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Research Papers

Aphelenchoididae nematodes in a centennial *Pinus pinea* tree, and a review of *Bursaphelenchus* species from this host

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Summary. A "monumental" centennial *Pinus pinea* L. tree of public interest with severe wilting symptoms was felled in Coimbra, Portugal. A survey was carried out to detect *Aphelenchoididae* nematodes (including *Bursaphelenchus* spp.) in the tree tissues. Nematode isolates were characterised/identified based on species-specific morphological characteristics, and on molecular data. *Bursaphelenchus xylophilus* was not detected. Three other *Bursaphelenchus* species (*B. arthuri*, *B. fungivorus* and *B. sexdentati* Type II) were found co-occurring in the tree. Other *Aphelenchoididae* nematodes, *Potensaphelenchus stammeri* and *Cryptaphelenchus* sp., were also identified. An annotated checklist of *Bursaphelenchus* spp. on *P. pinea* is also presented, demonstrating the wide variability of *Bursaphelenchus* species in this host. These results have shown that centennial wilted pine trees can be reservoirs of nematode diversity impacting forest health.

Keywords. Nematode diversity, stone pine, urban tree.

INTRODUCTION

Centennial trees play important ecological roles contributing to climate change mitigation by sequestering large amounts of carbon (Stephenson *et al*., 2014). Their unique dendrometric characteristics, such as cavities, dry and hollow branches, intricate crowns, and complex branching, also create biodiversity hotspots that support a wide variety of organisms, including fungi, insects, plants, bacteria, and nematodes (Lindenmayer, 2017; Paillet *et al*., 2017; Azuma *et al*., 2022). Beyond their ecological significance, these trees can hold historical, cultural, and religious values (Dafni, 2006; Lopes *et al*., 2019; Rivers *et al.*, 2022). In recent years, however, many of these trees in Europe have been lost due to natural and human-induced factors such as fires, invasive plant species, abandonment, improper pruning, a lack of protective legislation, or pathogens (San-Miguel-Ayanz *et al.*, 2012; Stara and Tsiakiris, 2019).

In 1938, Portugal introduced the "Trees of Public Interest Law," establishing one of the earliest tree protection laws in Europe. Over the course of the 20th century, this law evolved, leading to the creation of a National Registry, which catalogues and classifies 100-year-old trees of public interest as "monumental trees" and "living monuments" (Lopes *et al.*, 2019). Due to their age and size, upon their senescence and eventual death, these may become reservoirs for invasive pathogens, such as bacteria, fungi, insects, and nematodes (Stara and Tsiakiris, 2019). To prevent the spread of these pathogens to adjacent areas, dead and affected trees are removed from the field.

The present study examined a classified "monumental" centennial stone pine (*Pinus pinea* L.) with severe wilting symptoms, felled at the Health Sciences Campus of the University of Coimbra, Portugal. *Pinus pinea* is a medium-sized coniferous tree widely distributed across the Mediterranean basin, from Portugal to Syria. It is most abundant in southwestern Europe, particularly in the Iberian Peninsula, southern France, and Italy (Viñas *et al.*, 2016).

The primary economic value of *P. pinea* are its nutritious and edible seeds (pine nuts), with secondary uses in furniture and rosin production. Additionally, stone pine trees stabilise sand dunes, and are commonly used as ornamental trees in warm European regions. As for other Mediterranean pines, stone pine is vulnerable to forest fires but is considerably less fire-sensitive than other Mediterranean pines due to its thick bark and high crown without low branches. Moreover, *P. pinea* is a slow-growing species with some resistance to pests and diseases (Viñas *et al.*, 2016). Nevertheless, it is susceptible to several fungal diseases, including blister rust (*Cronartium flaccidum*), twisting rust (*Melampsora populnea*), needle rust (*Coleosporium tussilaginis*), diplodia tip blight (*Diplodia sapinea*), and Pestalotiopsis disease (*Pestalotiopsis pini*) (Fady *et al.*, 2004; Viñas *et al.*, 2016; Silva *et al.*, 2020). Severe damage can also be caused by insects such as boring beetles (*Ernobius* spp.), snout moths (*Dioryctria* spp.), pine tortoise scale (*Toumeyella parvicornis*) and western conifer seed bug (*Leptoglossus occidentalis*) (Bracalini *et al.*, 2013; Özçankaya *et al.*, 2013; EPPO, 2015; Viñas *et al.*, 2016; Sousa *et al.*, 2017; Farinha *et al.*, 2018).

Regarding the species *Monochamus galloprovincialis*, the European insect vector of the quarantine pinewood nematode (PWN), *Bursaphelenchus xylophilus*, the European pine species *P. pinaster* Aiton (maritime pine), *P. nigra* J. F. Arnold (black pine), *P. sylvestris* L. (Scots pine), and *P. halepensis* Mill. (Aleppo pine) are also hosts of this insect (EPPO, 2024). For *P. pinea*, laboratory tests have shown that adult *M. galloprovincialis* feed on fresh shoots, but this host was the least preferred species among other European pines tested, with few eggs laid, and few or no young adults emerging (Sanchez-Husillos *et al.*, 2013; Naves *et al.*, 2006). Furthermore, *P. pinea* volatile compounds are not attractive for *M. galloprovincialis* that is particularly attracted to alpha-pinene. Compared to other European pines, *P. pinea* has a greater proportion of limonene and consequently lower levels of alpha- and beta-pinene, which may explain its reduced attractiveness to insects (Pajares *et al.*, 2004; Ibeas *et al.*, 2007; Rodrigues *et al.*, 2017; Gaspar *et al.*, 2020; van Halder *et al.*, 2022).

Pines susceptibility to PWN infection varies among species, with *P. pinea* being one of the less susceptible species (EPPO, 2024). Artificial PWN inoculation assays on *P. pinea* seedlings have consistently demonstrated its low susceptibility (Nunes da Silva *et al.*, 2015; Menéndez-Gutiérrez *et al.*, 2017; Pimentel *et al.*, 2017; Estorninho *et al.*, 2022). Contrasting to other European pine species, *P. pinea* showed almost no wilting symptoms and maintained physiological performance under stressful conditions such as high temperatures and low water availability (Estorninho *et al.*, 2022). Resistance to PWN infection could be linked to drought-resistance of *P. pinea*, which is associated with slow growth rates, constitutive defences and high production of phenolics and tannins (Awada *et al.*, 2003; Tapias *et al.*, 2004; Pimentel *et al.*, 2017).

Santos *et al.* (2012), using high-throughput sequencing, analysed the responses of *P. pinaster* and *P. pinea* to PWN infection, and showed that there was upregulation of genes involved in activating plant defence mechanisms, including general stress response genes such as ricin B-related lectin, phytoalexins like chalcone synthase, antioxidant enzymes (e.g. malic oxidoreductase), and disease resistance proteins that protect against a variety of plant pathogens. Resistant pine species demonstrate defence mechanisms including reactive oxygen species (ROS) detoxification, increased cell wall lignification related genes, and the production of secondary metabolites (polyphenols, terpenes flavonoids, stilbenoids, tannins), which hinder PWN mobility and survival (Modesto *et al.*, 2022; Rodrigues *et al.*, 2024).

Although Portugal has extensive stone pine forests, no cases of PWN infection or significant attacks of *M. galloprovincialis* have been reported on *P. pinea* in the field. However, other *Bursaphelenchus* species, which are mycetophagous and insect-dispersed, have been previously recorded to be associated with *P. pinea*, including *B. hylobianum*, *B. leoni*, *B. minutus*, *B. pinasteri*, *B. sexdentati*, *B. teratospicularis* and *B. tusciae* (Ryss *et al.*, 2005; Errico *et al.*, 2015).

In the present study, a dendrochronological analysis of the classified "monumental" centennial *P. pinea* tree, which had severe wilting symptoms, was carried out by determining the tree age and the *Aphelenchoididae* diversity within the tree. Five nematode isolates were obtained from this tree and characterised using morphological and molecular characterisation. An updated checklist of *Bursaphelenchus* spp. reported on *P. pinea* is also presented in this paper.

MATERIALS AND METHODS

Dendrochronological characterisation of the Pinus pinea *tree*

The "monumental" *P. pinea* displaying severe wilting symptoms (Figure 1 A) was felled at the Health Sciences Campus (40°13'07"N, 8°25'02"W) of the University of Coimbra, Portugal.

A circular cross-section from the base of the main trunk (Figure 1 B) was cut from the felled tree with a chainsaw for dendrochronological examination. A mechanical hand-held circular sander was used to prepare the wood surface along the longest section radius (Figure 1 B) until the tree-ring limits were clearly visible. Tree rings were visually identified using standard dendrochronology methods (Stokes and Smiley, 1996), and the age of the tree was determined.

Wood sampling

Wood samples (100 g each) were collected from four different zones of the trunk, using an electric drill with a 20 mm diam. bit, and several branches were cut into small pieces (≈ 1 cm) using pruning scissors (EPPO, 2023). The samples, from the trunk and branches, were combined for nematode extraction.

Nematode extraction and nematode isolate establishment

Nematodes were extracted from the wood samples using the tray method (Whitehead and Hemming, 1965; EPPO, 2023) and were observed under an Olympus CKX41SF inverted stereomicroscope (Olympus). Ten nematodes (males and females), exhibiting the same morphological characteristics, were selected, individually picked, and transferred to malt extract agar (MEA) plates colonised with *Botrytis cinerea*. The plates were then incubated at 25°C, and five nematode isolates were established. These were maintained by transferring small culture plugs containing nematodes to new MEA plates colonised with *B. cinerea* at 3-week intervals (Fonseca *et al.*, 2008). Nematodes from the five isolates were collected by washing the *B. cinerea* plates with sterilised distilled water, for subsequent morphological and molecular characterisation.

Figure 1. (A) The *Pinus pinea* tree exhibiting severe wilting symptoms at Health Sciences Campus of the University of Coimbra, Portugal. (B) Main trunk cross section of the tree.

Morphological characterisation

Female and male nematodes from each isolate were individually selected and placed on glass microscope slides in drops of water. The slides were heated (to kill the nematodes), and the nematodes were immediately photographed with a microscope DM 2500; Leica, using a digital camera (DFC 450; Leica). Nematode genus identification was based on the morphological characters of females and males.

Molecular characterisation/identification

DNA extraction and amplification of ITS rDNA regions

Nematode DNA was extracted from mixed developmental nematode stages collected from MEA culture plates using the DNeasy Blood and Tissue Mini Kit (Qiagen), following the manufacturer's instructions, and were quantified using Nanodrop 2000C (ThermoScientific). The Internal Transcribed Spacer (ITS) rDNA regions containing partial 18S and 28S and complete ITS1, 5.8S and ITS2 sequences were amplified using 50 ng of extracted DNA and 1 U of Dream Taq DNA polymerase (Fermentas) in 1× Dream Taq buffer, 0.2 mM each dNTP, and 1 μM primers 18SF 5′-CGTAACAAGGTAGCTGTAG-3′ (Ferris *et al.* 1993) and 28SR 5′-TTTCACTCGCCGTTACTAAGG-3′ (Vrain, 1993). PCR reactions were carried out in a Thermal Cycler (Bio-Rad), with an initial denaturation step of 95°C for 2.5 min, followed by 40 reaction cycles each of 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min.

Restriction fragment length polymorphism analysis

The resulting amplification products were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel), according to manufacturer's instructions, and were used for restriction fragment length polymorphism (RFLP) analysis for *Bursaphelenchus* species characterisation/identification, according to Burgermeister *et al.* (2009) and EPPO (2023). The RFLP analysis of amplified ITS rDNA PCR products was carried out for isolates BfungPt2 and BsexPt2, using a combination of eight restriction endonucleases: *Afa*I, *Alu*I, *Hae*III, *Hinf*I, *Msp*I, *Mfe*I, *BsrB*I and *Hpy*188I (Amersham Biosciences), with restriction reactions performed according to the manufacturer's instructions. Restriction products were separated by electrophoresis on 2% agarose gel, fragment sizes were estimated via Hyper Ladder II (Bioline), and these were compared to previously described RFLP patterns. When the species identification was not possible from analysis of the RFLP patterns, the ITS rDNA PCR product was sequenced.

Sequencing and phylogenetic analyses

For the isolate CryPt1, which was morphologically characterised as *Cryptaphelenchus* sp., the ITS rDNA PCR product was sequenced in both strands in an Automatic Sequencer 3730xl under BigDye™ terminator cycling conditions at Macrogen Company (Seoul, Korea), using the same primers as used in the PCR. Sequence analyses were carried out using BioEdit (Hall, 1999). Homologous sequences in the databases were searched by BLAST (Altschul *et al*., 1997), and selected sequences were used for sequence alignment. Phylogenetic analyses were carried out in MEGA 11 (Tamura *et al*., 2021) by the Neighbor-Joining method (Saitou and Nei, 1987) with 1000 replications of bootstrap (Felsenstein, 1985) and Jukes-Cantor as substitution model (Jukes and Cantor, 1969), with all ambiguous positions removed for each sequence pair (pairwise deletion option), using the ITS rDNA sequence alignments.

RESULTS

Pinus pinea *dendrochronology*

The identification and quantification of the tree rings in the circular wood section from the base of the main trunk of the diseased tree indicated that the tree was of approx. 160 years old.

Nematode isolates

Five nematode isolates, corresponding to five different species, were designated as BaPt1 (*B. arthuri*); BfungPt2 (*B. fungivorus*); BsexPt2 (*B. sexdentati*); PsPt1 (*Potensaphelenchus stammeri*) and CryPt1 (*Cryptaphelenchus* sp.). These isolates have been maintained at the Laboratory of Nematology (NEMATO-lab), Department of Life Sciences, University of Coimbra, Portugal.

Morphological, morphometrical and molecular characterisation of PsPt1 were described by Silva *et al.* (2023), and of BaPt1 by Silva *et al.* (2024). Morphological and molecular characterisation/identifications of the other three isolates (BfungPt2, BsexPt2 and CryPt1) are presented below.

Morphological characterisation

Nematodes from the five isolates had main characteristic of *Aphelenchoididae* as described by Nickle (1970), including: i) body slender with variable length; ii) lips often slightly offset; iii) stylet with knobs ranging from absent, to slightly thickened, to well-developed; iv) oesophagus with overlapping glands; and v) spicules characteristically rosethorn-shaped with caudal papillae.

Nematodes of the isolates BfungPt2 and BsexPt2 had main morphological characters of the *Bursaphelenchus* genus (Ryss *et al*., 2015; EPPO, 2023), including: i) vermiform; ii) cephalic region offset by a constriction; iii) stylet length with weak basal knobs; iv) strong median bulb oval to quadrangular; v) oesophageal glands overlapping the intestine; vi) female with vulva posterior, with or without an anterior vulval flap, rounded tail or with a smooth or mucronate terminus; and vii) male with hook-like spicules with a prominent rostrum, and a capitulum, sometimes with a cucullus in the distal end, and a ventrally arched tail terminus.

The main morphological diagnostic characters of the nematodes of the isolate BfungPt2 were: i) cephalic region offset by a constriction with six lips; ii) stylet with weak basal knobs; iii) median bulb well developed (Figure 2 A); iv) males with ventrally curved tails with compact spicules without cucullus (Figure 2 B); v) females with vulvae without flaps and long ventrally bent tails (Figure 2, C and D). These characteristics are in accordance with those described for *B. fungivorus* (Franklin and Hooper, 1962; Ambrogioni *et al*., 2003; Arias *et al*., 2005; Oliveira *et al*., 2011; Fonseca *et al*., 2014; Torrini *et al*., 2020b).

The main morphological diagnostic characters for isolate BsexPt2 nematodes were: i) cephalic region offset by a constriction with six lips; ii) stylet with weak basal knobs, median bulb well developed (Figure 3 A); iii) male spicules each with a sharply pointed rostrum, and well-developed broadly truncate condylus with a small cucullus (Figure 3 B); and iv) females each with a small vulval flap and with a gradually tapering tail (Figure 3, C and D). These characteristics are in accordance with those previously described for *B. sexdentati* (Lange *et al*., 2006; 2008; Penas *et al*., 2008; Slonin *et al*., 2018). According to Lange *et al*. (2006) and considering the condylus and female tail shapes, isolate BsexPt2 belongs to *B. sexdentati* Type II (South European isolates).

Nematodes of isolate CryPt1 had main morphological diagnostic characters of *Cryptaphelenchus* (Figure 4) (Pedram, 2017; Gu *et al*., 2024), including: i) short body length (≤ 500 µm), C-shaped, not slender; ii) lips rounded forming a cap, slightly offset; iii) stylet delicate (≤ 10 µm), with small rounded basal knobs (Figure 4 A); iv) males with arcuate spicules, and prominent and narrow rostrum; condylus well developed; v) bursa absent and tail gradually attenuated to point (Figure 4 B); and vi) females with vulva posterior (V ≥ 75%) without vulvar flap, tail short and conical (Figure 4 C).

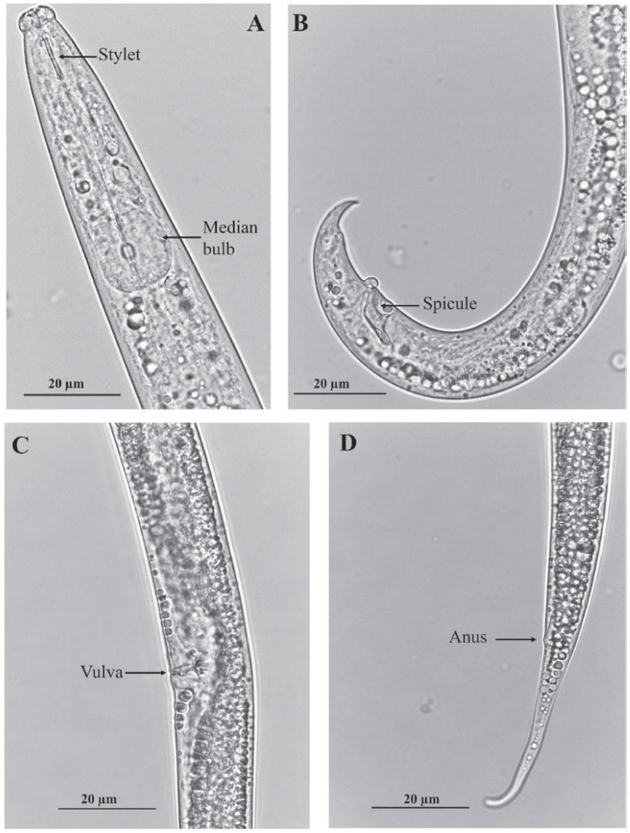

Figure 2. Light microscope photographs of the nematode isolate BfungPt2. (A) Anterior region. (B) Male tail. (C) Female vulvar region. (D) Female tail.

Molecular characterisation/identification

Amplification of ITS rDNA region amplifications from isolate BfungPt2 yielded a single product of approx. 1050 bp. The restriction endonuclease patterns with five enzymes (*Afa*I, *Alu*I, *Hae*III, *Hinf*I and *Msp*I) (Figure 5 A) agree with patterns previously reported for *B. fungivorus* (Arias *et al*., 2005; Burgermeister *et al*., 2009; Oliveira *et al*., 2011; Fonseca *et al*., 2014), confirming the identifications of isolate BfungPt2 as *B. fungivorus*.

Isolate BsexPt2 ITS rDNA region amplification yielded a single product of approx. 960 bp. ITS-RFLP analysis with the five enzymes (*Afa*I, *Alu*I, *Hae*III, *Hinf*I and *Msp*I) produced a restriction pattern similar to

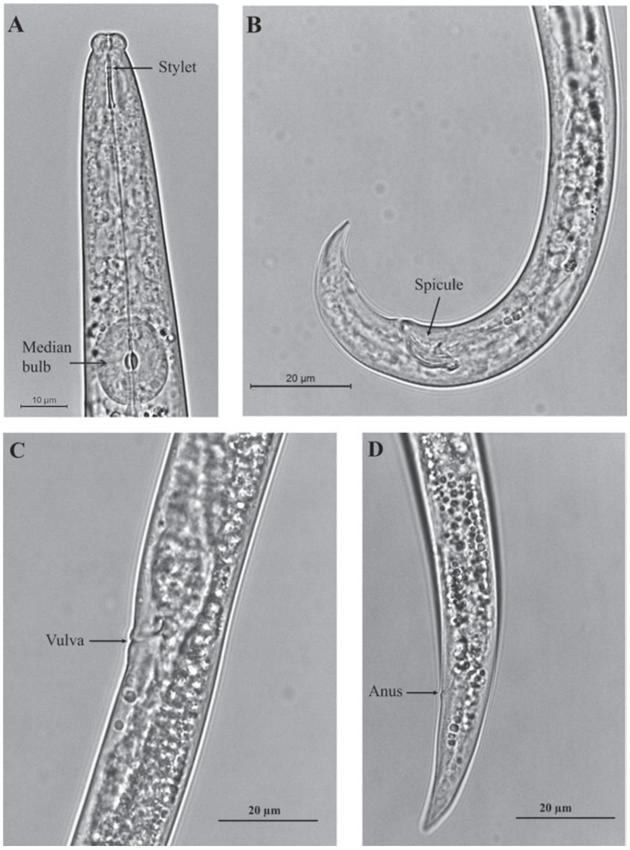

Figure 3. Light microscope photographs of the nematode isolate BsexPt2. (A) Anterior region. (B) Male tail. (C) Female vulvar region. (D) Female tail.

B. sexdentati isolates (Figure 5 B) (Penas *et al.*, 2006; Burgermeister *et al.*, 2009; Dayi *et al.*, 2014). *Bursaphelenchus sexdentati* Type I isolates could not be distinguished from Type II isolates using these five restriction enzymes. Three additional restriction enzymes, *Mfe*I (isochizomer of *Mun*I), *BsrB*I (isochizomer of *Mbi*I) and *Hpy*188I were selected according to Lange *et al.* (2006, 2008). Isolate BsexPt2 had a restriction pattern correspondent to the southern European isolates (Type II) (Figure 5 C).

After sequencing, an 813 bp sequence corresponding to the CryPt1 rDNA region, containing partial 18S and 28S and complete ITS1, 5.8S and ITS2 sequences, was submitted to the NCBI database under accession number PQ408586. The phylogenetic analysis, from multiple sequence alignment of sequences available in GenBank of *Cryptaphelenchus* species, showed that isolate CryPt1 was included in a large phylogenetic group corresponding to *Cryptaphelenchus* species. This group included *C. minutus*, *C. orientalis*, *C. recticaudatum* and *C. tumidus*, and other unidentified *Cryptaphelenchus* species from Israel, forming a separate phylogenetic group from the other isolates analysed (Figure 6).

DISCUSSION

After the detection of *B. xylophilus* (EPPO A2 quarantine list) associated with *P. pinaster* in the Setúbal peninsula, Portugal, in 1999 (Mota *et al.*, 1999), official surveys have been conducted to evaluate nematode diversity associated with *Pinus* spp. In Portugal, *B. xylophilus* was also reported in *P. nigra* (Inácio *et al.*, 2015) and *P. sylvestris* (Fonseca *et al.*, 2024), and in Spain, in *P. pinaster* (Abelleira *et al.*, 2011) and *P. radiata* D. Don (Zamora *et al.*, 2015).

In Portugal, from 1999 to the present, several other *Bursaphelenchus* species have been reported in *P. pinaster*, including *B. hellenicus*, *B. hylobianum*; *B. leoni*, *B. mucro-*

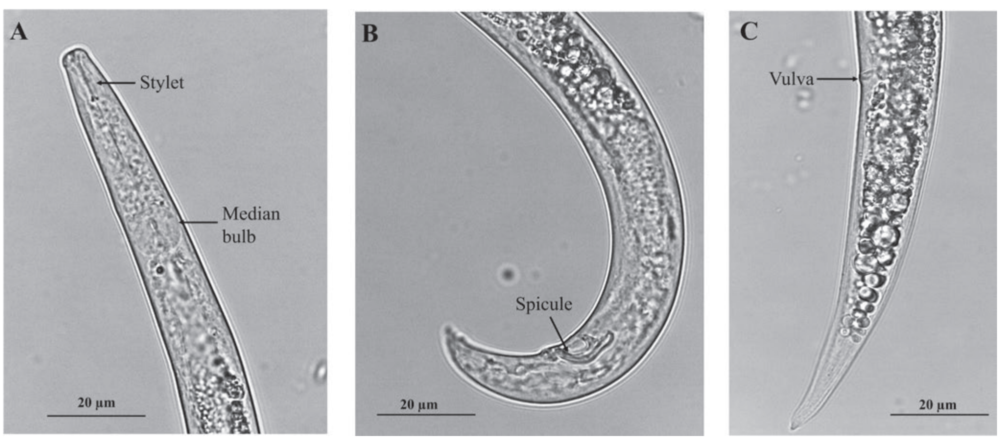

Figure 4. Light microscope photographs of the nematode isolate CryPt1. (A) Anterior region. (B) Male tail (C) Female vulvar region and tail.

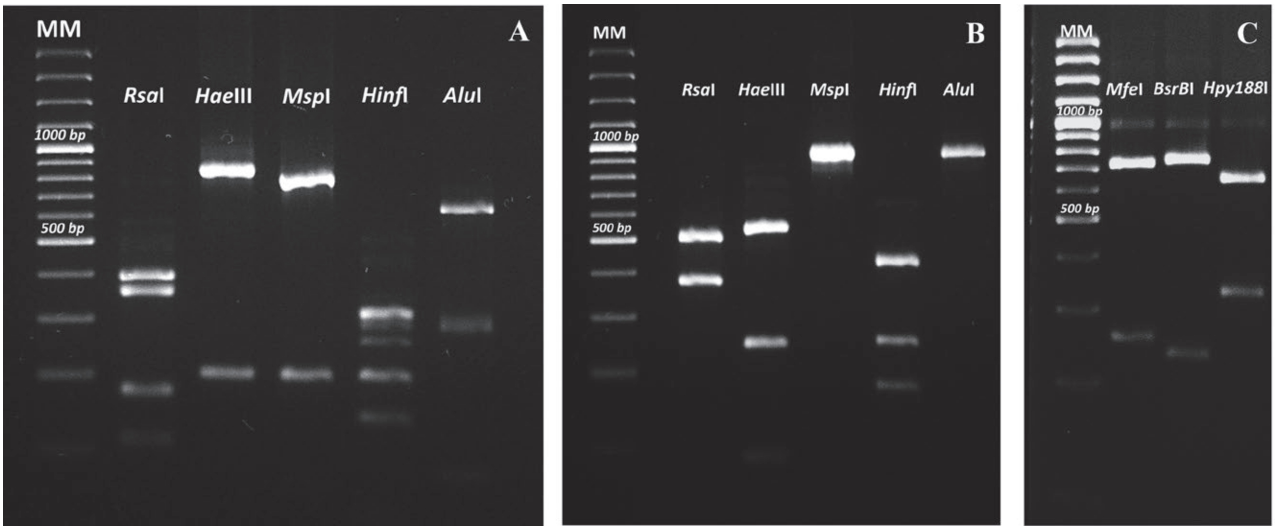

Figure 5. ITS-RFLP patterns of two *Bursaphelenchus* isolates. (A) Isolate BfungPt2. (B) and (C) Isolate BsexPt2. MM is the DNA size marker (Hyper Ladder II; Bioline).

natus, *B. pinasteri*, *B. pinophilus*, *B. sexdentati*, *B. teratospicularis*, *B. tusciae* (Penas *et al*., 2004, 2006), *B. fungivorus* (Fonseca *et al*., 2014) and *B. luxurosiae* (Inácio *et al*., 2017). From *P. pinea*, nine *Bursaphelenchus* species have been reported in the Mediterranean region (Table 1).

Species of *Bursaphelenchus* are primarily mycetophagous and include nematodes that are associated with insects and dead or dying coniferous trees (Ryss *et al*., 2005). In forestry areas, the most significant vectors of *Bursaphelenchus* spp. belong to *Cerambycidae*, *Curculionidae*, *Scolytidae*, and *Buprestidae* (Linit *et al*., 1983; Linit, 1988).

In Portugal, three *Bursaphelenchus* species have now been identified in association with *P. pinea*: i) *B. arthuri* (Silva *et al*., 2024); ii) *B. fungivorus* isolate BfungPt2; and iii) *B. sexdentati* isolate BsexPt2 (this study) (Table 1). These species, as mycetophagous, can be maintained in the laboratory in fungus cultures.

Bursaphelenchus arthuri was first detected in Portugal and Europe associated with *P. pinea*, in 2024 (Silva *et al*., 2024). This nematode was previously reported in coniferous packaging wood imported from Taiwan, South Korea, United States of America, China, and Japan (Burgermeister *et al*., 2005; Gu *et al*., 2006, 2008).

Bursaphelenchus fungivorus was first reported in the United Kingdom, in rotting gardenia buds (*Gardenia* sp.) infected by *Botrytis cinerea* (Franklin and Hooper, 1962). It was later found in: i) a bark-based growing medium in Germany (Braasch *et al*., 1999); ii) coniferous bark imported from the Czech Republic and Russia (Braasch *et al*., 2002); iii) Chinese *Callitris columellaris* F. Muell. packaging wood imported to Italy (Ambrogioni *et al*., 2003); iv) *Pinus* species, in Spain, associated with the bark beetle *Orthotomicus erosus* (Arias *et al*., 2004; 2005); v) wood chips and sawdust in Germany (Schonfeld *et al*., 2008); vi) fibrous husk of *Cocos nucifera* L. in Brazil (Oliveira *et al*., 2011); vi) *P. pinaster* bark in Portugal (Fonseca *et al*., 2014); and vii) associated with *Crocus sativus* L. in Italy (Torrini *et al*., 2020b). The present report is the first for presence of *B. fungivorus* on *P. pinea*.

Bursaphelenchus sexdentati is a cosmopolitan species with broad geographic range across European pine forests. This nematode has been divided into two distinct Types based on morphological characteristics, molecular features, and geographical distribution: Type I has been mainly found in Central Europe (Germany, Switzerland); and Type II is mostly distributed in Southern Europe (Cyprus, Greece, Portugal, Spain) (Lange *et al*., 2006, 2008). The isolate BsexPt2 is of the Type II (Lange *et al*., 2006; 2008; Penas *et al*., 2006; Burgermeister *et al*., 2009; Dayi *et al*., 2014). In the Mediterranean region, *B. sexdentati* has been associated with several pine species, including *P. brutia* Ten., *P. halepensis*, *P. nigra*, *P. pinea*, *P. pinaster*, *P. radiata*, and *P. sylvestris*, which are typically colonised by bark beetles (Errico *et al*., 2015). *Bursaphelenchus sexdentati* has also been described as vectored by insects such as *Ips sexdentatus*, *Tomicus piniperda*, and *O. erosus*. Furthermore, *O. erosus* has been reported as a vector for other *Bursaphelenchus* species, including *B. erosus, B. fuchsi*, *B. minutus* and *B. fungivorus* (Slonim *et al*., 2018).

Three *Bursaphelenchus* species were found co-existing within the *P. pinea* tree assessed in the present study. The co-existence of multiple *Bursaphelenchus* species within a single tree has also been reported by Caroppo

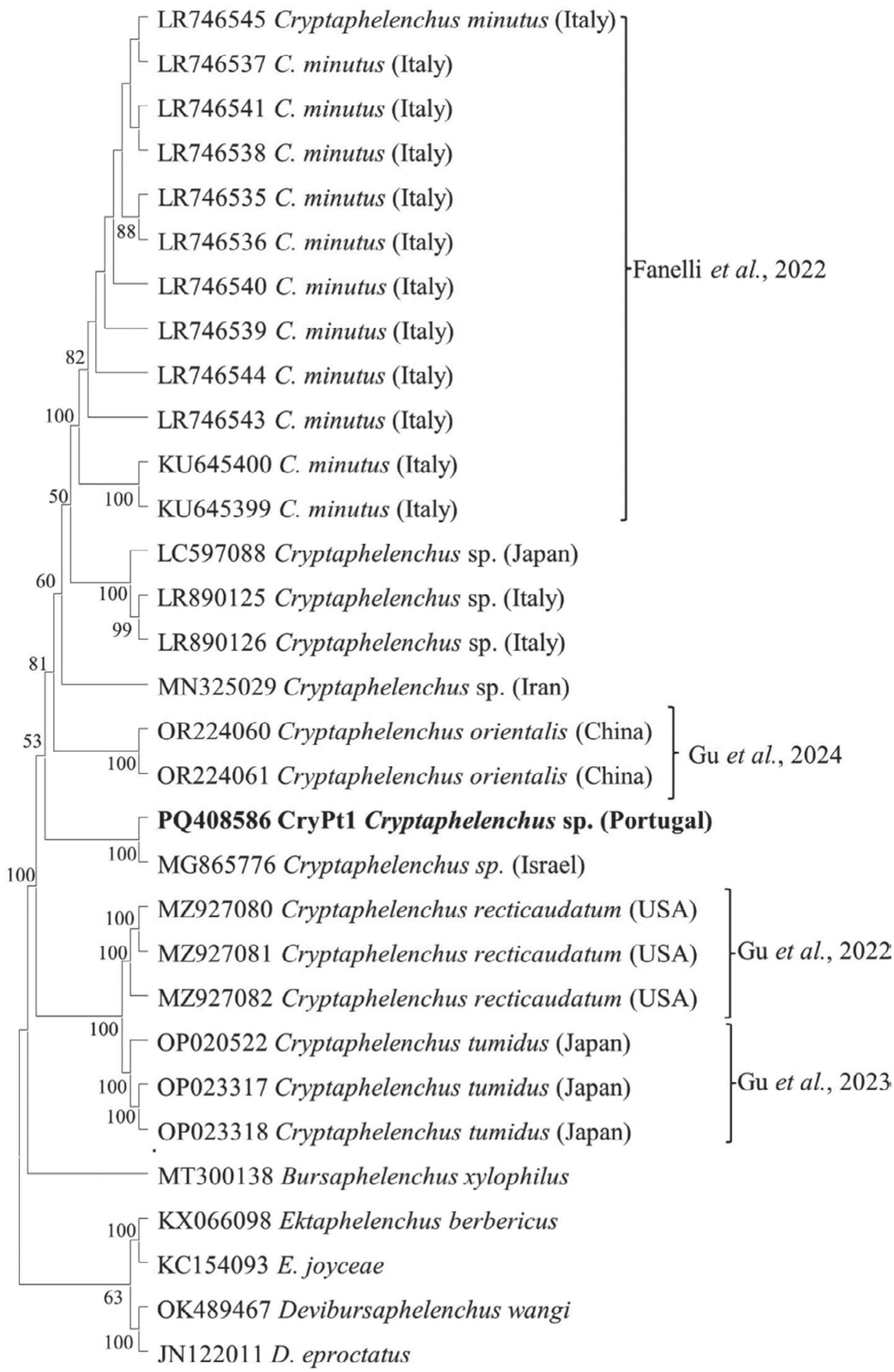

Figure 6. Phylogenetic tree generated by the Neighbour-Joining method using the multiple sequence alignment of 5.8S gene, ITS1 and ITS2 regions and partial regions of 18S and 28S genes sequences conducted in MEGA11. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. Evolutionary distances were computed using the Jukes-Cantor method. The analysis involved 31 nucleotide sequences with all ambiguous positions removed for each sequence pair (pairwise deletion option).

Table 1. *Bursaphelenchus* species associated with *Pinus pinea*.

Bursaphelencaahus species	Country	Reference
B. arthuri	**Portugal**	Silva *et al.* (2024)
B. fungivorus	**Portugal**	**The present study**
B. hylobianum	Spain	Escuer *et al.* (2004)
B. leoni	Cyprus	Philis (1996); Philis and Braasch (1996)
	Italy	Ambrogioni *et al.* (1994); Palmisano and Ambrogioni (1994); Ambrogioni and Caroppo (1998); Caroppo *et al.* (1998); Carletti (2008); Torrini *et al.* (2020a)
	Spain	Arias *et al.* (2004); Escuer *et al.* (2004)
B. minutus	Italy	Carletti (2008); Torrini *et al.* (2017
B. pinasteri	Spain	Escuer *et al.* (2004)
B. sexdentati	Italy	Ambrogioni and Caroppo (1998); Caroppo *et al.* (1998); Carletti (2008); Torrini *et al.* (2020a)
	Portugal	**The present study**
	Spain	Arias *et al.* (2004); Escuer *et al.* (2004)
B. teratospicularis	Italy	Ambrogioni and Caroppo (1998); Caroppo *et al.* (1998)
	Spain	Arias *et al.* (2004)
B. tusciae	Italy	Ambrogioni and Palmisano (1998); Carletti (2008); Torrini *et al.* (2020a)

et al. (1998), Braasch (2001) and Karmezi *et al.* (2022). Despite the *Bursaphelenchus* species reported, other nematode isolates of *Cryptaphelenchus* (this study) and of *Potensaphelenchus stammeri* (Silva *et al.*, 2023) were characterised and identified.

Cryptaphelenchus genus is known for its fungal-feeding species and has received little attention due to its small body size, resulting in fewer species being described. However, in the past decade, interest in *Cryptaphelenchus* species has grown, leading to the identification of more than 20 nominal species in countries including Germany, Italy, Iran, the United States of America, Japan, and China (Pedram, 2017; Fanelli *et al.*, 2022; Gu *et al.*, 2022; 2023; 2024). *Cryptaphelenchus* species have been isolated directly from bark or wood packaging materials and have been extracted from insect bodies (Kanzaki *et al.*, 2021; Gu *et al.*, 2022, 2023, 2024). Isolate CryPt1 showed the main morphological characters of this genus, belongs to the phylogenetic group of *Cryptaphelenchus* species, and clustered with an unidentified species from Israel (Fanelli *et al.*, 2022; Gu *et al.*, 2022, 2023, 2024). Further morphological, morphometrical and molecular characterisation is required to identify the CryPt1 isolate.

Potensaphelenchus stammeri was also found associated with the *P. pinea* tree. This is the first report of this nematode in Portugal and in *P.* pinea (Silva *et al.*, 2023). This nematode was first isolated from the insect *Spondylis buprestoides* and was described as *Aphelenchoides stammeri* (Körner, 1954). Later, it was found widely distributed in German coniferous forests in *S. buprestoides* breeding sites and in trunks of declining *Pinus* and *Picea* trees (Braasch, 1998), in China from PWN infected *P. massoniana* Lamb. (Huang and Ye, 2006), in Slovenia from *P. sylvestris* (Urek *et al.*, 2007), and in Turkey and China from *P. massoniana* (Dayi *et al.*, 2019; Gu *et al.*, 2021).

CONCLUSIONS

The present study has assessed the diversity of *Aphelenchoididae* nematodes within a classified "monumental" centennial *P. pinea* tree and has reported three *Bursaphelenchus* species co-existing simultaneously with two other *Aphelenchoididae* nematodes. This knowledge emphasises the role of large, catalogued trees as biodiversity hotspots for nematodes from different trophic groups, including plant parasites. The enduring stability of these habitats supports nematofauna, insect galleries, as well as bacteria and fungi. Diseased and dead trees should be removed from plant communities to prevent pathogen spread to nearby healthy areas. These "monumental" trees impact forest health, nutrient cycling, and microbial communities in forest areas.

AUTHORS CONTRIBUTIONS

Luís Fonseca (conceptualisation, methodology, writing, review, and editing of the manuscript, research supervision, project administration, funding acquisition), Hugo Silva (methodology, writing, review, and editing of the manuscript), Joana M. S. Cardoso (meth-

odology, writing, review, and editing of the manuscript), Ricardo M. C. da Costa (methodology, writing, review, and editing of the manuscript, funding acquisition), Filipe Campelo (methodology, writing, review, and editing of the manuscript), Joana Vieira (methodology, writing, review, and editing of the manuscript), and Isabel Abrantes (conceptualisation, writing, review, and editing of the manuscript, supervision, funding acquisition).

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DATA AVAILABILITY

Genomic data presented in this study related to the *Cryptaphelenchus* sp. isolate can be found in NCBI database under the accession number PQ408586.

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