Plant Pathol. J. 24(2) : 159-163 (2008)

The Plant Pathology Journal

A Simple PCR-RFLP for Idenficiation of *Bursaphelenchus* spp. Collected from Korea

Hyerim Han*, Bo Young Han, Yeong Jin Chung and Sang Chul Shin

Division of Forest Insect Pests and Diseases, Korea Forest Research Institute, Seoul 130-012, Korea

(Received on March 31, 2008; Accepted on May 15, 2008)

Accurate identification of pine wood nematode, *Bursaphelenchus xylophilus* is a prerequisite to diagnose the pine wilt disease. However, a fungivorous nematode, *B. mucronatus* is highly similar to *B. xylophilus* and it is difficult to differentiate these two species by morphological features. A molecular diagnosis method, ITS-RFLP was applied for the identification of *B. xylophilus* and *B. mucronatus* from Korea. Genomic DNA was extracted from a single individual nematode and ITS DNA was amplified by PCR. The size of PCR product was approximately 900 bp and the sequence data were obtained after cloning. Amplified ITS was digested by 5 different restriction enzymes (Rsa I, Hae III, Msp I, Hinf I, and Alu I) and provided a discriminatory profile for *B. xylophilus* and *B. mucronatus*. Besides, *B. mucronatus* was determined to have 2 different genotypes, East Asian type and European type also clearly separated by Rsa I and Hae III digestion. European type of *B. mucronatus* is recently collected from *Pinus koraiensis* and has not been reported before. ITS sequnce data were analyzed by Restriction Mapper program and the result supported ITS-RFLP pattern. These data indicated that PCR-RFLP method is an accurate and simple way for identification of *Bursaphelenchus* species.

Keywords **:** *Bursaphelenchus xylophilus*, *B. mucronatus*, identification, ITS, PCR-RFLP, pine wood nematode

Pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970, is the causal agent of pine wilt disease and is associated with cerambycid beetles, particularly *Monochamus* spp. (Mamiya, 1972). The nematode was first reported at North America by the different genus name, *Aphelenchoides xylophilus*, but pine wilt disease was first described in Japan in early 1900's. Subsequently, the plant disease has occurred in other East Asian countries such as Taiwan (Tzean & Jan, 1985), China (Cheng, 1983), and Korea (Yi et al., 1989) and also recently in Portugal, Europe (Mota et al., 1999). Pine wilt disease has devastated particularly in pine forest and induced economic loss in forest industry.

In Korea, pine wilt disease was first detected in Gumjung Mt., Busan city in 1988. It has been almost 20 years since first disease was reported and now the area of pine wilt disease occupied approximately 7,800 ha over 65 cities in Korea. Most damaged trees are red pine tree (*Pinus densiflora*) and black pine tree (*P. thunbergii*) which are dominant species of *Pinus* in Korea. Recently, however, the disease also occurred in Korean pine tree (*P. koraiensis*) that is usually in forest plantation area located in northern area of South Korea around Gyeonggi and Gangwon Provinces. In Korea, coniferous trees including *Pinus* species highly susceptible to pine wilt disease take about 35% of the total forest area. Pine tree is an important natural source not only in forestry economy but also in national emotion in Korea.

Fast and accurate identification of the species of *B. xylophilus* is an essential step to diagnose pine wilt disease and to make a strategy for its management. Over 70 species of *Bursaphelenchus* have been reported (Burgermeister et al., 2005) and they are morphologically very similar to each other. Especially *B. mucronatus* belonging to 'xylophilus-group' is almost identical to *B. xylophilus* in morphology except only for mucro in tail. However, *B. mucronatus* is a common species found in already dead or dying trees with no or little pathogenecity. Therefore, accurate identification of pine wood nematode is prerequisite to diagnose the pine wilt disease.

Identification of *Bursaphelenchus* species has been relied on morphological characteristics; however, it requires high expertise on nematode taxonomy. Especially it is much more difficult to identify juveniles that carry few morphological features informative to species identification. Molecular biological methods have been introduced to supplement the morphological diagnosis of pine wood nematode and ITS in ribosomal DNA was determined as a useful marker gene (Bergermeister, 2005; Kanzaki and Futai, 2002; Matsunaga and Togashi 2004; Zheng et al., 2003). Therefore, in this study we introduce PCR-RFLP method to identify and differentiate closely related *Bursaphelenchus* species collected from pine trees in Korea.

*Corresponding author.
Phone) +82-2-961-2654, FAX) +82-2-961-2657
E-mail) hrhan@forest.go.kr

Materials and Methods

Nematode collection. Four *Bursaphelenchus* isolates, each two of *B. xylophilus* and *B. mucronatus*, were selected for this experiment. Two *B. xylophilus* isolates were originally collected from *Pinus densiflora* in Jinju, Gyeongnam province (Bx-DJ) and from *P. koraiensis* in Gwangju, Gyeonggi Province (Bx-KG), respectively. One *B. mucronatus* isolate came from *P. thunbergii* in Jeju, Jeju province (Bm-TJ) and the other from *P. koraiensis* in Pocheon, Gyenonggi Province (Bm-KP). Nematodes were extracted from wood chip by Bearmann's funnel method (Ayoub, 1977) and inoculated on a lawn of *Botrytis cinerea* cultured on potato dextrose agar. The plate was incubated at 25°C in a chamber for 5 days. Successful cultures were maintained on *Botrytis cinerea* cultured on steamed-sterilized barley-corn at 25°C for 10 days and then stored at 6°C in a refrigerator until used. A control species was *B. conicaudatus* provided by Dr. Kanzaki, Natsumi in Forestry and Forest Products Research Institute in Japan.

Genomic DNA extraction. Nematode was picked up from *B. cinerea* culture plate and washed with deionized sterile water for 3 times. A single female nematode was transferred into a 1.5 ml micro-centrifuge tube containing cell lysis buffer and proteinase K. The tube was incubated in water bath at 55°C for 12 hrs. The rest steps followed the protocols provided from QIAGEN DNeasy tissue kit (QIAGEN, Inc., Valencia, CA). Final volume of extracted DNA was 20 µl and each 5 µl was used as a template DNA for PCR.

PCR amplification. PCR was performed in a total 50 µl of reaction mixture containing PCR buffer (10 mM Tris-HCl, 40 mM KCl, 2.0 mM $MgCl_2$, pH 9.0), 250 µM dNTP, 0.6 µM of each primer, 2.0 U of Taq DNA polymerase (TaKaRa Bio Inc. Shiga, Japan), and 2 ng of DNA template. The primer set selected in this study was designed by Ferris et al. (1993) and Vrain (1993) and the target amplified region was ITS-1, 5.8S, and ITS-2 of ribosomal DNA. The forward primer was 5'-CGT-AAC-AAG-GTA-GCT-GTA-G-3' (Ferris et al., 1993) and the reverse primer was 5'-TTT-CAC-TCG-CCG-TTA-CTA-AGG-3' (Vrain, 1993). The ITS region was successfully amplified in 40 reaction cycles; denaturation for 1 min at 94°C, annealing for 1 min at 55°C, extension for 2 min at 72°C, including initial denaturation 2.5 min at 94°C and final 5 min extension at 72°C. PCR program was conducted in a DNA thermal cycler (Bio-Rad, US/PTC-0220), and the PCR products (5 µl) were analyzed by 1.5% agarose gel electrophoresis.

Restriction digestion. Five different restriction enzymes, Alu I, Hae III, Hinf I, Msp I, and Ras I, were selected for ITS-RFLP. Five to 10 units of each endonuclease was mixed with each reaction buffer and PCR product, and the final volume reached to 20 µl with water. For appropriate enzymatic digestion reaction, the mixture was incubated at 37°C in a water bath for 3 h. The results of PCR-RFLP were confirmed by 2.0% agarose gel electrophoresis.

DNA Recombination. Recombinant DNA techniques, including restriction enzyme digestion, ligation, bacterial transformation, and plasmid DNA preparation were performed by standard methods (Sambrook et al., 1989). For the cloning of ITS, PCR products were ligated into the vector plasmid pCR2.1-TOPO, following the protocol provided by the manufacturer (Invitrogen, Carlbad, CA). For the analysis of positive clones, the selected colonies were cultured in LB medium containing 50 µg/ml ampicillin. After 16 hrs of incubation, plasmids were extracted using Promega mini prep Kit (Promega, Madison, WI). The extracted pure plasmids were analyzed by EcoRI restriction analysis. After analysis of positive clones, the inserted DNA was sequenced on both strands using an Applied Biosystem ABI PRISM 3730XL Analyzer at Macrogen company. Obtained sequence data was analyzed for the sizes of restriction by using RestrictionMapper version 3 (http://www.restrictionmapper.org/).

Results

PCR product. The amplified PCR product was ranged from 900 bp-1.0 kb in size. There was no size difference between *B. xylophilus* and *B. mucronatus* by elencrophoresis. The PCR products of *B. xylophilus* and *B. mucronatus* showed approximately 900 bp whereas the control species, *B. conicaudatus*, showed somewhat bigger size of 1.0 kb (Fig. 1). According to ITS sequence data the exact sizes were calculated as 925 bp for *B. xylophilus* (Bx-DJ, Bx-KG), 919 bp for *B. mucronatus* (Bm-KP), and 918 bp for *B. mucronatus* (Bm-TJ) (Table 2). Therefore, minor

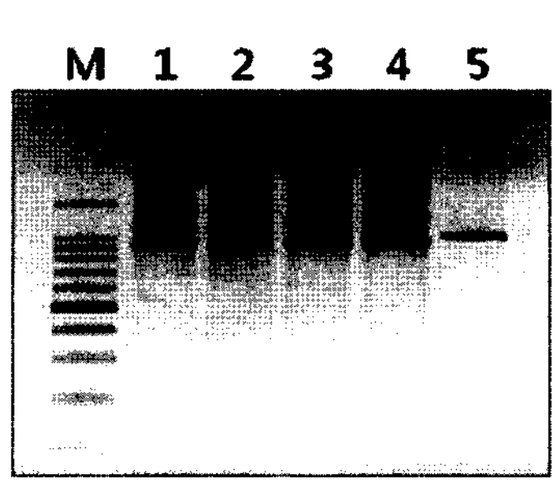

Fig. 1. PCR amplified size of ITS from different species of *Bursaphelenchus*. M: 1 kb molecular marker, 1: *B. xylophilus* (Bx-DJ), 2: *B. xylophilus* (Bx-KG), 3: *B. mucronatus* (Bm-TJ), 4: *B. mucronatus* (Bm-KP), 5: *B. conicaudatus*.

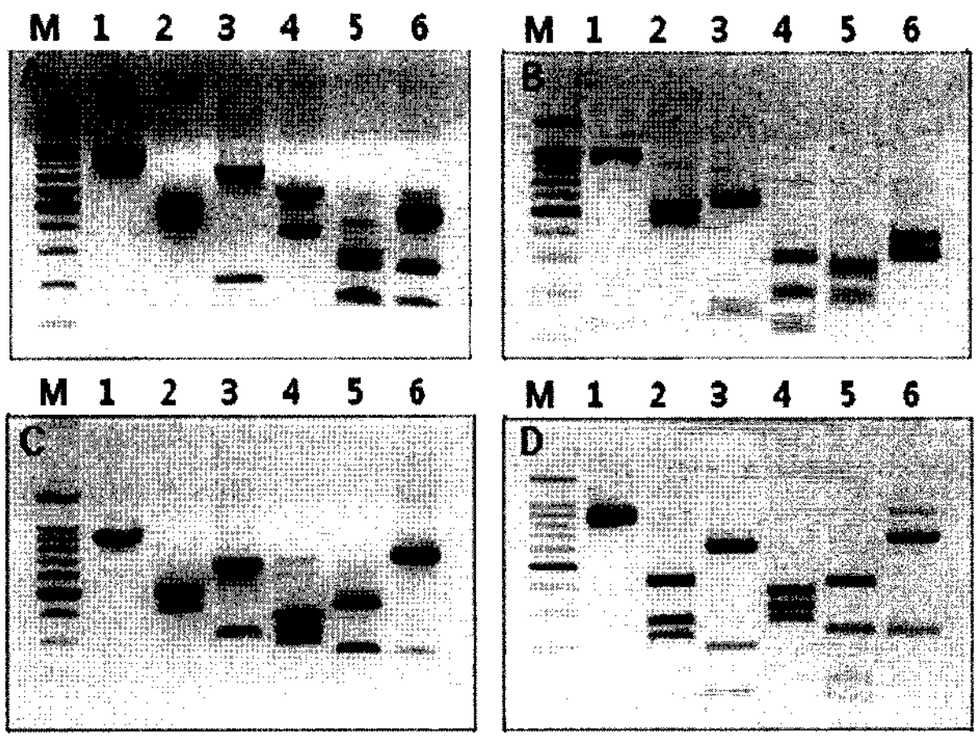

Fig. 2. ITS-RFLP patterns from different species of *Bursaphelenchus* in Korea. A: *B. xylophilus*, B: *B. conicauddatus*, C: *B. mucronatus* (East Asia type), D: *B. mucronatus* (European type). M: 1 kb molecular marker, 1: total PCR product, 2: Rsa I, 3: Hae III, 4: Mp I, 5: Hinf I, and 6: Alu I.

variation in the ITS size between *B. xylophilus* and *B. mucronatus* was detected, but it was unrecognizable in agarose gel electrophoresis.

PCR-RFLP. Three different patterns were observed from each two isolates of *B. xylophilus* and *B. mucronatus* by ITS-RFLP (Fig. 2). The two isolates of *B. xylophilus*, Bx-DJ and Bx-KG, were identical in their ITS-RFLP although they were originated from different hosts. However, two isolates of *B. mucronatus*, Bm-KP and Bm-TJ, showed two different types of ITS-RFLP patterns (Fig. 2C & D) and they were

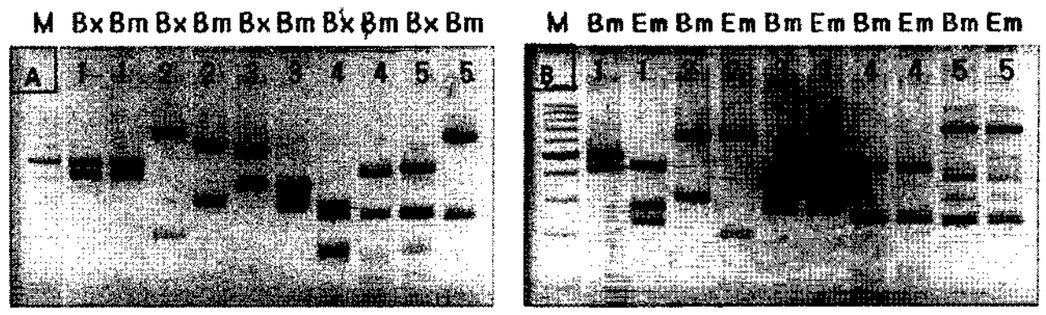

Fig. 3. Comparisons of ITS-RFLP patterns between closely related *Bursaphelenchus* species. A. ITS-RFLP patterns of *B. xylophilus* and *B. mucronatus* (East Asia type). B. ITS-RFLP patterns of *B. mucronatus* (East Asian type) and *B. mucronatus* (European type). Bx represents *Bursaphelenchus xylophilus*, Bm represents *B. mucronatus* (East Asia type), and Em represents *B.mucronatus* (European type). M: 1 kb molecular marker, 1: Rsa I, 2: Hae III, 3: Mp I, 4: Hinf I, and 5: Alu I.

discriminated by Rsa I and Hae III enzyme digestion (Fig. 3B). Bm-KP isolate collected from *P. koraiensis* was turned out to be a European type whereas Bm-TJ isolate from *P. thunbergii* was an East Asian type of *B. mucronatus*. *B. xylophilus* and European type of *B. mucronatus* was discriminated by all five different enzymes (Fig. 2A, D) and *B. xylophilus* and East Asia type of *B. mucronatus* was distinguished by four restriction enzymes except for the Rsa I (Fig. 3A). Table 1 shows the calculated restricted fragment size of ITS sequence by each five enzymes, which matched the PCR-RFLP result from gel electrophoresis. Therefore, ITS-RFLP is useful for species diagnosis of *Bursaphelenchus* and also for the intraspecific variation of *B. mucronatus*.

Discussion

Investigation of ITS-RFLP for species identification of

Table 1. Fragment sizes of ITS-RFLP for *Bursaphelenchus* spp. by both electrophoresis and sequence analysis

Nematode species	PCR product (bp)	Restriction enzymes (bp)[a]				
		Rsa I	Hae III	Msp I	Hinf I	Alu I
B. xylophilus (Bx-DJ & Bx-KG)	925	**485**[*], **417**, 22	**728**, **197**	**565**, **360**	**266, 233**, 141, 139	**435, 238**, **142,** 96, 13
B. mucronatus (Bm-TJ)	919	**486**, **411**, 22	**620**, **299**	**354**, **302**, **263**	**408**, **232**, **120**, 86, 49, 24	**674**, **232**, 13
B. mucronatus (Bm-KP)	918	**413**, **263**, **220**, 22	**618**, **195**, **105**	**356**, **303**, **259**	**405**, **232**, **121**, 87, 49, 25	**671**, **234**, 13
B. conicaudatus	972	**506**, **444**, 22	**565**, **156, 150**, 60, 41	**275**, **184, 182**, **112, 110, 109**	**253, 239**, **183, 166**, 79, 28, 24	**361**, **307, 291**, 13

[a]Sizes of DNA fragments were calculated by RestrctionMapper program based on the sequences of ITS from each species.
[*]Bold numbers are more than 100 bp that is visible fragment band in 2.0% agarose gel.

Bursaphelenchus was tried firstly in 1998 (Hoyer et al., 1998), and later in 2005 reported on the ITS-RFLP patterns for 26 species of *Bursaphelenchus* including two genotypes of *B. mucronatus* (Burgermeister et al., 2005). Burgermeister et al. (2005) selected a set of five enzymes which were distinctive enough to separate different species of *Bursaphelenchus*. As a simple discrimination method, a species-specific primer set was introduced for just two species of *B. xylophilus* and *B. mucronatus* (Matsunaga and Togashi, 2004). The principle of this method was to discriminate size differences of PCR product which was completed by just one time PCR reaction. However, the method was limited to two species of *Bursaphelenchus* and it was not sensitive enough to detect intraspecific variations of *B. mucronatus*.

Intraspecific variations of *B. xylophilus* have been reported in Japan (Iwahori et al., 1998; Iwahori et al., 2004). *B. xylophilus* was divided into pathogenic and non-pathogenic groups which were also differentiated by ITS-RFLP analysis. In Korea, we surveyed more than 30 geographical isolates of *B. xylophilus* as a preliminary work but, there were no variations in morphology and ITS, D2D3 rDNA sequence data. We assume the subspecies structure of *Bursaphelenchus* in Korea is quite different from that in Japan.

On the other hand, two genotypes of *B. mucronatus* were detected in Korea. *B. mucronatus* is known to be distributed much wider than *B. xylophilus* (Mamiya and Enda, 1979), and it is supposed to be an endemic species in Japan. Genetic variations of *B. mucronatus* have been proved by sequence variations of heat shock protein (Beckenbach, 1992), RAPD (Braasch et al., 1995), ITS-RFLP (Hoyer et al., 1998; Zheng et al., 2003), and chromosome numbers (Bolla and Boschert, 1993). Currently it is confirmed that *B. mucronatus* populations are divided into two groups, East Asian type and European type. The distribution of East Asian type is predominant to East Asia and European type is prevalent in Europe and Siberia (Braasch et al., 2000); however, both types are reported in Japan (Iwahori et al., 1998), in Siberia (Braasch et al., 2001), and in Germany (Braasch et al., 1999). Therefore, two genotypes of *B. mucronatus* are spread to world wide and it is also occupying pine forest in Korea.

Accurate identification of pine wood nematode is prerequisite step to diagnose pine wilt disease. Phenotypic traits relying on morphology, however, is not sufficient to differentiate closely related *Bursaphelenchus* species. ITS-RFLP is a useful method to 'xylophilus group' of *Bursaphelenchus* especially for the differentiation of *B. xylophilus* from *B. mucronatus*. Two species of *Bursaphelenchus* in Korea were successfully identified by ITS-RFLP and an intraspecific variation of *B. mucronatus* was also detected. This method will be applied for further investigation of new or unrecorded species of *Bursaphelenchus* distributing in Korea, which will provide fundamental information to understand taxonomic structure of *Bursaphelenchus* in Korean forest environment.

References

Ayoub, S. M. 1977. Plant nematology in agricultural training aid. Department of Food and Agriculture, Division of Plant Industry, Sacramento, CA.

Beckenbach, K., Smith, M. J. and Webster, J. M. 1992. Taxonomic affinities and intra- and interspecific variation in *Bursaphelenchus* spp. as determined by polymerase chain reaction. *J. Nematol.* 24:140-147.

Bolla, R. I. and Boschert, M. 1993. Pinewood nematode species complex: interbreeding potential and chromosome number. *J. Nematol.* 25:227-238.

Braasch, H., Burgermeister, W. and Patrika, K. H. 1995. Differentiation of three *Bursaphelenchus* species by means of RAPD-PCR. *Nachrichtenbl. Deut. Pflanzenschutzd.* 47:310-314.

Braasch, H., Burgermeister, W., Harmey, M. A., Michalopoulos-Skarmoutsos, H., Tomiczek, Ch. and Caroppo, S. 2000. Pest risk analysis of pine wood nematode related *Bursaphelenchus* species in view of South European pine wilting and wood imports from Asia. *Final Report of EU Research Project Fair CT* 95-0083.

Braasch, H., Tomiczek, Ch., Metge, K., Hoyer, U., Burgermeister, W., Wulfert, I. and Schonfield, U. 2001. Records of *Bursaphelenchus* spp. (Nematoda: Pasatistaphelenchidae) in coniferous timber imported from the Asian part of Russia. *For. Pathol.* 1:129-140.

Burgermeister, W., Metge, K., Braasch, H. and Buchbach, E. 2005. ITS-RFLP patterns for differentiation of 26 *Bursaphelenchus* (Nematoda: Parasitaphelenchidae) and observations on their distribution. *Russ. J. Nematol.* 13:29-42.

Cheng, H. R. 1983. The occurrence of a pine wilting disease caused by nematode found in Nanjing. *For. Pest Dis.* 4:1-5.

Ferris, V. R., Ferris, J. M. and Faghihi, J. 1993. Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. *Fundam. Appl. Nematol.* 16:177-184.

Kanzaki, N. and Futai, K. 2002. A PCR primer set for determination of phylogenetic relationships of *Bursaphelenchus* species within the xylophilus group. *Nematology* 4:35-41.

Hoyer, V. U., Burgermeister, W. and Braasch, H. 1998. Identification of *Bursaphelenchus* species (Nematoda, Aphlenchoididae) on the basis of amplified ribosomal DNA (ITS-RFLP). *Nachrichtenbl. Deut. Pflanzenschutzd* 50:273-277.

Iwahori, H., Tsuda, K., Kanzaki, N., Izui, K. and Futai, K. 1998. PCR-RFLP and sequencing analysis of ribosomal DNA of *Bursaphelenchus* nematodes related to pine wilt disease. *Fundam. Appl. Nematol.* 21:655-666.

Iwahori, H., Kanzaki, N., Izui, K. and Futai, K. 2004. *Bursaphelenechus xylophilus* and *B. mucronatus* in Japan: where are they from? *Nematol. Monogr. Perspect.* 2:793-803.

Mamiya, Y. 1972. Pine wood nematode, *Bursaphelenchus ligni-*

colus Mamiya and Kiyohara, as causal agent of pine wilting disease. *Rev. Plant Prot. Res.* 5:46-60.

Mamiya, Y. and Enda, N. 1979. *Bursaphelenchus mucronatus* n. sp. (Nematoda: Aphlenchoididae) from pine wood and its biology and pathogenicity to pine trees. *Nematologica* 25:353-361.

Matsunaga, K. and Togashi, K. 2004. A simple method for discriminating *Bursaphelenchus xylophilus* and *B. mucronatus* by species-specific polymerase chain reaction primer pairs. *Nematology.* 6:273-277.

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

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning: A laboratory manual, vols I-III. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Steiner, G. and Buhrer, E. M. 1934. *Aphelenchoides xylophilus* n. sp., a nematode associated with blue-stain and other fungi in timber. *J. Agric. Res.* 48:946-951.

Tzean, S. and Jan, S. 1985. The occurrence of pine wood nematode, *Bursaphelenchus xylophilus*, in Tiwan. *Proceedings of the 6th ROC symposium of electron microscopy,* pp. 38-39 (Abstr.).

Vrain, T. C. 1993. Restriction fragment length polymorphism separates species of the Xiphinema americanum group. *J. Nematol.* 25:361-364.

Yi, C., Byun, B., Park, J., Yang, S. and Chang, K. 1989. First finding of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle and its insect vector in Korea. *Research Reports of the Forestry Research Institute Seoul* 38:141-149.

Zheng, J., Subbotin, S. A., He, S., Gu, J. and Moens, M. 2003. Molecular characterisation of some Asian isolates of *Bursaphelenchus xylophilus* and *B. mucronatus* using PCR-RFLPs and sequences of ribosomal DNA. *Russ. J. Nematol.* 11:17-22.