Russian Journal of Nematology, 2005, 13 (2), 115 - 121

Duplex PCR based identification of *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970

Liqin Jiang*, Jingwu Zheng*, Lieven Waeyenberge, Sergei A. Subbotin*** and Maurice Moens**, ******

***Institute of Biotechnology, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310029, China, e-mail: jwzheng@zju.edu.cn**
****Crop Protection Department, Agricultural Research Centre, Burg. Van Gansberghelaan 96, 9820, Merelbeke, Belgium,**
*****Institute of Parasitology of Russian Academy of Sciences, Leninskii Prospect 33, Moscow 119071, Russia and Department of Nematology, University of California, Riverside, CA 92521, USA,**
****** Department of Crop Protection, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium.**

Accepted for publication 2 August 2005

Summary. A duplex PCR combining species-specific primers and universal primers was developed to identify *Bursaphelenchus xylophilus*. The design of the specific primers was based on differences in the Internal Transcribed Spacer sequences of rRNA between *Bursaphelenchus xylophilus* and other *Bursaphelenchus* species. The method was validated using 24 populations of different *Bursaphelenchus* spp. and four other nematode species. The duplex PCR generated a specific amplicon of 580 bp for all populations of *B. xylophilus* and a control amplicon, obtained after amplification of D2-D3 expansion fragments of the 28S rRNA gene, of about 770 bp. All non *B. xylophilus* samples generated only the 770 bp fragment. Compared to other molecular methods, duplex PCR is more rapid, reliable and cheaper. It allows the detection of single specimen of *B. xylophilus* in a sample.

Key words: *Bursaphelenchus xylophilus*, *B. mucronatus*, diagnostics, duplex PCR, species-specific primer, rRNA.

The pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 (Nematoda: Aphelenchoididae), is the causal agent of pine wilt disease and is found in North America (Nickle *et al.*, 1981), East Asia (Mamiya, 1988; Yang, 2003) and Europe (Mota *et al.*, 2002). In China, Japan and Korea, the nematode causes serious economic damage to several coniferous species (Mamiya, 1988; Yang, 2003). Due to its damaging character, PWN is listed quarantine organism in the EU.

The genus *Bursaphelenchus* includes about 70 species of which approximately three-quarters live in conifer trees (Burgermeister *et al.*, 2005). Identification of *Bursaphelenchus* species is accomplished on the basis of both morphological and morphometric characteristics. However, several closely related species show great morphological similarity. *Bursaphelenchus mucronatus* is such a species; however, it is considered to be non-virulent to pine trees (e.g. Mamiya, 1984; Evans *et al.*, 1996; Bolla & Wood, 1999). Obviously, exact identification of *B. xylophilus* is of paramount importance both for quarantine purposes and other control strategies.

Several molecular techniques have been used to identify and differentiate *Bursaphelenchus* species: DNA probes (Bolla & Weaver, 1988; Webster *et al.*, 1990, Abad *et al.*, 1991; Harmey *et al.*, 1994), RAPD (Braasch & Burgermeister, 1995; Zheng *et al.*, 1998; Wang *et al.*, 2001), PCR-RFLP (Hoyer *et al.*, 1998; Iwahori *et al.*, 1998; Zheng *et al.*, 2003; Burgermeister *et al.*, 2005), and PCR with specific primers (Liao *et al.*, 2001; Kang *et al.*, 2004; Matsunaga & Kogashi, 2004). The latter technique is particularly attractive as the species is identified after a single reaction, decreasing both diagnostic time and cost. Species-specific primers have been applied widely in diagnostics of plant pathogens including fungi (e.g. Bridge *et al.*, 1998),

Table 1. Species and populations of nematodes used in this study.

Species	Code	Population origin	Source (ID)
Bursaphelenchus xylophilus	1	Nanjing, Jiangsu , China	J. Zheng
	2	Zhenhai, Zhejiang, China	J. Zheng
	3	Xiangshan, Zhejiang, China	J. Zheng
	4	Maanshan, Anhui, China	J. Zheng
	5	Japan	J. Zheng
	6	Japan	J. Zheng
	7	Japan	P. Castagnone-Sereno
	8	USA	P. Castagnone-Sereno
	9	USA	J. Zheng
	10	Canada	P. Castagnone-Sereno
B. mucronatus	11	Taiwan, China	J. Zheng
	12	Fuyang, Zhejiang, China	J. Zheng
	13	Japan	J. Zheng
	14	Korea	J. Zheng
	15	Zunyi, Guizhou, China	J. Zheng
	16	Japan	J. Zheng
	17	Japan	P. Castagnone-Sereno
	18	France	P. Castagnone-Sereno
	19	Greece	J. Zheng
B. eggersi	20	France	P. Castagnone-Sereno
B. glochus	21	France	P. Castagnone-Sereno
Bursaphelenchus sp. 1	22	Hongkong, China	J. Zheng
Bursaphelenchus sp. 2	23	unknown	H. Li
Bursaphelenchus sp. 3	24	unknown	H. Li
Pratylenchus sp.	25	Hangzhou, Zhejiang China	J. Zheng
Tylenchorhynchus sp.	26	Hangzhou, Zhejiang China	J. Zheng
Helicotylenchus dihystera	27	Hangzhou, Zhejiang China	J. Zheng
Xiphinema brevicollum	28	Hangzhou, Zhejiang China	J. Zheng

bacteria (e.g. Louws *et al.*, 1999) and nematodes (e.g. Mulholland *et al.*, 1996; Bulman & Marshall, 1997; Fullaondo *et al.*, 1999; Setterquist *et al.*, 1996; Subbotin *et al.*, 2001; Amiri *et al.*, 2002). With respect to the identification of *B. xylophilus*, Liao *et al.* (2001) were the first to report species-specific primers. Later, Kang *et al.* (2004) designed species-specific primer sets based on sequence differences in the Intergenic Spacer of rDNA. Recently, Matsunaga and Kogashi (2004) discriminated *B. xylophilus* from *B. mucronatus* by a PCR with two species-specific primers, each one generating a DNA fragment different for the two species.

PCR identification using a single pair of specific primers may fail because of poor DNA extraction or the conditions of the reaction itself. In this paper we describe a rapid and reliable method for the diagnosis of *B. xylophilus* using a duplex PCR with an original specific primer. A first pair of universal primers verifies the presence of template nematode DNA in the sample and the success of PCR, whereas the second pair, the species-specific primers, identifies *B. xylophilus*.

MATERIAL AND METHODS

Nematode populations. Twenty-eight nematode species and populations were used to validate the technique (Table 1). The *Bursaphelenchus* populations, identified up to species level on the basis of their morphology and morphometrics, were either obtained from pinewood packaging imported from Greece, Japan, Korea, or the United States of America, collected from pine trees in China, or provided by colleagues. They were all maintained on *Botrytis cinerea* grown on potato dextrose agar (PDA) at 25°C. Nematode species of other genera were extracted from soil samples at different locations in Hangzhou, Zhejiang, P.R. China.

DNA extraction. For each sample, one to four specimens were transferred into a drop of 20 µl double distilled water on a clear glass slide and cut into fragments. The fragments, suspended in 10 µl water, were transferred into a 0.2 ml Eppendorf tube containing 8 µl Worm Lysis Buffer (125 mM KCl, 25 mM Tris-Cl pH 8.3, 3.75 mM MgCl2, 2.5 mM DTT and 1.125% Tween 20) and 2 µl proteinase K (600 µg ml-1). The tubes were incubated at 65°C for 1 h followed by 10 min at 95°C and finally centrifuged (1.5 min; 16100 g). The DNA suspension was used immediately for PCR or stored at −20°C for further study

Duplex PCR. Four primers were used in the PCR reaction. The first set contained the universal primers D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG) and D3B (5'-TCG GAA GGA ACC AGC TAC TA-3'), which amplify a fragment of the 28S rRNA gene (De Ley *et al.*, 1999; Amiri *et al.*, 2002; Zheng *et al.*, 2003). The second set included the species-specific primers BZ2 (5'-TCA CGA TGA TGC GAT TGG TG -3') and BF3 (5'-AGA AGA TAT TGG TCG CGG AA-3'), which in combination amplify a fragment of the ITS1, the 5.8S rRNA and a fragment of the ITS2 (Fig. 1).

DNA suspension (2 µl) was added to the PCR reaction mixture containing 2.5 µl 10 X PCR buffer, 1.5 µl 25 mM MgCl2, 0.5 µl 10 mM dNTP (Sangon, Shanghai, China), 1 µl 40 µM each primer (synthesized by Shanghai Sangon Biological Engineering Technology and Service Company, Shanghai, China), 1.5 U Taq Polymerase (Sangon, Shanghai, China) and double distilled water to a final volume of 25 µl.

Amplification was performed in a PTC-150 MiniCycler (MJ Research Inc, Waltham, MA, USA). The DNA amplification program consisted of 4 min 94°C, 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, and a final elongation step of 72°C for 5 min. After DNA amplification, 2 µl of the PCR product was run on a 1% agarose gel, separated by electrophoresis (120V, 2.5h), stained with ethidium bromide, visualized and photographed under UV-light. To confirm the results, the experiments were repeated three times.

Direct sequencing and PCR-RFLP of species-specific fragment. To verify the amplicon specificity of the PCR-products obtained with the species-specific primers, the amplicons were purified and sequenced using the primers BZ2 and BF3. The purified PCR products were also digested with the restriction enzymes *Hha*I or *Msp*I according to the manufacture's instructions. To compare the digested DNA with the pattern obtained after virtual digestion it was loaded on a 1.5% agarose gel, separated by electrophoresis (120V, 2.5h), stained with ethidium bromide, visualized and photographed under UV-light. Procedures for obtaining PCR amplified products and the endonuclease digestion of these products were repeated three times to verify the results.

RESULTS AND DISCUSSION

For the development of the duplex PCR two sets of primers were selected: the first one for verifying the success of the PCR and the second one for the specific detection of *B. xylophilus*. PCR with the universal primers, D2A and D3B, amplified a single fragment of about 770 bp for all populations (data not shown). This set of primers indicates the presence of template nematode DNA in the sample and, as a consequence, the quality of the performance of the PCR. Primer combinations with D3B were shown to be very useful for the amplification of a 28S rRNA gene fragment of many nematode species (Al-Banna et al., 1997; Thomas *et al.*, 1997, Subbotin *et al.*, 2001). The obtained amplicon did not vary in length among the nematode taxa examined, proving that it can be used as a control fragment.

The second set of primers, BZ2 and BF3 identified using DNAstar software, was designed after alignment of earlier obtained ITS sequences of *B. xylophilus* and *B. mucronatus* (Zheng *et al.*, 2003) with ITS sequences from other *Bursaphelenchus* species deposited in the GenBank or NemATOL databases: *B. xylophilus* (AY347912), *B. mucronatus* (AY347916), *B. luxuriosae* (AY850162), *B. singaporiensis* (AB097864), *B. fraudulentus* (AB067757), and *B. conicaudatus* (AB067758). The position of the primers corresponds to bp 31 - 50 and 576 - 595 on the sequence align

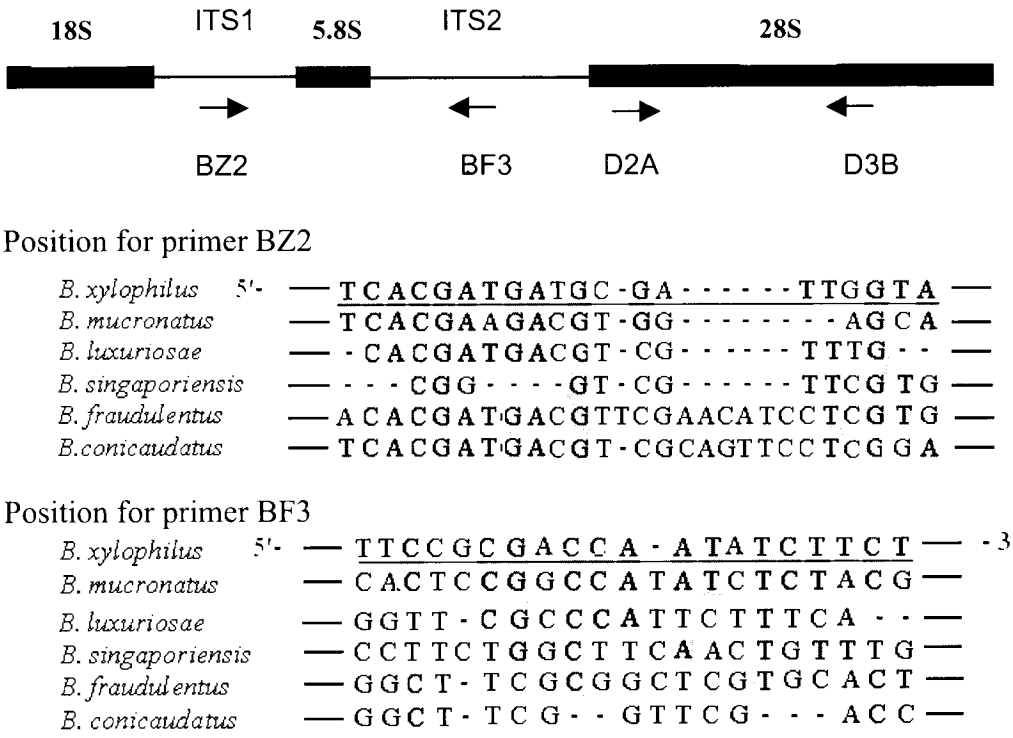

Fig. 1. Schematic drawing showing positions of four primers in the rDNA genes used in the duplex PCR for identification of *Bursaphelenchus xylophilus* and alignment used for development of the species-specific primer(s). The aligned sequence fragments of *B. xylophilus*, *B. mucronatus*, *B. luxuriosae*, *B. singaporiensis*, *B. fraudulentus*, *B. conicaudatus*. The position of the species-specific primers is underlined. Shaded parts represent identical residues; - shows absence of nucleotide.

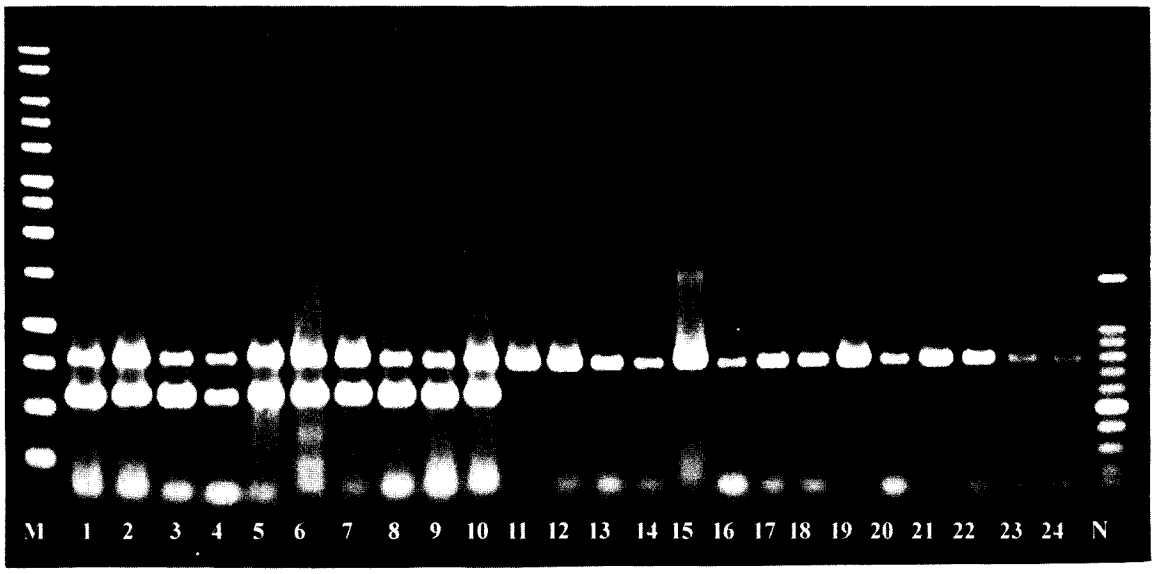

Fig. 2. Duplex PCR with BZ2 and BF3 species-specific primers for the detection of *Bursaphelenchus xylophilus*. M: 1kb DNA marker, N: 100 bp DNA marker; 1-10: *B. xylophilus*; 11-19: *B. mucronatus*; 20: *B. eggersi*; 21: *B. glochus*; 22: *Bursaphelenchus* sp. 1; 23: *Bursaphelenchus* sp. 2; 24: *Bursaphelenchus* sp. 3.

ment of *B. xylophilus* and *B. mucronatus*. PCR with these primers amplified a fragment of 580 bp of the ITS1, 5.8S and ITS2, also of single nematodes (data not shown). This primer combination was further used in duplex PCR with the set of universal primers.

As expected, the duplex PCR with samples containing specimens of *B. xylophilus* populations yielded two distinct fragments (580 and 770 bp), while all other nematode populations generated only one fragment of 770 bp (Fig. 2). The species-specific primers amplified a 580 bp fragment only from *B. xylophilus* populations; that fragment was not obtained from all other *Bursaphelenchus* species (Fig. 1). Nematode populations from other genera failed to generate a similar fragment (data not shown).

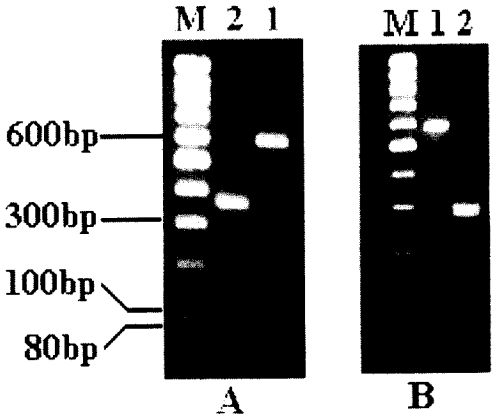

Fig. 3. Restriction fragments of amplicon obtained after PCR of *Bursaphelenchus xylophilus* with species specific primers BZ2 and BF3. A: *HhaI*; B: *MspI* (M: 100bp DNA marker, 1: undigested PCR fragment; 2: digested PCR fragment)

The specificity of the amplicon was confirmed by RFLP and direct sequencing. Digestion with *Hha*I generated three distinct fragments of about 70, 130, 380 bp (Fig. 3A); digestion with *Msp*I yielded two fragments of about 295 and 285 bp (Fig. 3B). The RFLPs obtained with both restriction enzymes were identical with the predicted RFLP patterns after virtual digestion of the published sequence (Fig. 3). Comparison of the sequence of the amplicon obtained with *B. xylophilus* specific primers with the known ITS sequence for *B. xylophilus* showed complete homology (data not shown). It can be concluded that the PCR amplified specific fragment is a fragment of the *B. xylophilus* 28S rRNA gene.

Precise identification of *B. xylophilus* using morphological features demands skill and time. Although molecular strategies like PCR-RFLP are well developed and may support the identification of PWN (Zheng *et al.*, 2003; Hoyer *et al.*, 1998; Burgermeister, 2005), duplex PCR has several advantages as it is time-saving, more sensitive and less expensive. Duplex PCR overcomes the disadvantage of PCR with a single pair of species-specific primers and allows the detection of a single *B. xylophilus* juvenile or adult.

ACKNOWLEDGEMENTS

The research was supported by Zhejiang Provincial Natural Science Foundation of China (No.300267) and Science and Technology Department of Zhejiang Province of China (No.2002C34003). We thank Drs Castagnone-Sereno and Li for providing some of the *Bursaphelenchus* populations

REFERENCES

Abad, P., Tares, S. & Bruguier, N. 1991. Characterization of the relationships in the pinewood nematode species complex (PWNSC) (*Bursaphelenchus* spp.) using a heterologous unc-22 DNA probe from *Caenorhabditis elegans*. *Parasitology* 102: 303-308.

Al-Banna, L., Williamson, V. & Gardner, S.L. 1997. Phylogenetic analysis of nematodes of the genus Pratylenchus using nuclear 26S rDNA. *Molecular Phylogenetic and Evolution* 7: 94-102.

Amiri, S., Subbotin, S.A. & Moens, M. 2002. Identification of the beet cyst nematode *Heterodera schachtii* by PCR. *European Journal of Plant Pathology* 108: 497-506.

Bolla, R.I. & Wood, R. 1999. Pathogenicity and potential for spread of pinewood nematode. In: Sustainability of pine forests in relation to pine wilt and decline. (K. Futai, K. Togashi. & T. Ikeda Eds). pp. 3-13. Kyoto, Japan, Shokado Shoten.

Bolla, R.I. & Weaver, C. 1988. Genomic differences among pathotypes of *Bursaphelenchus xylophilus*. *Journal of Nematology* 20: 309-316.

Braasch, H. & Burgermeister, W. 1995. Differentiation of three *Bursaphelenchus* species by means of RAPD-PCR. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* 47: 310-314.

Bridge, P.D., Arora, D.K., Reddy, C.A. & Elander, R.P. 1998. *Applications of PCR in Mycology*. Wallingford, UK, CAB International, 357 pp.

Bulman, S.R. & Marshall, J.W. 1997. Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New Zealand Journal of Crop and Horticultural Sciences* 25: 123-129.

Burgermeister, W., Metge, K., Braasch, H. & Buchbach, E. 2005. ITS-RFLP patterns for differentiation of 26 *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) and observations on their distribution. *Russian Journal of Nematology*: 29-42.

De Ley, P., Félix, M.A., Frisse, L.M., Nadler, S.A., Sternberg, P.W. & Thomas, W.K. 1999. Molecular and morphological characterization of two reproductively isolated species with mirror image anatomy (Nematoda: Cephalobidae). *Nematology* 2: 591-612.

Evans, H.F., McNamara, D.G., Braasch, H., Chadoeuf, J. & Magnusson, K. 1996. Pest risk analysis (PRA) for the territories of the European Union (as PRA area) on *Bursaphelenchus xylophilus* and its vectors in the genus *Monochamus*. *EPPO Bulletin* 26: 199-249.

Fullaondo, A., Brarrena, E., Viribay, M., Barrena, I., Salazar, A. & Ritter, E. 1999. Identification of potato cyst nematode species *Globodera rostochiensis* and *G. pallida* by PCR using specific primer combinations. *Nematology* 1: 157-163.

Harmey, J.H. & Harmey, M.A. 1994. DNA profiling of *Bursaphelenchus* species. *Gene* 145: 227-230.

Hoyer, V.U., Burgermeister, W. & Braasch, H. 1998. Identification of *Bursaphelenchus* species (Nematoda, Aphelenchoididae) on the basis of amplified ribosomal DNA (ITS-RFLP). *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes*. 50: 273-277.

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

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 the identification of the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). *Nematology* 6: 279-285.

Liao, J.L., Zhang, L.H. & Feng, Z.X. 2001. Reliable identification of *Bursaphelenchus xylophilus* by rDNA amplification. *Nematologia Mediterranea* 29: 131-135.

Louws, F.J., Rademaker, J.L.W. & De Bruijn, F.J. 1999. The three Ds of PCR-based genomic analysis of phytobacteria: diversity, detection, and disease diagnosis. *Annual Review of Phytopathology* 37: 81-125.

Mamiya, Y. 1984. The pine wood nematode. In: *Plant and insect nematodes*. (W.R. Nickle Ed.). pp. 589-628. New York, Marcel Dekker.

Mamiya, Y. 1988. History of pine wilt disease in Japan. *Journal of Nematology* 20: 219-226.

Matsunaga, K. & 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. & Sousa, H. 2002. First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* 1: 747-734.

Mulholland, V., Carde, L., O'Donnell, K.L., Fleming, C.C. & Powers, T.O. 1996. Use of the polymerase chain reaction to discriminate potato cyst nematode at the species level. In: Marshall, C. (Ed.). *Proceedings of diagnostics in crop production symposium*. Tarnham, UK, British Crop Production Council, pp. 247-252.

Nickle, W.R., Golden, A.M., Mamiya, Y. & Wergin, W.P. 1981. On the taxonomy and morphology of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer 1934) Nickle 1970. *Journal of Nematology* 13: 385-392.

Setterquist, R.A., Smith, C.K., Jones, R. & Fox, G.E. 1996. Diagnostic probes targeting the major sperm protein gene that may be useful in the molecular identification of nematodes. *Journal of Nematology* 28: 414-421.

Subbotin, S.A., Peng, D. & Moens, M. 2001. A rapid method for the identification of the soybean cyst nematode *Heterodera glycines* using duplex PCR. *Nematology* 3: 365-371.

Thomas, W.K., Vida, J.T., Frisse, L.M., Mundo, M. & Baldwin, J.G. 1997. DNA sequences from formalin-fixed nematodes: integrating molecular and morphological approaches to taxonomy. *Journal of Nematology* 29: 250-254.

Wang, L., Wang, Y., Yang, B., Hu, X. & Yu, S. 1998. Studies on relationships of *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus* by RAPD. *Acta Phytopathologica Sinica* 31: 225-229.

Webster, J.M., Anderson, R.V., Baillie, D.L., Beckenbach, K., Curran, J. & Rutherford, T.A. 1990. DNA probes for differentiating isolates of the pinewood nematode species complex. *Revue de Nematology* 13: 255-263.

Yang, B.J. 2003. The history, dispersal and potential threat of pine wood nematode in China. *Nematology Monographs & Perspectives* 1: 21-24.

Zheng, J., Subbotin, S., He, S., Gu, J. & Moens, M. 2003. Molecular characterization of some Asian isolates of *Bursaphelenchus xylophilus* and *B. mucronatus* using PCR-RFLPs and sequences of ribosomal DNA. *Russian Journal of Nematology* 11: 17-22.

Zheng, J., Xu, J., Wu, Y. & Li, D. 1998. RAPD fingerprinting on inter- and infra-species of *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus*. *Journal of Zhejiang Agricultural University* 24: 597-601.