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Species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Parasitaphelenchidae) associated with maritime pine in Portugal

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Summary – Species of *Bursaphelenchus* associated with maritime pine, *Pinus pinaster*, from Portugal – within and outside the quarantine restricted demarcated zone of *B. xylophilus* – are described and characterised both morphologically (LM and SEM) and with the use of molecular biology (ITS-RFLP). A new staining method for spicules is proposed. Species include *B. hellenicus*, *B. hylobianum*, *B. leoni*, *B. pinophilus*, *B. sexdentati*, *B. tusciae*, *B. teratospicularis*, *B. xylophilus* and *Bursaphelenchus* sp. 1. *Bursaphelenchus hylobianum* was collected from the insect *Hylobius* sp. The most frequent species in the demarcated zone, besides *B. xylophilus*, was *Bursaphelenchus* sp. 1. Morphological characterisation is compared with the original descriptions and discussed. The differentiation between *B. pinophilus* and *B. sexdentati* is not clear in the literature and is discussed. Since differentiation of *B. xylophilus* (mucronate form) from *B. mucronatus*, and *B. pinophilus* from *B. sexdentati*, as well as their juvenile forms, is almost im possible on the basis of morphological features, a molecular approach based on ITS-RFLPs was used. 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. Restriction profiles of the amplified products generated species-specific differences, leading to the unambiguous identification of isolates belonging to *B. xylophilus*, *B. mucronatus*, *B. sexdentati*, *B. tusciae* and *B. hylobianum*.

Keywords – diagnostics, identification, ITS-RFLP, morphology, *Pinus pinaster*, survey.

During a survey conducted within a national Praxis XXI research project no. 11 189/98, 'Survey and study of *Bursaphelenchus* and other nematode species associated with cerambycid insects in pine trees in Portugal', the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle 1970, was detected in March of 1999, associated with maritime pine (*Pinus pinaster* Aiton). This represented the first report of this important pest and pathogen in Portugal and Europe (Mota *et al*., 1999).

Official authorities implemented an intensive survey of the region where the nematode was detected and later expanded it to the rest of the country within a National Programme of Pinewood Nematode Control (PROLUNP, Programa Nacional de Luta contra o Nemátode do Pinheiro – http://www.dgf.min-agricultura.pt/prolunp/html/home-final.htm). As a result, this A1 quarantine organism was confirmed as being restricted within an area in the Setúbal region to the southeast of Lisbon. This infested area has been precisely delimited by annual surveys and a buffer zone, about 20 km wide, was established and included in a demarcated zone subject to restrictive quarantine measures. Since then, in addition to an intensive annual survey of the demarcated zone, the survey programme has also been carried out in the rest of the country (Mota & Vieira, 2003).

About 25 species of *Bursaphelenchus* associated with conifers have been reported in Europe (Braasch, 2001; Mota & Vieira, 2004). Some of these species, such as *B. mucronatus* Mamiya & Enda, 1979 and *B. xylophilus*, display similar morphological features which may con-

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found precise identification, a crucial step in establishing quarantine measures.

Morphology is an essential component of nematode differentiation and, in many cases, provides a rapid and unambiguous species diagnosis. However, this identification is highly dependent on the experience and interpretative skills of the researcher. In some groups of nematodes, morphological characters are only a first approach and require confirmation by more accurate methods. Differences in nucleic acid sequences, as revealed by means of molecular biology techniques such as ITS-RFLP (Hoyer *et al.*, 1998; Braasch *et al*., 1999), may help to characterise each species and complement its morphological description. Furthermore, DNA-based methods provide an attractive solution to problems associated with morphological identification, since they do not rely on the expression of the genome and are independent of environmental influence or developmental stage.

The purpose of this research was to survey and identify the *Bursaphelenchus* species associated with maritime pine in Portugal by means of morphological and molecular analysis (PCR-ITS RFLP).

Materials and methods

During the annual surveys carried out by PROLUNP, a total of 4810 samples of pine wood material from trees displaying symptoms of pine wilt disease, corresponding to a small proportion of all samples, were examined for *Bursaphelenchus* species in the nematology laboratory of EAN. Nematodes were extracted using a modified Baermann funnel technique. In addition, 62 specimens of *Hylobius* sp., collected from *Pinus pinaster*, were crushed in a Syracuse dish and examined under a stereoscopic microscope for possible *Bursaphelenchus* dauer juveniles. These were inoculated into 15 cm pine branch segments and *Botrytis cinerea* Petri dishes, and incubated for 1 month at 26°C.

Nematode identification was based on observations of the main morphological characters, particularly vulval flap, shape of spicules, female tail (Braasch, 2001) and head shape using light (LM) and scanning electron microscopy (SEM). For spicule observation, a new staining method was tested with the use of Rotring® Brilliant Ultramarine Blue ink (stock solution = one 0.5 ml cartridge in 20 ml of acetic acid; prepare a final solution by diluting 1 ml of stock solution in 10 ml of lactophenol). Live nematodes were transferred to a drop of this solution and heated briefly over an alcohol lamp. This staining method was applied to *B. hellenicus* Skarmoutsos, Braasch & Michalopoulou, 1998 and *B. sexdentati* which have inconspicuous spicules. For SEM studies, nematodes were fixed in a mixture of 4% gluteraldehyde/2% formaldehyde for several days, post-fixed in 2% OsO_4 overnight, dehydrated in an ethanol series, critical point dried and sputter coated with gold (Eisenback, 1985). Observations were made with a Jeol 35 SEM.

After extraction from wood, aliquots of one to ten nematodes were stored for DNA extraction. Nematodes were heated at 95°C for 5 min, homogenised on a glass slide with a micro pestle (Eppendorf®) and DNA obtained using the DNeasy Tissue Kit (Quiagen®). This procedure was applied to different nematode life stages, namely adult, dauer juvenile, propagative juvenile and resistant juvenile.

The ITS regions of rDNA were amplified using primers F194 and P5368 as described by Ferris *et al*. (1993) and Vrain (1993), respectively. All polymerase chain reactions were performed in a final volume of 50 μl using 10 ng/μl of template DNA, 1 μM of each primer, 0.2 μM of dNTPs (Invitrogene®), 2 U of *Taq* DNA polymerase (Invitrogene®), 1× Reaction Buffer (Invitrogene®) and 1.25 mM of MgCl2 (Invitrogene®). The reaction mixture was overlaid with sterile mineral oil to prevent evaporation during PCR cycling. A Stratagene® Robocycler was used for amplification and the reaction consisted of one denaturation step at 94°C for 1 min, 35 cycles with denaturation at 94°C for 1 min, annealing at 51°C for 1 min, polymerisation at 72°C for 2 min and a final extension step at 72°C for 5 min. After PCR, 5 μl of amplified product was analysed by electrophoresis in a 1% agarose gel. Data analysis was performed using the Kodak® 1D 2.0 system and 100 bp DNA Ladder (Invitrogene®) as a molecular size marker.

Restriction analysis of ITS regions was performed with *Alu*I, *Hae*III and *Rsa*I restriction endonucleases (Invitrogene®), using an aliquot of 4 μl of the PCR product and 10 U of each enzyme, according to the manufacturer's instructions. Fragments were resolved by electrophoresis in a 2% agarose gel and data were analysed as described above.

Results

The geographic distribution of *Bursaphelenchus* species in Portugal is presented in Table 1 and Figure 1. Morphological identification was based on original and other descriptions. The species are illustrated by light and scan-

Table 1. *Origin and location of* Bursaphelenchus *species in Portugal.*

No.	Species	Host	Location
1	*B. hellenicus*	*Pinus pinaster*	Samora Correia, Santarém Alcácer do Sal, Setúbal Melides, Sines Castro Daire, Viseu
2	*B. hylobianum*	*Hylobius* sp.	Leiria
3	*B. leoni*	*P. pinaster*	Ovar, Aveiro Sertã, C. Branco Oliveira Hospital, Coimbra Oeiras, Lisboa Marco de Canaveses, Porto Ferreira Zêzere, Santarém Rio Maior, Santarém S. Correia, Santarém Aiana, Setúbal Alcácer do Sal, Setúbal Azeitão, Setúbal Casal do Marco, Setúbal Grândola, Setúbal Santana, Setúbal Melides, Sines Sabrosa, Vila Real Castro Daire, Viseu Nelas, Viseu Mangualde, Viseu
4	*B. mucronatus*	*P. pinaster*	Figueira da Foz, Coimbra
5	*B. sexdentati*	*P. pinaster*	Figueira da Foz, Coimbra Póvoa do Varzim, Porto S. Correia, Santarém Grândola, Setúbal Mangualde, Viseu
5a	*Bursaphelenchus* spp.*	*P. pinaster*	Aveiro, Aveiro Caminha, Braga Fafe, Braga Paredes de Coura, Braga Sertã, C. Branco O. Hospital, Coimbra Guadalupe, Évora Vendas Novas, Évora Faro, Faro Pinhel, Guarda Pedrógão Grande, Leiria Loures, Lisboa Oeiras, Lisboa Rio Maior, Santarém Salvaterra Magos, Santarém Canha, Setúbal Carvalhal, Setúbal Montijo, Setúbal Melides, Sines Sabrosa, Vila Real Vila Real, Vila Real Castro Daire, Viseu Mangualde, Viseu Moimenta da Beira, Viseu Nelas, Viseu
6	*B. teratospicularis*	*P. pinaster*	Sertã, C. Branco Sertã, C. Branco Biscainho, Santarém Azeitão, Setúbal Grândola, Setúbal Marateca, Setúbal Melides, Sines Santo André, Sines Sabrosa, Vila Real Mangualde, Viseu
7	*B. tusciae*	*P. pinaster*	Figueira da Foz, Coimbra Faro, Faro Coruche, Santarém F. Salvaterra, Santarém Grândola, Setúbal Melides, Sines Castro Daire, Viseu Mangualde, Viseu
8	*B. xylophilus*	*P. pinaster*	Infested zone
9	*Bursaphelenchus* sp. 1	*P. pinaster*	Vila Flor, Bragança Cabrela, Évora Ota, Lisboa Biscainho, Santarém Coruche, Santarém F. Salvaterra, Santarém S. Magos, Santarém Samora Correia, Santarém S. do Mato, Santarém S. Estêvão, Santarém Amora, Setúbal Alcácer do Sal, Setúbal Carvalhal, Setúbal Comporta, Setúbal Corroios, Setúbal C. Caparica, Setúbal Feijó, Setúbal Grândola, Setúbal Santiago Cacém, Setúbal S. Martinho, Setúbal Melides, Sines Castro Daire, Viseu

* *B. pinophilus* or *B. sexdentati* (subject to confirmation; see Discussion for more details).

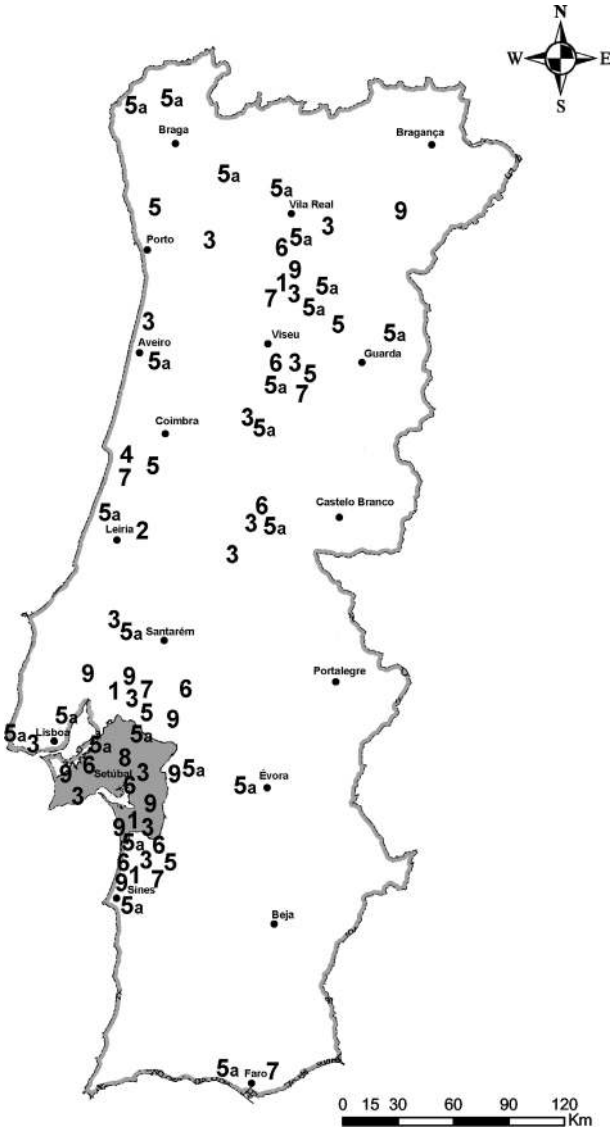

Fig. 1. *Occurrence of* Bursaphelenchus *species in continental Portugal. 1:* B. hellenicus*; 2:* B. hylobianum*; 3:* B. leoni*; 4:* B. mucronatus*; 5:* B. sexdentati*; 5a:* Bursaphelenchus *spp.; 6:* B. teratospicularis*; 7:* B. tusciae*; 8:* B. xylophilus*; 9:* Bursaphelenchus *sp. 1 (see Table 1 for details of locations).*

ning electron microscopy (Figs 2-10). Besides *B. xylophilus* (Fig. 9), other *Bursaphelenchus* species were collected and identified from pine wood, namely: *B. hellenicus* (Fig. 2); *B. leoni* Baujard, 1980 (Fig. 4); *B. mucronatus* (Fig. 5); *B. teratospicularis* Kakuliya & Devdariani, 1965 (Fig. 7); *B. tusciae* Ambrogioni & Palmisano, 1998 (Fig. 8); *B. sexdentati* Rühm, 1960 (Fig. 6) and *Bursaphelenchus* sp. 1 (Fig. 10). The presence of *B. pinophilus* Brzeski & Baujard, 1997 in Portugal was previously confirmed by Braasch (2001). ITS-RFLP analysis of some populations suspected to be *B. sexdentati* and/or *B. pinophilus* produced the characteristic pattern for *B. sexdentati*. However, it was not always possible to distinguish clearly between *B. pinophilus* and *B. sexdentati*, the nematodes in such cases being referred to as *Bursaphelenchus* spp. One of the species found, *Bursaphelenchus* sp. 1, was very similar to *B. pinasteri* Baujard, 1980, although it exhibits some characters, such as head and spicule shape and number of incisures in lateral fields, identical to *B. hofmanni* Braasch, 1998. Morphological characters used to diagnose the different *Bursaphelenchus* species are listed and compared in Table 2.

The staining method with blue ink allowed the spicule shape of *B. hellenicus* and *B. sexdentati* to be clearly seen (Figs 2, 6).

The number of samples in which each *Bursaphelenchus* species was found is presented in Table 3. Populations levels were usually low. Apart from *B. xylophilus*, the most frequent species in the Demarcated Zone was *Bursaphelenchus* sp. 1, while *B. mucronatus* was only found in one sample.

Nine *Hylobius* sp. contained dauer juveniles of *Bursaphelenchus* sp. under the elytra. Adult nematodes collected from *B. cinerea* plates and pine branches, previously inoculated with these dauer juveniles, were identified as *B. hylobianum* Korenchenko, 1980 (Fig. 3). As in the original description from Russia (Korenchenko, 1980), this nematode was found in Portugal associated with *Hylobius* beetles.

In addition to morphological observations, ITS-RFLP analysis was employed for confirmation and differentiation of *B. xylophilus*, *B. mucronatus*, *B. sexdentati*, *B. tusciae* and *B. hylobianum*. Amplification of ITS regions yielded a single DNA fragment of 950 bp for *B. xylophilus*, *B. mucronatus* and *B. tusciae*, of 1100 bp for *B. sexdentati* and of 1150 bp for *B. hylobianum*. Subsequent analysis of ITS regions with *Alu*I, *Hae*III and *Rsa*I endonucleases produced characteristic restriction profiles that clearly differentiated these five *Bursaphelenchus* species (Fig. 11). All collected mucronate forms of *B. xylophilus* individuals displayed the characteristic restriction pattern of this species, thereby allowing for their differentiation from *B. mucronatus*. Similarly, all propagative and resistant juveniles displayed the specific *B. xylophilus* pattern. Specific patterns were also obtained for dauer juveniles of both *B. xylophilus* and *B hylobianum*.

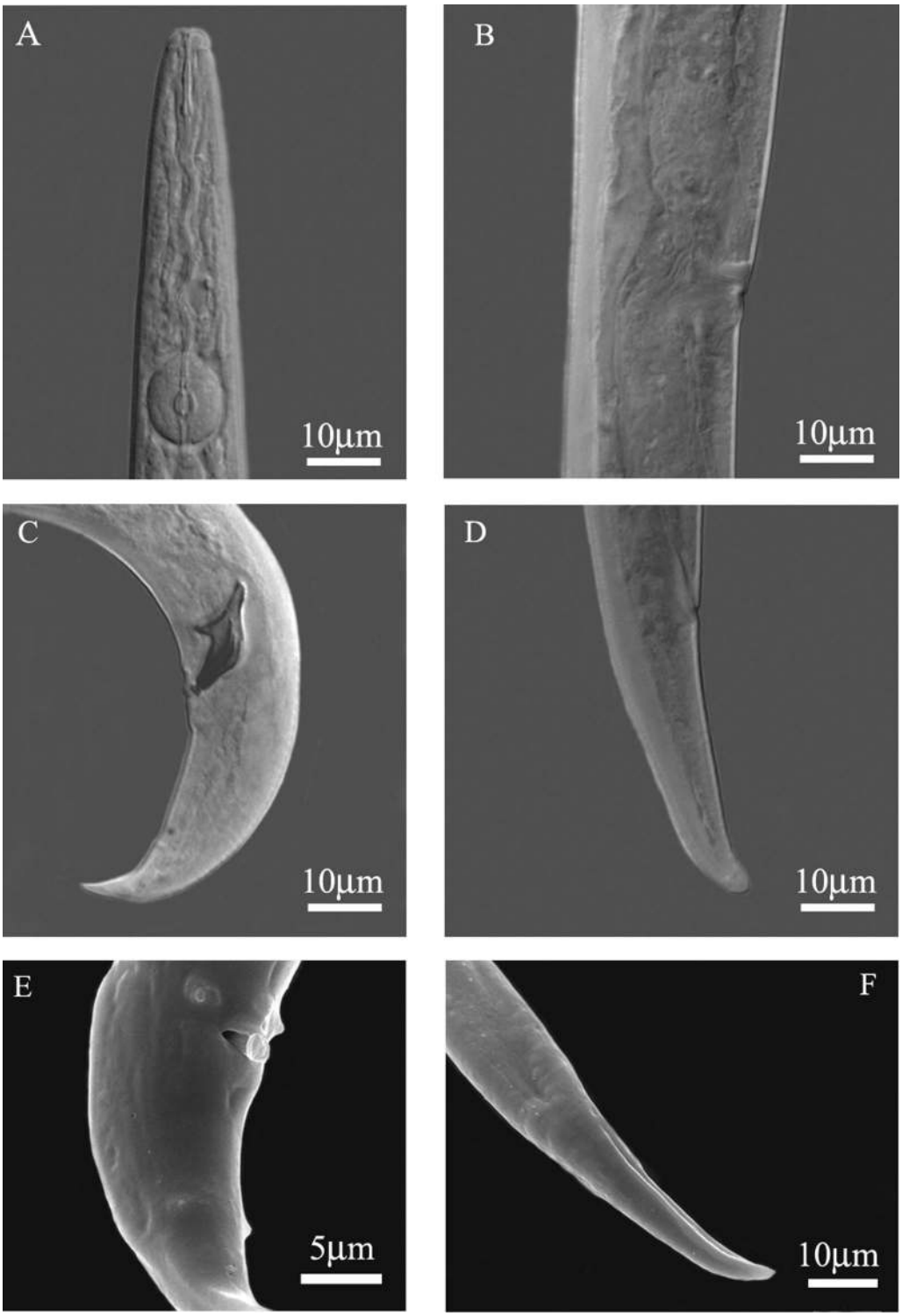

Fig. 2. Bursaphelenchus hellenicus. *A: Light micrograph (LM) of anterior region; B: LM of vulval region; C: LM of male tail (stained spicule); D: LM of female tail; E: Scanning electron micrograph (SEM) of male tail showing caudal papillae and protracted cucullus of spicules; F: SEM of female tail.*

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

Species	Cuticle annulation	No. of lateral incisures	Lip region	Vulva	Female tail	Spicule	Male caudal papillae
B. hellenicus	Fine	3	High, rounded, set off by constriction	Anterior lip slightly extended to form very small flap	Conoid with more a less rounded terminus, slightly ventrally bent	Small, rosethorn-shaped; rostrum prominent, blunt; apex well developed, elongate, bluntly rounded; distinct disc-like cucullus	Single pre-anal papilla; one pair adanal; one ventral post-anal and smaller, ventral, pair at beginning of bursa (not always visible)
B. hylobianum	Fine	2	High, rounded, set off by constriction	Anterior lip slightly extended to form small, distinct, flap	Conoid, gradually tapering with terminus bluntly rounded to acute	Robust, rosethorn-shaped, strongly curved; prominent rostrum not sharply pointed; apex well developed; disc-like cucullus (not always distinct)	Single ventral preanal papilla; one pair adanal; one pair post-anal and one small pair at beginning of bursa
B. leoni	Fine	3	Rounded, well set off by deep constriction	Anterior lip extended to form short flap; area posterior to flap often swollen	Very long, conoid, terminus variable in shape, finely rounded, sometimes slightly digitate or with a slight constriction	Medium to large with distinct, pointed, rostrum; apex well developed and dorsally hooked; distal end with slight hook-like process (not always distinct)	Single ventral pre-anal papilla; one pair adanal; one pair post-anal and one small pair at beginning of bursa
B. mucronatus	Fine	4	Rounded, set off by constriction	Anterior lip forming long flap overlapping vulva	Subcylindrical, rounded, with very long mucron	Large, limb long and strongly curved with transverse bar of capitulum almost parallel to shaft axis; apex bluntly rounded, rostrum prominent and pointed; distinct disc-like cucullus	Single ventral pre-anal papilla, one pair adanal and two contiguous post-anal pairs
B. sexdentati Bursaphelenchus spp.*	Fine	4	Rounded, set off by constriction	Anterior lip slightly extended to form small flap; post vulval swelling often present	Conoid, gradually tapering, with variable rounded or slightly digitate terminus	Rosethorn-shaped, medium size; rostrum prominent, pointed; apex well developed, broadly rounded-squared; distinct knob-like cucullus	Single ventral pre-anal papilla; one pair subventral adanal; two post-anal pairs

Table 2. *(Continued).*

Species	Cuticle annulation	No. of lateral incisures	Lip region	Vulva	Female tail	Spicule	Male caudal papillae
B. teratospicularis	Coarse	Not known	Flattened, slightly set off by weak constriction	Vulval lips only protruding very slightly; flap absent	Conical, with broadly rounded terminus	Mitten-shaped with pointed rostrum; apex well developed, bluntly rounded often forming a dorsally directed curve to shaft; distal tip dorsally curved, no cucullus	Adanal pair; one post-anal subventral pair at beginning of bursa
B. tusciae	Fine	3	Rounded, set off by distinct constriction	Anterior lip slightly extended to form small flap	Very long, conoid, often ventrally bent with variable terminus (rounded or slightly digitate, usually curved and hook-like)	Relatively long, centre of capitulum depressed; rostrum prominent, pointed; apex small, rounded (often dorsally bent like a small hook); no cucullus	Single ventral pre-anal papilla; one pair pre-anal; one pair ventral post-anal; one small ventral pair at beginning of bursa
B. xylophilus	Fine	4	Rounded, set off by constriction	Anterior lip forming long flap overlapping vulva	Subcylindrical with rounded terminus occasionally bearing short mucron	Large, limb long and strongly curved with transverse bar of capitulum almost parallel to shaft axis; apex bluntly rounded; rostrum prominent and pointed; distinct disc-like cucullus	Single ventral pre-anal papilla; one pair adanal and two contiguous post-anal pairs
Bursaphelenchus sp. 1	Fine	3	High, rounded, slightly set off by weak constriction	Anterior lip forming a small flap	Conoid, narrowing abruptly just behind anus and gradually tapering to pointed terminus	Rosethorn-shaped not strongly curved; rostrum prominent, more or less pointed; apex rounded, almost as long as rostrum; distinct cucullus absent	Single ventral pre-anal papilla; two subventral pairs, one adanal; one post-anal near tail terminus

[*] *B. pinophilus* or *B. sexdentati* (subject to confirmation; see Discussion for more details).

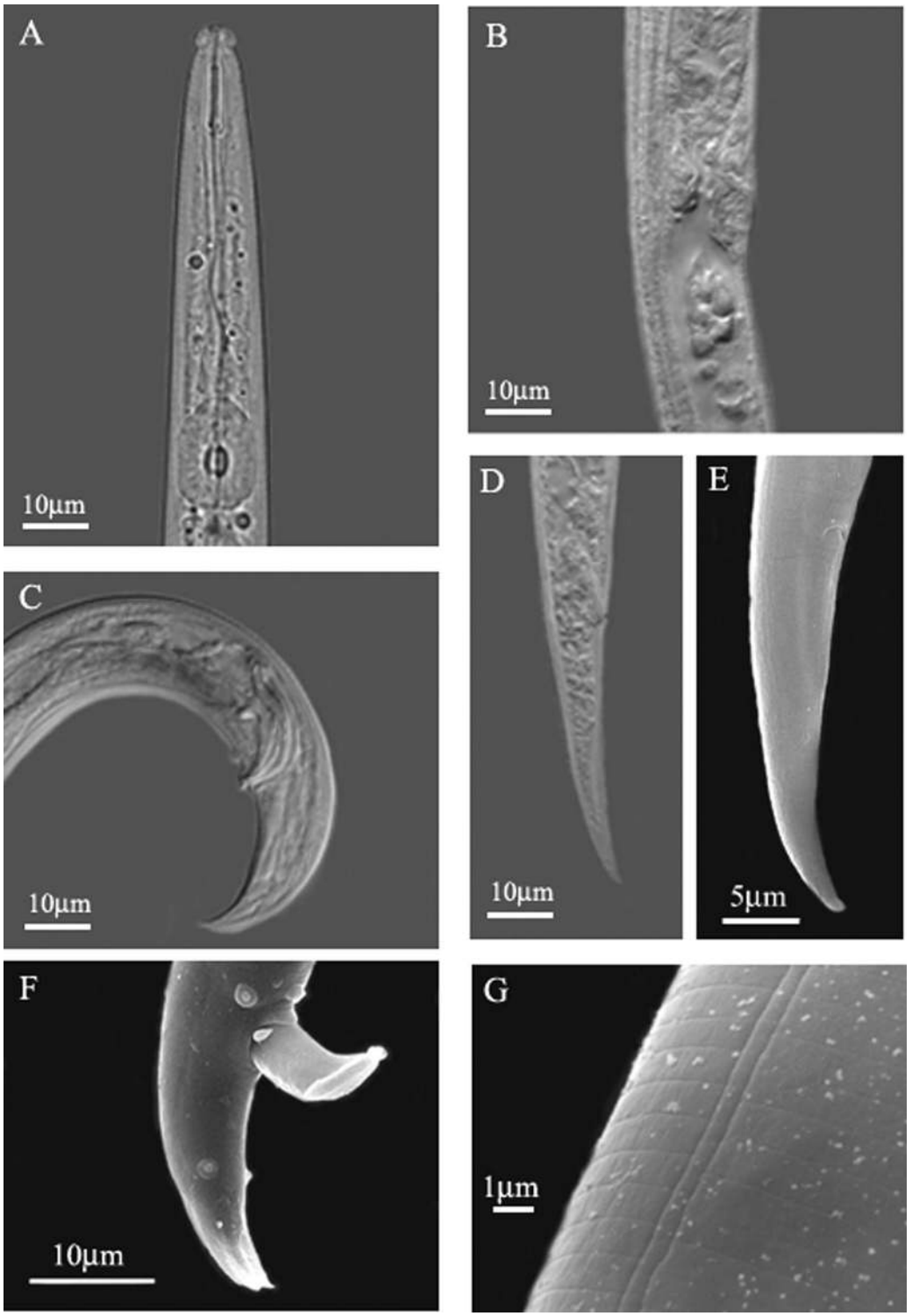

Fig. 3. Bursaphelenchus hylobianum. *A: Light micrograph (LM) of anterior region; B: LM of vulval region; C: LM of male tail; D: LM of female tail; E: Scanning electron micrograph (SEM) of female tail; F: SEM of male tail with protracted spicule and caudal papillae; G: SEM of lateral field.*

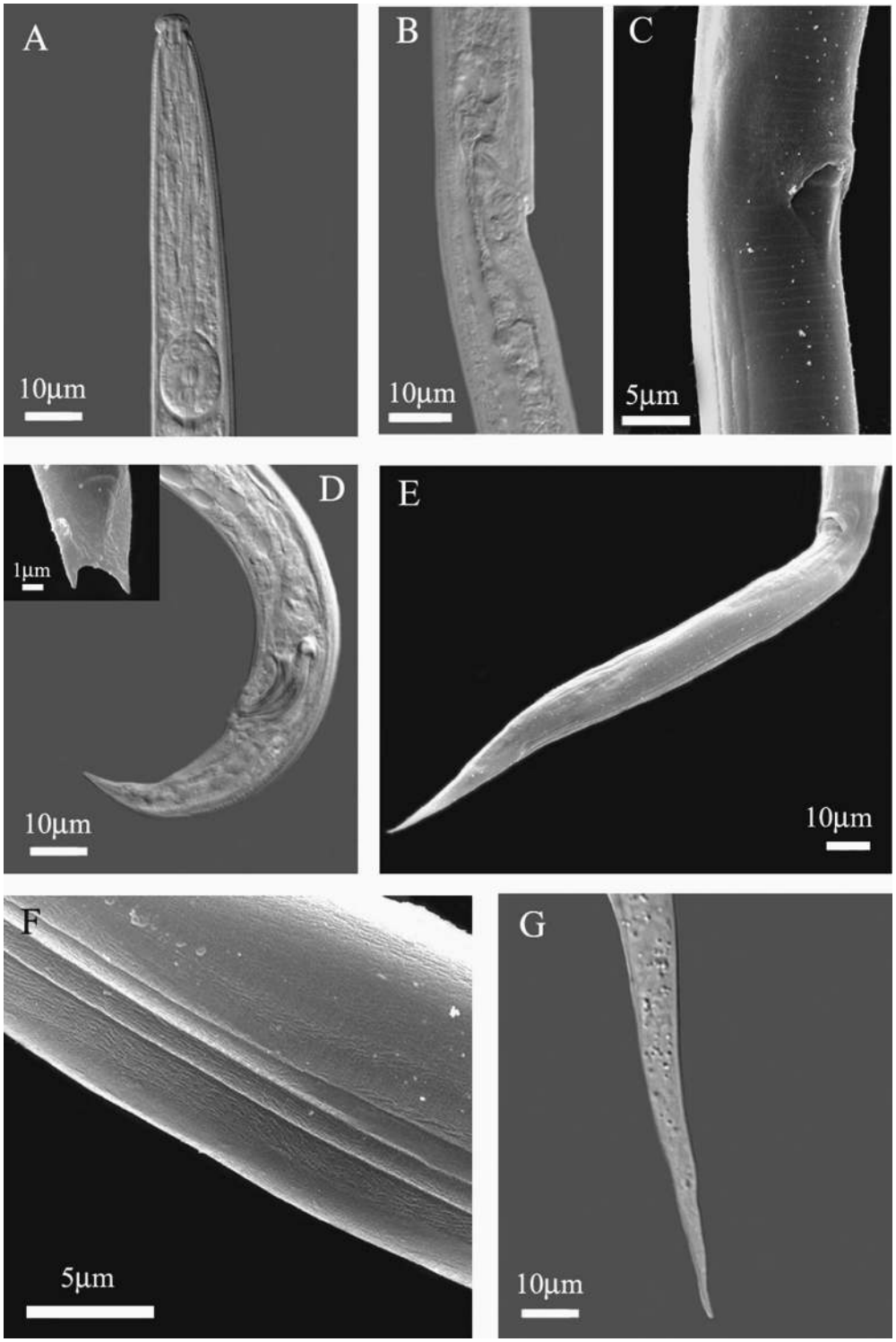

Fig. 4. Bursaphelenchus leoni. *A: Light micrograph (LM) of anterior region; B: LM of vulval region; C: Scanning electron micrograph (SEM) of vulval region; D: LM of male tail; inset: SEM of bursa; E: SEM of female tail; F: SEM of lateral field; G: LM of female tail.*

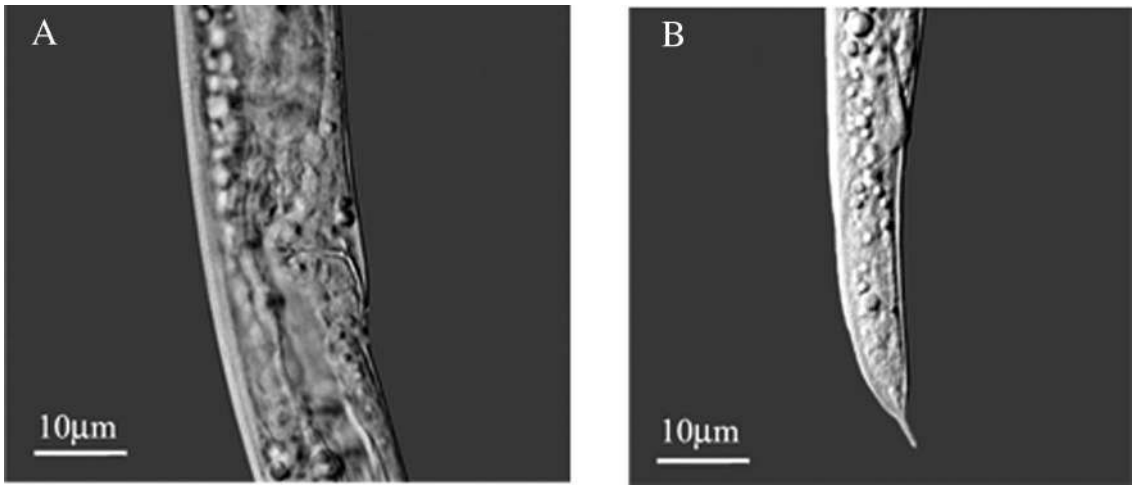

Fig. 5. Bursaphelenchus mucronatus. *A: Light micrograph (LM) of vulval region; B: LM of female tail.*

Table 3. *Number of wood samples containing species of* Bursaphelenchus *within the National Survey programme and the Demarcated Zone.*

Bursaphelenchus species	National Survey (1999-2002) 1129 samples	Demarcated Zone (1999-2003) 3681 samples
B. hellenicus	1	4
B. hylobianum	0	0
B. leoni	30	15
B. mucronatus	1	0
B. sexdentati	5	–
Bursaphelenchus spp.*	24	17
B. teratospiculatis	4	11
B. tusciae	7	10
B. xylophilus	0	1422**
Bursaphelenchus sp. 1	3	134

* *B. pinophilus* or *B. sexdentati* (subject to confirmation; see Discussion for more details).

** Results of a partial survey; for more detailed information see: http://www.dgf.min-agricultura.pt/prolunp/html/home-final.htm

Discussion

The greater number of *Bursaphelenchus* species found in northern and central Portugal reflect the higher density of maritime pine forest from those regions whereas the large number of species within the demarcated zone simply reflects a more intensive survey.

Tail and head shape, appearance of the vulval region and particularly the shape and comparative size of the spicules were sufficient for the separation of most species. The presence of round-tailed females with a well developed vulval flap, together with males having the typical spicule shape, provided a definitive morphological identification for *B. xylophilus*.

Differentiation based solely on morphological characters is not reliable for some species. Some Portuguese *B. xylophilus* populations have mucronate-tailed females, a feature which may result in confusion of this species with *B. mucronatus*. ITS-RFLP analysis, however, discriminates the mucronate form of *B. xylophilus* from *B. mucronatus* (Tarès *et al*., 1992).

Concerning the differentiation of the species *B. sexdentati* and *B. pinophilus*, Rühm (1960), in the original description of *B. sexdentati*, did not mention the presence of a cucullus. Brzeski and Baujard (1997) did not diagnose *B. pinophilus* in comparison to *B. sexdentati*, although Braasch (2001) distinguished the two species on the basis of the presence of a cucullus in *B. pinophilus*. However, using SEM techniques, Ambrogioni and Caroppo (1998) showed *B. sexdentati* to have a distinct cucullus. Although, most of our populations exhibited spicules with a distinct cucullus, something which would seem to indicate that they belong to *B. pinophilus*, ITS-RFLP analysis of these same populations produced the characteristic pattern for *B. sexdentati*. In addition to clarifying morphological identification of nematodes, this molecular method also facilitates the identification of juvenile stages of *Bursaphelenchus* species, thereby eliminating the need for

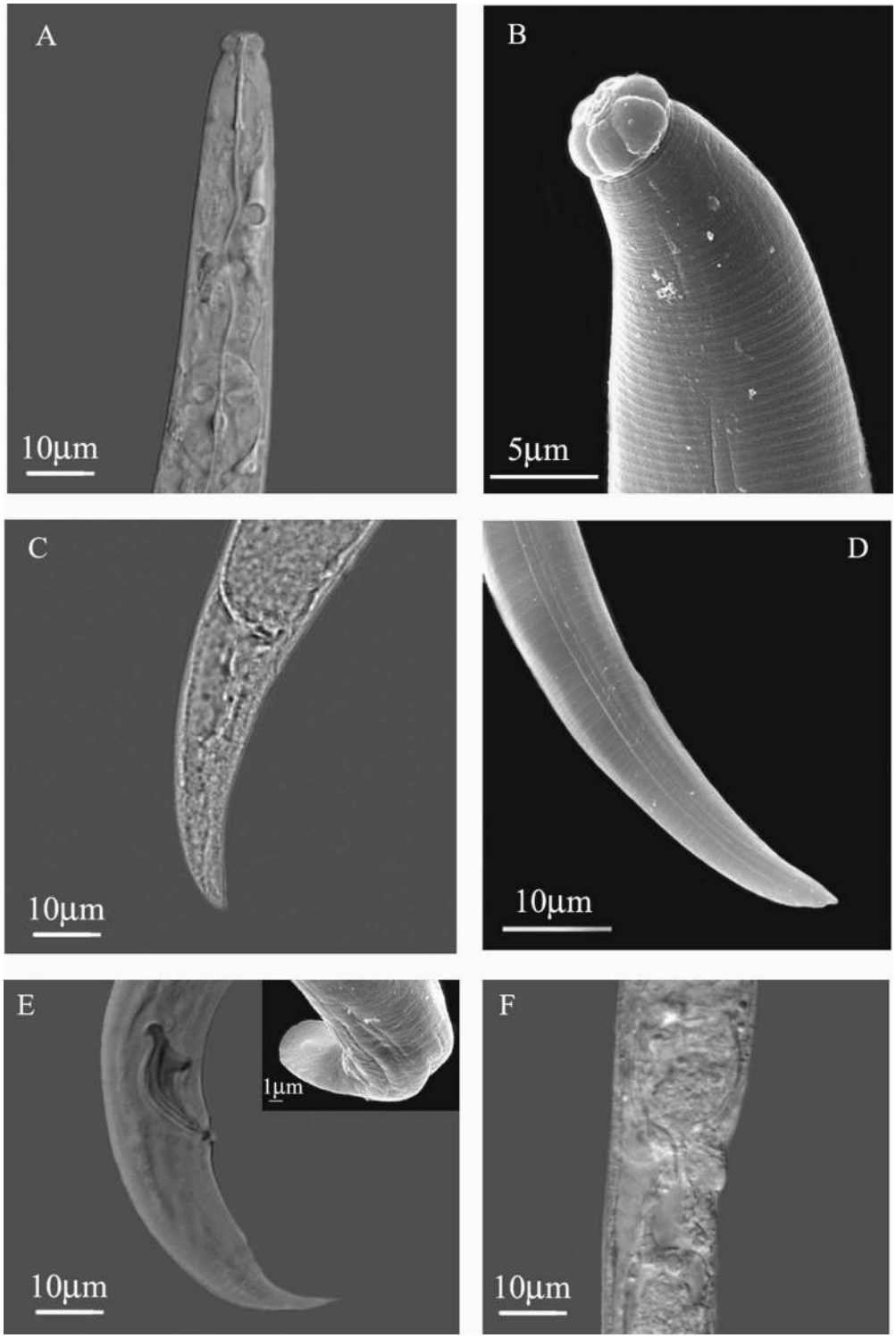

Fig. 6. Bursaphelenchus sexdentati. *A: Light micrograph (LM) of anterior region; B: Scanning electron micrograph (SEM) of anterior region; C: LM of female tail; D: SEM of female tail; E: LM of mail tail (stained spicule); inset: SEM of bursa; F: LM of vulval region.*

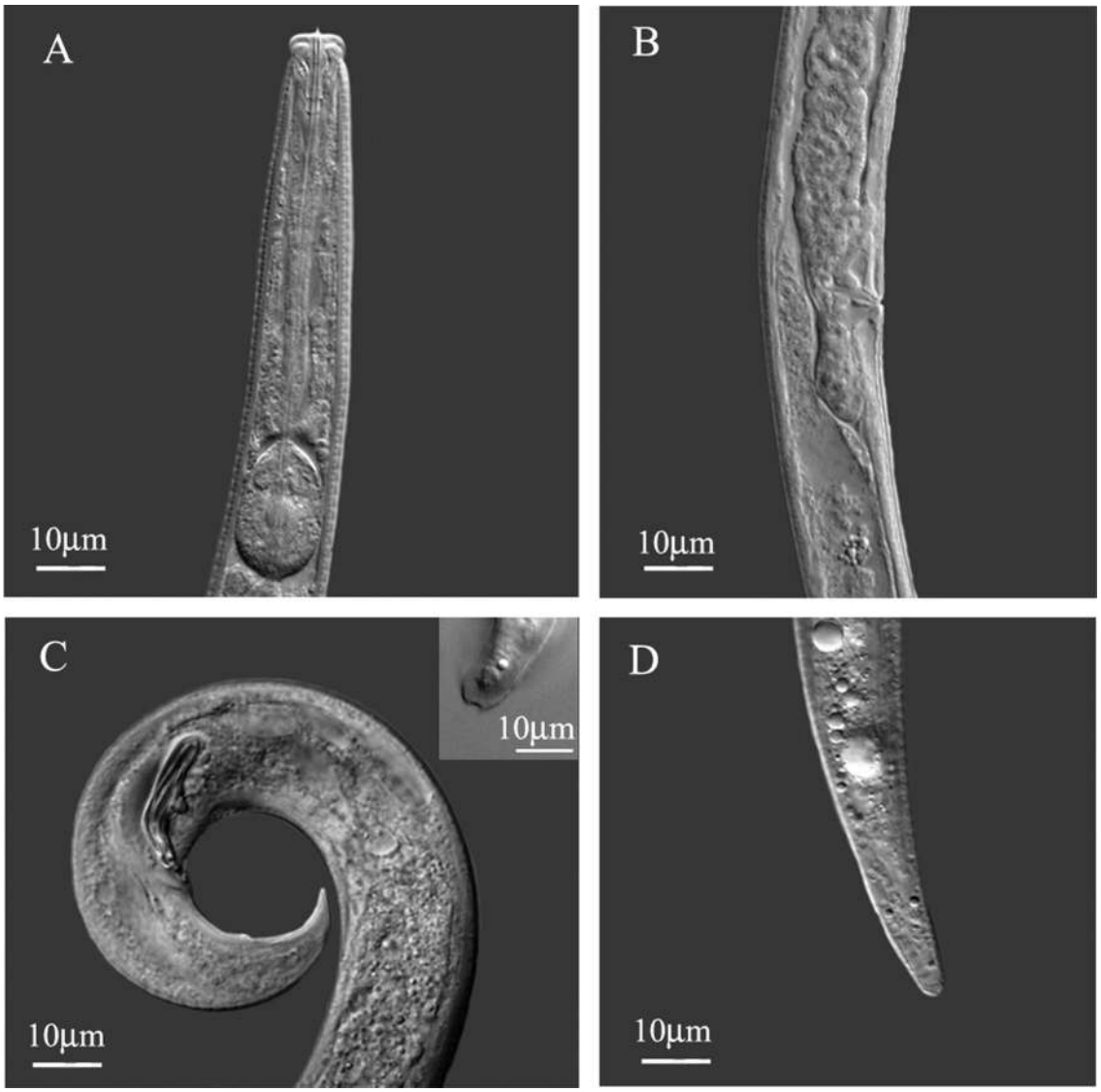

Fig. 7. Bursaphelenchus teratospicularis. *A: Light micrograph (LM) of anterior region; B: LM of vulval region; C: LM of male tail; inset: LM of bursa; D: LM of female tail.*

culturing to the adult stages and increasing both the speed and reliability of the diagnosis. Furthermore, considering the abundance of rDNA in the genome, this method can be effectively applied to individual nematodes, a significant advantage.

As confirmed by the results of this investigation, ITS-RFLP analysis is a more direct approach for identification of *Bursaphelenchus* species, since specific patterns of restriction fragments have been obtained for each of the five species studied. Although preliminary species determination can be reached by estimation of the number and size of the restriction fragments obtained (Braasch *et al*., 2001), the use of one reference sample for each species under consideration is strongly recommended. In fact, apart from some inaccuracy that may occur in the estimation of fragment size under different electrophoretic conditions, unoptimised restriction conditions may lead to partial or incomplete digestions making it difficult for correct species allocation of otherwise unidentified isolates.

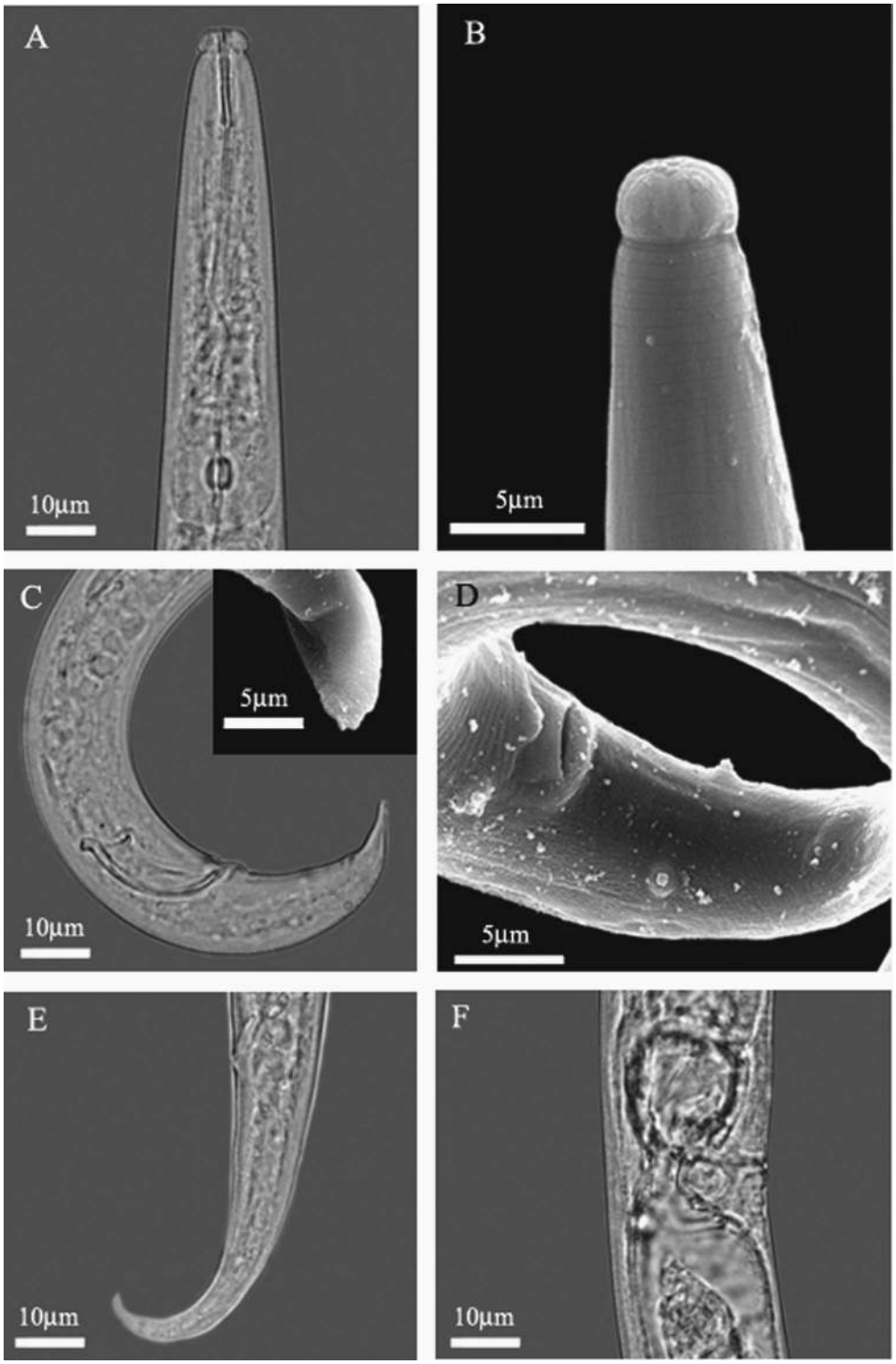

Fig. 8. Bursaphelenchus tusciae. *A: Light micrograph (LM) of anterior region; B: Scanning electron micrograph (SEM) of anterior region; C: LM of male tail; inset: SEM of bursa; D: SEM of male tail showing caudal papillae; E: LM of female tail; F: LM of vulval region.*

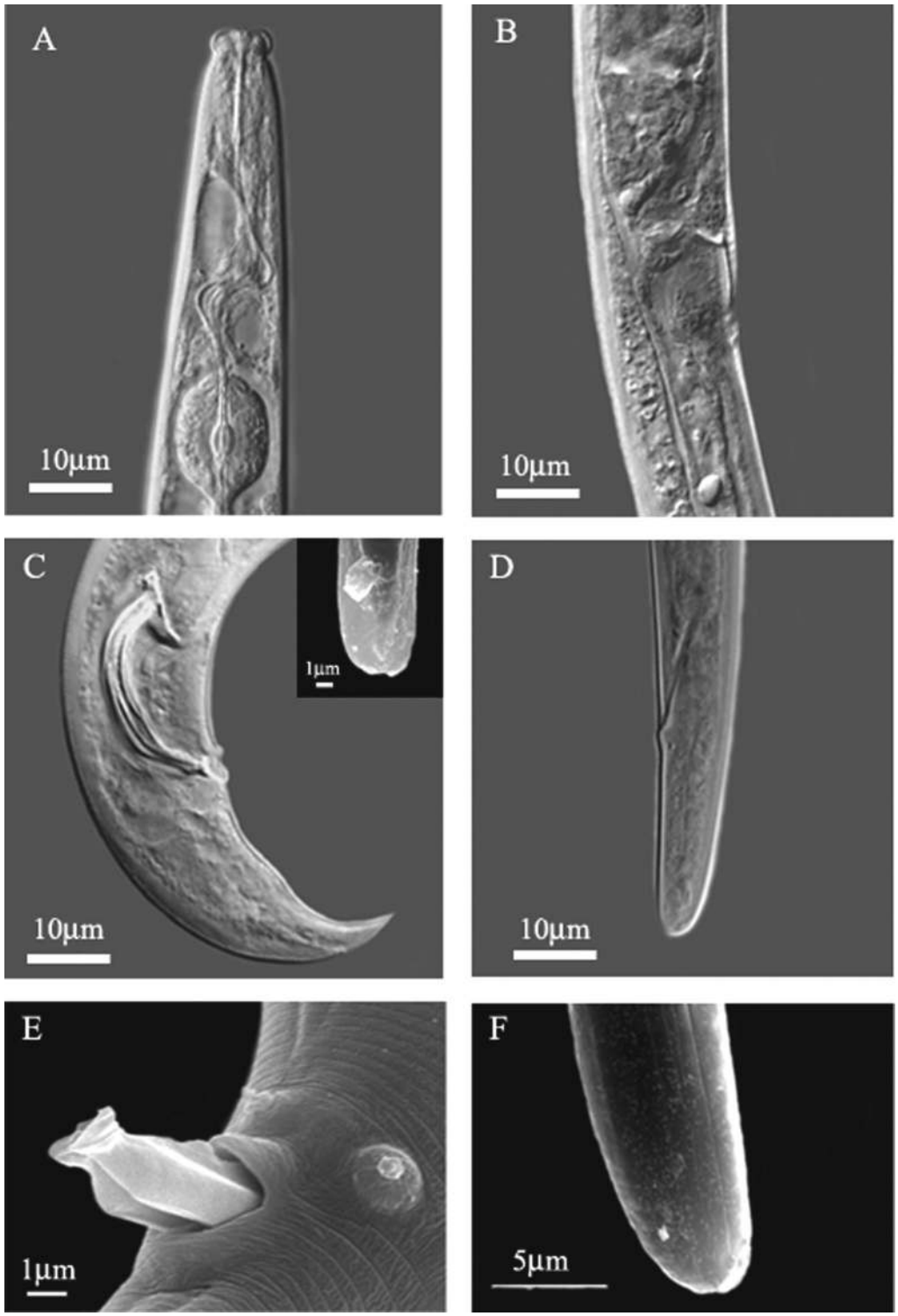

Fig. 9. Bursaphelenchus xylophilus. *A: Light micrograph (LM) of anterior region; B: LM of vulval region; C: LM of male tail; inset: Scanning electron micrograph (SEM) of bursa; D: LM of female tail; E: SEM of protracted spicule and cucullus; F: SEM of female tail tip.*

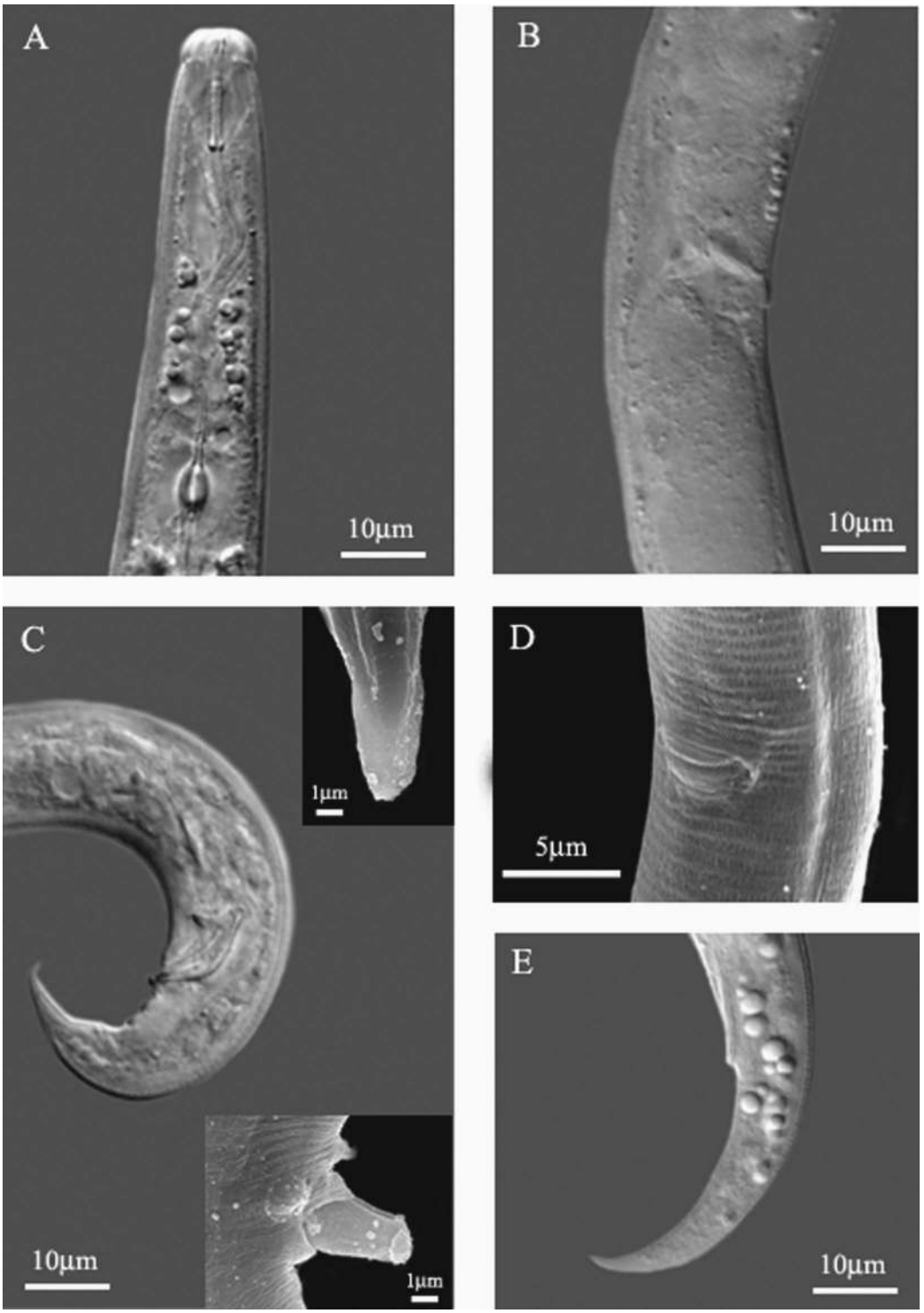

Fig. 10. Bursaphelenchus *sp. 1. A: Light micrograph (LM) of anterior region; B: LM of vulval region; C: LM of male tail; inset: Scanning electron micrograph (SEM) of bursa (top) and SEM of spicule protracted and cucullus (bottom); D: SEM of vulval region; E: LM of female tail.*

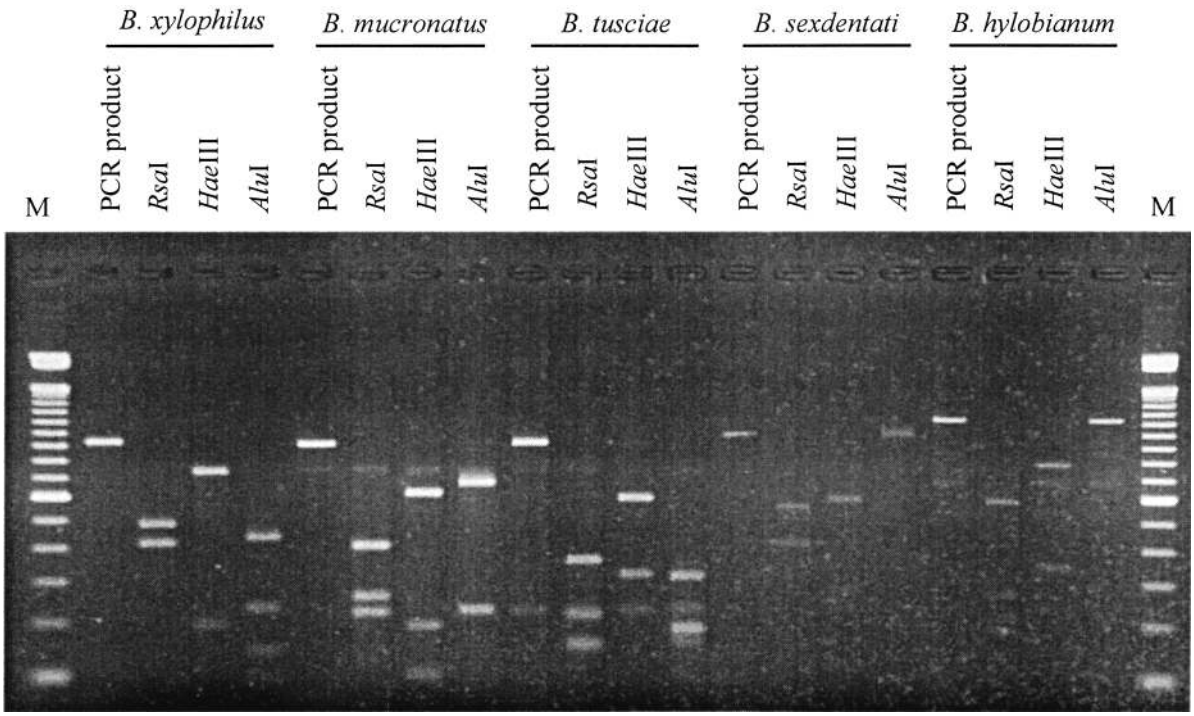

Fig. 11. *ITS-RFLP patterns of* Bursaphelenchus *isolates. Restriction fragments were obtained by digestion of the amplified ITS region of rDNA with the three named enzymes. M: 100 bb Marker (Invitrogene).*

Regardless of the underlying structural basis of polymorphism among populations, it is apparent that these genetic differences can discriminate within populations and are convenient diagnostic markers. Overall, ITS-RFLP has been established as a powerful and reproducible tool in the differentiation of many *Bursaphelenchus* species (Hoyer *et al*., 1998; Braasch *et al*., 1999) and has become an important feature in nematode diagnosis. ITS versatility, specificity, ease of experimental manipulation and availability of ITS databases should be increasingly applied in nematology.

This study, based on both morphological and molecular analysis, confirmed the identity of several *Bursaphelenchus* species reported from Portugal for the first time by Penas *et al*. (2002). Penas *et al*. (2002) had identified *B. hofmanni* from Portugal, although more recent studies have called into question the reliability of this initial identification, the species now being referred to as *Bursaphelenchus* sp. 1.

The ITS-RFLP patterns, as presented in this paper, were done whenever nematode numbers allowed for such analysis, or when morphological identification was doubtful. A more detailed characterisation of all Portuguese species, including morphology, morphometrics and DNA analysis is being conducted and will soon be published. *Bursaphelenchus hylobianum*, found for the first time in Europe (Penas *et al*., 2002) and never previously observed in pine wood from natural stands, was successfully reared in maritime pine branch segments and may therefore be considered as a potential associate of *Pinus pinaster*. Other *Bursaphelenchus* species, in addition to *B. hylobianum*, were found associated with potential insect vectors (unpubl.).

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