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RELIABLE IDENTIFICATION OF *BURSAPHELENCHUS XYLOPHILUS* BY rDNA AMPLIFICATION

by

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Summary. PCR direct identification and PCR-RFLP analysis from single nematodes were employed to discriminate *Bursaphelenchus xylophilus* from *B. mucronatus*. For PCR direct identification, two specific primer sets from ITS amplified a 220 bp and 330 bp fragment from DNA of *B. xylophilus*, respectively. But no amplification band was obtained from DNA of *B. mucronatus*. Restriction patterns of PCR-RFLP have revealed that HinfI and MspI could be used to discriminate *B. xylophilus* from *B. mucronatus*. Both methods employed here could be used to identify single specimens of *B. xylophilus* sensitively and accurately. It is suggested that PCR direct analysis is more convenient than other techniques. PCR-RFLP analysis could be used as an alternate method to confirm the result of PCR direct analysis when necessary.

The pine wilt disease is the most serious threat to pine forests in China. It presently occurs in Jiangsu, Anhui, Shandong, Zhejiang, Guangdong, Hongkong and Taiwan, and causes pine trees to die rapidly and in large numbers. *Bursaphelenchus xylophilus* is considered to be the main causal agent of the disease while *B. mucronatus* is non-pathogenic. Distinguishing between these two related species, based solely on morphological characters, can sometimes be inconclusive because the characters of some isolates are similar and at the same time variable. Therefore more accurate and stable methods are needed, especially for pine wood quarantine.

To identify the *Bursaphelenchus* species more accurately and elucidate the phylogenetic relationship among different isolates, some nematologists have developed molecular biological techniques such as DNA probe analysis (Bolla *et al.*, 1998; Webster *et al.*, 1990; Abad *et al.*, 1991; Tares *et al.*, 1992; Harmey and Harmey, 1993), DNA sequencing analysis (Beckenbach *et al.*, 1992; Iwahori *et al.*, 1998; Beckenbach *et al.*, 1999), RAPD (Eraasch *et al.*, 1995; Irdani *et al.*, 1995), PCR-RFLP (Hoyer *et al.*, 1998; Iwahori *et al.*, 1998) and PCR detection (Xu *et al.*, 1998). Among these methods, RAPD, PCR detection and PCR-RFLP are easier and cheaper. However, they need at least several nematodes for each diagnosis. In this study, two practical PCR-based methods, PCR direct analysis and PCR-RFLP analysis, from single nematodes were developed.

Materials and methods

Nematode populations. The nine isolates of *B. xylophilus* (Steiner *et* Buhrer, 1934) Nickle, 1970, examined originated from China, Japan and the United States, while the five isolates of *B. mucronatus* Mamiya *et* Enda, 1979, from China, Japan, France and Norway (Table I). The

nematodes were reared on *Pestalotia* sp. on PDA at 25 °C for about ten days. Propagated nematodes were collected by the Baermann funnel method.

DNA Extraction. DNA of single nematodes was extracted by a modification of a method used by Moens and Waeyenberge (personal communication). A single nematode was placed and cut into small pieces with a medical knife in a 20 µl drop of double distilled water on a glass slide. The nematode suspension was quickly transferred to a 200 µl Eppendorf containing 8 µl cold WLB solution { 2.5 mM DTT, 1.125% Tween 20, 0.025% gelatin, 2.5 x PCR buffer [125 mM KCl, 25 mM Tris-HCl (PH 8.3), 3.75 mM $MgCl_2$]}. Addition of 2 µl proteinase K (1 mg/ml) brought the final volume to 20 µl. The tube was then kept in a freezer at -70 °C for 10 min, incubated at 65 °C for 1 hr and then at 95 °C for 10 min. The DNA suspension from centrifugation at 14000 rpm for 1 min could be used for PCR or stored at -20 °C.

ITS-rDNA amplification. PCR were performed in 25 µl reaction solution containing: 10xPCR buffer, 2.5 µl; 2.5 mM of dNTP, 2 µl; 25 mM of $MgCl_2$, 2.5 µl, 10 µm of each primer, 1 µl; 10 ng/µl of template DNA, 1 µl; double distilled water 14.9 µl; 5 U/µl of Taq polymerase (Taka Ra Biochemicals), 0.1 µl.

Five primers used in this study are listed in Table II (Fig. 1). Primer 1 and 2 were from Iwahori *et al.* (1998). Primer 3, 4 and 5 were constructed based on sequence differences in ITS (Zhang *et al.*, 2001).Two primer sets, primer 1 and primer 4, primer 5 and primer 3, were used to explore the method "PCR direct analysis", while primer 1 and primer 2 could be used for PCR-RFLP analysis.

The amplifications was conducted in a thermocycler (Perkin-Elmer GeneAmp PCR System

TABLE I - *Isolates and origins of* Bursaphelenchus *populations.*

Species Code	Isolate name	Origin
B. xylophilus		
1	GDZ	Dongguan, Guangdong, China
2	GDC	Dongguan, Guangdong, China
3	GHZ	Huizhou, Guangdong, China
4	JN	Nanjing, Jiangsu, China
5	JNZ	Nanjing, Jiangsu, China
6	ZD	Daishan, Zhejiang, China
7	J10	Nishiaizu, Fukushima, Japan
8	US9	Tucson, Arizona, USA
9	US10	Cloguet, minnesota, USA
B. mucronatus		
10	GHH	Huizhou, Guangdong, China
11	ZZ	Zhoushan, Zhejiang, China
12	J13	Yachiyo, Chiba, Japan
13	BmF	Foret de campet, Landes, France
14	BmN	Hanestad, Norway
Caenorhabditis spp.		
C	C	Huidong, Guangdong, China

TABLE II - *Primers used*

Primer	Sequence	Origin
1	5'-CGTAACAAGGTAGCTGTAG-3'	Iwahori, 1998
2	5'-TCCTCCGCTAAATGATATG-3'	Iwahori, 1998
3	5'-CAATTCACTGCGTTCTTC-3'	This study
4	5'-GCCAACTCAACAACAGCAC-3'	This study
5	5'-GATGATGCGATTGGTGACT-3'	This study

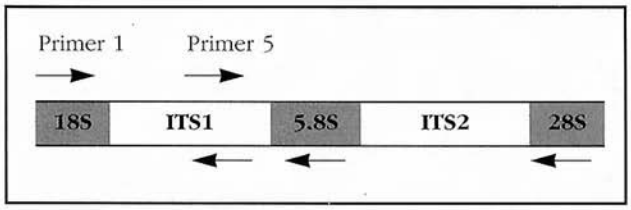

Fig. 1 - Location of primers for PCR on rDNA used.

9700). The reaction conditions consisted of 40 cycles with predenaturation at 94 °C for 1 min, denaturation at 94 °C for 45 s, annealing at 49 °C for 30 s, and polymerization at 72 °C for 1 min, with a final 10 min for incubation at 72 °C.

5 µl of amplified product mixed with 1 µl of dye maker solution (0.2% bromophenol blue, 0.5 g/ml sucrose) were analysed by electrophoresis in a 1.2% agarose gel in 1xTBE buffer for 30 min at 5 v/cm.

Restriction Enzyme Treatment. 17 µl of PCR product by primer 1 and primer 2 (Take Ra Ex Taq™ polymerase and higher concentration of primer was taken) mixed with 2 µl of 10x buffer were digested with 1 µl (10 U/µl) of restriction enzyme HinfI, MspI and AluI, respectively at 37 °C for 1 hr. Then 2 µl of 10x loading buffer were added into the reaction tube to end the restriction reaction. The DNA fragments thus generated were detected in 2% agarose gel.

Results

PCR direct analysis. With primer 1 and primer 4, a 220 bp fragment was amplified from *B. xylophilus*, while with primer 5 and primer 3, a 330 bp fragment was obtained. However, these primer sets did not amplify DNA from *B. mucronatus*, except for non-specific amplification from isolate GHH. No variations occurred among the different isolates of *B. xylophilus* (Fig. 2). This means that *B. xylophilus* could be differentiated from *B. mucronatus* by these two specific primer sets. Thus this method was named as "PCR direct analysis".

PCR-RFLP analysis. Amplified product with primer 1 and primer 2 was digested with the enzymes HinfI, MspI and AluI. Restriction patterns showed that HinfI obtained 260 bp and 140 bp of band from *B. xylophilus*. But 380 bp, 250 bp and 130 bp of band appeared from *B. mucronatus*. So 380 bp can be a specific band to allow differentiation between *B. xylophilus* and *B. mucronatus*. For MspI, it obtained 530

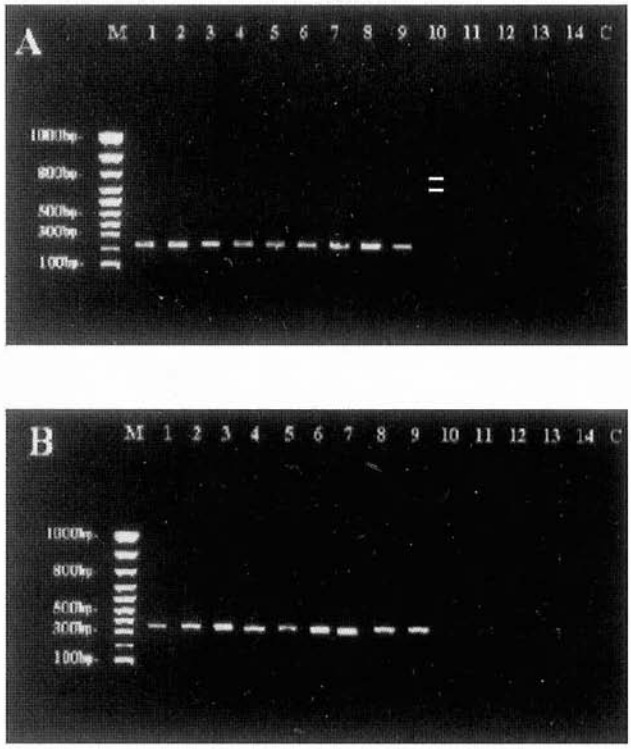

Fig. 2 - PCR direct analysis. Figure A shows the amplification of primer 1 and primer 4 (220 bp fragment from *Bursaphelenchus xylophilus*); Figure B shows the amplification of primer 5 and primer 3 (330 bp fragment from *B. xylophilus*).

bp and 360 bp of band from *B. xylophilus*, but 360 bp and 270 bp of band from *B. mucronatus*, thus 530 bp or 270 bp could be a specific band to discriminate *B. xylophilus* from *B. mucronatus*. However, Restriction patterrns revealed that AluI have different bands among the different isolates, and therefore it could not be used for identification (Table III, Fig. 3).

Discussion

Large quantities of nematodes are normally needed for a DNA-based study, but recently DNA isolation from a single nematode has proved possible for some plant-parasitic nematodes such as *Meloidogyne* (Harris *et al.*, 1990) and *Pratylenchus* (Orui, 1996). From this study it can be concluded that a single nematode can be reliably used to extract enough DNA for amplification. Therefore, the DNA-based technique is reliable in the identification of *Bursaphelenchus*.

Some nematologists used more than ten kinds of restriction enzymes to study rDNA of *B. xylophilus* and *B. mucronatus*, and found that only HinfI, MspI and AluI had the potential for identification (Iwahori *et al.*, 1998; Hoyer *et al.*, 1998). To simplify the process, 2% agarose gel was selected to replace 6% PAGE used by Iwahori *et al.* (1998) and resulted in some differences. HinfI and MspI were confirmed as useful restriction enyzymes for detecting the *Bursaphelenchus* species as described. However, the AluI restriction pattern showed that there was some intra-species variation so that it could not differentiate between *B. xylophilus* and *B. mucronatus* where 2% agarose gel was used.

PCR direct analysis is more convenient in nematode identification, but PCR-RFLP analysis could be used as an alternate method to confirm the result of PCR direct analysis when necessary.

TABLE III - *DNA fragment from PCR-RFLP analysis of ITS-rDNA from* Bursaphelenchus xylophilus *and* B. mucronatus.

Enzymes	*B. xylophilus*		*B. mucronatus*	
	GDZ GDC GHZ JN JNZ J10 US 10	ZD US9	GHH ZZ	J13 BmF BmN
AluI	450 bp	260 bp	610 bp	610 bp
	240 bp		400 bp	240 bp
			240 bp	
HinfI	260 bp	260 bp	380 bp	380 bp
	140 bp	140 bp	250 bp	250 bp
			130 bp	130 bp
MspI	530 bp	530 bp	360 bp	360 bp
	360 bp	360 bp	270 bp	270 bp

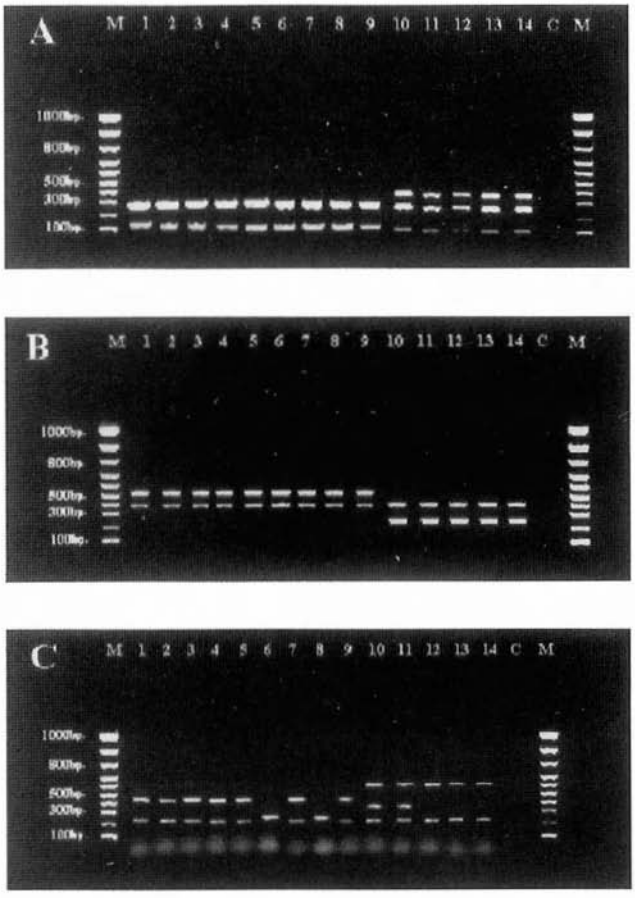

Fig. 3 - HinfI, MspI and AluI restriction enzyme digests of *Bursaphelenchus* ITS1-5.8S-ITS2 amplicon. A, B and C indicate HinfI, MspI and AluI, respectively.

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