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GENETIC ANALYSIS OF THREE *BURSAPHELENCHUS* SPECIES BY RANDOM AMPLIFIED POLYMORPHIC DNA[1]

by

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Summary. Random amplified polymorphic DNA (RAPD) technique was used to estimate the genetic relationships among three *Bursaphelenchus* species, *B. mucronatus*, *B. tusciae* and *B. sexdentati*. Nine isolates of *B. mucronatus*, three of *B. sexdentati* and one of *B. tusciae*, recently described in Italy, were examined. The nematodes, multiplied on *Botrytis cinerea* plates, were tested with 20 different primers. The differentiation obtained with RAPDs was fully in agreement with morphobiometrical data; the genetic similarity between the examined species is clearly revealed.

Morphology is an essential component of any higher level classification of nematodes and, in many cases, provides a rapid and unambigous diagnosis to species. However, the diagnostic morphological characters of some nematode groups are either obscure or display ontogenetic or enviromental variation with a consequent reduction in their diagnostic value (Curran *et al.*, 1985).

Among the molecular methods the random amplified polymorphic DNA (RAPD) technique has been established as a powerful tool in the analysis of genetic variation in many organisms (Black *et al.*, 1992; Caswell-Chen *et al.*, 1992). By these methods, arbitrary regions of the genome are amplified by random synthetic oligonucleotide primers. The DNA fragments obtained can then be compared by electrophoresis to reveal the degree of genetic similarity within and/or between populations.

The RAPD-PCR technique has already been used to differentiate the nematode species of different genera (Hahn *et al.*, 1994; Chacón *et al.*, 1994; Guirao *et al.*, 1995; Irdani *et al.*, 1995a; Irdani *et al.*, 1995a; Braasch *et al.*, 1995; Esquibet *et al.*, 1998; Schmitz *et al.*, 1998; Lamberti *et al.*, 1999).

In this study RAPD markers were employed to compare genetic variation in thirteen populations of *Bursaphelenchus* belonging to the species *Bursaphelenchus mucronatus* Mamiya *et* Enda, *B. sexdentati* Rühm and *B. tusciae* Ambrogioni *et* Marinari Palmisano.

Materials and methods

Table I presents the *Bursaphelenchus* isolates, with code, host tree and geographical origin analysed in this study. Nine *B. mucronatus*

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TABLE I - Bursaphelenchus *species and populations examined by RAPD-DNA.*

Species	Code	Host	Locality
B. sexdentati	IT 2 (w)	*Pinus pinaster*	Marina di Massa (Massa)
B. sexdentati	IT 9 (w)	*P. halepensis*	Piombino (Livorno)
B. sexdentati	GR-IT	*P. pinaster*	Thessaloniki, Greece
B. mucronatus	IT 4 (w)	*P. sylvestris*	Fenestrelle (Torino)
B. mucronatus	IT 5 (w)	*P. sylvestris*	Oulx (Torino)
B. mucronatus	IT 6 (w)	*P. nigra austriaca*	Varisella (Torino)
B. mucronatus	IT 7 (w)	*P. strobus*	Dronero (Cuneo)
B. mucronatus	IT 8 (w)	*P. sylvestris*	Monte Calvario (Cuneo)
B. mucronatus	IT 12 (i)*	*P. pinaster*	Prati di Prà (Genova)
B. mucronatus	IT 13 (w)	*P. pinaster*	Marina di Massa (Massa)
B. mucronatus	F2	*P. pinaster*	Landes, forêt de Campet, France
B. mucronatus	YACH	*P. densiflora*	Yachiyo, Chiba, Japan
B. tusciae	IT 14 (w)	*P. pinea*	Scopeti (Firenze)

* Isolate cited previously as ITA-1 (Irdani *et al.*, 1995b).

populations (seven Italian, one French, one Japanese), three *B. sexdentati* (two Italian, one Greek) and one Italian isolate of *B. tusciae* were examined. These nematodes were reared on a fungal mat of *Botrytis cinerea* Pers. grown on autoclaved malt agar (MA) in Petri dishes at 26 °C for about one month. Propagated nematodes were collected from the lid of the Petri dish, and concentrated by centrifugation at 5000 rpm for five minutes.

DNA was extracted from mixed life stages of the nematodes using the method of Dellaporta *et al.* (1983) with minor modifications. Nematodes were pelletted by centrifugation at 5000 rpm for two minutes and washed twice with sterile-water. Nematodes pellets corresponding to approximately 30 µl wet volume were briefly dried in a vacuum concentrator. The residue was suspended in 25 µl of water. Five microliters of 5X concentrated extraction buffer (500 mM Tris-HCl pH 8.0; 2.5 M NaCl; 250 mM Na-EDTA pH 8.0; 50 mM ß-mercaptoethanol) were added to the suspension of nematodes and homogenized on ice for three min with a micro pestle in a 1.5 ml Eppendorf. The micro pestle was then rinsed with 25 µl of 5X concentrated extraction buffer which was added to the homogenate. After addition of 75 µl of proteinase K (20 µg/ml), 87.5 µl of water were added to make up a final volume of 150 µl. This mixture was incubated for 60 min at 37 °C and for further 30 min at 55 °C with shaking. After addition of 10 µl of 20% SDS and mixing, the homogenate was incubated for 20 min at 65 °C, then 50 µl of 5 M potassium acetate pH 9.0 were added, and the sample incubated for 30 min. Then the supernatant was transferred to a fresh tube. One volume of ice-cold isopropanol was added and the mixture kept overnight at – 20 °C. DNA was pelletted by centrifugation (30 min at 10000 rpm/4 °C) and washed with 70% ethanol, centrifugated again, dried for 5 min in a vacuum concentrator and dissolved in 30 µl TE buffer (10 mM Tris-HCl pH 8.0; 0.1 mM Na-EDTA pH 8.0).

DNA concentrations were measured fluorimetrically using the fluorescent dye Hoechst 33258 and a DNA-fluorometer TKO-100 (Hoefer, San Francisco).

Random primers (ten base oligonucleotides with 50-70% G+C content and random se-

quence) were purchased from Genenco and Operon Kit A (Operon technologies, Atlanta, GA). The results reported here were obtained with random primers OPA1 (5'-CAG GCC CTT C-3'), OPA2 (5'-TGC CGA GCT G-3'), OPA4 (5'-AAT CGG GCT G-3'), OPA5 (5'-AGG GGT CTT G-3'), OPP2 (5'-TCG GCA CGA A-3') AH30 (5'-TGG TCA CTG T-3'), AG1 (5'-AGG TCA CTG A), AH29 (5'-TGG TGA CTG A) and AI2 (5'-ATC GCA CAC T).

PCR was carried out using a Perkin-Elmer 9600 thermocycler. The reaction mixture (40 µl) contained 15-20 ng of template DNA, 200 µM of each dNTP (Boehringer-Mannheim), 100 µM of a single random primer, 2.5 U of Taq-DNA polymerase (Boerhinger-Mannheim); 10X reaction mixture, autoclaved ultra-pure distilled water. The reaction mixture was heated for 2 min at 94 °C and submitted to 45 reaction cycles of 94 °C for 1 min, 35 °C for 2 min, and a final extension at 72 °C for 5 min. The rate of heating from 35 °C to 72 °C was regulated to 2 sec/°C. After the PCR, 10 µl aliquots of the reaction mixtures were resolved by electrophoresis in a 2% agarose gel and stained with ethidium bromide.

The pattern of stained DNA fragments were examined on a UV transilluminator and recorded by polaroid film.

Markers were scored for all different primers as present or absent and only amplified markers clearly present were included in the analysis.

Simple matching (Sneath and Sokal, 1973) was used to calculate a genetic similarity matrix. The matrix was used to perform hierarchical cluster analysis based on the unweighted pair-group method using arithmetic averages (UPGMA) implemented in the NTSYS ver. 1.3 package (Rohlf, 1987).

Results

More than twenty decamers of random primers were tested for their ability to differentiate among *B. mcuronatus*, *B. tusciae* and *B. sexdentati*. Some were unsuitable, due to weak DNA amplification, others produced informative patterns and were used for molecular analysis.

Initially, RAPD analyses were realized with the genomic DNAs of eight European and one Japanese *B. mucronatus* populations using five primers (OPA1, OPA2, OPA4, OPA5, OPP2). A total of 175 fragments were visualized across all the nine genotypes of *B. mucronatus* investigated (data not shown).

Then, the molecular experiments were also extended for a few isolates of *B. tusciae* and *B. sexdentati*.

The patterns obtained after amplification of the DNA from the 13 *Bursaphelenchus* genotypes with four primers (AH29, AH30, AG1, AI2) revealed more than 200 bands (Figs 1-4). Only fragments with high intensity and without doubt as to their presence or absence in the two RAPD replicates were taken into account.

With each primer, two to ten bands from 0.2-4.3 Kb were scored and a total of 224 reproducible fragments were counted. Most primers produced informative patterns of amplified DNA fragments revealing interspecific as well as intraspecific differences between isolates.

For reliable identification of these *Bursaphelenchus* species, certain amplified DNA fragments must obviously be distinctive and stable among all isolates of a given species. This condition was met for at least three primers (AH29, AG1, AI2) (Figs 1, 3, 4). In Fig. 1, RAPD pattern obtained with primer AH29 revealed a species-specific amplified DNA fragment permitting differentiation and identification of *B. sexdentati* from *B. mucronatus* and *B. tusciae*. One amplified fragment of approximately 0.3 Kb (indicated by <) was characteristic for all *B. sexdentati* isolates tested. With the AG1-RAPD profile (Fig. 3) all the *B. mucronatus* isolates, from Europe and Japan, could be clearly distinguished from the *B. tusciae* and *B. sexdentati* isolates on the basis of one fragment of approx. 1.1 Kb (indicated by >) and the lack of a 0.9 Kb amplified fragment, characteristic of both *B. tusciae* and

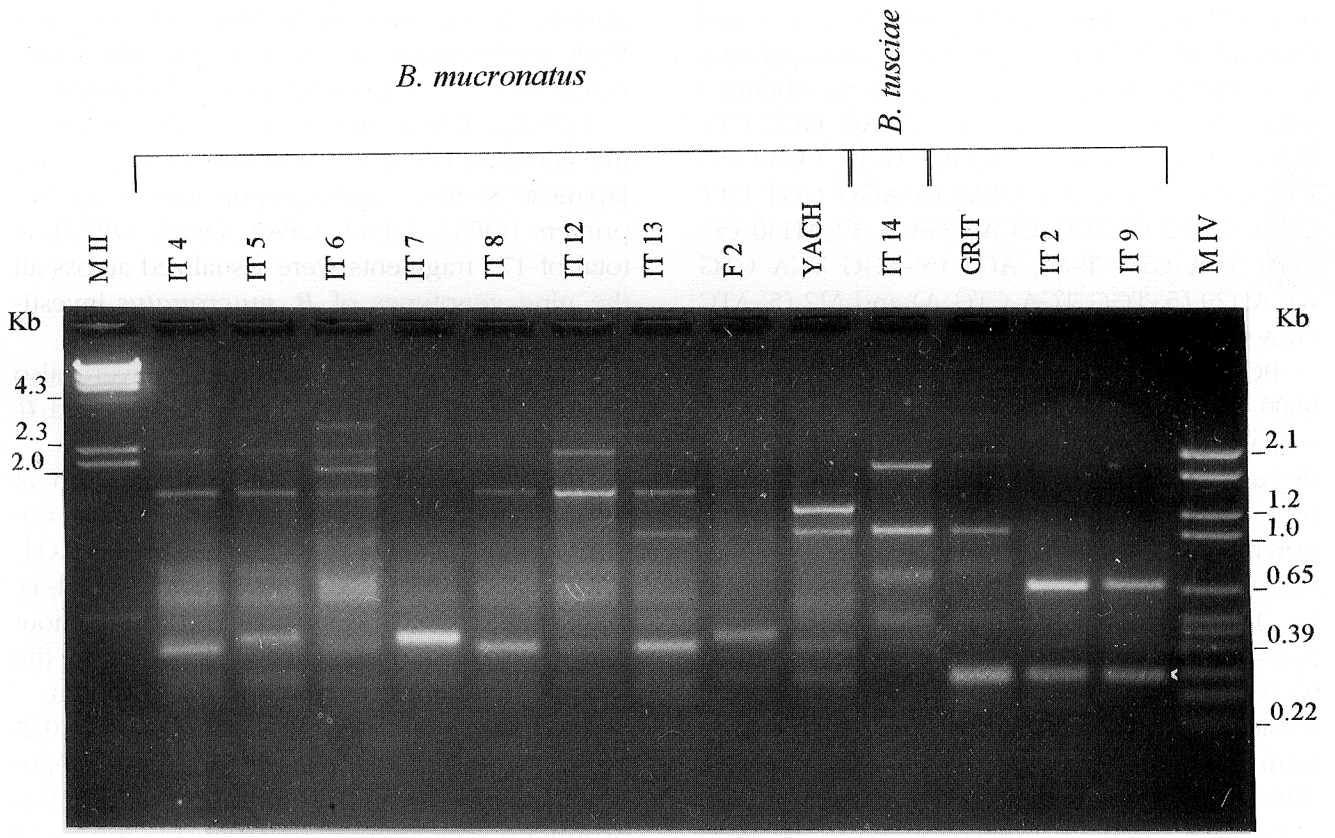


Fig. 1 - RAPD profiles of *Bursaphelenchus* species obtained with random primer AH29. Amplified DNA fragment characteristic for *B. sexdentati* is indicated by <.

B. sexdentati species (indicated by <). Another fragment of approx. 1.5 Kb (indicated by <) could be specific for *B. sexdentati* genetic characterization, but its real polymorphism should be confirmed with further investigations such as hybridization, since a band of the same molecular weight also seems to be present in some Italian *B. mucronatus* isolates.

Interestingly, RAPD-PCR employing random primer AI2 (Fig. 4) seems to be suitable for differentiation of the European *B. mucronatus* populations against that coming from Japan besides those of *B. sexdentati* and *B. tusciae*. The diagnostic fragment is clearly visible and of around 0.65 Kb (indicated by >).

Based on the patterns observed with the random primers AH29, AH30, AG1, AI2, a matrix of similarity indices was constructed (Fig. 5A) and subjected to the UPGMA analisys (Fig. 5B). The thirteen *Bursaphelenchus* populations, belonging to three different species, fell into two separate clusters, in perfect agreement with their European and Asian provenances. All the European species grouped together, but that of *B. mucronatus* was clearly separated from the other cluster containing the *B. tusciae* and *B. sexdentati* populations.

The clustering obtained revealed genetic relationships among the populations and species which are discussed below.

Moreover, solely for *B. mucronatus* populations it has been possible to calculate a second similarity matrix (Fig. 6A) taking into account previous RAPDs data realized with five primers

OPA1, OPA2, OPA4, OPA5, OPP-2. A dendrogram was drawn by scoring the 399 DNA bands obtained (Fig. 6B).

Discussion

Reliable differentiation between *B. xylophilus* and the closely related species *B. mucronatus* by means of RAPD-PCR has previously been described (Irdani *et al.*, 1995a, b); it clearly revealed the high level of polymorphism between these two species in accordance with other findings (Harmey and Harmey, 1993; Tarès *et al.*, 1994). Using this technique, individual patterns of amplified DNA fragments were obtained for each *Bursaphelenchus* isolate tested, and species-specific fragments could be identified by comparing several isolates for each *Bursaphelenchus* species. Species determination of new isolates is often difficult since it requires identification of species-specific fragments within the complex RAPD-pattern profiles. On the other hand, the species-specific fragments amplified by RAPD-PCR offer the interesting possibility of the genetic distance determination between the isolates. So, using RAPD-PCR methods we assessed the genetic relationship amongst three species of *Bursaphelenchus* on the basis of shared bands. In Fig. 5B the dendrogram shows two main branches containing, respectively, all of the European populations of *B. mucronatus*, *B. tusciae* and *B. sexdentati* and the second, only the *B. mucronatus* isolate from Japan. This indicates that the two groups are separated at a high level of genetic distance. The upper branch, is itself shifted in two other sub-branches, the first with populations belonging to Italian and French *B. mucronatus* which clusters closely, and the second well separated

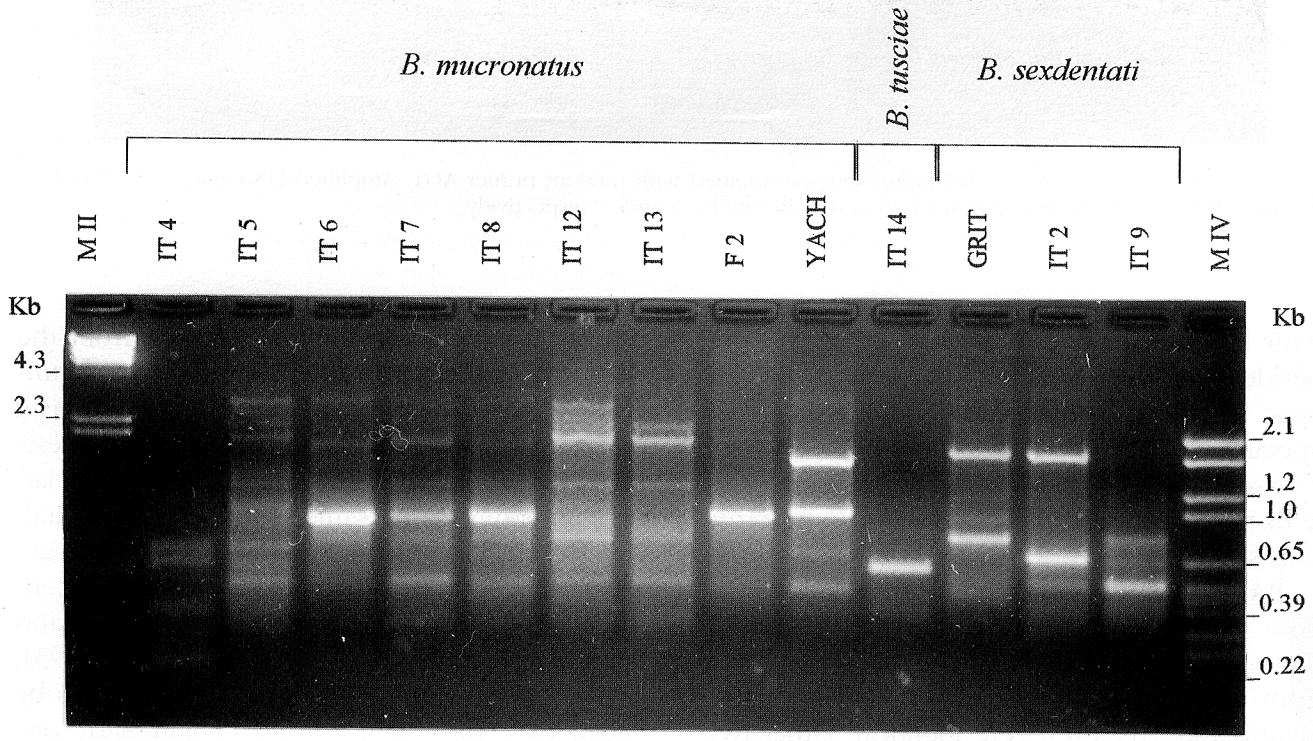


Fig. 2 - RAPD profiles of *Bursaphelenchus* species obtained with random primer AH30.

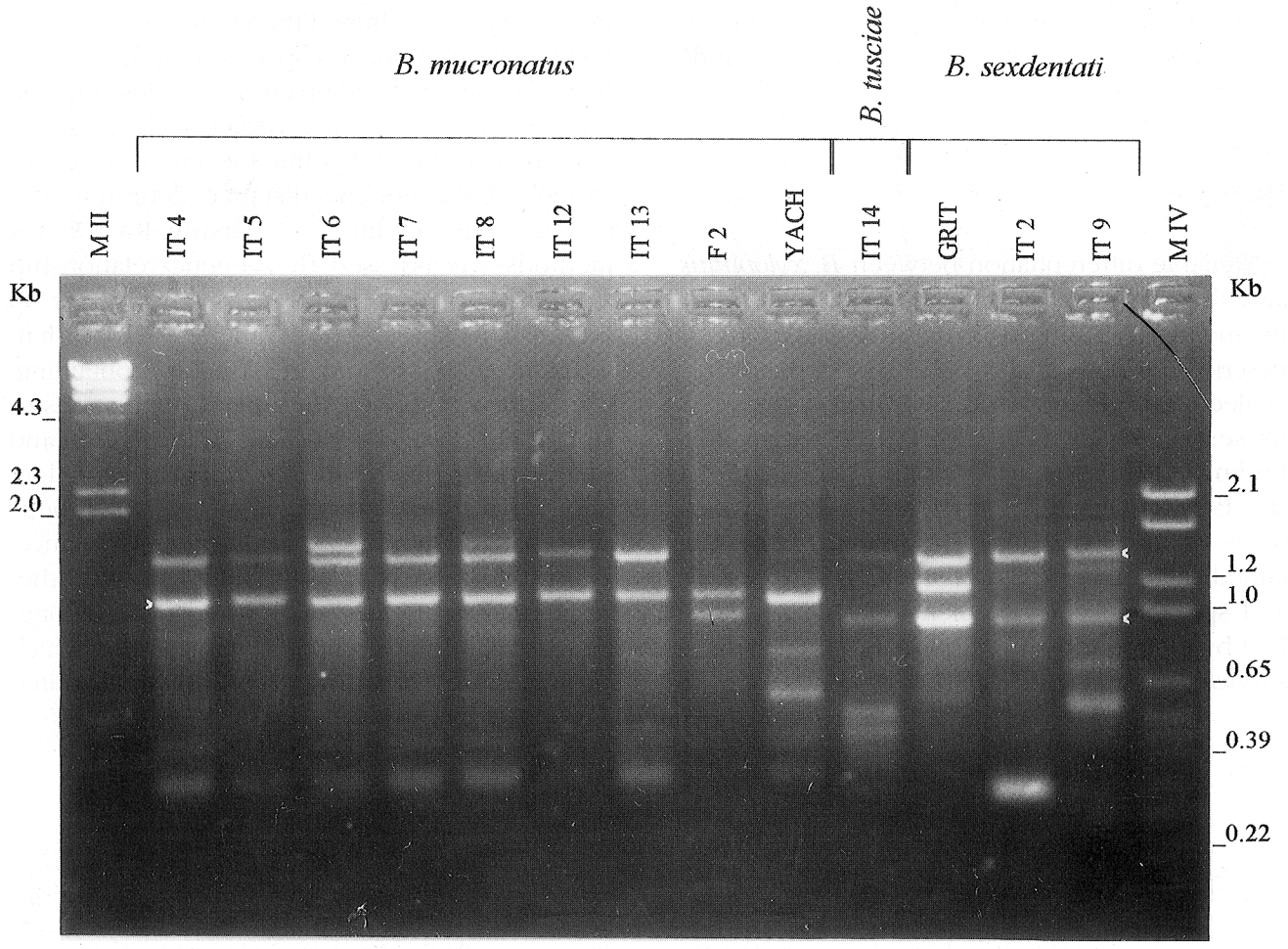


Fig. 3 - RAPD profiles of *Bursaphelenchus* species obtained with random primer AG1. Amplified DNA fragment characteristic for either *B. mucronatus* or *B. sexdentati* is indicated by > and <, respectively.

with Italian and Greek *B. sexdentati* isolates besides that of *B. tusciae*. A high degree of genetic similarity between these two last species appear evident. In the lower branch, the *B. mucronatus* from Japan is separated at high genetic distance from the other European *B. mucronatus* species.

RAPD-PCR analysis with four decamer random primers revealed that the Italian isolates IT4(w), IT5(w), IT7(w), IT12(i), IT13(w) coming from three different Italian regions (Piedmont, Liguria and Tuscany) were identical. Other two Italian isolates IT6(w) and IT8(w) from Piedmont appeared just slightly different from the previous group. The French isolate of *B. mucronatus* (F2), as already resulted previously (Irdani *et al.*, 1995a, b), confirms the hypothesis that the European populations are closely related, mapping in the same cluster of all the Italian *B. mucronatus*, although with a lower genetic similarity (Webster *et al.*, 1990; Beckenbach *et al.*, 1992; Riga *et al.*, 1992; Bolla and Boschert, 1993; Harmey and Harmey, 1993). While the Japanese isolate (Yach) appears to be genetically differentiated and evolutionarly distinct as expected for populations that are geo-

graphically so widely separated. This suggests that geographical origin may have an important effect on the genetic similarity of genotypes regardless of apparent morphological similarities. Distance calculations carried out with increasing numbers of RAPD bands scored (200-400 bands) indicates that evaluation of approx. 200 bands were sufficient to reach a constant value of genetic distance between intraspecific pairs of populations of *B. mucronatus* (Fig. 5B) since the dendrogram of genetic distances on the basis of 399 bands scored did not much change the final classification (Fig. 6B). The only exception is *B. mucronatus* IT13 that is positioned in a separate branch respect to all the other Italian *B. mucronatus* isolates. The two Italian *B. sexdentati* isolates cluster in two different branches, although close to each other, one with the Greek isolate and the other with *B. tusciae* isolate. In this latter case *B. tusciae*

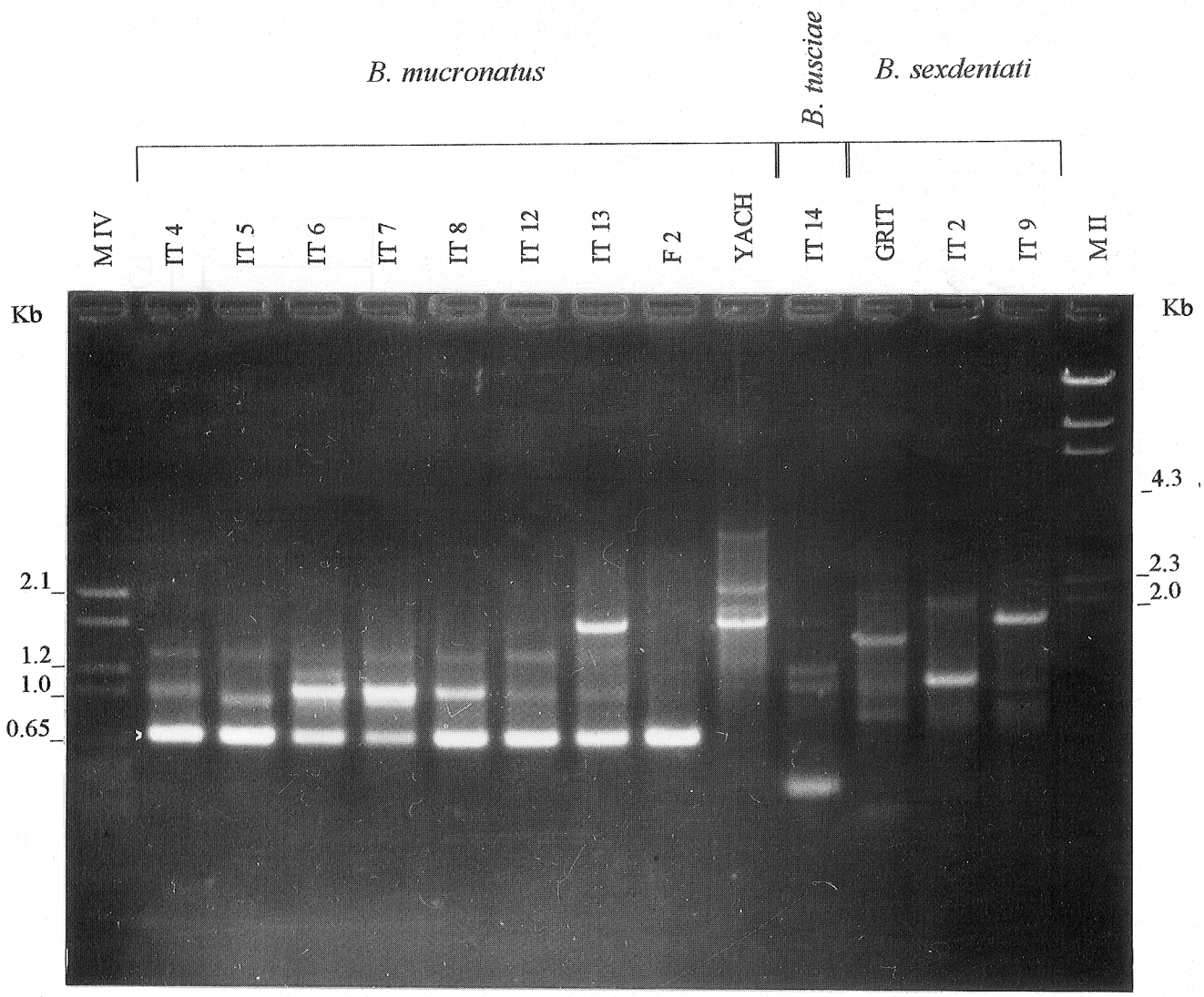
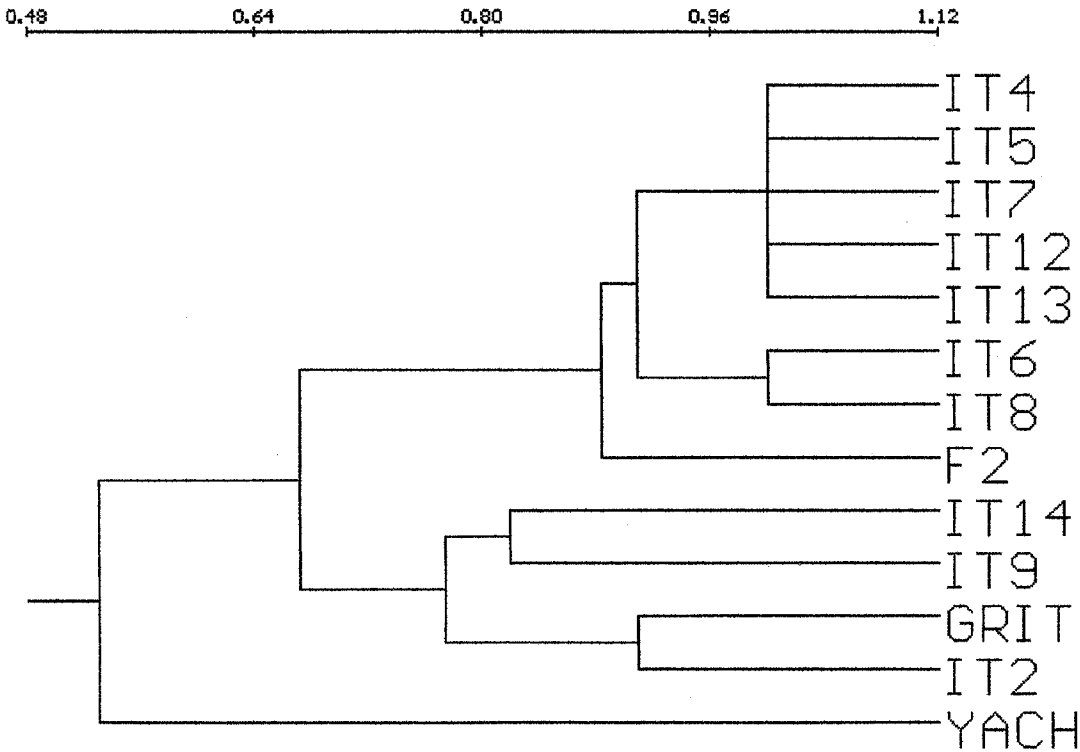


Fig. 4 - RAPD profiles of *Bursaphelenchus* species obtained with random primer AI2. Amplified DNA fragment characteristic for European *B. mucronatus* is indicated by >.

IT4	1.000												
IT5	0.808	1.000											
IT6	0.769	0.769	1.000										
IT7	0.769	0.885	0.846	1.000									
IT8	0.788	0.827	0.904	0.942	1.000								
IT12	0.808	0.808	0.846	0.923	0.942	1.000							
IT13	0.731	0.769	0.769	0.846	0.865	0.923	1.000						
F2	0.712	0.673	0.635	0.750	0.692	0.712	0.673	1.000					
YACH	0.519	0.558	0.481	0.596	0.577	0.558	0.558	0.692	1.000				
IT14	0.635	0.558	0.558	0.558	0.538	0.558	0.596	0.692	0.500	1.000			
GR-IT	0.596	0.596	0.596	0.673	0.654	0.635	0.635	0.731	0.538	0.654	1.000		
IT2	0.654	0.654	0.615	0.654	0.635	0.615	0.538	0.712	0.596	0.635	0.712	1.000	
IT9	0.654	0.615	0.538	0.615	0.596	0.615	0.538	0.673	0.519	0.673	0.712	0.808	1.000

A

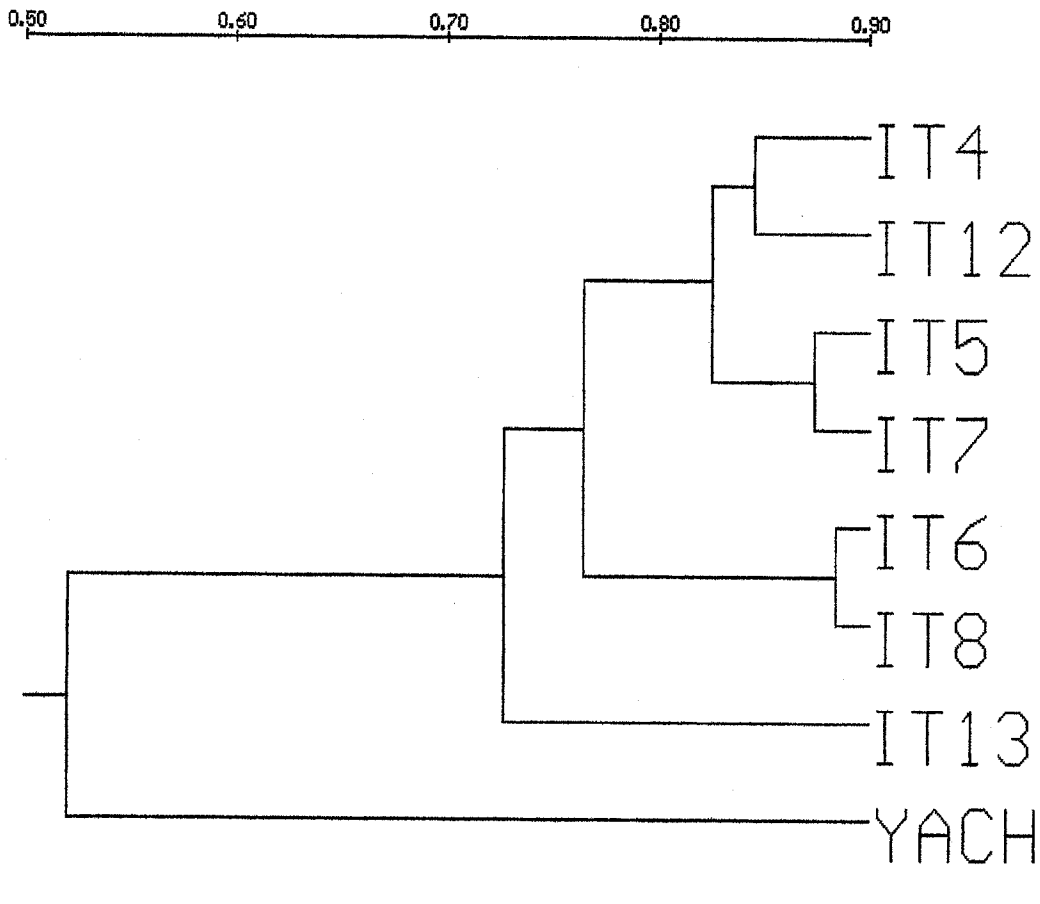


B

Fig. 5 - A, genetic similarity matrix; B, dendrogram of genetic similarity among 13 *Bursaphelenchus* species populations calculated on the basis of 224 bands scored.

IT4	1.000						
IT5	0.834	1.000					
IT6	0.699	0.689	1.000				
IT7	0.786	0.873	0.796	1.000			
IT8	0.737	0.747	0.883	0.873	1.000		
IT12	0.844	0.815	0.737	0.864	0.834	1.000	
IT13	0.689	0.679	0.679	0.747	0.737	0.825	1.000
YACH	0.504	0.533	0.475	0.563	0.533	0.543	0.48	1.000

A



B

Fig. 6 - A, genetic similarity matrix; B, dendrogram of genetic similarity among 8 *B. mucronatus* populations calculated on the basis of 399 bands scored.

shows a major genetic similarity with *B. sexdentati* respect to *B. mucronatus*. This might be expected because of the communality of some other important anatomical characters such as the absence of a mucron in the female tail and the cucullus in the male compared to *B. mucronatus*.

In conclusion, the data presented here again demonstrate the utility of RAPD markers for assessing genetic similarity and the identification of different species of nematodes.

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