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PCR-RFLP and sequencing analysis of ribosomal DNA of *Bursaphelenchus* nematodes related to pine wilt disease[1]

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Summary - A polymerase chain reaction - restriction fragment polymorphism (PCR-RFLP) analysis was used for the discrimination of isolates of *Bursaphelenchus* nematode. The isolates of *B. xylophilus* examined originated from Japan, the United States, China, and Canada and the *B. mucronatus* isolates from Japan, China, and France. Ribosomal DNA containing the 5.8S gene, the internal transcribed spacer region 1 and 2, and partial regions of 18S and 28S gene were amplified by PCR. Digestion of the amplified products of each nematode isolate with twelve restriction endonucleases and examination of resulting RFLP data by cluster analysis revealed a significant gap between *B. xylophilus* and *B. mucronatus*. Among the *B. xylophilus* isolates examined, Japanese pathogenic, Chinese and US isolates were all identical, whereas Japanese non-pathogenic isolates were slightly distinct and Canadian isolates formed a separate cluster. Among the *B. mucronatus* isolates, two Japanese isolates were very similar to each other and another Japanèse and one Chinese isolate were identical to each other. The DNA sequence data revealed 98 differences (nucleotide substitutions or gaps) in 884 bp investigated between *B. xylophilus* isolate and *B. mucronatus* isolate; DNA sequence data of *Aphelenchus avenae* and *Aphelenchoides fragariae* differed not only from those of *Bursaphelenchus* nematodes, but also from each other. To determine the phylogenic relationship of these species, the sequence data of the 5.8S gene of rDNA were examined. Cluster analysis revealed a monophyletic relationship between *A. fragariae* and *Bursaphelenchus* nematodes, both belonging to the superfamily Aphelenchoidea, and also showed an unexpected kinship between *A. avenae* and Tylenchina nematodes. © Orstom/Elsevier, Paris

Résumé - ***Utilisation de la réaction en chaîne des polymérases/polymorphisme des fragments de restriction (PCR-RFLP) et de l'analyse séquentielle de l'ADN ribosomal chez les nématodes du genre*** **Bursaphelenchus** ***associés au dépérissement des pins*** - La réaction en chaîne des polymérases/polymorphisme des fragments de restriction (PCR-RFLP) a été utilisée pour séparer des isolats du nématode *Bursaphelenchus*. Les isolats de *B. xylophilus* examinés provenaient du Japon, des USA, de Chine et du Canada, et ceux de *B. mucronatus* du Japon, de Chine et de France. L'ADN ribosomal contenant le gène 5.8S, les segments de transcription interne 1 et 2, et les segments partiels des gènes 18S et 28S ont été amplifiés par PCR. La digestion des produits amplifiés provenant de chaque isolat à l'aide de douze endonucléases de restriction et l'examen des données en RFLP qui en découlent révèlent, par une analyse en grappe, une séparation significative entre *B. xylophilus* et *B. mucronatus*. Parmi les isolats de *B. xylophilus* examinés, les isolats pathogènes du Japon, ceux de Chine et des USA étaient tous identiques, tandis que les isolats non pathogènes du Japon étaient légèrement distincts et que ceux du Canada formaient une grappe séparée. Parmi les isolats de *B. mucronatus*, deux isolats provenant du Japon étaient très semblables ; de même un autre isolat du Japon et un isolat de Chine étaient identiques. Les données provenant des séquences d'ADN montrent 98 différences (substitutions nucléotidiques ou séparations) dans les 884 paires de bases examinées chez les isolats de *B. xylophilus* et *B. mucronatus*. Les données provenant des séquences d'ADN chez *Aphelenchus avenae* et *Aphelenchoides fragariae* diffèrent non seulement de celles des *Bursaphelenchus* mais aussi entre elles. Afin de préciser les relations phylogéniques de ces espèces, les données séquentielles du gène 5.8S provenant de l'ADN ribosomal ont été examinées. L'analyse en grappe révèle une relation monophylétique entre *A. fragariae* et *Bursaphelenchus*, appartenant l'un et l'autre à la superfamille des Aphelenchoidea ; cette analyse a également montré une parenté inattendue entre *A. avenae* et les Tylenchina. © Orstom/Elsevier, Paris

Keywords: *Bursaphelenchus xylophilus*, *B. mucronatus*, DNA sequence, nematode, PCR-RFLP, phylogenic relationship, pine wilt disease.

Bursaphelenchus xylophilus (Steiner & Buhrer) Nickle, the causal agent of pine wilt disease, has been reported from several countries in North America and East Asia, while *B. mucronatus* Mamiya & Enda is a non-pathogenic related species from Western and Northern Europe, Russia, and East Asia. A mucro on the tail tip of adult females discriminates *B. mucronatus* from *B. xylophilus* (Mamiya & Enda, 1979). De Guiran and Bruguier (1989) showed incomplete reproductive isolation between these two species, *i.e.*,

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the French *B. mucronatus* isolate produced fertile offspring when crossed with a Japanese or North American *B. xylophilus* isolate, whereas the cross between Japanese *B. xylophilus* and Japanese *B. mucronatus* did not. On the basis of the mating study, they proposed a "supraspecies" to categorize the populations of pinewood nematode with incomplete reproductive isolation, and hypothesized that the isolates of Japanese and North American *B. xylophilus* derived from a common ancestor originating from Western Europe. A similar close relationship between *B. xylophilus* and *B. mucronatus* was proposed from the result of DNA analysis with dot blot techniques, and this group of *Bursaphelenchus* nematodes was called the 'pinewood nematode species complex' or PWNSC (Rutherford *et al.*, 1990; Webster *et al.*, 1990).

Presumably *B. xylophilus* was introduced to Japan from North America (de Guiran & Bruguier, 1989). On the other hand, considering the far wider distribution in Japan of *B. mucronatus* compared with *B. xylophilus* (Mamiya & Enda, 1979), *B. mucronatus* is supposed to be indigenous to Japan and to have been present there before the introduction of *B. xylophilus*.

Several attempts have been made to elucidate the phylogenetic relationship among *Bursaphelenchus* nematodes (Bolla *et al.*, 1988; Webster *et al.*, 1990; Abad *et al.*, 1991; Beckenbach *et al.*, 1992; Riga *et al.*, 1992; Tarès *et al.*, 1992, 1993, 1994; Harmey & Harmey, 1993, 1994). For example, Tarès *et al.* (1992) found three geographical subgroups in *B. xylophilus*: the US, Canadian, and Japanese subgroups. They found a closer relationship between the US and Japanese isolates and suggested that *B. xylophilus* reached Japan probably from the US but not from Canada. They also showed that Japanese *B. mucronatus* isolates were very different from French and Norwegian isolates. Beckenbach *et al.* (1992) found significant sequence variation in the heat shock protein gene between Japanese and European *B. mucronatus* and advocated separate species status for these two populations of *B. mucronatus*. Based on mating potential and chromosome number (Bolla & Boschert, 1993), Harmey and Harmey (1993) identified one Canadian isolate as *B. mucronatus* although this isolate was previously believed to be *B. xylophilus*.

The previous studies using several methods of DNA analysis have clearly demonstrated, *i*) the existence of two groups within the PWNSC, *i.e.*, the *B. xylophilus* group and the *B. mucronatus* group, and *ii*) a distinct difference between West European and Japanese *B. mucronatus* populations. However, the phylogenetic relationship among intraspecific isolates is still controversial, and further study is strongly needed.

Ribosomal DNA (rDNA) has been examined as a useful region for classifying eukaryotes at various taxonomic levels. Within rDNA, there are coding and non-coding regions. The non-coding regions such as internal transcribed spacer (ITS) are variable because they are not translated, which means that they are free from selection pressure. Therefore, the ITS regions of rDNA can be used for comparison at or below the species level.

In this study, we applied RFLP analysis of PCR amplified ITS regions as a simple and convenient method for the phylogenetic analysis and diagnosis of *Bursaphelenchus* nematodes within the PWNSC. To define the phylogenetic position of *Bursaphelenchus* in the order Aphelenchida, we performed DNA sequencing analysis of ITS regions of the rDNA of *B. xylophilus*, *B. mucronatus*, *Aphelenchus avenae,* and *Aphelenchoides fragariae.*

Materials and methods

NEMATODES

Five Japanese (pathogenic: S10, S6-1, T4, and non-pathogenic: C14-5, OK-2), five North American (MO, BC, FIDS, Q52A, St.J), and one Chinese (BxC) isolates of *B. xylophilus*, and three Japanese (M, Hh, Un), one Chinese (BmC) and one French (F1) isolates of *B. mucronatus* were examined. *Aphelenchus avenae*, *Aphelenchoides besseyi*, *A. fragariae*, and *A. ritzemabosi* were also examined as control species of Aphelenchida. For the sequence analysis of the ITS regions of rDNA, two *B. xylophilus* isolates (S10, C14-5), two *B. mucronatus* isolates (M, F1), *A. avenae*, *A. besseyi*, *A. fragariae,* and *A. ritzemabosi* were tested. Table 1 shows the isolate names and the geographical origin of these nematodes. These nematodes were reared on a fungal mat of *Botrytis cinerea* grown on autoclaved barley grain at 20°C for about 1 month. Propagated nematodes were collected by the Baermann funnel method and concentrated nematode suspension containing *ca* 10 000-50 000 nematodes was poured into a 1.5 ml micro test tube. If necessary, nematodes in the tube were frozen by immersion in liquid nitrogen and preserved at -20°C until used.

DNA EXTRACTION

Genomic DNA was extracted by a modification of a described method (Harmey & Harmey, 1993). For this method, 200 µl of 2× lysis buffer (200 mM NaCl, 200 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0, 2% SDS, 2% β-mercaptoethanol, 200 µg/ml proteinase K) were added to 200 µl of a nematode suspension containing approximately 5×10^4-10^5 nematodes in 1.5 ml micro test tube. The mixture was incubated at 65°C for 30 min with occasional mixing. Then, the DNA was extracted with an equal volume (400 µl) of phenol, followed by 400 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and 400 µl of chloroform:iso-

Table 1. *Isolates and origins of nematodes used in this study.*

Species	Code	Isolate name	Origin	Source
Bursaphelenchus xylophilus	1	S10	Shimane, Japan	H. Iwahori
	2	S6-1	Ibaraki, Japan	H. Iwahori
	3	T4	Iwate, Japan	T. Kiyohara
	4	C14-5	Chiba, Japan	H. Iwahori
	5	OK-2	Okinawa, Japan	T. Kiyohara
	6	BxC	Nanjing, China	B. Yang
	7	MO	Minnesota, United States	T. Kiyohara
	8	BC	British Columbia, Canada	T. Kiyohara
	9	FIDS	British Columbia, Canada	J. R. Sutherland
	10	St.J	New Brunswick, Canada	J. R. Sutherland
	11	Q52A	Quebec, Canada	J. R. Sutherland
Bursaphelenhcus mucronatus	1	M	Kyoto, Japan	H. Iwahori
	2	Hh	Hiroshima, Japan	S. Jikumaru
	3	Un	Nagasaki, Japan	T. Kiyohara
	4	BmC	Sichuan, China	B. Yang
		F1	Saint Symphorien, France	G. de Guiran
Aphelenchus avenae	a		Iwate, Japan	H. Okada
Aphelenchoides besseyi	b		Shizuoka, Japan	T. Nishizawa
Aphelenchoides fragariae	f		Shizuoka, Japan	T. Nishizawa
Aphelenchoides ritzemabosi	r		Shizuoka, Japan	T. Nishizawa

amyl alcohol (24:1). Each time, the aqueous phase was transferred to a new tube. The DNA was finally recovered by ethanol precipitation as follows. The DNA solution received 1/10 volume of 3 M sodium acetate, pH 4.6, and two volumes of 99.5% ethanol (–20°C) and kept at –80°C for 20 min, then centrifuged at 13 000 rpm (*ca* 13.8 × 10^3 *g*) for 10 min. The precipitated DNA pellet was washed twice with 70% ethanol (–20°C), then dried and resuspended in 100 µl of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). A 5 µl aliquot of the resulting DNA solution was diluted 500 times. After spectrophotometrical determination of its concentration, the ratio between the absorbancy at 260 and 280 nm was determined and the purity of the extracted DNA was confirmed. The DNA in TE buffer was diluted with autoclaved distilled water to the concentration of 10 ng/µl and used as PCR template.

PCR AMPLIFICATION

All polymerase chain reactions were performed in 50 µl reaction mixtures containing: 10 ng/µl of template DNA, 3 µl; 10 µM of each primer, 2.6 µl; 2.5 mM of dNTP, 8 µl; 2 U/µl of Taq polymerase, 0.4 µl (TaKaRa Biomedicals); 10× reaction buffer, 5 µl (TaKaRa Biomedicals); autoclaved ultra pure distilled water, 28.4 µl; a drop of mineral oil. The sequence of the forward primer, 5'-CGTAACAAGGTAGCTGTAG-3' (VRF1) and the reverse primer, 5'-TCCTCCGCTAAATGATATG-3' (VRF2) was derived from the data of Ferris *et al.* (1993). The amplified region began near the end of the 18S gene and terminated a short distance into the 28S gene, and included the ITS regions (ITS1, ITS2) and the 5.8S gene between them (Fig. 1).

The amplification was done in a thermocycler (TaKaRa PCR Thermal Cycler) and the reaction conditions consisted of 40 cycles with denaturation at 94°C for 45 s, annealing at 49°C for 30 s, and polymerization at 72°C for 1 min, and a final extension at 72°C for 10 min.

After PCR was completed, 5 µl of amplified product mixed with 1 µl of 6× dye marker solution (0.25% bromophenol blue, 30% glycerol) were analyzed by electrophoresis in a 1.2% agarose gel (DOTITE Agarose II) in Tris-Acetate-EDTA (TAE) buffer (40 mM Tris-HCl, 20 mM acetic acid, 2 mM EDTA, pH 8.1) for 30 min at 100 V. To examine the generation of PCR product, the gel was stained with 2.5 mg/l ethidium bromide for 15 min, and photographed under UV light.

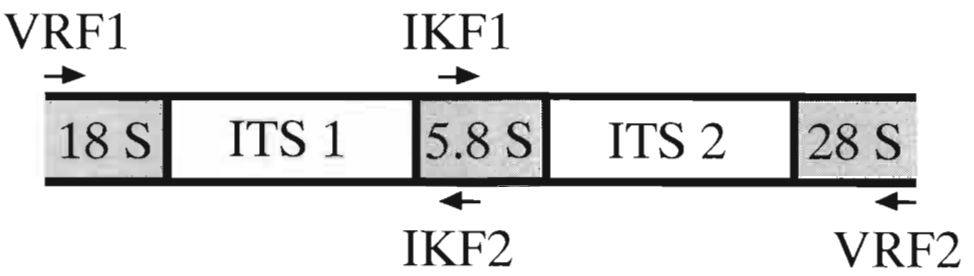


Fig. 1. *Locations of the primers for PCR and sequencing primers on rDNA used in this study. The regions in between the rDNA genes (18S, 5.8S, and 28S) are the internal transcribed spacer ITS 1 and ITS 2; the 18S and 28S genes are truncated.*

RESTRICTION ENZYME TREATMENT

Four µl of PCR product mixed with 0.5 µl of 10× buffer were digested with 0.5 µl (4-20 U) of each of twelve restriction enzymes (*Acc*II, *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Mse*I, *Msp*I, *Rsa*I, *Sau*3AI, and *Sau*96I at 37°C, or *Taq*I at 65°C) overnight. The DNA fragments thus generated were then separated by electrophoresis in a 6% polyacrylamide gel in Tris-Borate-EDTA (TBE) buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3) for 80 min at 100 V. The gel was stained with ethidium bromide, visualized, and photographed under UV light as in the case of agarose gel.

DNA SEQUENCING

Purified PCR products were used for DNA sequencing using a ABI PRISM™ 310 Genetic Analyzer with a reaction kit (Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer). The PCR product was purified by recovering the rDNA fragment after 1.2% agarose gel electrophoresis. Sequencing primers were the same as ones used in PCR amplification (VRF1 and VRF2). In addition, primers IKF1 (5'-GGGTCGATGAAGAACGCAG-3') and IKF2 (5'-CTGCGTTCTTCATCGACC-3') designed on the preliminary sequence data of 5.8S rDNA of *B. xylophilus* (isolate S10) were also used for sequencing (Fig. 1).

DATA ANALYSIS

The results of RFLP were analyzed by the method of the similarity matrix of Nei and Li (1979) and the genetic similarity between the nematode isolates was estimated. Then, a dendrogram was constructed by an unweighted pair-group method using arithmetic averages (UPGMA) cluster analysis.

Sequence data were aligned using a computer program GENETYX-MAC (v. 7.3). For comparative purposes, rDNA sequence data of other nematode species, such as *Caenorhabditis elegans*, *Strongylus vulgaris*, *Nacobbus aberrans*, *Heterodera avenae*, from GenBank (Accession numbers X03680, Z70737, U71375 and U12389, respectively) were added. The aligned sequences were used for phylogenic analysis with the most parsimonious method, and tree robustness was determined by bootstrap analysis.

Results

PCR AMPLIFICATION

For both *B. xylophilus* and *B. mucronatus*, PCR amplification of the ITS regions of rDNA generated only one fragment of *ca* 870 bp (Fig. 2A). The corresponding fragments of rDNA of *A. avenae*, *A. besseyi*, *A. fragariae*, and *A. ritzemabosi* are shown in Fig. 2B with those of *B. xylophilus* and *B. mucronatus*. Their sizes were approximately 680 (*A. avenae*), 870 (*A. besseyi*), 700 (*A. fragariae*), 870 (*A. ritzemabosi*) bp.

PCR-RFLP ANALYSIS

To detect the sequence variation in the amplified products among isolates and/or species of *Bursaphelenchus*, *Aphelenchus* and *Aphelenchoides* nematodes, RFLP analysis was carried out. The PCR products were digested with each of twelve restriction enzymes and the size (bp) of the resulting DNA fragments was determined (Table 2).

For several of the digestions, the sums of fragment sizes were greater than 870 bp. This could be attributed to inaccuracy of estimation of fragment sizes, and not to heterogeneity in the ITS regions because sequence data of the region did not include any variability within each isolate. When sequence data become available for all isolates, we will be able to predict more precisely the fragment sizes even when they are too small for detection on polyacrylamide gel.

The restriction patterns with *Mse*I and *Sau*3AI could not distinguish *B. xylophilus* from *B. mucronatus*. On the other hand, *Alu*I, *Hin*fI, and *Msp*I distinguished these two *Bursaphelenchus* species, but no variability could be detected among isolates of either species. Remarkable RFLPs were found when the

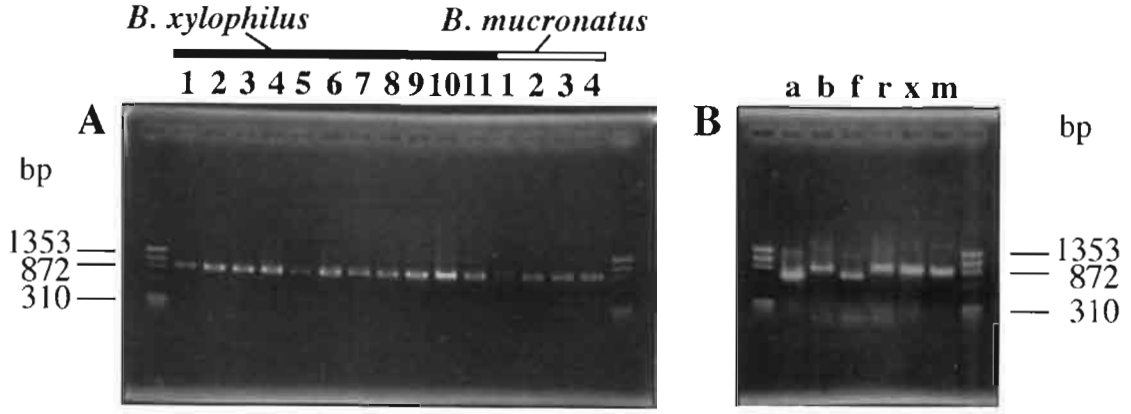


Fig. 2. *Amplification bands of polymerase chain reaction (PCR) products on a 1.2% agarose gel. Templates of total DNA were extracted from A:* Bursaphelenchus *isolates (Lanes were numbered according to the isolate numbers given in Table 1) and from B:* Aphelenchus avenae *(a),* Aphelenchoides besseyi *(b),* A. fragariae *(f),* A. ritzemabosi *(r),* B. xylophilus *(x) and* B. mucronatus *(m). The size in base pairs (bp) was estimated from ϕX174 phage DNA digested with HaeIII marker and indicated on the left (A) or on the right (B).*

Table 2. *DNA fragment sizes (bp) from PCR-RFLP analysis of ITS regions of rDNA from fifteen* Bursaphelenchus *nematode isolates,* Aphelenchus avenae, *and* Aphelenchoides fragariae.

Enzymes	*B. xylophilus*				*B. mucronatus*			*A. avenae*	*A. fragariae*
	S10, S6-1, T4, BxC, MO	C14-5, OK-2	Q52A	BC, FIDS, St.J	M	Hh	Un, BmC		
*Acc*II	260, 215, 170, 115, 75	260, 250, 170, 115, 110	260, 215, 170, 120, 110	260, 250, 170, 140, 110	370, 320, 170	370, 320, 290, 170	370, 320, 200	590, 140	720
*Alu*I	445, 240, 110, 105	445, 240, 110, 105	445, 240, 110, 105	445, 240, 110, 105	595, 235	595, 235	595, 235	500, 215	310, 260, 185
*Dde*I	700	700	700	700	700	700	700	385, 270, 105	285, 250, 165
*Hae*III	670, 165	670, 165	670, 165	670, 165	585, 165, 115	585, 165, 115	585, 260	585, 125	730
*Hha*I	380, 170, 135, 120	380, 270, 170, 120	380, 170, 150, 135, 121	480, 270, 170	**270**, 170, 110, 105	270, 205, 170, 110, 105, 75	270, 235, 195, 105	275, 235, 220	540, 270
*Hin*fI	270, 255, 150, 135	270, 255, 150, 135	270, 255, 150, 135	270, 255, 150, 135	385, 245, 130, 95, 50	385, 245, 130, 95, 50	385, 245, 130, 95, 50	440, 245	415, 225, 160
*Mse*I	**355**, 170	**355**, 170	**355**, 170	**355**, 170	**355**, 170	**355**, 170	**355**, 170	275, 250, 110, 60	170, **140**, **80**
*Msp*I	530, 360	530, 360	530, 360	530, 360	360, 280, 265	360, 280, 265	360, 280, 265	555, 110, 90	710
*Rsa*I	440, 415	440, 415	440, 415	440, 415	415, **230**	415, **230**	440, 415	675	590, 175
*Sau*3AI	510, 325	510, 325	510, 325	510, 325	510, 325	510, 325	510, 325	315, 195, 185	450, 245
*Sau*96I	870	870	870	870	870	870	870	600, 150	700
*Taq*I	275, 215, 170, 125, 115	275, 215, 170, 125, 115	275, 215, 170, 140, 130	275, 215, 140, 130, 115	215, 200, 165, 125, 115, 100	215, 200, 165, 125, 115, 100	215, 200, 165, 125, 115, 100	190, 130, **120**, 75, 60, 55	360, 335, 75

Fragment sizes are approximate estimation. Some small fragments less than 100 bp may be overlooked. Bold numbers are superimposed fragments.

rDNA fragment was digested with *Acc*II, *Hae*III, *Hha*I, *Rsa*I and *Taq*I. The differences in restriction patterns could be used to discriminate, not only *B. xylophilus* from *B. mucronatus*, but also some isolates of each species from the others. Fig. 3A, B, C show the typical restriction patterns, with *Mse*I, *Hin*fI, and *Hha*I, respectively. Digestion with *Dde*I and *Sau*96I showed only one fragment after electrophoresis and these enzymes did not seem to have a restriction site in the rDNA of the nematodes examined from RFLP data. However, the sequence data showed the presence of the *Dde*I restriction site in the conservative 28S region of the rDNA. The smaller remaining fragment, estimated to be *ca* 50 bp in size, could not be detected on polyacrylamide gel, and so we were unable to observe the restriction site of *Dde*I. Strictly speaking, however, we can not conclude that all of the isolates examined have a *Dde*I site, because we have not sequenced them all. In the case of *Sau*96I digestion, no restriction site was found from any of the sequence data.

In the case of *A. avenae* and *A. fragariae*, the RFLP patterns with all restriction enzymes tested were so different from each other and from those of *Bursaphelenchus* nematodes that the method of Nei and Li (1979) could not be applied to determine the phylogenic relationship among these nematodes. In the case of *A. besseyi* and *A. ritzemabosi*, however, the sum of the fragment sizes obtained by the restriction enzyme treatment was much larger than the size of the corresponding rDNA. When PCR amplification was applied to these nematodes, two rDNAs with different sizes were often obtained, as reported for *A. besseyi* and *A. arachidis* (Ibrahim *et al.*, 1994). This suggests the presence of polymorphic sequences in the rDNA regions of these species. Therefore, the above-mentioned phylogenic analysis was inapplicable to these *Aphelenchoides* species, and these two species were not used for DNA sequencing either.

Cluster analysis

Using the method of Nei and Li (1979), genetic distances were estimated for fifteen isolates of *Bursaphelenchus* from the RFLP data given by twelve restriction enzymes, and a dendrogram was drawn from the resulting distance matrix. The dendrogram (Fig. 4) indicated a critical separation between *B. xylophilus* and *B. mucronatus*. Among *B. xylophilus* isolates, Japanese (S10, S6-1, T4), Chinese (BxC), and US (MO) pathogenic isolates were identical to each other, but were slightly distinct from Japanese nonpathogenic isolates (C14-5, OK-2) (all the pathogenic isolates additionally had two *Acc*II and two *Hha*I restriction sites). Canadian isolates (BC, FIDS, St.J) formed another cluster within *B. xylophilus* with one exception (Q52A), which was somewhat closer to the Japanese isolates.

Among the *B. mucronatus* isolates, two Japanese isolates (M, Hh) were similar to each other (isolate M additionally had one *Acc*II and one *Hha*I restriction sites). Another Japanese isolate (Un) and a Chinese isolate (BmC) were identical to each other.

The DNA sequencing of ITS regions of rDNA revealed 98 differences (nucleotide substitutions or gaps) between one *B. xylophilus* isolate (S10) and one *B. mucronatus* isolate (M), six differences between two *B. xylophilus* isolates (S10, C14-5), and two differences between two *B. mucronatus* isolates (M, F1), mainly in the ITS2 region (Fig. 5A). Sequence data from *A. avenae* and *A. fragariae* differed not only from those of *Bursaphelenchus* nematodes, but also from each other (Fig. 5B). Therefore, for these four nematode species, it was difficult to align sequences strictly throughout ITS regions. To determine the phylogenic relationship of these species, the sequence data of the 5.8S rDNA region only, which is fairly conservative, was examined. Data on the corresponding region of *Heterodera avenae*, *Nacobbus aberrans*, *Strongylus vulgaris*, and *Caenorhabditis elegans* obtained from GenBank were also used for alignment (Fig. 6). The dendrogram obtained from these sequence data of 5.8S rDNA showed the monophyletic relationship between *A. fragariae* and *Bursaphelenchus* nematodes, both belonging to superfamily Aphelenchoidea, and also showed an unexpected kinship between *A. avenae* and Tylenchida nematodes (Fig. 7).

Discussion

Remarkable progress in molecular biological techniques has enabled identification and classification of several plant parasitic nematodes. As for *Bursaphelenchus* nematodes, some of which are related to pine wilt disease, molecular biological techniques such as Southern blotting analysis (Bolla *et al.*, 1988; Webster *et al.*, 1990; Abad *et al.*, 1991; Tarès *et al.*, 1992; Harmey & Harmey, 1993) and DNA sequencing analysis (Beckenbach *et al.*, 1992) have been carried out to elucidate the phylogenetic relationships among isolates with different geographical origins. These methods, however, require well-trained technicians and are expensive, whereas RAPD (Irdani *et al.*, 1995) and PCR-RFLP analyses are both easier and cheaper. Using the PCR-RFLP method, we examined the phylogenetic relationship among *Bursaphelenchus* isolates within the so-called PWNSC. We also used DNA sequencing analysis for four isolates of *Bursaphelenchus* nematodes, one *Aphelenchus*, and one *Aphelenchoides* species to understand their phylogenetic relationships.

PCR-RFLP analysis with twelve restriction enzymes revealed that three Japanese pathogenic (S10, S6-1,

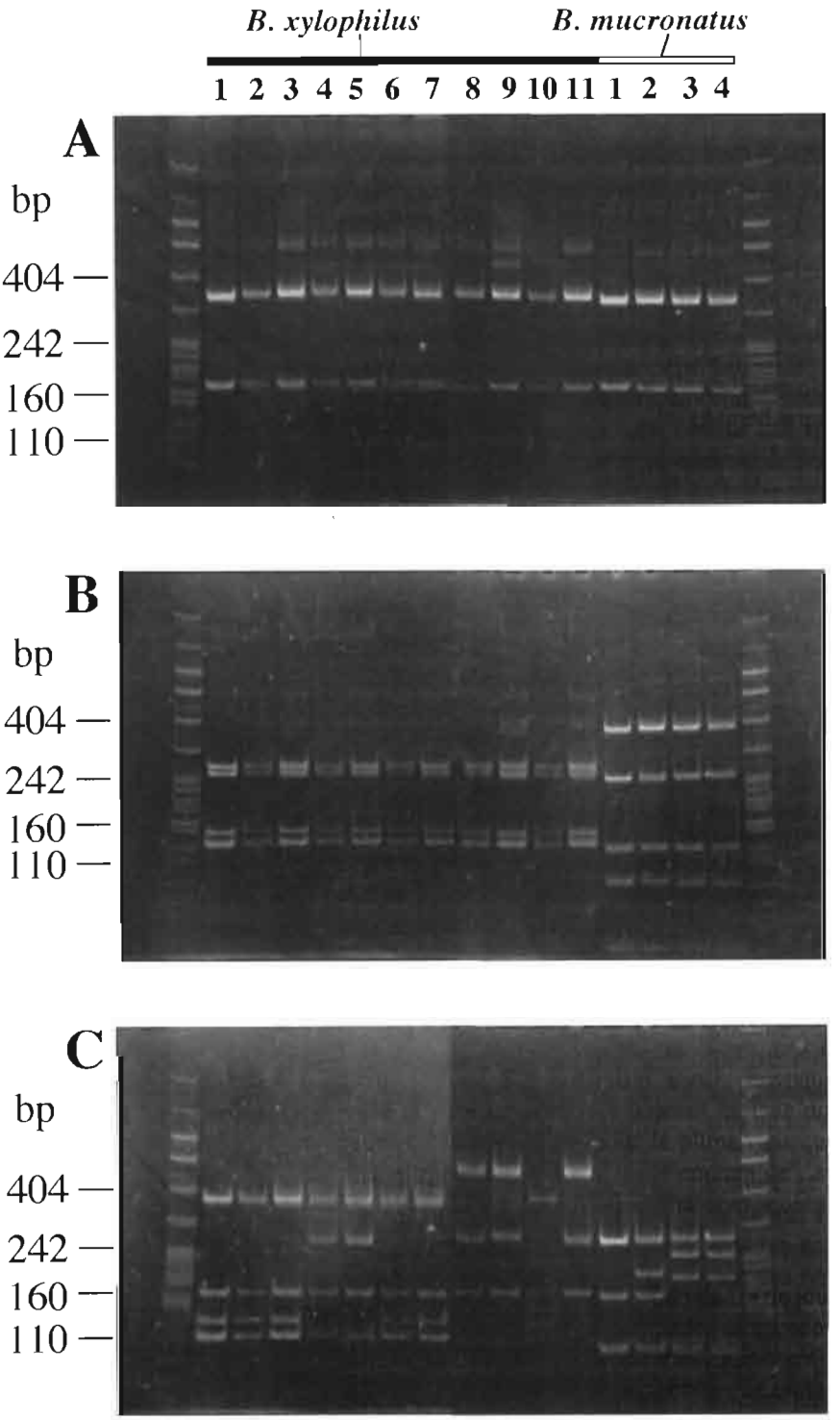


Fig. 3. *Typical PCR-RFLP patterns of the PCR-amplified ITS regions of* Bursaphelenchus *nematode isolates on a 6% polyacrylamide gel digested with A:* Mse*I ; B:* Hin*fI; C:* Hha*I. Lanes were numbered according to the isolate numbers given in Table 1. The size in base pairs (bp) were estimated from pBR322-* Msp*I digest marker and indicated on the left.*

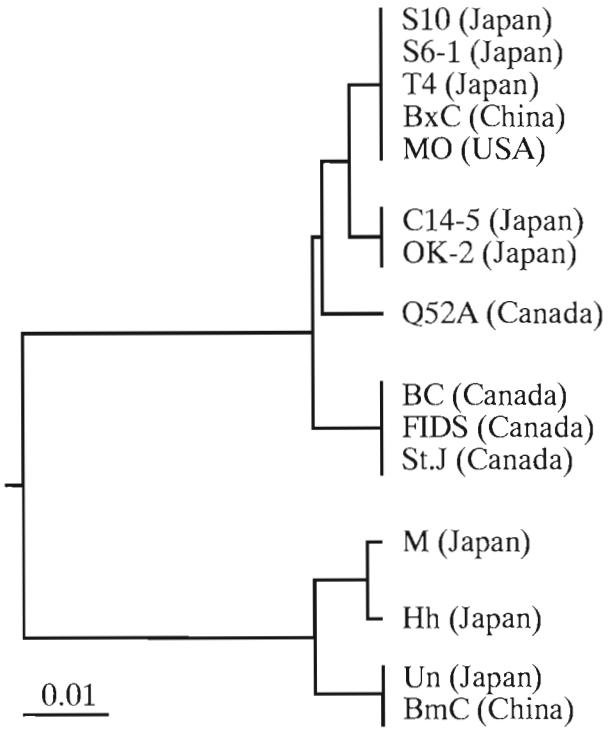


Fig. 4. *Relationship of fifteen isolates of* Bursaphelenchus. *Isolates S10, S6-1, T4, BxC, MO, C14-5, OK-2, Q52A, BC, FIDS, and St.J are* B. xylophilus. *Among these, isolates C14-5 and OK-2 are nonpathogenic. Isolates M, Hh, Un, and BmC are* B. mucronatus. *The country of origin of each isolate is given in parenthesis. The dendrogram was generated based on the UPGMA cluster analysis from genetic distance (showing in scale) as calculated in Nei and Li (1979).*

T4), one Chinese (BxC), and one American (MO) isolates were identical. This indicates that these isolates are closely related to each other and may have derived from a common ancestral population. This also suggests that they have not yet been established as separate isolates after they were introduced into East Asia, perhaps from North America, approximately less than 100 years ago. Japanese nonpathogenic isolates (C14-5, OK-2) were slightly different from Japanese pathogenic isolates. These nonpathogenic isolates, therefore, may have a different origin, or might have been exposed to a selection pressure different from the selection pressure acting on pathogenic isolates. Here, 'pathogenicity' is defined as the potential to kill susceptible pine trees, although the existence of pathogenic gene(s) has not yet been verified. Among Canadian isolates, BC, FIDS, and St.J were distinct from other isolates, while Q52A was close to Japanese isolates. The present genetic data suggests genetic diversity among Canadian isolates.

The present results support the hypothesis that *B. xylophilus* was introduced to Japan from North America (de Guiran & Bruguier, 1989), probably from the US (Tarès *et al.*, 1992; Harmey & Harmey 1993). Only one US (MO) isolate was available for our experiment. Thus, further experiments with additional US isolates are needed to ascertain the precise origin of the Japanese isolates.

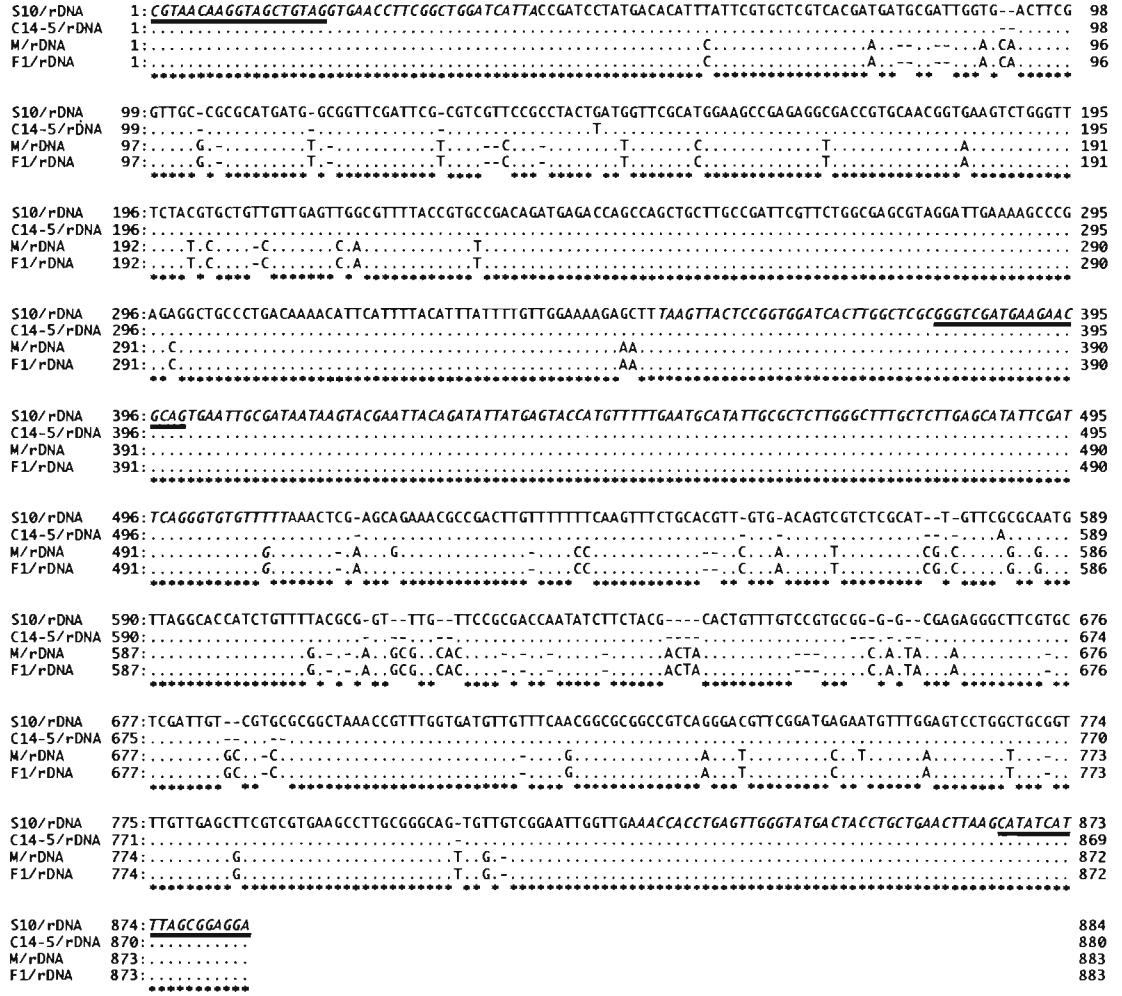
The four *B. mucronatus* isolates examined can be classified into two groups, Japanese M and Hh, and Japanese Un and Chinese BmC, although the first two isolates were not completely identical to each other. Thus, *B. mucronatus* isolates seemed to be much more diversified than *B. xylophilus*, as five isolates of pathogenic *B. xylophilus* examined were completely identical to each other in RFLP patterns, irrespective of their geographical origins.

Sequence data of rDNA ITS regions indicated that *i*) *B. mucronatus* was distinct from *B. xylophilus*; *ii*) a French isolate (F1) could not be distinguished from Japanese *B. mucronatus* isolates although authors (Beckenbach *et al.*, 1992; Tarès *et al.*, 1992; Harmey & Harmey; 1993) found some genetic difference between the Japanese isolate and the French isolate (F1) based on different methods of DNA analyses. The reason for these contradictory results could be attributed to the fact that different nematode isolates were examined. This suggests the presence of at least two genealogical isolates of *B. mucronatus*, European and East Asian, in Japan. To elucidate the route of their migration and the process of their evolution, more *Bursaphelenchus* isolates should be examined from a DNA-based view point.

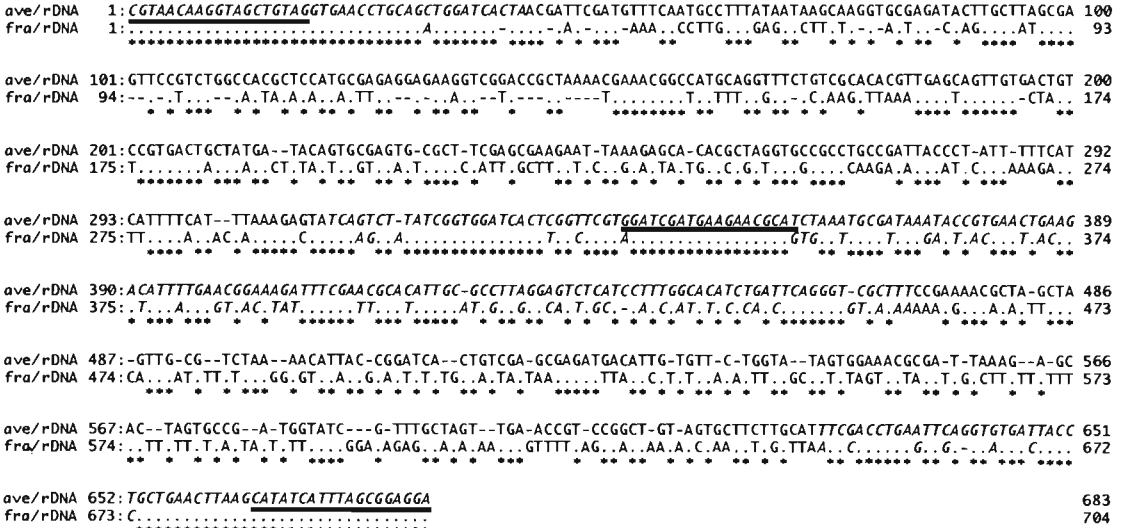
The dendrogram (Fig. 7) based on the sequence analyses on the 5.8S regions of rDNA showed that *A. fragariae*, *B. xylophilus*, and *B. mucronatus* were clustered into one group, and thus seemed to be closely related to each other. The resulting diagram supports the morphological classification (Goodey, 1960; Nickle, 1970) that distinguishes *Bursaphelenchus* species from *Aphelenchoides* species by characteristics of male tail region (bursa) and the shape and

Fig. 5. *Comparison of nucleotide sequences of ribosomal DNA of A:* Bursaphelenchus xylophilus *isolates S10 and C14-5 and* B. mucronatus *isolates M and F1, and B:* Aphelenchus avenae *(ave) and* Aphelenchoides fragariae *(fra) (Underlined sequences are the sequencing primers; italicized bases are in the 18S, 5.8S and 28S; asterisks indicate nucleotide identical throughout the species compared; dashes indicate gaps introduced to maximize the alignment).*

A



B



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B.xylophilus(S10)   1:TAAG--TTACTCCGGTGGATCACTTGGCTCGCGGGTCGATGAAGAACGCAGTGAATTGCG 58
B.mucronatus(M)     1:....--.....................................................  58
A.avenae            1:.C..TC..-A..-...........C..T...T..A..............TCT..A....  58
A.fragariae         1:C...-C.ATA..-..................TA.A.....................  58
H.avenae            1:CT..TC..-A..-...........C......T..A................CC..C....  58
N.aberrans          1:CT..TC..-A..-...........C......TA.T................CT..C....  58
S.vulgaris          1:CT..-C..CAG.-.A......GG.C.AT.....TA........A.......CA.GC....  58
C.elegans           1:CT..-C..CAG.-.A......GG...CA...A.TA................CTTGC....  58
                        **  *    * * ******  * *  ***    ******** ******      ****

B.xylophilus(S10)  59:ATAATAAGTACGAATTACAGATA--TTATGAGTACC-ATGTTTTT-GAATGCATATTGCG 114
B.mucronatus(M)    59:.......................--..........-........-.............. 114
A.avenae           59:....AT.CCGT...C.GA...C.--..T...ACGG--.AAGA...C...C...C..... 114
A.fragariae        59:T...G..TC.............--........GA.-..A.-...C..T.........A 114
H.avenae           59:.....T...G....C.G....A.CC..--..AC..--.AAC-...C.......C..... 113
N.aberrans         59:.....T...GT...C.G....AGCA..--...C..AG.ATC-...-.......C..... 114
S.vulgaris         59:T..T.T.CC.......G....CGC-...-....GGTG.AA.-...-...C.....--... 112
C.elegans          59:T..C.T.CC.......G....CGC-...-....GGTG.AA--...C...C.....--..A 112
                       **   *    *** *  ***    **  **      *    *** **  *** *  **

B.xylophilus(S10) 115:CTCTT-GGG-CTTTGC-TC---TTGAGCATATTCGATTCAGGGTGTGTTTTT        160
B.mucronatus(M)   115:.....-...-.....-..---.............................G..        160
A.avenae          115:.C-..A..A-G.C.CA-..C--..TG...C..CT..........-CGC...-        159
A.fragariae       115:TCG..-...CA.....-..A--.C-GAT...CC.A.C..........A.AA-        160
H.avenae          114:.CA..-..A-G..ACA-..CA-..--...CGCCT.G.........-CG..A--        156
N.aberrans        115:.CA..-..A--...CGG..CA-..-G...CG..T.G.........-CG-.A--        157
S.vulgaris        113:.CG..-...--...---..CC-..CG...CG.CT.G.............---..G..        153
C.elegans         113:.CAACT...C.------..CAG..-G.T.CG.CT.G.............---..G..        153
                          **          **   *     *        ******     *
```

Fig. 6. *Comparison of nucleotide sequences of 5.8S ribosomal DNA of* Bursaphelenchus xylophilus *(isolate S10),* B. mucronatus *(isolate M),* Aphelenchus avenae, Aphelenchoides fragariae, Heterodera avenae, Nacobbus aberrans, Strongylus vulgaris, *and* Caenorhabditis elegans *(Data of the latter four species were from GenBank; asterisks indicate nucleotides identical throughout the species compared; dashes indicate gaps introduced to maximize the alignment).*

size of the spicule. *A. avenae* and two Tylenchina species were grouped into one cluster, but the lower value in the bootstrap proportion suggests that this does not indicate a phylogenetic relationship. However, there can be no doubt about the monophyly of these four aphelench species because morphological characters, such as orifice of dorsal oesophageal gland in postcorpus anterior to the central cuticular valvular apparatus (Goodey, 1960; Nickle, 1990; Siddiqi, 1980, 1986) and morphology of anal aperture (Siddiqi, 1980, 1986), are common to these four species. To confirm the phylogenic relationship among these highly developed plant-parasitic nematodes, further information on various parts of genomic or mitochondrial DNA is needed from more nematode species including species of Diplogasterida.

PCR-RFLP analysis is a useful method not only for unequivocal identification of nematode species, but also for the determination of species-specific DNA markers as a useful tool for such identification. Also, the nucleotide sequence data will certainly result in marked advances in nematode systematics as it already has with other organisms. So far, for such DNA-based studies, several thousands of nematodes must be processed for each diagnostic. Recently, however, DNA isolation from a single juvenile has been proved possible for some plant-parasitic nematodes (Harris *et al.*, 1990; Cenis, 1993; Powers & Harris, 1993; Orui, 1996). However, the details of the intra-specific variability of nematodes still remains to be studied.

The present taxonomic relationships among species of the genus *Bursaphelenchus* or the order Aphelenchida was obtained from sequence analyses of the ITS regions of rDNA. Since this region consisting of 683 to 884 base pairs is a rather small part of the genomic DNA, the present findings may not be quite identical with those obtained previously using other parts of the genomic DNA. More information on genomic or mitochondrial DNA of various *Bursaphelenchus* nematodes needs to be accumulated to clarify the phylogenic relationships among these nematodes, and to elucidate the geographical origin of *Bursaphelenchus* nematodes, the route of their worldwide distribution, and the mechanisms of their speciation.

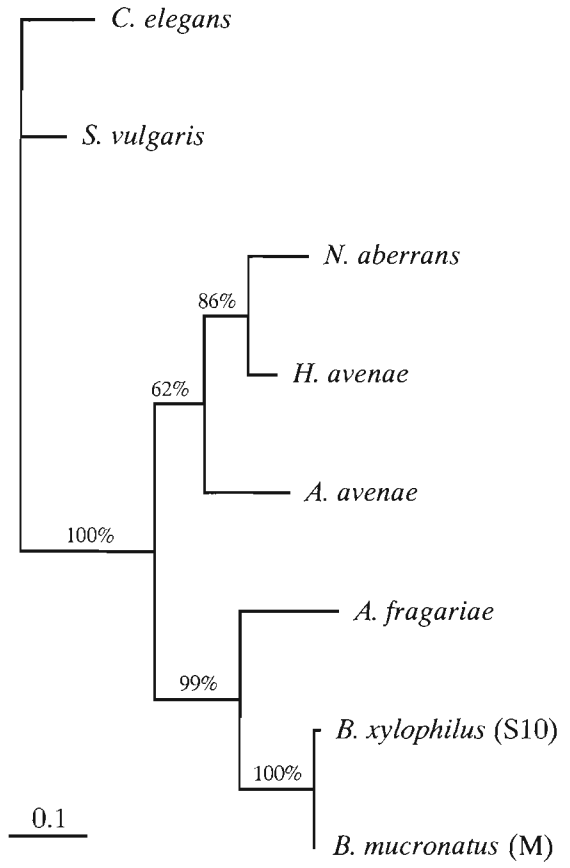

Fig. 7. *Relationship of eight species of nematodes,* Caenorhabditis elegans *and* Strongylus vulgaris *(Rhabditida),* Nacobbus aberrans *and* Heterodera avenae *(Tylenchida), and* Aphelenchus avenae, Aphelenchoides fragariae, Bursaphelenchus xylophilus, *and* B. mucronatus *(Aphelenchida) (Dendrogram was obtained with the most parsimonious method and the alignment of the sequences of the 5.8S rDNA shown in Fig. 6; the percentages represent the proportion of 1000 bootstrap replications. Genetic distance was shown in scale).*

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