *Bursaphelenchus xylophilus*, *B. mucronatus*, ribosomal DNA, polymerase chain reaction-restriction fragment length polymorphism, identification

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CHEN Fengmao, YE Jianren (✉), WU Xiaoqin
College of Forest Resources and Environment, Nanjing Forestry University, Nanjing 210037, China
E-mail: jrye@njfu.edu.cn

CHEN Fengmao, TANG Jian
Anhui Forestry Department, Hefei 230031, China

1 Introduction

The restriction fragment length polymorphism (RFLP) of internal transcribed spacers (ITS) of ribosomal DNA (rDNA) has been widely employed as a useful method for identifying and differentiating many plant parasitic nematodes such as root-knot nematodes (*Meloidogyne* spp.) (Zijlstra et al., 1995), cyst nematodes (*Heterodera* spp.) (Ferris et al., 1993, 1995; Thiery and Mugniery, 1996; Orui, 1997; Szalanski et al., 1997; Subbotin, 1999; 2000; Peng et al., 2003), root rot nematodes (*Pratylenchus* spp.) (Waeyenberge et al., 2000), stem nematodes (*Ditylenchus* spp.) (Wendt et al., 1993) and dagger nematodes (*Xiphinema* spp.) (Vrain et al., 1992). Pine wood nematode (*Bursaphelenchus xylophilus*) and *Bursaphelenchus mucronatus* were also no exceptions and these two nematodes could be differentiated well by RFLP analysis from their rDNA-ITS regions (Iwahori et al., 1998; Lu et al., 2001). But as an identification method, the restriction enzyme, which could differentiate *B. xylophilus* and *B. mucronatus*, is not always convenient for judging when used for sample inspection. Based on previous studies, the aim of this study was to find some restriction enzymes which digest one species of nematodes but not the other and should be a more accurate, clear and convenient identification method for *B. xylophilus* and *B. mucronatus* and not misleading when used during an inspection.

2 Materials and methods

2.1 Nematode isolates and DNA extraction

A total of nine isolates of nematodes were studied. There were five isolates of *B. xylophilus* and four *B. mucronatus*, identified as GZ01, GZ02, AH01, US01, CAN01, X02, X03, JAP01 and KOR01. Their geographic origins are listed in Table 1.

DNA from a single nematode was extracted following the methodology by Chen et al. (2005).

Table 1 Geographic origins of *B. xylophilus* and *B. mucronatus*

No.	Species	Code	Origin	Host
1	*B. xylophilus*	GZ01	Guangzhou, China	*Pinus massoniana*
2	*B. xylophilus*	GZ02	Guangzhou, China	*P. thunbergii*
3	*B. xylophilus*	AH01	Anhui, China	*P. massoniana*
4	*B. xylophilus*	US01	USA	Packaging wood
5	*B. xylophilus*	CAN01	Canada	Packaging wood
6	*B. mucronatus*	X02	Anhui, China	Packaging wood
7	*B. mucronatus*	X03	Anhui, China	Packaging wood
8	*B. mucronatus*	JAP01	Japan	Packaging wood
9	*B. mucronatus*	KOR01	Korea	Packaging wood

2.2 Polymerase chain reaction (PCR) amplification of ITS regions

2.2.1 Internal transcribed spacer- Polymerase chain reaction (ITS-PCR) primers

Primers used in this study followed the method of Iwahori et al. (1998). The sequence of the forward primer, beginning near the end of the 18S gene, is 5′-CGTAACAAGGTAGCTGTAG-3′ and the reverse primer, terminating at a short distance into the 28S gene, is 5′-TCCTCCGCTAAATGATATG-3′.

2.2.2 Internal transcribed spacer- Polymerase chain reaction (ITS-PCR) reaction volumes

The PCR amplification was performed in 50 μL reaction volumes containing 5 μL of a 10 × PCR buffer, 5 μL of dNTP (2.5 mmol/L), 4 μL of $MgCl_2$ (25 mmol/L), 2 U AmpliTaq DNA polymerase, 2 μL of each prime (20 μmol/L), 4 μL of template DNA (20 ng/mL) and 27.6 μL of sterile distilled water. Amplification was performed on a PTC-200 (MJ Research).

2.2.3 Internal transcribed spacer- Polymerase chain reaction (ITS-PCR) procedures

The reaction settings were as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturing for 50 s at 94°C, annealing for 40 s at 49°C, extension for 1 min at 72°C and a final extension for 7 min at 72°C. After DNA amplification, 6 μL of amplified products were analyzed by 1.5% (w/v) agarose gel electrophoresis for 80 min at 100 V. Then the gel was stained for 15 min with ethidium bromide and viewed under UV light (BIO-RAD Laboratries, Italy).

2.3 Restriction fragment length polymorphism (RFLP) analysis

2.3.1 Restriction enzymes selection

Three kinds of restriction enzymes, *Dra* I, *Xho* I and *Msp* I, were chosen to digest the ITS region-amplified products of rDNA of *B. xylophilus* and *B. mucronatus* based on the sequence of the ITS region of *B. xylophilus* by Iwahori et al. (1998) (including the ITS1, ITS2, 5.8S gene and parts of the 18S and 28S genes adjacent to the spacer regions); *Apa* I, *Msp* I, and *Sal* I were chosen according to the sequence of the ITS region of *B. mucronatus* by Zheng et al. (2003) (including the ITS1, ITS2, and 5.8S gene).

2.3.2 Restriction fragment length polymorphism (RFLP) reaction volumes

The 10 μL reaction volumes comprised 8 μL of ITS region-amplified products, 1.5 μL of a 10 × reaction buffer and 0.5 μL of a restriction enzyme (10 U). A drop of paraffin oil was added to the surface in case of evaporation after all components were added to the tube and mixed well.

2.3.3 Restriction fragment length polymorphism (RFLP) reaction conditions

The mixture was incubated at 37°C overnight to digest the PCR products. Then the mixture, containing *Dra* I, *Apa* I, *Msp* I and *Sal* I, was incubated at 65°C for 20 min and the mixture containing *Xho* I was incubated at 80°C for 20 min to end the reaction. Finally, 8 μL restricted DNA fragments were resolved by electrophoresis in a 1.8% (w/v) agarose gel, stained with ethidium bromide, viewed and photographed under UV light.

3 Results and analysis

3.1 The amplified products of rDNA-ITS region of *B. xylophilus* and *B. mucronatus*

The PCR amplified results of the rDNA-ITS region are shown in Fig. 1. Lanes 5–9 are *B. xylophilus* isolates, the total lengths of PCR-amplified region were about 890 bp and their moving rates in agarose gel were consistent. Lanes 1–4 are *B. mucronatus* isolates and their moving rates, which were different from *B. xylophilus* isolates, were consistent within themselves. They were about 930 bp in length.

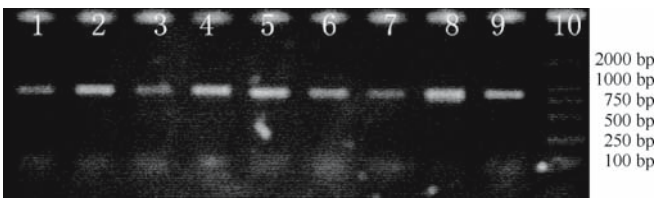

Fig. 1 PCR-amplified internal, transcribed spacers of *B. xylophilus* and *B. mucronatus*
Lanes 1–4: X02, X03, JAP01 and KOR01, respectively; 5–9: GZ01, GZ02, AH01, US01 and CAN01, respectively; 10: DNA Marker DL2000 (from Co. TaKaRa).

3.2 Restriction fragment length polymorphism (RFLP) analysis

3.2.1 Digestion with *Dra* I

Restriction fragments obtained by the digestion of the amplified ITS region of rDNA with *Dra* I are shown in Fig. 2A. Digestion with *Dra* I produced two fragments of about 510 and 380 bp for *B. xylophilus* groups, whereas *Dra* I could not digest the ITS products of *B. mucronatus* populations. Therefore, *B. xylophilus* groups could be differentiated from *B. mucronatus* groups by using the *Dra* I enzyme.

3.2.2 Digestion with *Apa* I

Digestion with *Apa* I is shown in Fig. 2B. The amplified ITS regions of rDNA of all *B. xylophilus* and *B. mucronatus* isolates could not be digested with the *Apa* I enzyme. The ITS domains of *B. mucronatus* isolates showed one *Apa* I cleavage site (GTGCAC) according to Zheng et al. (2003); therefore, the regions of *B. mucronatus* isolates should, theoretically, be digested by the *Apa* I enzyme. But an undigested band of about 930 bp was detected for all *B. mucronatus* isolates in our study. Probably there is heterogeneity between the ITS regions of *B. mucronatus*. This question needs to be further studied using more *B. mucronatus* isolates. It is at least clear that *B. xylophilus* groups cannot be differentiated from *B. mucronatus* groups by using the *Apa* I enzyme.

3.2.3 Digestion with *Msp* I

Digestion with *Msp* I is shown in Fig. 2C. For *B. xylophilus* isolates, GZ01, AH01 and US01 yielded two fragments of 530 and 360 bp with *Msp* I enzyme, whereas an undigested band of about 890 bp was detected for GZ02. It also showed that there is heterogeneity between the ITS regions of *B. xylophilus*. For *B. mucronatus* isolates, three bright fragments of 340, 290 and 180 bp were detected, which were analyzed by the Bio-Rad Quantity One software (BIO-RAD Laboratories, Italy). Although the RFLP patterns of *B. xylophilus* isolates were different from those of *B. mucronatus* isolates with *Msp* I enzyme, detection with the *Msp* I enzyme is not desirable because it produces more RFLP fragments.

3.2.4 Digestion with *Sal* I

Digestion with *Sal* I is shown in Fig. 2D. All *B. xylophilus* isolates could not be digested, whereas all *B. mucronatus*

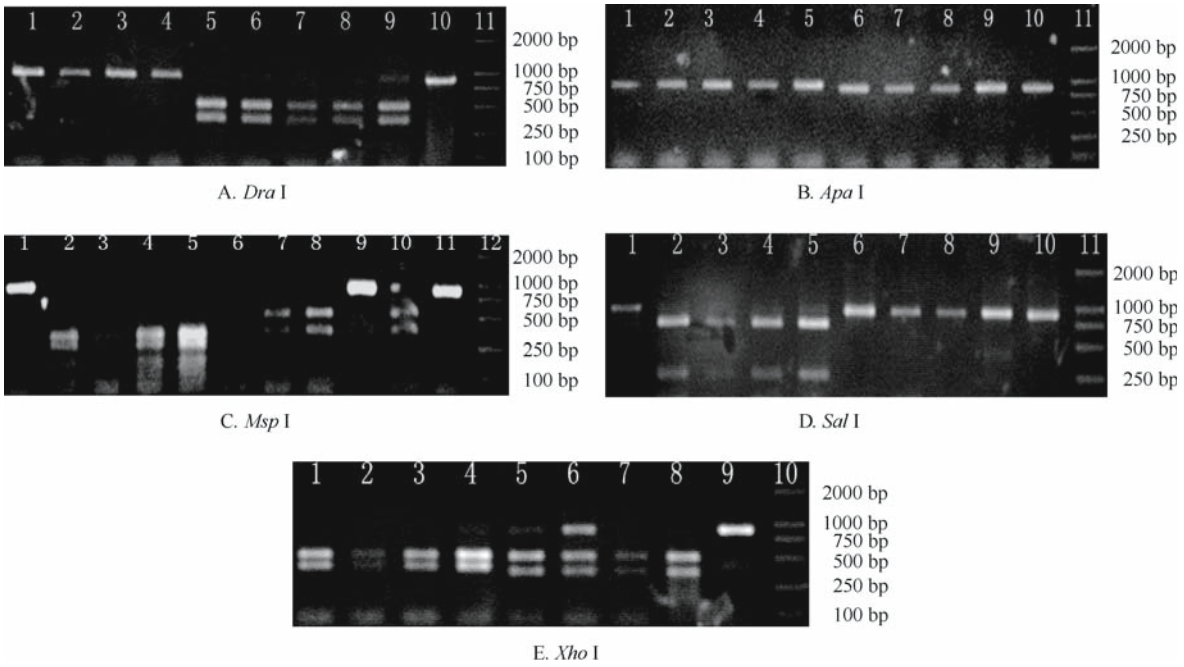

Fig. 2 Restriction fragments of amplified rDNA-ITS regions of *B. xylophilus* and *B. mucronatus* digested by five restriction endonucleases
A: *Dra* I. Lane 1–4: X02, X03, JAP01 and KOR01 respectively; 5–9: GZ01, GZ02, AH01, US01 and CAN01 respectively; 10: Undigested PCR product of *B. xylophilus*; 11: DNA Marker DL2000 (from Co. TaKaRa).
B: *Apa* I. Lane 1: Undigested PCR product of *B. mucronatus*; 2–5: X02, X03, JAP01 and KOR01 respectively; 6–10: GZ01, GZ02, AH01, US01 and CAN01respectively; 11: DNA Marker DL2000 (from Co. TaKaRa).
C: *Msp* I. Lane 1: Undigested PCR product of *B. mucronatus*, 2–5: KOR01, JAP01, X03 and X02 respectively: 6–10: CAN01, US01, AH01, GZ02 and GZ01 respectively; 11: Undigested PCR product of *B. xylophilus*, 12: DNA Marker DL2000 (from Co. TaKaRa).
D: *Sal* I. Lane 1: Undigested PCR product of *B. mucronatus*; 2–5: X02, X03, JAP01 and KOR01 respectively; 6–10: GZ01, GZ02, AH01, US01 and CAN01respectively; 11: DNA Marker DL2000 (from Co. TaKaRa).
E: *Xho* I. Lane 1–4: X02, X03, JAP01 and KOR01 respectively; 5–8: GZ02, AH01, US01 and CAN01 respectively; 9: Undigested PCR product of *B. xylophilus*; 10: DNA Marker DL2000 (from Co. TaKaRa).

isolates yielded two fragments of about 720 and 220 bp base pairs with the *Sal* I enzyme. Hence, *B. xylophilus* isolates could be clearly differentiated from *B. mucronatus* isolates by using the *Sal* I enzyme.

3.2.5 Digestion with *Xho* I

Digestion with *Xho* I is shown in Fig. 2E. The digestion of the ITS region PCR products of *B. xylophilus* and *B. mucronatus* with the *Xho*I enzyme produced two bright fragments of 520 and 370 bp and 540 and 400 bp, respectively. The *Xho* I digestion of the ITS region fragments of *B. xylophilus* and *B. mucronatus* yielded two similar-sized fragments. Clearly, this enzyme is not suitable for use alone to differentiate these two species. Besides, there was no *Xho* I cleavage site (CTCGAG) on the ITS region of *B. mucronatus* according to Zheng et al. (2003), but *B. mucronatus* isolates X02, X03, JAP01 and KOR01 could be digested with the *Xho* I enzyme in this study.

4 Conclusions and discussion

The total lengths of the rDNA-ITS regions of *B. xylophilus* were about 890 bp and those of *B. mucronatus* about 930 bp; although the lengths of the same region of *B. xylophilus* and *B. mucronatus* were 880–884 bp and about 900 bp according to Iwahori et al. (1998) and Lu et al. (2001) with the same primers. The cause of this discrepancy needs further investigation.

We used five restriction endonucleases, *Dra* I, *Apa* I, *Msp* I, *Sal* I, and *Xho* I, in our study. Digestion of the amplified products of each nematode isolate with five restriction endonucleases revealed the following results: 1) *Dra* I digestion of ITS products of *B. xylophilus* populations yielded two fragments, of 510 and 380 bp, whereas *Dra* I could not digest the ITS products of *B. mucronatus* populations; 2) the amplified ITS regions of rDNA of all *B. xylophilus* and *B. mucronatus* isolates could not be digested with the *Apa* I enzyme; 3) digested production of four *B. xylophilus* populations with *Msp* I yielded two fragments, of 530 and 360 bp, except for GZ02, which could not be digested, whereas *B. mucronatus* populations yielded three bright fragments of 340, 290, and 180 bp; 4) all *B. xylophilus* isolates could not be digested, although all *B. mucronatus* isolates yielded two fragments of about 720 and 220 bp with the *Sal* I enzyme; 5) the digestion of the ITS region PCR products of *B. xylophilus* and *B. mucronatus* with the *Xho* I enzyme produced two bright fragments of 520 and 370 bp and 540 and 400 bp respectively. The restriction endonucleases, *Dra* I and *Sal* I could be used for identification of *B. xylophilus* and *B. mucronatus*. Because the results of the digestion of *B. xylophilus* and *B. mucronatus* showed such sharp differences, it was very easy to identify and convenient to apply.

As for *Msp* I, the results of this study were a little different from those of Iwahori et al. (1998). The digestion of the ITS regions of *B. xylophilus* isolates with the *Msp* I enzyme, GZ01, AH01 and US01 yielded two fragments of 530 and 360 bp, which were consistent with those of Iwahori et al. (1998), whereas Lu et al. (2001) obtained RFLP fragments of 520 and 330 bp. In our experiment, the amplified product of the ITS region of *B. xylophilus* isolate, GZ02, could not be digested with the *Msp* I enzyme. It showed that there was heterogeneity between the ITS regions of *B. xylophilus* isolates, which was not reported in the studies of Iwahori et al. (1998) and Lu et al. (2001). For *B. mucronatus* isolates, three bright fragments of 340, 290 and 180 bp were detected, although the RFLP patterns of *B. xylophilus* isolates were different from those of *B. mucronatus* isolates with the *Msp* I enzyme. Detection with the *Msp* I enzyme was not a desirable method because it produced more RFLP fragments. Since the amplified products of rDNA of all *B. xylophilus* and *B. mucronatus* isolates could not be digested with *Apa* I, this enzyme could not be used alone to differentiate between these two species. Because *Xho* I yielded similar types of PCR-RFLP for *B. xylophilus* and *B. mucronatus* isolates, this enzyme was not suitable either for differentiating *B. xylophilus* and *B. mucronatus*. Although the *Xho* I cleavage site (CTCGAG) did not exist in the sequence of the ITS region of *B. mucronatus* according to Zheng et al. (2003), four *B. mucronatus* isolates, X02, X03, JAP01 and KOR01 could be digested to yield two fragments in our study. The total lengths of these two fragments were approximately consistent with those of the ITS region of *B. mucronatus*.

From the digestion results with *Msp* I, *Apa* I and *Xho* I, we know that there may be heterogeneity between the ITS regions of *B. xylophilus* and *B. mucronatus* isolates. Similarly, it was reported that some ITS regions of the *Heterodera avenae* population from Morocco could be digested with *Rsa*I, while others could not, according to Peng et al. (2003). Because of heterogeneity, perhaps we can analyze the different types of *B. xylophilus* and *B. mucronatus* isolates using the PCR method of the rDNA-ITS region. This was not carried out here because of the limited supply of samples in this study.

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