ITS-RFLP patterns for differentiation of 26 *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) and observations on their distribution

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Summary. RFLP analysis of the internal transcribed spacer (ITS) regions of ribosomal DNA has become a valuable tool for differentiation of *Bursaphelenchus* species. Restriction cleavage of amplified rDNA using five restriction enzymes (*Rsa*I, *Hae*III, *Msp*I, *Hinf*I and *Alu*I) has provided species-specific ITS-RFLP patterns for 26 species of *Bursaphelenchus* and two intraspecific types so far, five of which are presented here for the first time. The technique has proved very valuable for differentiation of the pinewood nematode, *B. xylophilus*, from other *Bursaphelenchus* species during plant quarantine inspections. ITS-RFLP analysis has also been used effectively during investigations on the distribution of *Bursaphelenchus* species. ITS-RFLP patterns have been employed as additional identification criteria in the description of several new *Bursaphelenchus* species.

Key words: *Bursaphelenchus xylophilus*, *Bursaphelenchus* species identification, internal transcribed spacer, molecular diagnosis, restriction fragment length polymorphism, pinewood nematode (PWN), taxonomy.

The genus *Bursaphelenchus* includes about 70 species which occur predominantly in North America, Europe and Asia. Approximately three-quarters of them live in conifer trees, and one species, the pinewood nematode, *Bursaphelenchus xylophilus*, is the causal agent of the pine wilt disease. Identification of *Bursaphelenchus* species was originally accomplished only on the basis of morphological features (Tarjan & Aragon, 1982; Yin *et al.*, 1988; Braasch, 2001). However, several closely related species show great morphological similarity. More recently, DNA-based molecular identification techniques have been developed which are very useful to supplement and confirm morphological affiliations.

Initial molecular diagnostic work has concentrated on differentiation of xylophilus group species such as *B. xylophilus*, *B. mucronatus* and *B. fraudulentus* with respect to the close morphological similarity of these species and the economic importance of *B. xylophilus*. Two species-specific DNA probes (pBx6 and pBm4), derived from the non-transcribed spacer region of ribosomal RNA genes, were used in dot blot hybridisation and RFLP analysis (Webster *et al.*, 1990). Species-specific RFLP patterns were also obtained using a heterologous unc-22 DNA probe from *Caenorhabditis elegans* (Abad *et al.*, 1991). A repetitive DNA fragment (X14) cloned from *B. xylophilus* was used to obtain isolate-specific DNA fingerprints after RFLP analysis (Harmey & Harmey, 1993). A cloned satellite DNA from *B. xylophilus* formed the basis of highly sensitive and specific detection of the pinewood nematode in dot blot hybridisation and PCR (Tarès *et al.*, 1993; 1994). Two PCR methods using species-specific primers derived from different ribosomal DNA sequences have been described recently (Matsunaga & Togachi, 2004; Kang *et al.*, 2004).

Attempts were also made to extend molecular

diagnosis to a larger number of *Bursaphelenchus* species. RFLP analysis of the internal transcribed spacer (ITS) regions of ribosomal DNA has become an efficient tool for species differentiation of many organisms including fungi (White *et al.*, 1990), insects (Armstrong *et al.*, 1997) and nematodes (Vrain, 1993; Ferris *et al.*, 1993; Zijlstra *et al.*, 1995; Jones *et al.*, 1997). Since 1998, species-specific ITS-RFLP patterns for 21 *Bursaphelenchus* species have been described in several publications as listed in Tab. 1. In the present work, ITS-RFLP patterns of five additional species are introduced (Tab. 1). A total of 26 species of *Bursaphelenchus* and two intraspecific types of *B. mucronatus* can now be identified on the basis of their ITS-RFLP patterns. This demonstrates the remarkable scope of the technique and its usefulness for differential diagnosis and taxonomy. In addition, recent studies on the distribution of *Bursaphelenchus* species and their introduction to new areas have been greatly facilitated by ITS-RFLP analysis, as outlined in the present article.

MATERIAL AND METHODS

The geographic and host origins of the *Bursaphelenchus* isolates used are listed in Tab. 1. After extraction from their original source or from *Botrytis cinerea*/malt agar cultures by means of a modified Baermann funnel technique, the nematodes were sedimented by centrifugation, washed with water and used for DNA extraction.

DNA was extracted from mixed life stages of nematodes (adult females and males, juveniles). In previous investigations, several DNA extraction methods were employed involving DNA purification by alcohol precipitation (Hoyer *et al.*, 1998; Braasch & Burgermeister, 2002), reversible adsorption to a silica-based membrane (Schmitz *et al.*, 1998) or to magnetic beads (Mota *et al.*, 1999; Braasch *et al.*, 1999). Using these techniques, sufficient DNA for amplification by PCR was reliably extracted from a minimum of 5 to 10 nematodes. When DNA was extracted from single nematodes, sufficient DNA for PCR was obtained with about 30 to 80% of samples in an extraction experiment. The success rate of DNA extractions from single nematodes has recently been increased to almost 100% by application of a micro version of the silica-based membrane adsorption method, following the protocol for isolation of genomic DNA from tissues provided with the QIAamp DNA Micro Kit (Qiagen) as detailed below.

Nematode samples (1 to 30 specimens) were placed in 5 µl of water using Eppendorf tubes and frozen at −20°C until extraction. The sample was thawed, mixed with 10 µl of buffer ATL (Qiagen) and homogenized in the Eppendorf tube using a micropestle (Eppendorf). Buffer ATL (170 µl) and 20 µl proteinase K solution (>600 mAU/ml) were added on rinsing the pestle, the sample was mixed and incubated at 56°C for 3 h. Then 200 µl buffer AL (Qiagen) containing 1 µg carrier RNA (Qiagen) was added and the sample was mixed by pulse-vortexing for 15 s. Ethanol (200 µl) was added, the sample was pulse-vortexed for 15 s, transferred to a QIAamp MinElute column and centrifuged at 6 000 g for 1 min. The flow-through was discarded, and the column washed two times, first by adding 500 µl buffer AW1 (Qiagen) and centrifuging at 6 000 g for 1 min, then by adding 500 µl buffer AW2 (Qiagen) and centrifuging at 6000 g for 1 min. Then the column was centrifuged at 20 000 g for 3 min to dry the membrane. For elution of adsorbed DNA, the column was placed in a clean Eppendorf tube, and 20 µl (for single nematode extraction) up to 100 µl (for extraction of up to 30 nematodes) of water was applied to the membrane. The sample was incubated for 10 min at room temperature and centrifuged at 20 000 g for 1 min. The eluate containing extracted DNA was stored at −20°C until use. DNA concentration was determined fluorimetrically using a DyNA Quant 200 fluorometer (Hoefer/Pharmacia) and the fluorescent dye, Hoe 33258.

ITS-RFLP analysis was carried out as described previously (Hoyer *et al.*, 1998; Mota *et al.*, 1999). A segment of nematode rDNA containing the internal transcribed spacer regions ITS1 and ITS2 was amplified by PCR using forward primer 5'-CGT-AAC-AAG-GTA-GCT-GTA-G-3' (Ferris et al., 1993) and reverse primer 5'-TTT-CAC-TCG-CCG-TTA-CTA-AGG-3' (Vrain, 1993). The PCR mixture (50 µl) contained 0.6 µM of each primer, 2 units Taq DNA polymerase (Stratagene or Fermentas), 10 mM Tris-HCl pH 8.8, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM dNTPs (Roche) and 2 ng DNA template. Amplification was carried out using a Perkin Elmer 9600 thermocycler, employing an initial denaturation at 94°C for 2.5 min, 40 reaction cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 5 min. After completion of the PCR, 5 µl aliquots of the reaction mixture were resolved by electrophoresis in a 1.8% agarose gel and DNA fragments were visualized by staining in 1 µg per ml ethidium bromide. Suitable aliquots of the amplified DNA were digested with 3 units of the restriction endonucleases *Alu*I, *Hae*III, *Hin*fI, *Msp*I

and *Rsa*I, following the manufacturer's instructions. Restriction fragments were resolved by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide.

RESULTS AND DISCUSSION

ITS-RFLP patterns of 26 species of *Bursaphelenchus* and two intraspecific types of *B. mucronatus* are shown in Fig. 1. For each species or intraspecific type, the PCR product and DNA restriction fragments obtained with *Rsa*I, *Hae*III, *Msp*I, *Hinf*I and *Alu*I were separated according to size by agarose gel electrophoresis. The patterns of *B. aberrans*, *B. lini*, *B. paracorneolus*, *B. pinasteri* and *B. seani* are shown here for the first time, whereas the patterns of the other species have been published previously (quoted in Tab. 1) and are included for comparison. The size of PCR products and DNA restriction fragments achieved with the methods described above and estimated by visual comparison with DNA markers are presented in Table 2.

Among the species investigated, PCR products ranging from 850 bp to 1350 bp were obtained, permitting initial diagnostic differentiation. The patterns of restriction fragments obtained using the same set of five enzymes for all species are sufficiently distinct to secure species identification. As shown in earlier investigations, identical species-specific ITS-RFLP patterns were obtained from different isolates (Hoyer *et al.*, 1998) and from individual specimens (Braasch *et al.*, 1999; Iwahori *et al.*, 2000) of the same species. Species identification can also be carried out on non-adult stages including dauer juveniles, which lack morphological features essential for species diagnosis. The technique has proved very valuable in recent investigations on the distribution and, in some cases, on the genetic relationships of *Bursaphenchus* species as detailed below.

Using ITS-RFLP analysis, the pinewood nematode, *B. xylophilus*, can be distinguished easily from species with a very high degree of morphological similarity and the same hosts, i.e. *B. mucronatus* and *B. fraudulentus*. Although *B. xylophilus* can be recognized by the rounded tail terminus of females, there are strains of *B. xylophilus* in North America with mucronate female tail. Cultured *B. xylophilus* isolates normally have only round-tailed females, whereas isolates obtained from infested wood or from inoculated trees may contain females with mucros of variable lengths. However, females with the typical rounded tail terminus are also present in these populations (Braasch, 1996). Some *B. fraudulentus* strains and some European strains of *B. mucronatus* have very short mucros and are difficult to distinguish from each other and from *B. xylophilus*. Due to this complex situation, application of ITS-RFLP analysis is strongly recommended whenever suspected specimens of the quarantine pest *B. xylophilus* are detected in previously unknown areas of distribution. The first record of *B. xylophilus* in Portugal and its initial distribution at the time of detection were confirmed by ITS-RFLP analysis (Mota *et al.*, 1999). During plant quarantine inspections of coniferous packaging wood imported to Germany from non-European countries where the pinewood nematode is known to occur, the infestation with *B. xylophilus* was confirmed by ITS-RFLP analysis in six cases since 2001 (W. Burgermeister & E. Buchbach, unpublished).

In the case of *B. mucronatus*, a European type and an East Asian type can be distinguished by ITS-RFLP analysis on the basis of restriction fragments obtained with *Rsa*I and *Hae*III (Hoyer *et al.*, 1998). With both types, a PCR product of the same size of 950 bp and identical restriction fragment patterns with *Msp*I, *Hinf*I and *Alu*I are obtained, indicating a close genetic relationship. As suggested by Beckenbach *et al.* (1999) on the basis of DNA sequence analyses of the ITS regions, the European and Japanese *B. mucronatus* should be considered as intraspecific genetic variants rather than separate species. However, only two provenances from Japan were investigated by Beckenbach *et al.* (1999), and the real distribution of the two types was apparently not known. A clear subdivision of various *B. mucronatus* populations into two groups has been confirmed recently based on the ITS sequence analysis by Zheng *et al.* (2003) and Iwahori *et al.* (2004).

Examination of more than 60 *B. mucronatus* isolates from different origins by ITS-RFLP analysis has provided additional information on the distribution of both types (Braasch *et al.*, 2000). The East Asian type of *B. mucronatus* is found predominantly in East Asia, whereas the European type has its main distribution in Europe and Siberia. However, several investigations employing ITS-RFLP analysis have demonstrated unexpected areas of distribution of these types: both types were shown to occur in Japan by Iwahori *et al.* (1998). The East Asian type was recorded in three cases in Siberia (Braasch *et al.*, 2001) and in two cases in Germany (Braasch *et al.*, 1999). The European type was shown to occur in coniferous packaging wood from China

(Tomiczek *et al.*, 2003). One of the mucronate Canadian isolates initially believed to be *B. xylophilus* was later identified as *B. mucronatus*, European type (Harmey & Harmey, 1993; Braasch *et al.*, 1995). Both types of *B. mucronatus* were isolated in southern Germany from trees at about 100 meters distance from each other, with no indication of hybridization (Braasch *et al.*, 1999). The observation that concordant ITS-RFLP patterns of these two types represent separate populations at different loci indicates that they may be separate species. In addition, detailed investigations have revealed minor morphological differences of the two types (Braasch *et al.*, 1998).

In spite of its close morphological similarity to *B. xylophilus* and *B. mucronatus*, *B. fraudulentus* exhibits rather different ITS-RFLP features. Its PCR product is larger (1030 bp as compared to 950 bp) and distinct restriction fragment patterns are obtained with each of the five enzymes used. Kanzaki & Futai (2003) demonstrated the closer relationship of *B. xylophilus* and *B. mucronatus* as compared to *B. fraudulentus* in two phylogenetic trees. *B. fraudulentus* was thought to be an inhabitant of deciduous trees (Rühm, 1956; Schauer-Blume & Sturhan, 1989). It was shown by ITS-RFLP analysis and RAPD-PCR, respectively, that *B. fraudulentus* is distributed in Europe (Braasch *et al.*, 1999), Asia (Braasch *et al.*, 2001) and North America (Braasch *et al.*, 1995) in coniferous as well as in deciduous trees. Two other species of the xylophilus group have not been found in conifers so far. *B. luxuriosae* was isolated from a cerambycid beetle (*Acalolepta luxuriosa*) and its host trees of family Araliaceae. *B. conicaudatus* was found on another longhorn beetle (*Psacothea hilaris*) and its host trees of family Moraceae. Both occur in Japan and were successfully differentiated from the other species of the group by ITS-RFLP (Kanzaki & Futai, 2003).

B. sexdentati is a widespread nematode in pine trees in Europe and especially in the Mediterranean area. It was repeatedly identified in Germany (Rühm, 1960; Braasch *et al.*, 1999), Greece (Skarmoutsos & Skarmoutsos, 1999), Italy (Ambrogioni & Caroppo, 1998), Spain (Abelleira *et al.*, 2003), Austria (Tomiczek, 2000), Russia (Vosilite, 1990) and Switzerland (Braasch *et al.*, 2004). In spite of the great morphological similarity of the *B. sexdentati* isolates, two morphotypes were consistently observed in samples from Germany, Austria, Greece and Italy, corresponding to two types of ITS-RFLP patterns. Their PCR products had the same size of about 1000 bp, and differences in DNA restriction patterns were only revealed with one enzyme (*Hae*III) out of the five enzymes used. Type 1 corresponded to the original description of *B. sexdentati* by Rühm (1960), whereas type 2 was described as *B. vallesianus*, with reference to recently isolated strains of this type from *Pinus sylvestris* in Valais, Switzerland (Braasch *et al.*, 2004) (Fig. 1). Both species occur in central and southern Europe since they have been identified by ITS-RFLP analysis in samples from Germany, Switzerland and Greece.

The ITS-RFLP patterns of three other species found in conifers, *B. borealis, B. poligraphi* and *B. pinophilus*, exhibit some common features among each other and to both *B. sexdentati* and *B. vallesianus*. In all cases, a PCR product of about 1000 bp is obtained which is not cleaved by the enzymes *Msp*I and *Alu*I. Differences in restriction fragment patterns are observed only with one or two of the remaining three enzymes used. The five species mentioned above have a number of morphological features in common and therefore have been subsumed to the sexdentati group (Braasch, 2001). Due to the high morphological species similarity and the lack of type material for the original descriptions, the identification of nematodes of the sexdentati group has been facilitated by introduction of ITS-RFLP analysis. Moreover, phylogenetic relationships between species and populations of the sexdentati group have been revealed by sequence analysis of amplified rDNA (Lange, Burgermeister, Metge & Braasch, in preparation).

B. fungivorus was initially found on rotting *Botrytis*-contaminated *Gardenia* buds in a glasshouse in England (Franklin & Hooper, 1962) and in glasshouse soil in Germany (Braasch *et al.*, 2002). It is the only representative of the fungivorus group known so far in Europe. The identity of this species with similar nematodes found in imported coniferous bark and wood was confirmed by ITS-RFLP analysis (Braasch *et al.*, 1999). The source for infestation in glasshouses obviously was coniferous bark added to the substrate used for growing ornamental plants. Therefore, *B. fungivorus* like *B. fraudulentus* has to be added to the *Bursaphelenchus* species living in coniferous trees. Other members of the fungivorus group like *B. thailandae* and *B. seani* live in East Asia and North America, respectively. *B. thailandae* was originally described from Thailand (Braasch & Braasch-Bidasak, 2002). The occurrence of the same species in packaging wood imported from China to Austria (Tomiczek *et al.*, 2003) was demonstrated by ITS-RFLP. *B. seani*

has a phoretic association with a solitary, soil-dwelling bee in North America (Giblin *et al.*, 1983). Its ITS-RFLP pattern supplements the earlier description of this species.

Within recent years, ITS-RFLP patterns have been introduced as additional identification criteria to the description of new *Bursaphelenchus* species, as discussed above for *B. vallesianus*. *B. rainulfi* was described from Malaysia, a country where no *Bursaphelenchus* species was known to occur so far. Using ITS-RFLP analysis, this species was clearly distinguished from the morphologically similar species *B. hellenicus*, *B. hylobianum* and *B. abietinus* (Braasch & Burgermeister, 2002). Likewise, Kanzaki & Futai (2003) successfully used ITS-RFLP analysis to differentiate *B. luxuriosae* sp. n. from other known species of the xylophilus group, i.e. *B. mucronatus*, *B. xylophilus*, *B. conicaudatus* and *B. fraudulentus* and from *B. abruptus* which is associated with a digger bee in North America and shows some morphological features similar to the xylophilus group species (Giblin-Davis *et al.*, 1993).

The initial descriptions of most *Bursaphelenchus* species listed in Tab. 1 were based only on morphological criteria. Due to incomplete early descriptions of species, lack of type material and close similarity of several *Bursaphelenchus* species, it is sometimes difficult to establish the real distribution of a species. An ITS-RFLP pattern provides each isolate investigated with an invariable identity permitting successful comparisons with isolates from other places. Even in case of uncertainty concerning the species affiliation of an isolate, the "molecular fingerprint" makes it comparable with further findings. Therefore, ITS-RFLP analysis has greatly facilitated identification of closely related species and revealed new information on their distribution. This is further illustrated by the examples discussed below.

B. tusciae is morphologically very similar to *B. eggersi*. Both were found at several places in Germany, and ITS-RFLP patterns were an important tool for their differentiation. *B. leoni* was isolated in Greece, Italy, Austria and Germany (Braasch *et al.*, 2000). Surprisingly, it was also recorded from South Africa and confirmed by ITS-RFLP (Braasch *et al.*, 1998; Hoyer et al., 1998). Taking into consideration that the natural distribution of *Bursaphelenchus* species seems to be restricted to the northern hemisphere, the occurrence in a pine plantation in South Africa may be explained by import of the nematode with infested wood from its area of distribution in Europe. Dauer juveniles of *B. abietinus* were found on the vectors *Pityokteines spinidens*, *P. vorontzowi* and *P. curvidens* (Coleoptera, Scolytidae) and cultivated on *Botrytis* to adults. After establishment of the pattern, the taxonomic identity of many isolates from these vectors was recognized by ITS-RFLP analysis without the necessity to cultivate them.

ITS-RFLP analysis has disclosed the widespread occurrence of *B. hylobianum*. It was originally described from northeastern Russia (Korenchenko, 1980), was then found in coniferous wood imported to Germany from Russia (Braasch *et al.*, 2001) and later isolated from pines in Thailand (Braasch & Braasch-Bidasak, 2002). Surprisingly, Penas *et al.* (2004) found this species on the vector *Hylobius* sp. in Portugal and confirmed the species affiliation by ITS-RFLP using three enzymes (*Alu*I, *Hae*III, *Rsa*I). This is the only record of *B. hylobianum* in Europe so far.

B. lini was morphologically described from Chinese pine trees as an exceptional *Bursaphelenchus* species sharing some characters with Ektaphelenchidae (Braasch, 2004). Its special taxonomic status may reflect the unusual size of the PCR product (1350 bp), which is the largest one among the *Bursaphelenchus* species investigated.

B. aberrans described by Fang *et al.* (2002) from Guandong Province in China, was reported from Thailand (Braasch & Braasch-Bidasak, 2002) and has repeatedly been found in packaging wood from China (Tomiczek *et al.*, 2003). Its ITS-RFLP pattern shown in Fig. 1 was obtained from a sample taken by Gu Jianfeng (China) from Japanese packaging wood (unpublished). Recently, a similar species, *B. sinensis*, was described by Marinari Palmisano *et al.* (2004) from Chinese packaging wood. Further investigations, and especially morphological and molecular comparisons with type material of *B. aberrans*, may reveal whether the ITS-RFLP pattern shown for *B. aberrans* in Fig. 1 represents this species or *B. sinensis*.

Experience has shown that a considerable number of species of *Bursaphelenchus*, including two intraspecific types, can be distinguished reliably by ITS-RFLP analysis using the five restriction enzymes described in this article (Fig. 1). ITS-RFLP patterns of all these species are distinct, also permitting differentiation of morphologically very similar species. Consequently, the technique has also proved valuable for differentiation of the quarantine pest *B.*

Table 1. *Bursaphelenchus* isolates examined by ITS-RFLP analysis in the present work.

Bursaphelenchus species	Origin and code of isolate examined	Host/vector of isolate examined	Reference for initial ITS-RFLP
B. aberrans Fang, Kan & Jun, 2002	Japan (65.2)	Coniferous packaging wood imported to China	10
B. abietinus Braasch & Schmutzenhofer, 2000	Austria, Vorarlberg, Bludenz (AT-28i)	*Pityokteines curvidens*	5
B. abruptus Giblin-Davis, Mundo-Ocampo, Baldwin, Norden & Batra, 1993	USA, Maryland (NE12/98)	*Anthophora abrupta*	7
B. borealis Korenchenko, 1980	Germany, Mecklenburg-Vorpommern, Eggesin (DE-8w)	*Pinus sylvestris*	3
B. conicaudatus Kanzaki, Tsuda & Futai, 2000	Japan (184.2)	*Ficus carica*	7
B. eggersi Rühm, 1956	Germany, Brandenburg, Caputh (DE-21i)	*Hylurgops palliatus*	3
B. fraudulentus Rühm, 1956	Germany, Berlin (Bf Berlin)	*Quercus* spec	1, 3
B. fungivorus Franklin & Hooper, 1962	Czechia (CZ-DE-1w)	Coniferous bark	3
B. hellenicus Skarmoutsos, Braasch & Michalopoulou, 1998	Greece, Thessaloniki (GR-2w)	*Pinus brutia*	3
B. hofmanni Braasch, 1998	Germany, Thuringia, Georgenthal (DE-6w)	*Picea abies*	3
B. hylobianum Korenchenko, 1980	Russia (Asian part) (RU-DE-16w)	*Larix sibirica*	4
B. leoni Baujard, 1980	Austria, Burgenland, Grosshöflein (AT-23w)	*Pinus nigra*	1, 3
B. lini Braasch, 2004	China, Nanjing (65.1)	*Pinus thunbergii* *Pinus massoniana*	10
B. luxuriosae Kanzaki & Futai, 2003	Japan, Nara Pref. (57.1)	*Acalolepta luxuriosa*	7
B. mucronatus Mamiya & Enda, 1979 (East Asian type)	Germany, Thuringia, Bad Salzungen (DE-5w)	*Picea abies*	1, 2
B. mucronatus Mamiya & Enda, 1979 (European type)	Germany, Brandenburg (H320/01B)	*Pinus sylvestris*	1, 2
B. paracorneolus Braasch, 2000	Germany, Bavaria, Rauheck (DE-14w)	*Picea abies*	3, 10
B. pinasteri Baujard, 1980	Germany, Brandenburg, Trebitz (H523/04)	*Pinus sylvestris*	10
B. pinophilus Brzeski & Baujard, 1997	Portugal (PT-2w)	*Pinus pinaster*	8
B. poligraphi Fuchs, 1937	Germany, Lower Saxony, Torfhaus (DE-17w)	*Picea abies*	3
B. rainulfi Braasch & Burgermeister, 2002	Malaysia, Kuala Lumpur (NE30/00)	*Pinus caribaea*	5
B. seani Giblin & Kaya, 1983	USA, California (NE14/98)	*Anthophora bomboides stanfordiana*	10
B. sexdentati Rühm, 1960	Germany, Hessen, Darmstadt (DE-29w)	*Pinus sylvestris*	1, 3
B. thailandae Braasch & Braasch-Bidasak, 2002	Thailand, Pai-Maehongson region (RC-AT-7w)	*Pinus merkusii*	6
B. tusciae Ambrogioni & Palmisano, 1998	Italy, Florence, Scopeti (IT-14w)	*Pinus pinea*	9, 10
B. vallesianus Braasch, Schunfeld, Polomski & Burgermeister, 2004	Switzerland, Valais (ST223B)	*Pinus sylvestris*	8
B. xylophilus (Steiner & Buhrer, 1934), Nickle 1970	China, Nanjing (NE12/02)	*Pinus thunbergii*	1, 2

1: Hoyer *et al.* (1998). 2: Iwahori *et al.* (1998). 3: Braasch *et al.* (1999). 4: Braasch *et al.* (2001). 5: Braasch & Burgermeister (2002). 6: Tomiczek *et al.* (2003). 7: Kanzaki & Futai (2003). 8: Braasch *et al.* (2004). 9: Penas *et al.* (2004). 10: This article.

Table 2. Approximate size of DNA fragments observed in ITS-RFLP analysis of *Bursaphelenchus* species.

Bursaphelenchus species	PCR product (bp)	Restriction fragments (bp)				
		*Rsa*I	*Hae*III	*Msp*I	*Hinf*I	*Alu*I
B. aberrans	980	410 120	690 290	380 350 240	440 280 210	550 190 120
B. abietinus	1070	610 270 180	670 230 170	530 400 160	530 210 120	580 220
B. abruptus	1300	750 (180) (120)	900 (130)	850 150	500 320 220 (90)	700 230 120
B. borealis	1000	290 220 130	560 350 120	1000	450 290 230	1000
B. conicaudatus	980	510 450	550 160	290 200 120	270 190 90	380 310
B. eggersi	950	380 180	950	620 310	450	380 190
B. fraudulentus	1030	560 470	340 290 150 110	340 290 130	310 260 160	470 390 180
B. fungivorus	1070	410 380 180 120	880 210	820 210	310 230 170	680 (450) 300
B. hellenicus	1080	610 290 180	530 390 160	690 390	520 320 220	330 280 180 (130)
B. hofmanni	1050	560 490	910 140	380 300 130	350 230 120	360 300 280
B. hylobianum	1150	590 280	790 360	360 310 180	490 270 240 120	1150
B. leoni	850	790	590 260	690 160	480 160 100	590 (490) 400 180
B. lini	1350	420 310	890 430	1350	810 600 510	900 420
B. luxuriosae	950	500 420	750 160 50	450 240 130	270 240 170	600 320

Table 2. (cont.). Approximate size of DNA fragments observed in ITS-RFLP analysis of *Bursaphelenchus* species.

Bursaphelenchus species	PCR product (bp)	Restriction fragments (bp)				
		*Rsa*I	*Hae*III	*Msp*I	*Hinf*I	*Alu*I
B. mucronatus European type	950	410 290 230	620 220 110	370 310 280	410 250 130 90	700 250
B. mucronatus East Asian type	950	500 410	620 310	370 310 280	410 250 130 90	700 250
B. paracorneolus	1020	300 170 140	1020	630 270 120	230 210 190 130	760 260
B. pinasteri	1050	560 450	1050	630 340	270 220 120	340 280 130
B. pinophilus	1000	430 340 210	600 280 120	1000	400 290 220 90	1000
B. poligraphi	980	430 340 210	530 340 110	980	470 290 220	980
B. rainulfi	1050	270 180 160 120	1050	690 390	510 210 110	340 200 100
B. seani	950	510 440	950	950	510 430	590 280 100
B. sexdentati	1000	550 410	590 280 120	1000	470 280 210	1000
B. thailandae	900	500 400 (330)	900	900	400 230 210	540 290
B. tusciae	950	390 220 170	610 340	510 320 140	470	310 240 200 180
B. vallesianus	1000	570 420	890 120	1000	480 290 220	1000
B. xylophilus	950	500 420	730 200	570 380	270 260 140	460 250 140 100

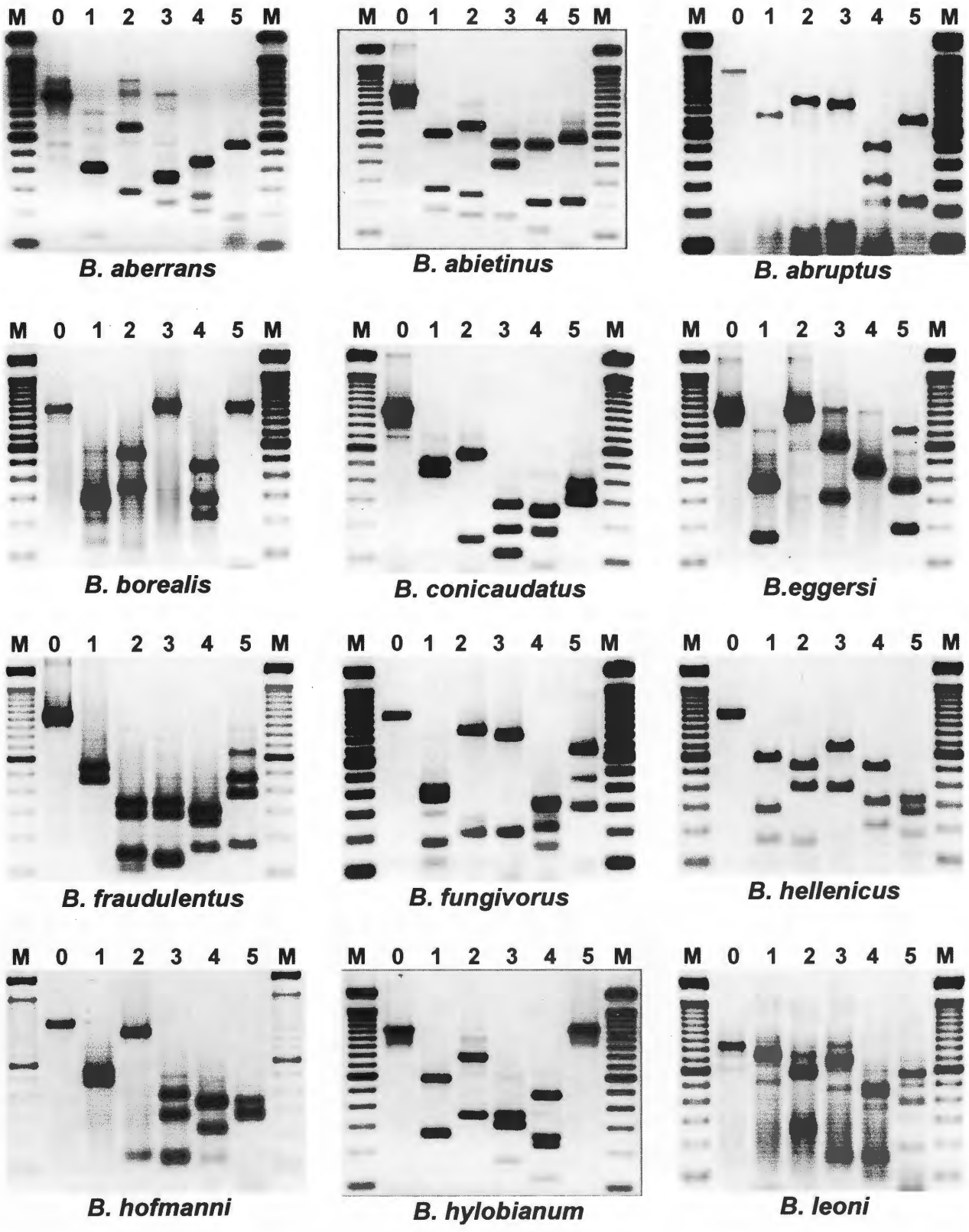

Fig. 1. ITS-RFLP patterns of 26 *Bursaphelenchus* species including two intraspecific types of *B. mucronatus*. Restriction fragments were obtained by digestion of the amplified rDNA fragment (0) with *Rsa*I (1), *Hae*III (2), *Msp*I (3), *Hinf*I (4) and *Alu*I (5). M: DNA marker (100 bp ladder, Invitrogen Life Technologies).

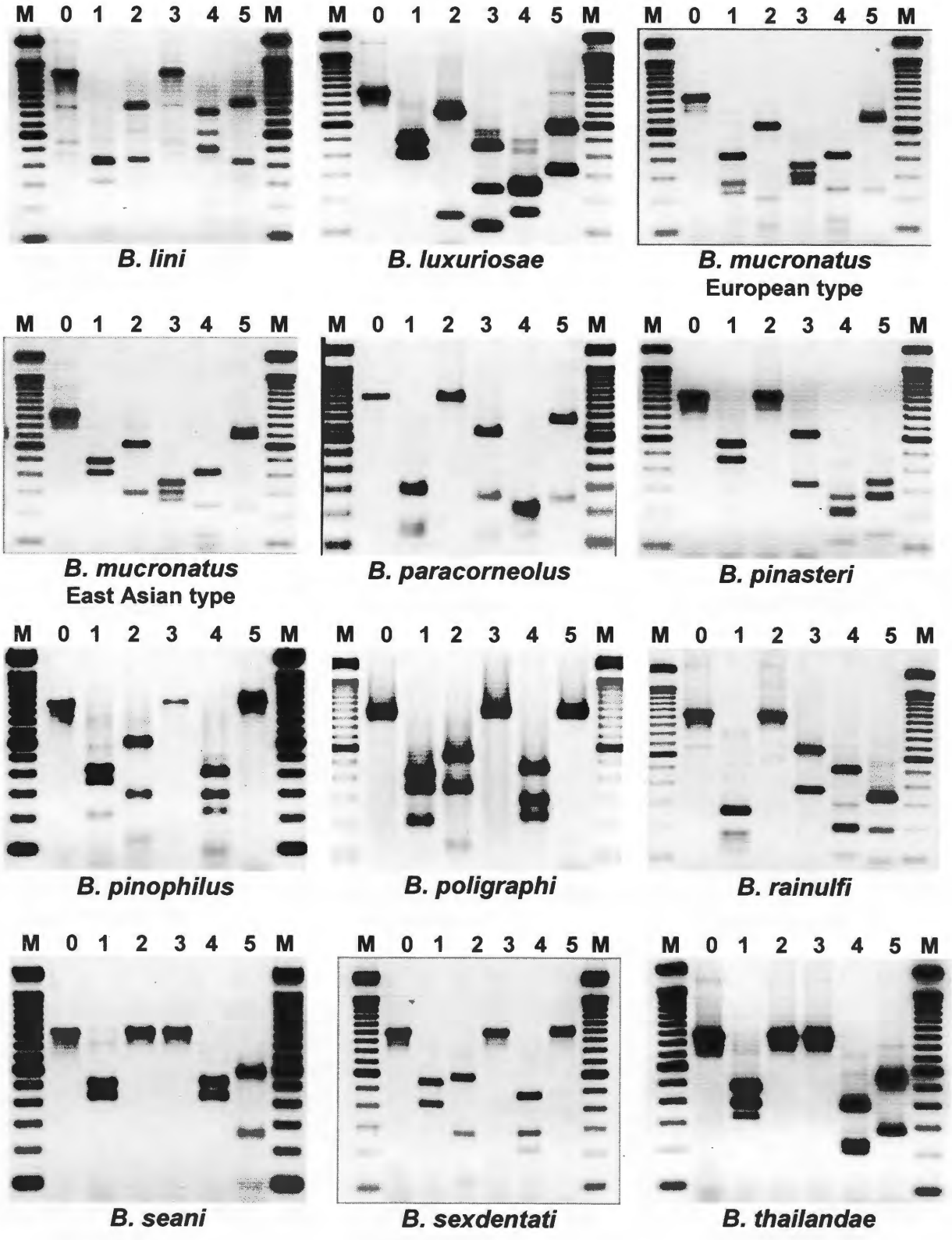

Fig. 1. (cont.) ITS-RFLP patterns of 26 *Bursaphelenchus* species including two intraspecific types of *B. mucronatus*. Restriction fragments were obtained by digestion of the amplified rDNA fragment (0) with *Rsa*I (1), *Hae*III (2), *Msp*I (3), *Hinf*I (4) and *Alu*I (5). M: DNA marker (100 bp ladder, Invitrogen Life Technologies).

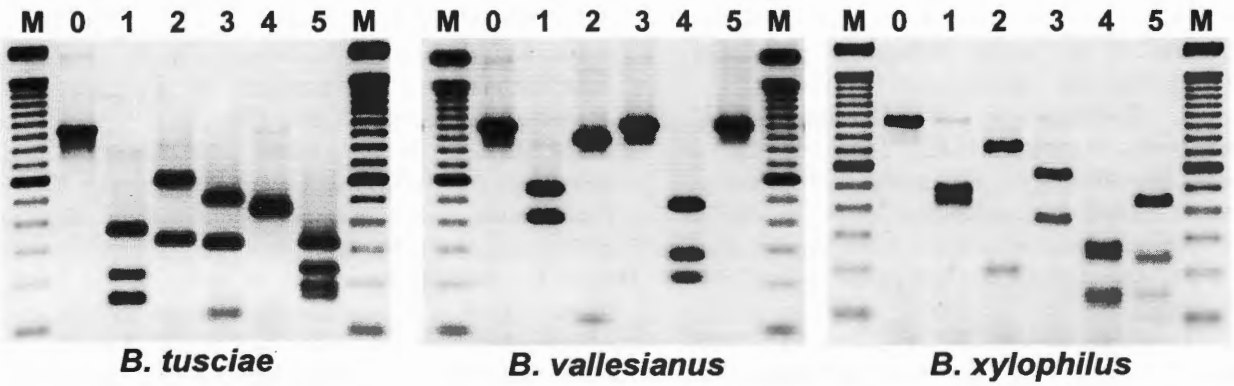

Fig. 1. (cont.) ITS-RFLP patterns of 26 *Bursaphelenchus* species including two intraspecific types of *B. mucronatus*. Restriction fragments were obtained by digestion of the amplified rDNA fragment (0) with *Rsa* I (1), *Hae*III (2), *Msp*I (3), *Hinf*I (4) and *Alu*I (5). M: DNA marker (100 bp ladder, Invitrogen Life Technologies).

investigated. Due to improved methods, more and more *Bursaphelenchus* species are being described, especially from East Asia, and ITS-RFLP is increasingly important in the recognition of their species status in comparison with morphologically similar species.

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Burgermeister W., Metge K., Braasch H., Buchbach E. Использование ITS-RFLP для дифференциации 26 видов Bursaphelenchus (Nematoda: Parasitaphelenchidae) и наблюдения по их распространению.

Резюме. Анализ ITS-участка рибосомальной ДНК представляет собой ценный инструмент для дифференциации видов *Bursaphelenchus*. Рестрикция 5-ю эндонуклеазами (*Rsa*I, *Hae*III, *Msp*I, *Hinf*I и *Alu*I) выявляет видоспецифичные ITS-RFLP спектры для 26 видов *Bursaphelenchus*, а также для двух интраспецифичных форм. Впервые представлены ITS-RFLP спектры для пяти видов. Эта методика оказалась особенно ценной для дифференциации нематоды сосновой древесины *B. xylophilus* от других видов *Bursaphelenchus* в процессе карантинных обследований. Ценным оказывается ITS-RFLP-анализ и для выявления географического распределения видов *Bursaphelenchus*. Предлагается использовать спектры ITS-RFLP в качестве дополнительной информации при описании нескольких новых видов *Bursaphelenchus*.