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SHORT COMMUNICATION

On the track of *Bursaphelenchus pinophilus* Brzeski and Baujard, 1997 (Nematoda: Aphelenchoididae) in Portugal

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Summary

This is the first report and characterization of *Bursaphelenchus pinophilus* in Portugal. This species was isolated from a young dying *Pinus pinaster* tree located in Valverde, in the Alentejo region. Nematodes were identified using several morphological diagnostic characters for this species (male spicule structure, number of lateral incisures, number and distribution of the male papillae, presence of female vulval flap and female tail shape) and confirmed using RFLP analysis of the internal transcribed spacer (ITS) regions of ribosomal DNA.

1 Introduction

In Portugal, the distribution of the genus *Bursaphelenchus* has been studied in detail for the past years (Mota et al. 1999; Penas et al. 2004, 2006a,b, 2007). Up to date, nine species have been reported and characterized both morphologically and molecularly, namely *B. hellenicus* Skarmoutsos, Braasch and Michalopoulou, 1998, *B. leoni* Baujard, 1980, *B. mucronatus* Mamiya and Enda, 1979, *B. pinasteri* Baujard, 1980, *B. sexdentati* Rühm, 1960, *B. teratospicularis* Kakuliya and Devdariani, 1965, *B. tusciae* Ambrogioni and Palmisano, 1998, *B. xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970, including a new species for the genus, *B. antoniae* Penas, Metge, Mota and Valadas, 2006. *Bursaphelenchus fungivorus* (Franklin & Hooper, 1962) Nickle, 1970, and *B. minutus* Walia, Negi, Bajaj and Kalia, 2003, have been also cited to Portugal but a detailed characterization will be presented in the future (Fonseca et al. 2012). In addition to these species, *B. pinophilus* Brzeski and Baujard 1997; has been stated to also occur in Portugal (A. C. Penas and H. Braasch, unpublished data, in Braasch 2001). However, the occurrence and distribution of this species in Portugal have remained ambiguous (see Penas et al. 2004, 2007), as this species morphologically resembles other species of the *sexdentati* group (e.g. *B. sexdentati*), no confirmation of the actual status has been provide so far, either by morphological or by molecular characterization. Recently, several *Bursaphelenchus* specimens were found associated with a young declining maritime pine tree (*Pinus pinaster* Aiton), growing in an oak tree stand (*Quercus suber* L.) located near the University of Évora campus, in Valverde (Alentejo region, Portugal). Our morphological and molecular analyses identified these specimens as *B. pinophilus* and thereby confirming the occurrence of this species in Portugal.

2 Material and methods

2.1 Wood sampling and nematode extraction

Wood samples (50 g each) were collected from different levels of the main trunk of a maritime pine tree displaying strong wilting symptoms, using a low-speed drill and kept in plastic bags until nematode extraction. Nematodes were extracted using the 'tray' method (Whitehead and Hemming 1965) and processed within 48 h. The nematodes obtained were used to produce temporary slides, and specimens were identified according to the main diagnostic characters of the genus *Bursaphelenchus* (Ryss et al. 2005; Braasch et al. 2009).

2.2 Molecular analysis

DNA extraction was carried out using a minimum of 10 specimens collected directly from wood samples, as described in Vieira et al. (2007). 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). The PCR mixture (total volume 25 μl) contained 1X buffer enzyme with $MgCl_2$, 0.5 mM $MgCl_2$, 0.6 μM of each primer, two units Taq DNA polymerase (Fermentas), 0.1 mM dNTPs (Fermentas) and 4 μl of DNA extract. A Biometra thermocycler was used for amplification, in agreement with the following PCR conditions: one initial denaturation step at 94°C for 2.5 min, followed by 40 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final extension step of 5 min 72°C. To generate the ITS-RFLP profiles, suitable aliquots of the amplified ITS region were digested for at least 3 h at 37°C using 2.5 U of each of the five restriction endonucleases (*Rsa*I, *Hae*III, *Msp*I, *Hin*fI and *Alu*I). These five restriction enzymes are known to generate species-specific ITS-RFLP profiles for the species of this genus (Burgermeister et al. 2009). Fragments were resolved by electrophoresis in a 2% agarose gel and stained with ethidium bromide.

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3 Results and discussion

Morphologically, the specimens collected (Fig. 1) followed the main characters of the original description of *B. pinophilus* (Brzeski and Baujard 1997). The males presented stout spicules (Fig. 1c, d) with well-developed rostrum, variously shaped condyles, with small cucullus, four lateral lines and seven caudal papillae (single ventral pre-anal papilla, one pair subventral adanal and two post-anal pairs); females presenting the anterior vulva lip slightly extended to form a small flap (Fig. 1 b), with a post-vulval swelling often present, tail conical terminally pointed, with more or less pronounced mucron (Fig. 1e–g). The ITS-RFLP patterns obtained (Fig. 2) are in agreement with the reference patterns established for this species (Burgermeister et al. 2009), complementing the morphological identification.

This species was initially described associated with *P. sylvestris* from Poland (Brzeski and Baujard 1997). Afterwards, this species has been found sporadically reported associated with *P. sylvestris* in Germany (unpublished data, in Braasch 2001), *P. nigra* in Turkey (Akbulut et al. 2008), *P. koraiensis* in Korea (Han et al. 2009) and *P. sylvestris* in the Czech Republic (Čermák et al. 2013).

The only known insect vector identified for this species is the bark beetle, *Pityogenes bidentatus* (Herbst, 1783) belonging to the subfamily Scolytinae (Čermák et al. 2012). In Portugal, this insect species has been associated with declining maritime pine trees, distributed predominantly in the branches and main trunk of the tree and often occurring in

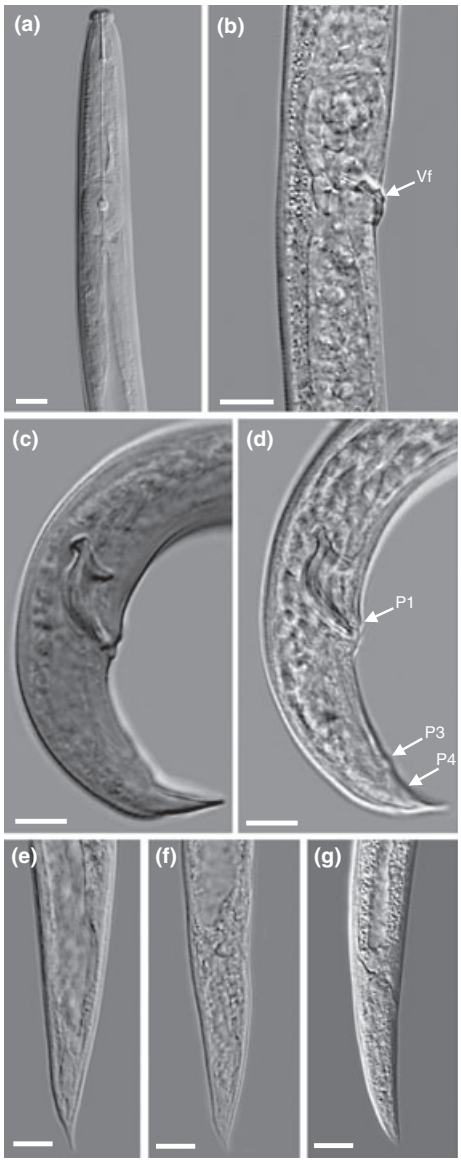

Fig. 1. Light micrograph of *Bursaphelenchus pinophilus*. (a) Anterior region. (b) Vulva region. Arrow points out the vulval flap (Vf). (c-d) Male tail in lateral view (P1, pre-anal single papilla, P3 and P4, post-anal papillae). (e-g) Variability of female tails (tail conical terminally pointed, with more or less pronounced mucron). Scale bars = 10 μm.

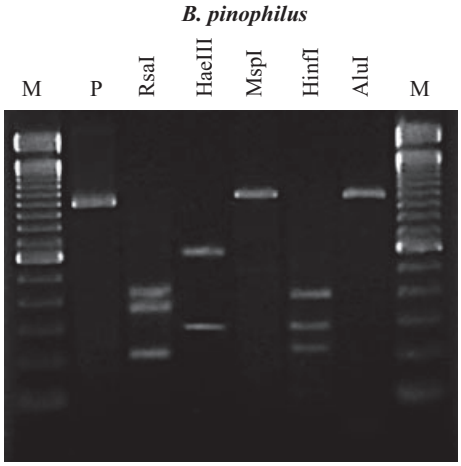

Fig. 2. ITS-RFLP profiles of *B. pinophilus*. Restriction fragments were obtained by digestion of the amplified rDNA fragment with five restriction enzymes (*Rsa*I, *Hae*III, *Msp*I, *Hinf*I and *Alu*I). M, molecular marker (100 bp ladder, Invitrogen Life Technologies). P, PCR product originated by the specific primers used herein.

association with the attack by other Scolytinae (Coleoptera: Curculionidae) species (Sousa et al. 2007). Interestingly, the nematode specimens found were collected from a pine tree displaying several insect holes and galleries (star-shaped galleries, typical of *P. bidentatus*); however, we were unable to collect any insect species from this tree and confirm the inherent insect vector. The association of *B. pinophilus* with declining or dying pine trees displaying insect attacks could suggest this species as a secondary agent on these dead trees. Surprisingly, the potential pathogenicity of other morphologically close species belonging to the *sexdentati* group (e.g. *B. vallesianus*) has been demonstrated in young *P. sylvestris* trees (Polomski and Rigling 2010). The pathogenicity effects of *B. vallesianus* were correlated with the increased drought water treatments, which could reach up to 100% tree mortality (Polomski and Rigling 2010). The levels of pathogenicity of *B. pinophilus* on pine trees still remain unclear as no pathogenicity tests have ever been performed for this species. Nevertheless, the potential threat of *B. pinophilus* (or other species belonging to the *sexdentati* group) to young pine trees should be taken into account in the future, especially in pine forest areas where these species are known to occur naturally.

Additionally to the above-mentioned *Bursaphelenchus* species found and characterized in Portugal, this study confirms the first record of *B. pinophilus* in the country.

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