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Molecular characterisation of some Asian isolates of *Bursaphelenchus xylophilus* and *B. mucronatus* using PCR-RFLPs and sequences of ribosomal DNA

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Summary. Five isolates of *Bursaphelenchus xylophilus*, four of which were collected from Chinese forests and one from Japan, as well as, four isolates of *B. mucronatus* extracted from pinewood packaging imported from Japan, Korea and from forests in China, were studied using PCR-RFLPs and sequences of the ITS and D2-D3 fragments of rDNA. RFLP analysis of the ITS region with restriction endonucleases revealed that *Cfo*I, *Hae*III, *Hin*fI, *Msp*I and *Taq*I yielded distinct patterns that separated *B. xylophilus* and *B. mucronatus*. Phylogenetic analyses of alignments of published and original sequences of both the ITS1 region and ITS1-5.8S-ITS2 region revealed that both *B. xylophilus* and *B. mucronatus* are composed of two groups of populations with different ITS types. The taxonomic status of these groups is discussed.

Key words: 28S rDNA, *Bursaphelenchus*, China, ITS-rDNA, Japan, phylogenetic analysis.

Bursaphelenchus xylophilus (Steiner & Buhrer, 1934) Nickle, 1970 is the causal agent of pine wilt disease and a quarantine organism in many countries (Rutherford *et al.*, 1990). The species has been reported from Asia (Japan, China, Korea) and North America (USA and Canada). In recent years it was also reported from Portugal, suggesting a potential threat to pine trees in Europe (Mota *et al.*, 1999). Since its first detection in China (Nanjing, Jiangsu province) in 1982, *B. xylophilus* was found in East and South China: Anhui, Guangdong, Hongkong, Shandong Taiwan and Zhejiang (Zhu *et al.*, 1995).

Since several species including *B. mucronatus*, a non-pathogenic but widely distributed species are morphologically similar to *B. xylophilus*, and are reported to occur in conifers (Mamiya, 1987), exact identification and discrimination of both species is crucial for pine wood quarantine. Several authors have reported molecular identification or discrimination of *B. xylophilus, B. mucronatus* and other *Bursaphelenchus* species using PCR-RFLP (Hoyer *et al.*, 1998; Iwahori *et al.*, 1998; Mota *et al.*, 1999; Braasch *et al.*, 1999, 2001; Liao *et al.*, 2001) or sequences of rDNA ITS (Iwahori *et al.*, 1998; Beckenbach *et al.*, 1999; Zhang *et al.*, 2001; Kanzaki & Futai, 2002).

In recent years, *B. xylophilus* has been frequently found in the forests of Zhejiang province, Eastern China, whereas *B. mucronatus* was detected in several samples taken from pinewood packaging imported from Japan and Korea. The present paper describes the results of analyses of PCR-RFLPs, and sequences of the ITS region and D2 and D3 of the 28S gene of populations of both species.

MATERIAL AND METHODS

Nematode samples and DNA extraction. Five

Table 1. Isolates and origin of nematodes used in this study.

Species	Code	Origin
Bursaphelenchus mucronatus	HG	Packaging wood from Korea
	HK	Packaging wood from Hong Kong, China
	FY	Tree sample from Fuyang, Zhejiang, China
	JP	Packaging wood from Japan
Bursaphelenchus xylophilus	NJ	Tree sample from Nanjing, Jiangsu, China
	T4	Tree sample from Japan
	NB	Tree sample from Ningbo, Zhejiang, China
	MA	Tree sample from Maanshan, Anhui, China
	XS	Tree sample from Xiangshan, Zhejiang, China

isolates of *B. xylophilus* and four isolates of *B. mucronatus* were used in this study (Table 1). For each isolate, one to five specimens were put into 15 µl of double distilled water on a glass slide and cut into two or three fragments. Ten µl of water with nematode fragments were transferred into a 0.5 ml Eppendorf tube, which contained 8 µl of nematode lysis buffer (125 mM KCl, 25 mM Tris-Cl pH 8.3, 3.75 mM MgCl2, 2.5 mM DTT, 1.125% Tween 20) and 2 µl of proteinase K (600 µg/ml). Tubes were incubated at 65°C for 60 min, thereafter at 95°C for 10 min.

PCR reaction. After centrifugation (13,000 rpm, 1 min), 2 µl of the DNA suspension was added to the PCR reaction mixture containing 2.5 µl 10X *Taq* incubation buffer, 5 µl Q-solution, 200 µM of each dNTP (Taq PCR Core Kit, Qiagen, Germany), 1.5 µM of each primer (synthesised by Life Technologies, Merelbeke, Belgium), 1 U Taq polymerase (Taq PCR Core Kit, Qiagen, Germany) and double distilled water to a final volume of 25 µl. The primers F194 (5'-CGTAACAAGGTAGCTGTAG-3') and 5368 (5'-TTTCACTCGCCGTTACTAAGG-3') and D2A (5'-ACAAGTACCGTGAGGGAAAGTTG) and D3B (5'-TCGGAAGGAACCAGCTACTA-3') were used for the PCR amplification of the ITS region (Hoyer *et al.*, 1998) and the D2-D3 expansion segments of the LSU rDNA (De Ley *et al.*, 1999), respectively. The amplification program consisted of 4 min at 94°C, 10 cycles of 30 seconds at 94°C, 1 min at 57°C, and 2 min at 72°C, and another 25 cycles of 15 seconds at 94°C, 45 seconds at 57°C and 2 min at 72°C, followed by a final elongation step of 10 min at 72° C. After DNA amplification, 2 µl of each PCR product was run on a 1% agarose gel.

RFLP analysis. Four to 6 µl of each PCR product of the ITS region were digested with one of the following restriction enzymes, *Cfo*I, *Dde*I, *Hae*III, *Hin*fI, *Msp*I, *Mva*I, *Rsa*I, and *Taq*I in the buffer stipulated by the manufacturer. The digested DNA was loaded on a 2% agarose gel, separated by electrophoresis (120V, 2h), stained with ethidium bromide, visualised on a 2011 Macrovue UV transilluminator, and photographed with a Kodak Digital Science 1 D system. Procedures for obtaining PCR amplified products and endonuclease digestion of these products were repeated several times to verify the results.

Direct sequencing of the PCR product. PCR products were purified using the QiAquick PCR Purification Kit (Qiagen, Germany) and sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems Benelux, Leusden, The Netherlands) according to the manufacturer's instruction with the same primers as those used in PCR amplification. The resulting products were purified using a Centriflex Gel Filtration Cartridge (Edge BioSystems Inc., Gaithersburg, MD, USA). Sequences were run on a 377 DNA Sequencer (PE Applied Biosystems, Warrington, UK). DNA sequences were edited with Chromas 1.45. The original sequences of the ITS and the D2-D3 fragment of 28S gene have been submitted to the GenBank database.

Sequence and phylogenetic analysis. The original ITS sequences and sequences of several populations of *B. xylophilus, B. mucronatus* and *B. conicaudatus* (outgroup taxon) obtained from the GenBank database or from published data (Iwahori *et al.*, 1998; Beckenbach *et al.*, 1999; Zhang *et al.*, 2001; Kanzaki & Futai, 2002) were aligned using Clustal X1.64. Alignments were generated for *i*) twenty-four sequences of the ITS1 region, and *ii*) eleven sequences of the ITS1-5.8S-ITS2 region. Alignments are available from the first author. Se-

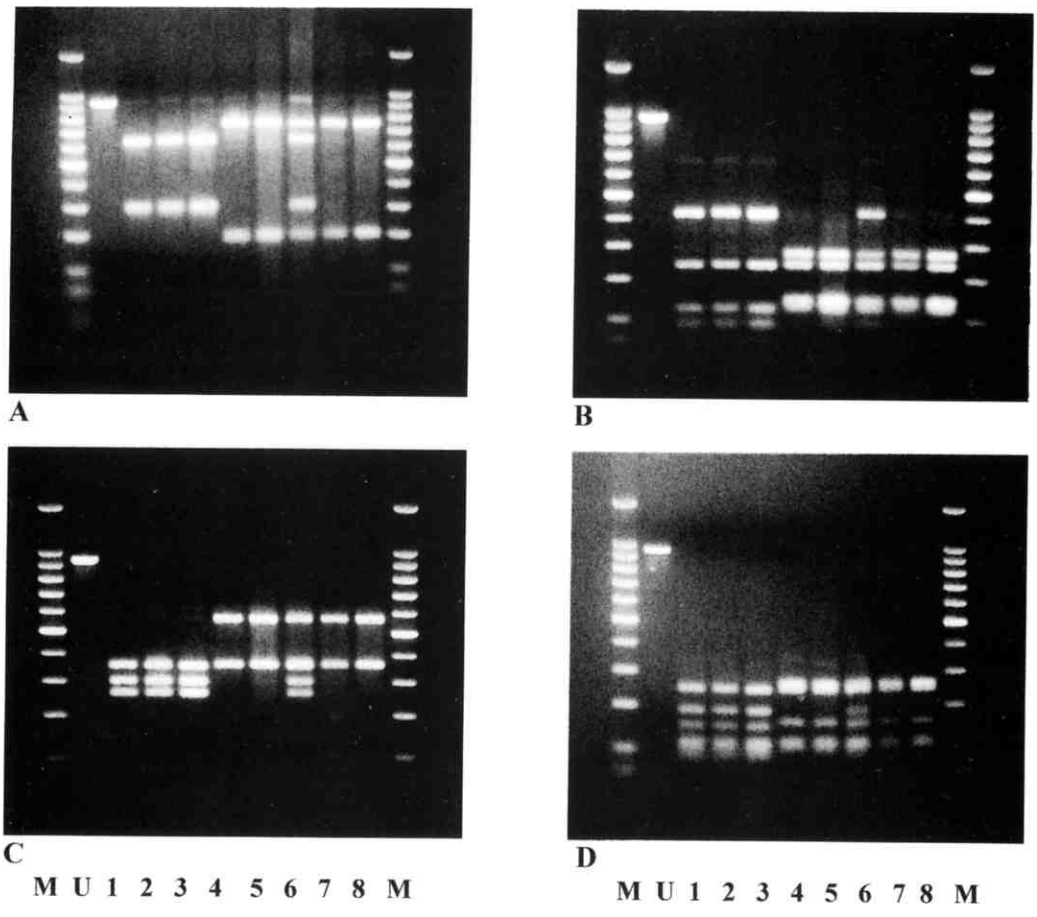

Fig. 1. Restriction fragments of amplified ITS regions digested by restriction enzymes. A: *Hae*III; B: *Hin*fI; C: *Msp*I; D: *Taq*I; M: 100 bp marker; U: undigested PCR product. 1-3: *Bursaphelenchus mucronatus* (1: HG, 2: HK, 3: FY); 4-8: *B. xylophilus* (4: NJ, 5: T4, 6: NB, 7: MA, 8: XS). For codes see Table 1.

quence alignments were analysed with an equally weighted maximum parsimony (MP) method using PAUP* 4.0b4a (Swofford, 1998). We used heuristic search setting with ten replicates of random taxon addition, tree bisection-reconnection branch swapping to seek for the most parsimonious trees. Gaps were treated as missing data. To obtain an estimation of the support for each node, a bootstrap analysis (100 replicates, heuristic search, and simple addition of sequence) was performed.

RESULTS

PCR-RFLP of ITS regions. The amplification of the ITS1-5.8S-ITS2 and flanking genes yielded for each isolate a single product of approximately 930 bp. RFLP of the ITS region revealed that five restriction enzymes *Cfo*I, *Hae*III, *Hin*fI, *Msp*I and *Taq*I generated distinct patterns that separated *B. xylophilus* from *B. mucronatus* (Fig. 1). The sample from Ningbo, Zhejiang province, China, contained a mixture of both species.

Sequence and phylogenetic analyses. We observed no differences in ITS-sequences within *B. mucronatus* isolates or within *B. xylophilus* isolates, respectively, sequenced in our study. The intraspecific pairwise sequence divergence of ITS1 for *B. mucronatus* ranged from 0 to 2.0 %; for the East Asian type from 0-0.03%, and for the European type was 0%. The pairwise sequence divergence for *B. xylophilus* ranged from 0 to 1.6%, and between the species from 9.3 to 11.0%. The divergence in the ITS1-5.8S-ITS2 alignment between *B. mucronatus* and *B. xylophilus* varied from 9.6 to 10.2 %. MP analysis of the ITS1 alignment yielded nine most parsimonious trees, the strict consensus tree is presented in Fig. 2A. Two main distinct clades were distinguished within *B. mucronatus*. Two groups of sequences were also observed within *B. xylophilus*. MP analysis of the ITS1-5.8S-ITS2 yielded two most parsimonious trees, one of them is given in Fig. 2B.

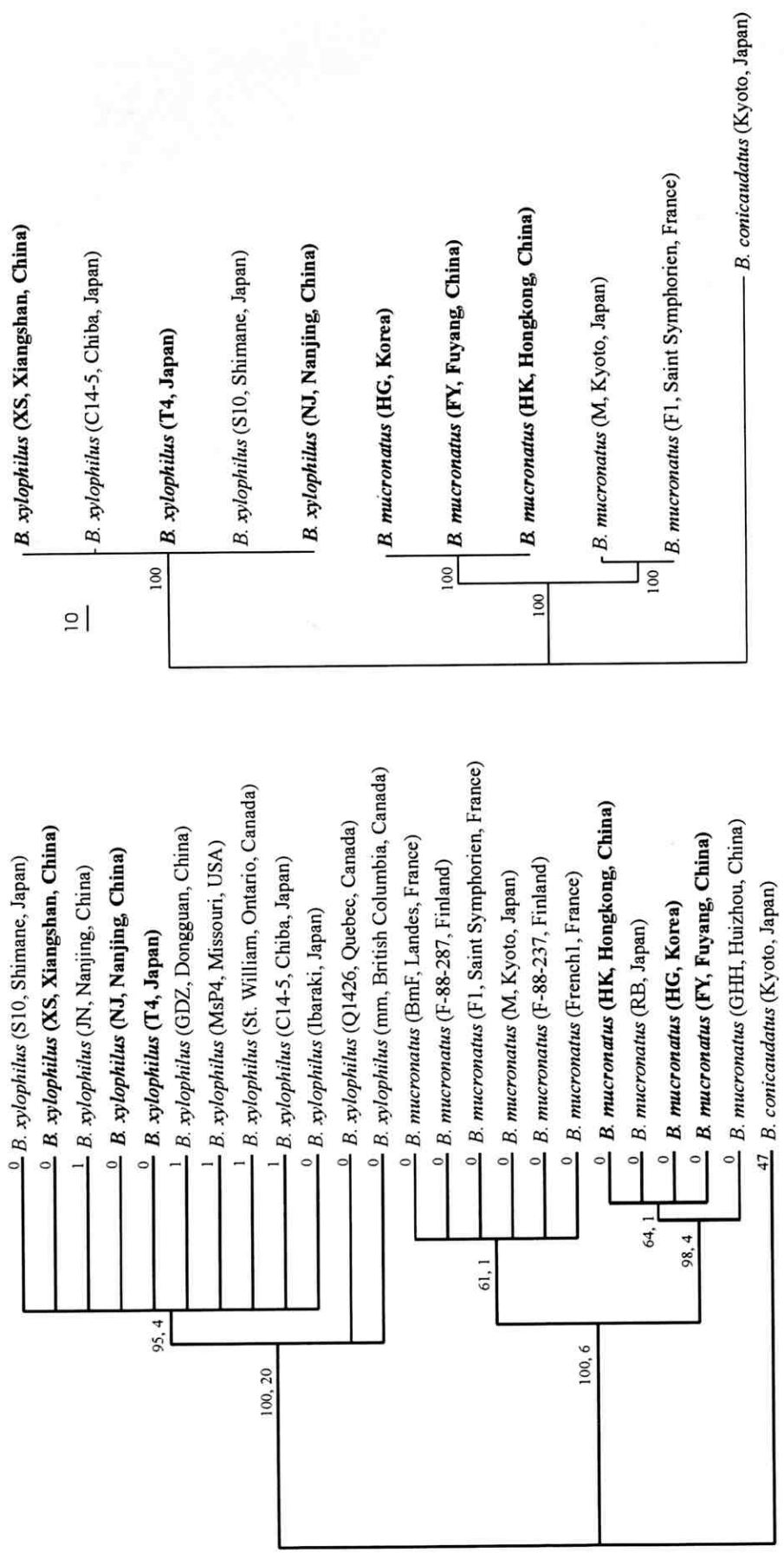

Fig. 2. **A**: Strict consensus of nine maximum parsimonious trees (tree length = 88, number of parsimony-informative characters = 34) obtained after analysis of alignment of the ITS1 region for *Bursaphelenchus* spp. **B**: One of two maximum parsimonious tree (tree length = 252, number of parsimony-informative characters = 85) obtained after analysis of the ITS1-5.8S-ITS2 region for *Bursaphelenchus* spp. Bootstrap values and nucleotide changes are given in appropriate clade. Original sequences are indicated in bold.

The fragment generated by the D2-D3 primers was about 740 bp. The *B. mucronatus* isolates differed from each other by only one nucleotide, whereas, sequences of *B. mucronatus* and *B. xylophilus* differed by 16 or 17 nucleotides.

DISCUSSION

Our study showed that ITS and D2-D3 sequences distinguished *B. mucronatus* and *B. xylophilus* from each other.

After analysing partial nucleotide sequences of the heat shock *hsp-70A* gene of 19 populations of *Bursaphelenchus* spp., Beckenbach *et al.* (1992) concluded that the Japanese population (RB) was quite distinct from the studied European populations of *B. mucronatus*. This distinction agreed with cross-hybridisation experiments in which a French and the Japanese population (RB) of *B. mucronatus* did not freely interbreed (Riga *et al.*, 1992). It contradicted, however, the report by De Guiran & Bruguier (1989) on the successful interbreeding of other European and Japanese populations. Beckenbach *et al.* (1992) explained this inconsistency by the use of different populations. Later, Iwahori *et al.* (1998) revealed the presence of two genotypes of *B. mucronatus* in Japan.

Further molecular analyses of *B. mucronatus* isolates using RFLP of the ITS and RAPD (Braasch *et al.*, 1995, 1999; Hoyer *et al.*, 1998) revealed the presence of two genotypes, an East Asian genotype widely distributed in China and Japan, and having restricted distribution in Europe (Germany and Russia), and a European genotype common in European forests but also recorded from forests in Siberia and the Russian Far East. The RFLP patterns obtained for all our isolates of *B. mucronatus* matched those for the East Asian genotype published by Hoyer *et al.* (1998) and Braasch *et al.*, (1999, 2001). The East Asian genotype is differentiated from the European one after ITS digestion with *Hae*III and *Rsa*I (Hoyer *et al.* 1998). Recently, Liao *et al.* (2001) observed RFLP patterns of Chinese *B. mucronatus* populations from Huizhou (GHH) and Zhoushan. Compared with other Asian populations, an additional restriction fragment was observed in these populations using *Alu*I. Our phylogenetic tree based on ITS1 sequences (Fig. 2A) visualises two genotypes and the separate position of the Huizhou (GHH) population.

After comparison of the ITS sequences from eleven *Bursaphelenchus* isolates, Beckenbach *et al.* (1999) concluded that ITS analysis does not support the separation of European and Japanese populations of *B. mucronatus* into distinct species-level taxa and placed the Japanese population (RB) within the *B. mucronatus* clade. The authors suggested that the previous results of *hsp-70* gene analyses may be artificial due to paralogous comparison. As these statements contradicted published RFLP-ITS data, we re-analysed all published ITS sequences of *B. mucronatus* and *B. xylophilus*. We excluded the ITS2 region for sequences of *B. mucronatus* populations published by Beckenbach *et al.* (1999), as it contained positions with ambiguous sequences. In both our ITS1 tree and ITS1-5.8S-ITS2 tree, two main groups corresponding to the East Asian and European ITS types are well defined for *B. mucronatus* isolates. This suggests that *B. mucronatus* is a species complex with at least two sibling species. In addition, Braasch *et al.* (1998) reported that females of European and East Asian genotypes could be differentiated by small differences in shape and length of their mucro.

Using DNA probes, Tarès *et al.* (1992) identified three geographical subgroups of *B. xylophilus* from the USA, Canada and Japan and detected a close relationship between the USA and Japanese subgroups. ITS-RFLPs obtained from eleven *B. xylophilus* isolates with twelve restriction enzymes revealed differences between some Canadian isolates and other *B. xylophilus* populations (Iwahori *et al.*, 1998). Within the *B. xylophilus* clade, the Quebecois (Q1426) and British Columbian (mm) isolates from Canada were placed basal to other isolates, when ITS1 (Beckenbach *et al.*, 1999, present study) or *hsp-70* (Beckenbach *et al.*, 1992) sequence data sets are considered. Substantial sequence divergence of these isolates from the others reflect their biological isolation, and suggests that these populations could belong to a sibling species. Assuming at least four species, instead of two, comprise the pinewood nematode species complex, a re-evaluation of previously published data on pathogenicity, chromosome number and cross-breeding tests of populations of *Bursaphelenchus* is required.

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Zheng, J., Subbotin, S.A., He, S, Gu, J. Moens M. Молекулярно-таксономические особенности некоторых азиатских изолятов *Bursaphelenchus xylophilus* и *B. mucronatus* по данным PCR-RFLP и анализа нуклеотидных последовательностей рибосомальной ДНК.

Резюме. С помощью PCR-RFLP и анализа нуклеотидных последовательностей ITS и D2-D3 участков рибосомальной ДНК изучены изоляты *Bursaphelenchus xylophilus* и *B. mucronatus.* Рестрикционный анализ ITS участка эндонуклеазами *Cfo*I, *Hae*III, *Hinf*I, *Msp*I и *Taq*I выявил существенные различия между *B. xylophilus* и *B. mucronatus.* Проведен филогенетический анализ секвенсов *Bursaphelenchus* spp.