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

Hideaki IWAHORI, Kaku TSUDA, Natsumi KANZAKI, Katsura IZUI and Kazuyoshi FUTAI

Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan.

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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 Japanese 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-

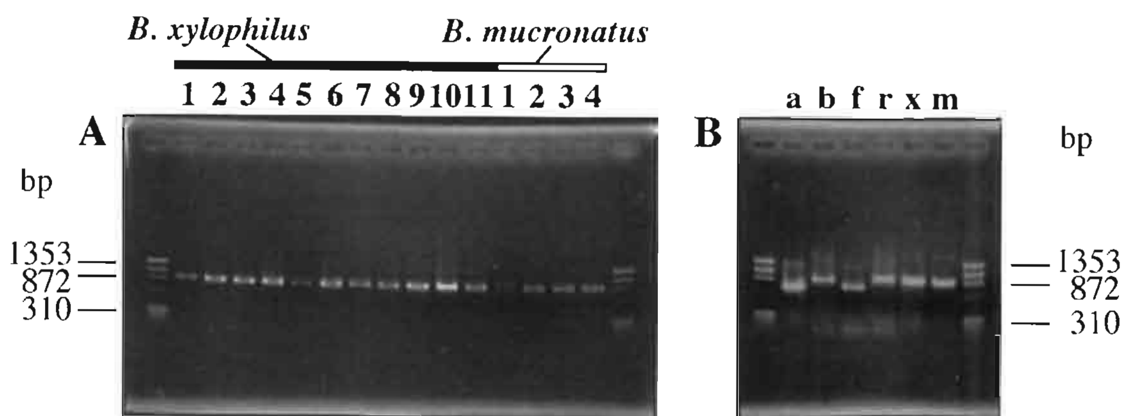

Fig. 2. Amplification bands of polymerase chain reaction (PCR) products on a 1.2% agarose gel. Templates of total DNA were extracted from A: Bursaphelenchus *isolates (Lanes were numbered according to the isolate numbers given in Table 1) and from B:* Aphelenchus avenae *(a),* Aphelenchoides besseyi *(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 *Bursaphe lenchus* 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,

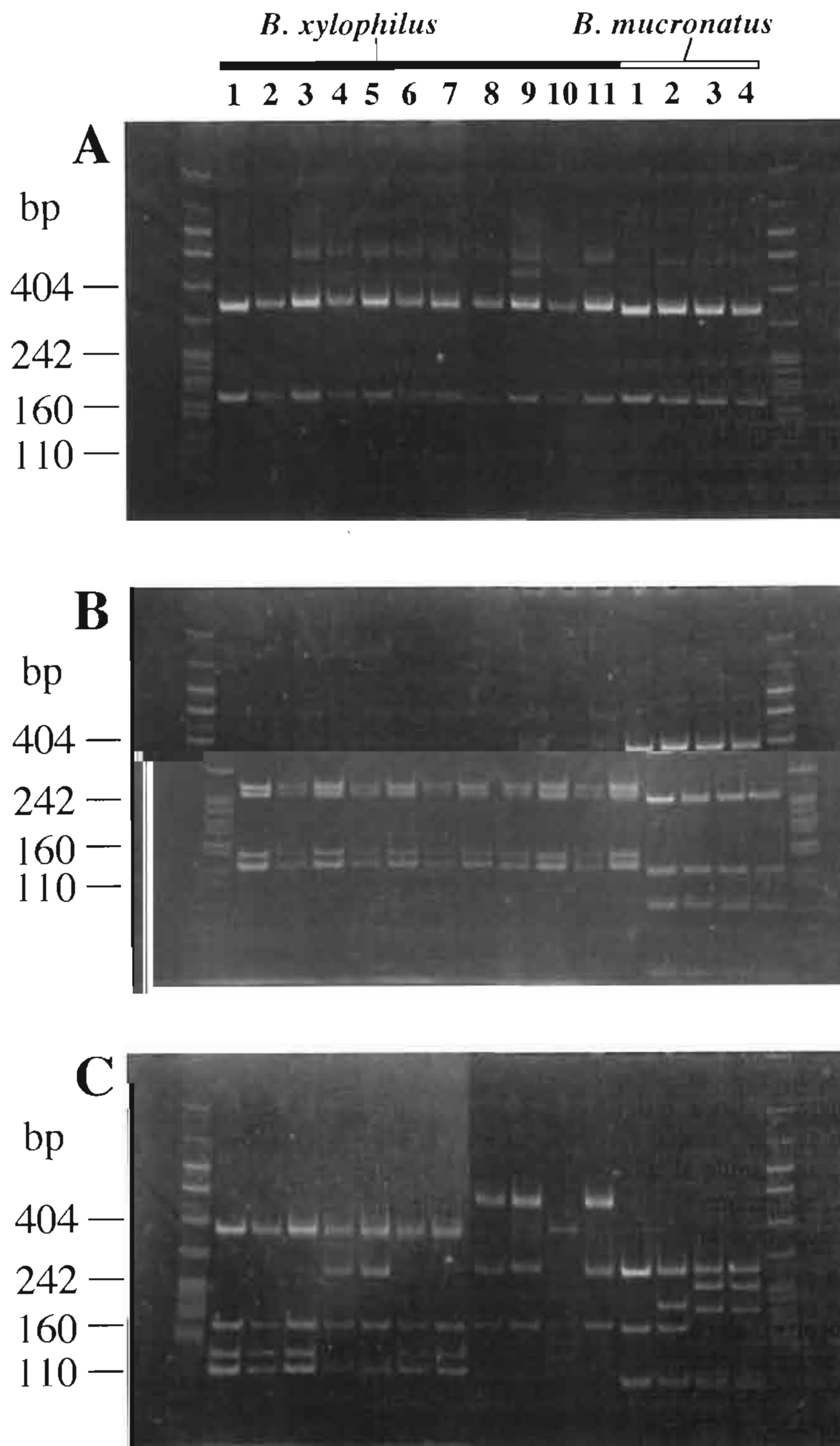

Fig. 3. Typical PCR-RFLP patterns of the PCR-amplified ITS regions of Bursaphelenchus *nematode isolates on a 6% polyacrylamide gel digested with A:* MseI *; B:* HinfI*; C:* HhaI*. Lanes were numbered according to the isolate numbers given in Table 1. The size in base pairs (bp) were estimated from pBR322-*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

A

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S10/rDNA      1:CGTAACAAGGTAGCTGTAGGTGAACCTTCGGCTGGATCATTACCGATCCTATGACACATTTATTCGTGCTCGTCACGATGATGCGATTGGTG--ACTTCG  98
C14-5/rDNA    1:............................................................................................--......  98
M/rDNA        1:............................................................C.................A..--..--...A.CA......  96
F1/rDNA       1:............................................................C.................A..--..--...A.CA......  96
                ************************************************************ ***************** **  **  *** *  ******

S10/rDNA     99:GTTGC-CGCGCATGATG-GCGGTTCGATTCG-CGTCGTTCCGCCTACTGATGGTTCGCATGGAAGCCGAGAGGCGACCGTGCAACGGTGAAGTCTGGGTT 195
C14-5/rDNA   99:.....-...........-............-................T................................................... 195
M/rDNA       97:.....G.-.........T.-...........T....--C..-........T......C.............T..............A........... 191
F1/rDNA      97:.....G.-.........T.-...........T....--C..-........T......C.............T..............A........... 191
                ***** * ********* * *********** ****   *** ***** ** ******* ************* ************** ***********

S10/rDNA    196:TCTACGTGCTGTTGTTGAGTTGGCGTTTTACCGTGCCGACAGATGAGACCAGCCAGCTGCTTGCCGATTCGTTCTGGCGAGCGTAGGATTGAAAAGCCCG 295
C14-5/rDNA  196:.................................................................................................... 295
M/rDNA      192:....T.C....-C.......C.A............T................................................................ 290
F1/rDNA     192:....T.C....-C.......C.A............T................................................................ 290
                **** * ****  ******* * ************ ****************************************************************

S10/rDNA    296:AGAGGCTGCCCTGACAAAACATTCATTTTACATTTATTTTGTTGGAAAAGAGCTTTAAGTTACTCCGGTGGATCACTTGGCTCGCGGGTCGATGAAGAAC 395
C14-5/rDNA  296:.................................................................................................... 395
M/rDNA      291:..C.................................................AA............................................. 390
F1/rDNA     291:..C.................................................AA............................................. 390
                ** **************************************************  *********************************************

S10/rDNA    396:GCAGTGAATTGCGATAATAAGTACGAATTACAGATATTATGAGTACCATGTTTTTGAATGCATATTGCGCTCTTGGGCTTTGCTCTTGAGCATATTCGAT 495
C14-5/rDNA  396:.................................................................................................... 495
M/rDNA      391:.................................................................................................... 490
F1/rDNA     391:.................................................................................................... 490
                ****************************************************************************************************

S10/rDNA    496:TCAGGGTGTGTTTTTAAACTCG-AGCAGAAACGCCGACTTGTTTTTTTCAAGTTTCTGCACGTT-GTG-ACAGTCGTCTCGCAT--T-GTTCGCGCAATG 589
C14-5/rDNA  496:......................-.........................................-...-...............--.-....A....... 589
M/rDNA      491:............G.......-.A...G..............-....CC............--..C...A.....T.........CG.C.....G..G... 586
F1/rDNA     491:............G.......-.A..................-....CC............--..C...A.....T.........CG.C.....G..G... 586
                ************ ******* * *** ************** ****  ************  ** *** ***** *********  * ****  ** ***

S10/rDNA    590:TTAGGCACCATCTGTTTTACGCG-GT--TTG--TTCCGCGACCAATATCTTCTACG----CACTGTTTGTCCGTGCGG-G-G--CGAGAGGGCTTCGTGC 676
C14-5/rDNA  590:.......................-..--...--.................................---.-.-............................ 674
M/rDNA      587:.................G.-.-.A..GCG..CAC....-.-..-.......-.......ACTA..........---.....C.A.TA...A.........-.. 676
F1/rDNA     587:.................G.-.-.A..GCG..CAC....-.-..-.......-.......ACTA..........---.....C.A.TA...A.........-.. 676
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S10/rDNA    677:TCGATTGT--CGTGCGCGGCTAAACCGTTTGGTGATGTTGTTTCAACGGCGCGGCCGTCAGGGACGTTCGGATGAGAATGTTTGGAGTCCTGGCTGCGGT 774
C14-5/rDNA  675:........--..--...................................................................................... 770
M/rDNA      677:........GC..-C.........................-....G..............A...T.........C..T......A........T...-.. 773
F1/rDNA     677:........GC..-C.........................-....G..............A...T.........C.........A........T...-.. 773
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S10/rDNA    775:TTGTTGAGCTTCGTCGTGAAGCCTTGCGGGCAG-TGTTGTCGGAATTGGTTGAAACCACCTGAGTTGGGTATGACTACCTGCTGAACTTAAGCATATCAT 873
C14-5/rDNA  771:.................................-.................................................................. 869
M/rDNA      774:.........G.......................T..G.-............................................................. 872
F1/rDNA     774:.........G.......................T..G.-............................................................. 872
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S10/rDNA    874:TTAGCGGAGGA                                                                                          884
C14-5/rDNA  870:...........                                                                                          880
M/rDNA      873:...........                                                                                          883
F1/rDNA     873:...........                                                                                          883
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B

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ave/rDNA      1:CGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCACTAACGATTCGATGTTTCAATGCCTTTATAATAAGCAAGGTGCGAGATACTTGCTTAGCGA 100
fra/rDNA      1:................................A.......-.....-.A.-...-AAA..CCTTG...GAG..CTT.T.-.-A.T..-C.AG....AT....  93
                ******************************** ******* **** * * ***   **   ***   **   * * *  * ** *  * ****  ****

ave/rDNA    101:GTTCCGTCTGGCCACGCTCCATGCGAGAGGAGAAGGTCGGACCGCTAAAACGAAACGGCCATGCAGGTTTCTGTCGCACACGTTGAGCAGTTGTGACTGT 200
fra/rDNA     94:--.-.T...--.A.TA.A.A..A.TT..--.-..A..--T.---..----T........T..TTT..G..-.C.AAG.TTAAA....T......-CTA.. 174
                  * * ***  * *  * * ** *  **  * ** **  *   **      ******** **   ** ** * *   *    **** ******     **

ave/rDNA    201:CCGTGACTGCTATGA--TACAGTGCGAGTG-CGCT-TCGAGCGAAGAAT-TAAAGAGCA-CACGCTAGGTGCCGCCTGCCGATTACCCT-ATT-TTTCAT 292
fra/rDNA    175:T.......A...A..CT.TA.T..GT..A.T....C.ATT.GCTT..T.C..G.A.TA.TG..C.G.T...G....CAAGA.A...AT.C...AAAGA.. 274
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ave/rDNA    293:CATTTTCAT--TTAAAGAGTATCAGTCT-TATCGGTGGATCACTCGGTTCGTGGATCGATGAAGAACGCATCTAAATGCGATAAATACCGTGAACTGAAG 389
fra/rDNA    275:TT....A..AC.A.....C.....AG..A...............T..C....A.................GTG..T....T...GA.T.AC...T.AC.. 374
                  **** **  * ***** *****  ** *************** ** **** *****************   ** **** ***  * *  *** *  **

ave/rDNA    390:ACATTTTGAACGGAAAGATTTCGAACGCACATTGC-GCCTTAGGAGTCTCATCCTTTGGCACATCTGATTCAGGGT-CGCTTTCCGAAAACGCTA-GCTA 486
fra/rDNA    375:.T...A...GT.AC.TAT......TT...T.....AT.G..G..CA.T.GC.-.A.C.AT.T.C.CA.C.......GT.A.AAAAA.G...A.A.TT... 473
                * *** ***  *  *  ******  *** *****  * ** **  * *  * * * * *  * * *  * *******  * *  * *** * *  ***

ave/rDNA    487:-GTTG-CG--TCTAA--AACATTAC-CGGATCA--CTGTCGA-GCGAGATGACATTG-TGTT-C-TGGTA--TAGTGGAAACGCGA-T-TAAAG--A-GC 566
fra/rDNA    474:CA...AT.TT.T...GG.GT..A..G.A.T.T.TG..A.TA.TAA.....TTA..C.T.T..A.A.TT..GC..T.TAGT..TA..T.G.CTT.TT.TTT 573
                  ***  *  * ***  *  ** ** * * * *  ** *   *  *****  ** * * ** * *  **  ** *    **  ** * *    *  *

ave/rDNA    567:AC--TAGTGCCG--A-TGGTATC---G-TTTGCTAGT--TGA-ACCGT-CCGGCT-GT-AGTGCTTCTTGCATTTCGACCTGAATTCAGGTGTGATTACC 651
fra/rDNA    574:..TT.TT.T.A.TA.T.TT....GGA.AGAG..A.A.AA...GTTTT.AG..A..AA.A.C.AA..T.G.TTAA..C......G..G.-..A...C.... 672
                **  *  * * *  * *  ****  *   ** * * ***     * ** **  * * *  ** * *   ** ****** ** * ** *** ****

ave/rDNA    652:TGCTGAACTTAAGCATATCATTTAGCGGAGGA                                                                     683
fra/rDNA    673:C...............................                                                                     704
                 *******************************
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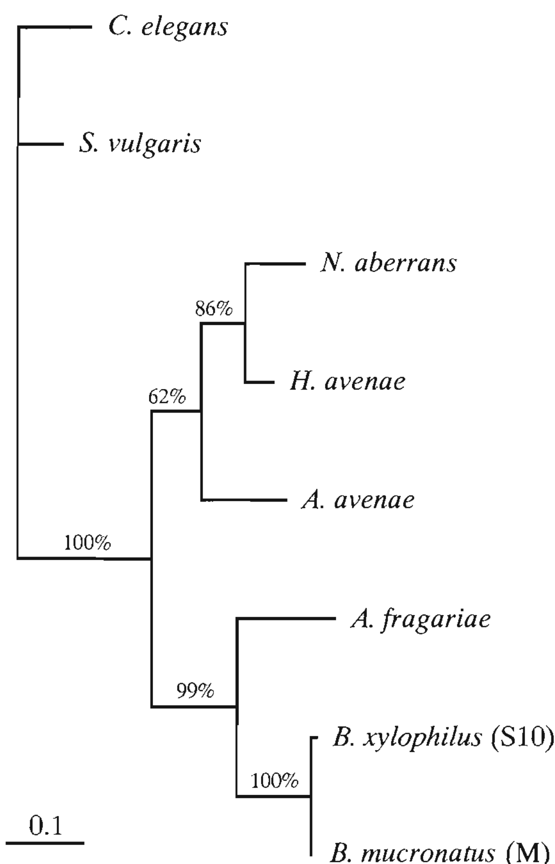

***Fig.** 7. Relationship of eight species of nematodes,* Caenorhabditis elegans *and* Strongylus vulgaris *(Rhabditida),* Nacobbus aberrans *and* Heterodera avenae *(Tylenchida), and* Aphelenchus avenae, Aphelenchoides fragariae, Bursaphelenchus xylophilus, *and* B. mucronatus *(Aphelenchida) (Dendrogram 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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