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Bursaphelenchus Fuchs, 1937 (Nematoda: Parasitaphelenchidae) species associated with *Pinus* species in northern Turkey

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Summary

A survey for *Bursaphelenchus* nematodes, associated with different conifer trees, was conducted in several forest areas in the northern regions of Turkey. Only pine trees (*Pinus nigra*, *P. pinaster* and *P. sylvestris*) yielded *Bursaphelenchus* specimens. Nematodes were identified using several morphological diagnostic characters of the genus (male spicule structure, number of lateral incisures, number and distribution of the male papillae, presence of female vulval flap), and confirmed by using RFLP analysis of the internal transcriber spacer (ITS) regions of ribosomal DNA. Three different species were identified from several sampled areas, namely *B. mucronatus*, *B. pinophilus* and *B. sexdentati*, representing a first report of the last two species for Turkey. The association of *B. pinophilus* with black pine (*P. nigra*) is herein reported for the first time.

Key words: *Bursaphelenchus* spp.; pine trees; morphology; ITS-RFLP analysis

Introduction

Within the past seven years, special relevance and attention has been given to the genus *Bursaphelenchus*, Fuchs 1937 particularly in European countries. The official measures imposed by the European Union force each country to proceed with national surveys to prevent the introduction of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970. The enforcement of these measures is a direct result of the first detection of this pathogenic species (A1 quarantine pest, according to EPPO) in Portugal, and in Europe (Mota *et al.*, 1999). The need for prevention and detection of *B. xylophilus*, from endemic conifer forests, and in wood product trade, has increased the number of species recorded worldwide, especially within the European and Asiatic continents (Ryss *et al.*, 2005).

In Turkey, a country that forms one of the boundaries between Europe and Asia, forest ecosystems play a very important role in the economy and occupy a considerable area (27 % of the country's territory). Total forestry area consists of over 21 million ha, represented by 54 % conifer trees, 36 % broad-leaved trees, and 10 % of mixed forest. Among conifers, four species of *Pinus* are predominant: the Turkish pine (*P. brutia* Ten.) spread mainly to the West and South West (Mediterranean region), covers an area of 5 420 524.6 ha; the Austrian pine (*P. nigra* Arnold) forming a forest of pure or mixed type, covers an area of 4 202 298.2 ha, spread over the mountainous areas of all coastal regions; Scots pine (*P. sylvestris* L.) in single or mixed forests, in the higher mountainous areas of North Anatolia and some areas in the inner and Southern regions, occupies an area of 1 239 578.2 ha; and Stone pine (*P. pinea* L.), a typical Mediterranean species, covers an area of 42 618.2 ha, mainly in West and Southern Anatolia (Critchfield & Elbert, 1966; Anonymous, 2006).

Besides the broad area occupied by these indigenous species, other introduced pines occupy a representative area (*P. pinaster* Aiton covers an area of 77 091.7 ha, and *P. radiata* D. Don an area of 47 ha), as well as other indigenous species (*Abies* spp. occupying 626 647.2 ha; *Cedrus* spp. occupying 417 188.5 ha; *Picea orientalis* (L) Link. occupying 297 396.5 ha and *Juniperus* spp. occupying 447 492.5 ha) (Anonymous, 2006). Despite the richness of the Turkish forest, the importation of industrial wood for consumption assumes special relevance, reaching almost 1 million m^3 of imported coniferous wood in 1999 (Anonymous, 2001).

Until recently, little information was available for the genus *Bursaphelenchus* in Turkey, corresponding to several short reports of this genus (Vieira *et al.*, 2003; Vieira *et al.*, 2004). More recently two new species have been described, namely *B. anatolius* Giblin-Davis, Hazir, Center, Ye, Keskin, Thorp & Thomas, 2005, associated with bees

of the genus *Halictus* (Giblin-Davis *et al.*, 2005), and *B. anamurius* Akbulut, Braasch, Baysal, Brandstetter & Burgermeister, 2007, associated with *Pinus brutia* (Akbulut *et al.*, 2007).

Due to the economic importance assumed by the PWN in Europe, associated with the large natural resources of Turkey, it was imperative to conduct some studies related to the species occurrence of this genus in the country. Therefore, a preliminary survey on the pinewood nematode was conducted in specific forest areas in the north of the country, special relevance being given to species belonging to the *xylophilus*-group of the genus *Bursaphelenchus* (Akbulut *et al.*, 2006). During this survey, and besides *B. mucronatus* Mamiya & Enda, 1979, several others specimens displaying morphological features of the genus *Bursaphelenchus* were collected but identified only at the genus level. In order to identify the species, further studies were carried out, and so a general morphological characterization (optical and scanning microscopy) and a molecular analysis (ITS-RFLP patterns) of the *Bursaphelenchus* species found in Turkey is provided in this paper.

Material and methods

Wood sampling and nematode isolation

In 2003 – 2004, a survey was conducted in the conifer forest areas of Ankara, Artvin, Düzce, Istanbul, Samsun and Trabzon, in Turkey (Fig. 1). Wood samples (40 – 80 g each) were collected from conifer trees (*Abies* spp., *Cedrus* spp., *Picea* spp. and *Pinus* spp.), displaying decline symptoms, at 1.5 m level of the trunk, using a drill and kept in polyethylene bags until nematode extraction. Nematodes were extracted using a modified Baermann funnel technique, and processed within 48 h. For those samples with significant numbers, the nematodes were inoculated on *Botrytis cinerea* Pars., growing in malt agar, and incubated for 2 weeks at 25 º C.

Morphological identification

Nematodes obtained from successful fungal cultures were fixed with hot formalin (4 %), processed to anhydrous glycerin and mounted in permanent slides according to the "express technique" described by Ryss (2003), and identified using an optical microscope (Olympus BX50). For scanning electron microscope (SEM) observations, using a JEOL 35 SEM, adult nematodes were fixed in a mixture of 4 % gluteraldehyde-2 % formaldehyde for 48 h, post-fixed in 2 % OsO_4 overnight, dehydrated in an ethanol series, followed by critical point drying and sputter coated with gold (Eisenback, 1985).

Molecular identification

From each fungal culture, 30 – 50 nematodes were collected into a small drop of water, in a 1.5 ml Eppendorf tube, and stored at -20 ºC for DNA analysis. The DNA extraction was carried out following Cenis (1993), with some modifications. After a brief centrifugation, 300 µl of extraction buffer (200 mM Tris-HCl, pH 8.0; 250 mM NaCl; 25 mM EDTA, and 0.5 % SDS) was added to the tube, and the nematodes were smashed with a conical pestle (Eppendorf). The lysate was incubated for 2 hours at 65 ºC. Then, 0.5 volume of 3 M sodium acetate (pH 5.2) was added, and stored on ice for 20 min. After centrifugation at 13 000 rpm, 4 ºC for 15 min., the supernatant was transferred to a new tube, the nucleic acids were precipitated with 1 volume of isopropanol at room temperature for 30 min. and pelleted by centrifugation at 13 000 rpm, at 4 ºC during 15 min. The pellet was washed in 500 µl of 70 % ethanol, centrifuge at 13 000 rpm, at 4 ºC during 10 min, and redissolved in 40 µl of TE buffer.

ITS-RFLP analysis was carried out following the method described in Hoyer *et al.* (1998). The ITS regions of rDNA were amplified using the forward primer 5'-CGTAACAAGGTAGCTGTAG-3' (Ferris *et al.*, 1993) and reverse primer 5'-TTTCACTCGCCGTTACTAAGG-3' (Vrain, 1993). All polymerase chain reactions were performed in a final volume of 50 µl, contained 1x reaction buffer (BIOPortugal), 1.5 mM $MgCl_2$, 0.1 mM dNTP's (Invitrogene), 0.6 µM of each primer (STABVIDA), 2 U of *Taq* DNA polymerase (BIOPortugal) and 2 ng DNA. For amplification a thermocycler was used employing the following steps: one initial denaturation at 94 ºC for 2.5 min., 40 reaction cycles of 94 ºC for 1 min., 55 ºC for 1 min., 72 ºC for 2 min., and a final extension at 72 ºC for 5 min.

The restriction analysis of the ITS regions was performed with *Alu*I, *Hae*III, *Hinf*I, *Msp*I and *Rsa*I restriction endonucleases, using an aliquot of 8.5 µl of the PCR product and 10 U of each enzyme. Fragments were resolved by electrophoresis in a 1.8 % agarose gel and stained with ethidium bromide.

Results

Species distribution

A total of 358 wood samples were collected from several conifer species (cedar, fir, pine, spruce) for the presence of *Bursaphelenchus* nematodes, in different geographic areas in the northern part of the country (Fig. 1). The greatest number of wood samples was collected from *P. nigra* (118 samples) and *P. sylvestris* (159 samples), corresponding to the species with a higher number of symptomatic trees in the sampled forest areas. Although several nematodes species were present in high numbers of wood samples,

Fig. 1. Localization of the sampled areas associated with the distribution of the major pine species trees sampled (dark grey areas: *P. nigra*; light grey areas: *P. sylvestris*), and distribution of *Bursaphelenchus* species. ○ : *B. mucronatus;* △ : *B. pinophilus*; ◇ : *B. sexdentati;* □: *Bursaphelenchus* specimens belonging to the *sexdentati*-group *sensu* Braasch (see text).

only a few samples contained *Bursaphelenchus* nematodes reared only from pine trees. The geographic localization of the *Bursaphelenchus* isolates found during this survey (Fig. 1), and associated *Pinus* host, is summarized in Table 1.

Table 1. Host tree and localization of the *Bursaphelenchus* isolates found in north regions of Turkey.

***Bursaphelenchus* sp.**	**Isolate code**	**Location**	**Host tree**
B. mucronatus	A-6	Artvin	*P. sylvestris*
	A-13	Artvin	*P. sylvestris*
	A-43	Artvin	*P. sylvestris*
	A-50	Artvin	*P. sylvestris*
	S-4	Düzce	*P. nigra*
	S-18	Düzce	*P. nigra*
	S-26	Düzce	*P. nigra*
	S-28	Düzce	*P. nigra*
	13Y-K	Düzce	*P. nigra*
	12-K	Düzce	*P. nigra*
	O-7	Düzce	*P. sylvestris*
B. pinophilus	T-I	Ankara	*P. nigra*
B. sexdentati	T-II	Ankara	*P. nigra*
	S-12	Düzce	*P. pinaster*
Bursaphelenchus sp.[1]	A-14	Artvin	*P. sylvestris*
	A-58	Artvin	*P. sylvestris*
	T-47	Trabzon	*P. sylvestris*

[1]Due to the reduced number of specimens and slide conditions, the species identification was not achieved, and only the species group is mentioned.

Morphological identification

All *Bursaphelenchus* isolates were separated based on the spicule structure type, and assigned to one of the morphological groups of species defined by Ryss *et al.* (2005) (Fig. 2), namely the *xylophilus*-group *sensu* Ryss *et al.* and the *piniperdae*-group *sensu* Ryss *et al*., in the last case, and to a more restricted number of species, assigned to the so called *sexdentati*-group *sensu* Braasch (2001).

Previously, the isolates belonging to the *xylophilus*-group were identified to species level, revealing the presence of *B. mucronatus* (Fig. 2F-I) from two sampled areas (Fig. 1) (Akbulut *et al.*, 2006). No other specimens belonging to this group, including *B. xylophilus*, were found in the collected wood samples. All other specimens, identified as *Bursaphelenchus* sp. 1 and *Bursaphelenchus* sp. 2 (Akbulut *et al.*, 2006), displayed the main diagnostic characters of the *sexdentati*-group *sensu* Braasch, i.e., four lateral lines, caudal papillae distributed as 1 single pre-anal, 1 pair adanal, and 2 pairs post-anal (1 before bursa, and 1 at the bursa), the typical spicules structure (spicules bent and relatively compact) (Fig. 2A), small vulval flap, with a protuberance behind vulva (Braasch, 2001). A more detailed morphological observation, based on the most important diagnostic characters (Table 2), confirmed the presence of two different species belonging to this group, namely *B. pinophilus* Brzeski & Baujard, 1997 (Fig. 2C-E) and *B. sexdentati* Rühm, 1960 (Fig. 2A-B).

While isolate T-1 conformed to the original morphological description of *B. pinophilus* (Brzeski & Baujard, 1997), in the case of *B. sexdentati* (isolates T-II and S-12) some differences can be observed in certain characters comparing with the original description given by Rühm (1960), i.e., a female tail gradually tapering, conical conoid (Fig. 2C) *vs.* a tail less conoid and bluntly rounded end described in the original description, the presence of cucullus (Fig. 2B) *vs.* the absence of cucullus in the male spicules. However, these isolates show the same morphological differences

Table 2. Diagnostic morphological characters for *Bursaphelenchus* species occurring in Turkey.

Species	**Spicule structure**	**Number of lateral incisures**	**Male caudal papillae**	**Female vulval flap**	**Female tail**
B. mucronatus	Spicule narrow, capitulum flattened, condylus small, lamina angular in last third, cucullus present	4	Single ventral pre-anal papilla, one pair subventral adanal, two post-anal pairs	Present, anterior lip forming long flap overlapping vulva	Subcylindrical, rounded, tail tip mucronate
B. pinophilus	Spicule stout, with well developed rostrum, variously shaped condylus, with small cucullus	4	Single ventral pre-anal papilla; one pair sub-ventral adanal; two post-anal pairs	Present, anterior lip slightly extended to form small flap; post vulval swelling often present	Tail conical terminally pointed, with more or less pronounced mucron
B. sexdentati	Spicule stout, rostrum sharply pointed, condylus well developed, broadly truncate, with distinct small cucullus[2]	4	Single ventral pre-anal papilla; one pair sub-ventral adanal; two post-anal pairs	Present, anterior lip slightly extended to form small flap	Tail gradually tapering, conoid with more or less finely rounded, slightly blunt

[2]According to the original description of Rühm (1960), *B. sexdentati* does not possess a cucullus at the spicule tip. However, this morphological character is reported for *B. sexdentati* "South European" type (Lange et al., 2006).

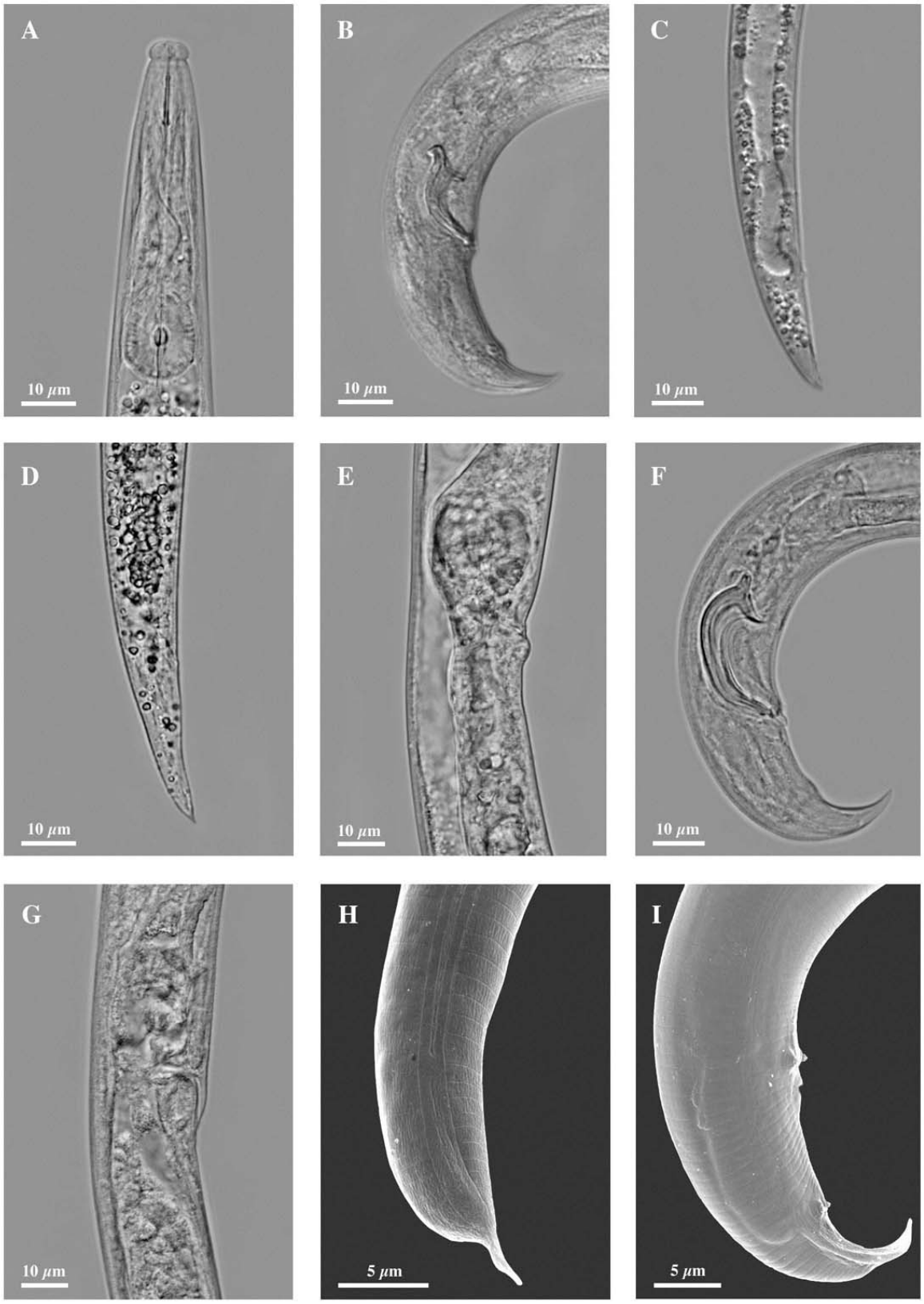

Fig. 2. *Bursaphelenchus sexdentati* (A-B)[3]. A: Light micrograph (LM) of male tail; B: LM of female tail. *B. pinophilus* (C-E). C: LM of vulva region; D: LM of male tail; E: LM of female tail. *B. mucronatus* (F-I). F: LM of male tail (spicule structure); G: LM of vulval region; H: Scanning electron micrograph (SEM) of female tail; I: SEM of male tail.

[3]These images do not correspond to the original morphological description of Rühm (1960), but to the morphological and molecular of *B. sexdentati* "South European" type (Lange et al., 2006).

Table 3. Approximate size of DNA fragments observed in ITS-RFLP analysis of *Bursaphelenchus* species.

Bursaphelenchus sp.	PCR product (bp)	Restriction fragments (bp)				
		*Rsa*I	*Hae*III	*Msp*I	*Hinf*I	*Alu*I
B. mucronatus	950	410	620	370	410	700
European type		290	220	310	250	250
		230	110	280	130	
					90*	
B. pinophilus	1000	430	600	1000	400	1000
		340	280		290	
		210	120*		220	
					90	
B. sexdentati	1000	550	590	1000	470	1000
		410	280		280	
			120*		210	

*Bands not clearly visible in the gels.

cited for other *B. sexdentati* isolates collected in Greece (Lange *et al.*, 2006), Italy (Ambrogioni & Caroppo, 1998) and Portugal (Penas *et al.*, 2004).

Previously, isolates A-14, A-58 and T-47 were also classified as *Bursaphelenchus* sp. 2 (Akbulut *et al.*, 2006), belonging to the *sexdentati*-group *sensu* Braasch. However, due to the limited number of specimens from each isolate (as well as the poor quality of the fixed material), and to the close morphological similarity among the species of this group, namely between *B. sexdentati* South-European type and *B. vallesianus*, the precise species identification was not clearly achieved, and therefore the occurrence of *Bursaphelenchus* specimens of the *sexdentati*-group *sensu* Braasch for these correspondent geographical areas is only mentioned.

Molecular identification

In order to complement and confirm the results obtained by morphological observations, a molecular analysis based on ITS-RFLP was performed, and the restriction patterns obtained were compared with the reference patterns established for the same species by Burgermeister *et al.* (2005).

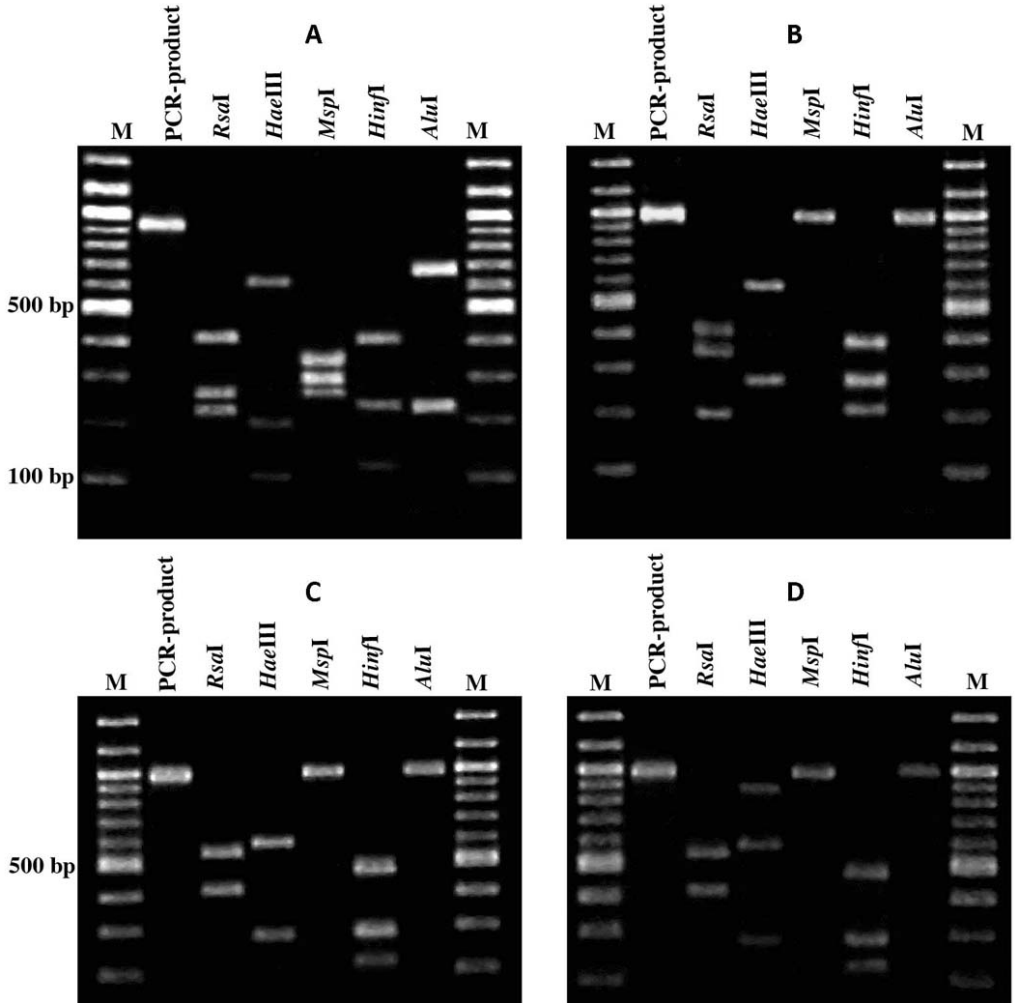

Fig. 3. ITS-RFLP patterns of *Bursaphelenchus* species. A: *B. mucronatus*. B: *B. pinophilus*. C: *B. sexdentati* isolate T-12. D: *B. sexdentati* isolate S-12. The enzymes used for the restriction digest are indicated above the corresponding line. M: DNA size marker (100 bp).

The ITS-RFLP profiles obtained confirm the morphological identification, revealing the presence of the three *Bursaphelenchus* species previously mentioned (Fig. 3). In the case of *B. mucronatus* (Table 3, Fig. 3A), the ITS-RFLP patterns obtained for the different geographic isolates reveal the European genotype of this species as shown by Burgermeister *et al.* (2005). Although isolate S-12 exhibits the same ITS-RFLP pattern as *B. sexdentati*, an additional weaker fragment of approximately 860 bp was obtained for the *Hae* III restriction enzyme (Fig. 3D), comparable to the results achieved by Lange *et al.* (2006), for a *B. sexdentati* isolate [IT-2(w)] collected in Italy.

Discussion

The genus *Bursaphelenchus* is widely distributed over the North Hemisphere, associated with the conifer tree distribution, mainly pine species. The intense surveys for the pinewood nematode clearly emphasize our knowledge relating to the number and species distribution worldwide. After the detection of *B. xylophilus* in Portugal (Mota *et al.*, 1999), up to thirty new species have been described for this genus, revealing high species diversity (Vieira *et al.*, 2006). While most species have been found associated with pines, the detection of new species in other types of host trees (broadleaf trees) (Kanzaki & Futai, 2005) and habitats (soil-dwelling bees, nitidulid beetles) (Giblin-Davis *et al.*, 2005; Giblin-Davis *et al.*, 2006) shows the high plasticity of the species that compose this genus. For detailed information on this subject see Ryss *et al.* (2005).

In Turkey, due to the large amount of forest resources (high biodiversity of suitable host species), associated with favorable biotic (insect vectors) and abiotic conditions (climate), the occurrence of a significant number of *Bursaphelenchus* species within the forest areas may be predictable. During this survey, three different species were found, constituting the first report and detailed description of these species for Turkey, although *B. mucronatus* has been previously reported (Akbulut *et al.*, 2006).

Up to date, *B. mucronatus* is the species with the widest known distribution within the Euro-Asian continents (for a detailed species distribution see Ryss *et al.*, 2005), and can be divided into two different types with regard to its genetic (ITS-RFLP) pattern (Burgermeister *et al.*, 2005). Nevertheless, in Turkey only the European type was found, even when considering two extreme regions of the country, i.e. a more European area (Düzce) and an Asiatic area (Artvin).

Regardless of the importance of the morphological diagnostic characters to identify the different species-group, the high morphological similarity in some closely related species within a specific group (e.g. *sexdentati*-group *sensu* Braasch) could make identification at the species level difficult. The species that compose the *sexdentati*-group *sensu* Braasch, share a high level of morphological similarities, especially among *B. pinophilus, B. sexdentati* and *B. vallesianus* Braasch, Schönfeld, Polomski & Burgermeister, 2004. In the case of *B. pinophilus* the morphological data obtained in this study follow the original description of Brzeski & Baujard (1997), and the same ITS-RFLP patterns published for this species (Burgermeister *et al.*, 2005; Lange *et al.*, 2006). The known distribution of this species is quite limited, reported only in Germany (Braasch, 2001), Poland (Brzeski & Baujard, 1997) and Portugal (Penas *et al.*, 2004). Although it has been reported to be associated with pine species (*P. sylvestris* and *P. pinaster*), it had never been associated with black pine (*P. nigra*).

On the other hand, *B. sexdentati* is one of the most wide spread species among the European continent, being the most frequently encountered *Bursaphelenchus* species in southern European countries. Regarding several studies where *B. sexdentati* has been reported (Ambrogioni & Caroppo, 1998; Penas *et al.*, 2004; Lange *et al.*, 2006), this species shows a significant morphological variability when compared with other species of the genus (e.g. the shape of the female tail). Likewise, it has been shown that in some cases, specimens could be wrongly identified as *B. sexdentati* (Braasch *et al.*, 2004). Recently, an integrated study postulated the differentiation of several *B. sexdentati* isolates in two major types, a "Central" and a "South" European types, based on both morphological and molecular analysis (ITS-RFLP with eight restriction enzymes, and sequence of the ITS regions) (Lange *et al.*, 2006). Therefore, the original description of *B. sexdentati* showing a bluntly rounded female tail, male spicules with a less pointed rostrum, and without cucullus, correspond to the "Central European" type of this species (Lange *et al.*, 2006), whereas the isolates herein reported (T-II and S-12) are closer to the morphology of the "South European" type, i.e., variable conoid female tail with more or less finely rounded, slightly blunt terminus, and the presence of a small cucullus at the male spicules.

The presence of the additional weak band in the profile generated by *Hae* III, in the isolate S-12, may be explained by some sequence heterogeneity of the ITS regions within this isolate, as has been recently shown for an Italian *B. sexdentati* isolate, where individuals or even the same specimen possess different ITS2 fragments, reflecting the appearance of minor bands in the ITS-RFLP patterns (Lange *et al.*, 2006).

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