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# Molecular and morphological characterisation of a new record of *Bursaphelenchus arthuri* (Nematoda: Aphelenchoididae) from a new host, *Pinus pinea*, in Europe

Hugo SILVA[1], Joana M.S. CARDOSO[1], Ricardo M.F. DA COSTA[2], Isabel ABRANTES[1], Luís FONSECA[1,3,*]

[1] *University of Coimbra, Centre for Functional Ecology-CFE, Associate Laboratory for Sustainable Land Use and Ecosystem Services-TERRA, Department of Life Sciences, Coimbra, Portugal*
[2] *University of Coimbra, Molecular Physical-Chemistry R&D Unit, Department of Chemistry, LAQV Requimte, Coimbra, Portugal*
[3] *FITOLAB – Laboratory for Phytopathology, Instituto Pedro Nunes (IPN), Coimbra, Portugal*
*Corresponding author. E-mail: luis.fonseca@uc.pt

**Summary.** Nematodes with morphological characters of *Bursaphelenchus arthuri*, a species of the Fungivorus group, first described in 2005 in China from imported coniferous packaging wood, were extracted from a centennial *Pinus pinea* tree with wilting symptoms in Coimbra, Portugal. Nematodes were transferred to *Botrytis cinerea* cultures to establish a nematode isolate which was characterised using morphological, morphometrical and molecular methods. Restriction fragment length polymorphism (RFLP) analysis carried out with five endonucleases of amplified internal transcribed spacer (ITS) of the rDNA region, revealed the characteristic restriction pattern described for *Bursaphelenchus arthuri*. Sequencing of the D2-D3 expansion region of the large subunit (LSU) of rDNA confirmed this identification. Phylogenetic analysis based on multiple sequence alignment of selected D2-D3 sequences showed that the Portuguese *B. arthuri* isolate formed a separate group, along with the other *B. arthuri* isolate from Taiwan available in GenBank, from those of other isolates of closely related nematodes species belonging to the Fungivorus group. This study is the first report of *B. arthuri* in Europe and from *P. pinea*.

**Keywords.** Centennial tree, D2-D3 expansion region sequencing, morphobiometrics, nematode, PCR-ITS-RFLP.

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**ORCID:**
HS: 0000-0002-0046-4716
JMSC: 0000-0002-8720-8845
RMFC: 0000-0002-5426-412X
IA: 0000-0002-8761-2151
LF: 0000-0001-7405-8916

## INTRODUCTION

*Bursaphelenchus* can be divided into several groups based on morphological and molecular analyses. The Xylophilus group includes the phytoparasitic species *Bursaphelenchus xylophilus* Steiner and Buhrer, 1934 (Nickle, 1970), the causal agent of pine wilt disease (EPPO, 2024). *Bursaphelenchus arthuri*

Burgermeister, Gu and Braasch, 2005, a mycetophagous nematode species first described in China from coniferous packaging wood imported from Taiwan and South Korea (Burgermeister *et al.,* 2005), belongs to the Fungivorus group, which comprises other species such as: 1) *B. hunti* (Steiner, 1935) Giblin-Davis and Kaya, 1983; 2) *B. sychnus* Rühm, 1956; 3) *B. steineri* Rühm, 1956; 4) *B. fungivorus* Franklin and Hooper, 1962; 5); *B. gonzalezi* Loof, 1964; 6) *B. seani* Giblin-Davis and Kaya, 1983; 7) *B. thailandae* Braasch and Braasch-Bidasak, 2002; 8) *B. willibaldi* Schönfeld, Braasch and Burgermeister, 2006; 9) *B. braaschae* Gu and Wang, 2010; 10) *B. kiyoharai* Kanzaki, Maehara, Aikawa, Masuya and Giblin-Davis, 2011; 11) *B. arthuroides* Gu, Wang and Zeng, 2012; 12) *B. parathailandae* Gu, Wang and Chen, 2012; 13) *B. tadamiensis* Kanzaki, Taki, Masuya and Okabe, 2012; 14) *B. penai* Kanzaki, Giblin-Davis, Carrillo, Duncan and Gonzalez, 2014; 15) *B. sycophilus,* Kanzaki, Tanaka, Giblin-Davisa and Davies 2014*;* 16) *B. kesiyae* Kanzaki, Aikawa, Maehara and Thu, 2016; 17) *B. rockyi* Wang, Fang, Maria, Gu and Ge, 2019; and 18) *B. suri* Kanzaki, Kruger, Greeff and Giblin-Davis, 2022 (Gu *et al.*, 2012; Wang *et al.*, 2019; Kanzaki *et al.,* 2014; 2016; 2022). Since the first detection of *B. arthuri* in China from coniferous packaging wood imported from Taiwan and South Korea, this nematode was later identified in imported wood from Republic of Korea, the United States of America, China, and Japan (Gu *et al.,* 2006; 2008).

In the present study, wood samples from an isolated *P. pinea* Linnaeus, 1753 tree displaying severe wilting symptoms were collected, and were assessed for presence of the EPPO A2 quarantine organism, *B. xylophilus* (the pinewood nematode), and for other *Bursaphelenchus* species. Nematodes resembling *B. arthuri* were separated from these samples and propagated in fungus cultures. Subsequent characterisation and identification were accomplished based on the morphological and morphometric characteristics of females and males and based on molecular characteristics of the ITS rDNA region using PCR ITS-RFLP and D2-D3 LSU rDNA sequencing. A phylogenetic analysis was also built with the multiple sequence alignment between D2-D3 LSU rDNA sequences available on databases.

## MATERIALS AND METHODS

### *Nematodes extraction and culture establishment*

Wood samples (trunk and branches) were collected from a centennial *P. pinea* tree showing wilting symptoms in Coimbra, Portugal. The samples were then cut into small pieces (< 1 cm wide). Nematodes were extracted from these pieces using the tray method (Whitehead and Hemming, 1965; EPPO, 2013) and were handpicked and transferred to *Botrytis cinerea* grown at 25°C on malt extract agar to obtain a nematode isolate, as described by Fonseca *et al.* (2008). The nematode isolate obtained was identified (see below) as *Bursaphelenchus arthuri* and designated as BaPt1.

### *Morphobiometrical characterisation*

Fifteen females and 15 males were killed by heat in a drop of water on a cavity glass slide, and were mounted in water, photographed, and their morphological and morphometric characters were analysed. Photographs were taken with a Leica DM 2500 bright field light microscope (Leica) using a LeicaDFC 450 digital camera (Leica). Measurements (Tables 1 and 2) were carried out using Microsystem LAS Interactive Measurement Software Version 4.0.0. (Leica) (Silva *et al.,* 2023).

### *DNA extraction and amplification of ITS-rDNA and D2-D3 rDNA regions*

DNA from nematodes collected from a culture plate (mixed developmental stages) was extracted with the DNeasy Blood and Tissue Mini Kit (Qiagen), following manufacturer's instructions, and were quantified using Nanodrop 2000C (ThermoScientific). The ITS rDNA region containing partial 18S and 28S and complete ITS1, 5.8S and ITS2 sequences was amplified using 50 ng of extracted DNA, 1× of NZYTaq II Master Mix (Nzytech), and 0.4 µM of the primers 18SF 5'-CGTAACAAGGTAGCTGTAG-3' (Ferris *et al.,* 1993; EPPO 2023) and 28SR 5'-TTTCACTCGCCGTTACTAAGG-3' (Vrain, 1993; EPPO, 2023). 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 of 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The resulting amplification product was used for RFLP analysis.

The D2-D3 expansion region of LSU rDNA was amplified in a 50 µL reaction using 50 ng of DNA, 1× NZYTaq II Master Mix (Nzytech), and 0.4 µM of primers D2A (5' ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') (Ley *et al.,* 1999). All reactions were conducted in a Bio-Rad Thermal Cycler, with an initial denaturation step of 95°C for 2.5 min, followed by 40 reaction cycles of 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The resulting amplification product was purified with the

NZYGelpure Kit (Nzytech), according to manufacturer instructions, and used for sequencing.

*Restriction fragment length polymorphism analysis*

RFLP analysis was carried out on the amplified ITS rDNA PCR product following Burgermeister *et al.* (2009) and EPPO (2023) protocols, using the five restriction endonucleases *Hinf*I, *Alu*I, *Hae*III, *Msp*I, and *Rsa*I (Amersham Biosciences), according to the manufacturer's instructions. The restriction products were separated by electrophoresis on 1.5% agarose gel.

*Sequencing and phylogenetic analysis*

The amplified D2-D3 LSU rDNA product was sequenced in both strands using an Automatic Sequencer 3730xl with BigDyeTM terminator cycling conditions, at Macrogen Company (Seoul, Korea), employing the same primers used in the PCR (above). Sequences obtained were analyzed with BioEdit (Hall, 1999). BLAST (Altschul *et al.,* 1997) was used to search for homologous sequences in the databases, and selected sequences were aligned using BioEdit. Phylogenetic analysis was carried out in MEGA 11 (Tamura *et al.,* 2021), using the Neighbor-Joining method (Saitou and Nei, 1987) with 1000 bootstrap replications, and employing the Jukes-Cantor substitution model. Ambiguous positions were removed from each sequence pair (pairwise deletion option) using the D2-D3 LSU sequences alignment.

## RESULTS

*Morphobiometrical characterisation*

Female and male nematodes (Figure 1, A to F) had key diagnostic morphological characters consistent with the original description of *B. arthuri* (Burgermeister *et al.,* 2005): heat relaxed form almost straight, only slightly ventrally arcuate when heat-killed; lip region offset by a constriction; head with six lips; stylet slender with only very slight basal swellings; median bulb rounded to elongated oval with conspicuous valve plates centrally placed (Figure 1 E); nerve ring located closely posterior to the median bulb; excretory pore position approx. half to one body diameter behind the median bulb at level with intestine. Female (Figure 1 A): vulva a transverse slit without flap; ovary outstretched; conspicuous post uterine branch extending over two-thirds of vulva to anus distance (Figure 1 B); anus slightly protruding; tail slightly ventrally bent and with finely rounded terminus (Figure 1 C). Male (Figure 1 D): tail region sharply curled ventrally when killed by heat; posterior anus lip slightly protruding; spicules paired and straight with a high rounded condylus and rostrum in the middle position; distal ends of spicules rounded without a cucullus; tail with a distinct, oval-shaped terminal bursa with a pointed terminus, which can be seen in dorso-ventral position; small tubercle observed close to the anus indicates presence of a single pre-anal papilla (Figure 1 F).

Morphometrics of the female and male nematodes were compared with other morphometric data for *B. arthuri* (Burgermeister *et al.,* 2005). Most of the morphometric data for the females and males were within the range of the nematodes measured in the original species description (Burgermeister *et al.,* 2005), apart of the total body length, and *a* and *c* ratios, which were smaller than those in the original description (Tables 1 and 2).

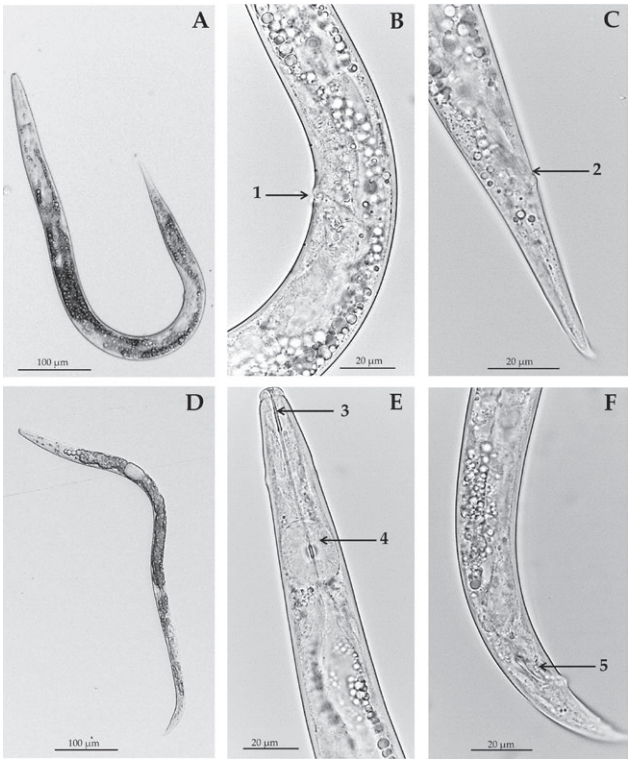


**Figure 1.** Light micrographs of the Portuguese *Bursaphelenchus arthuri* isolate (BaPt1). A; female (entire body): B; part of a female reproductive system; C; female tail: D; male (entire body): E; anterior region: F; male tail. 1; vulva: 2; anus: 3; stylet: 4; median bulb: 5; spicule.

**Table 1.** Morphometric data of *Bursaphelenchus arthuri* females from Portugal (BaPt1) and Taiwan. Values are means ± SD, and those in parentheses are minima and maxima.

| Character | Females | |
|---|---|---|
| | Portugal (n = 15) (BaPt1, this study) | Taiwan (n = 15) (Burgermeister *et al.*, 2005) |
| *Linear (µm)* | | |
| Body length (L) | 709.3 ± 55.9 (601.5–828.8) | 961.9 ± 62.4 (827–1069) |
| Greatest body width (GBW) | 24.4 ± 2.3 (21.3–29.5) | ----- |
| Stylet length | 15.0 ± 0.7 (14.1–16.8) | 16.4 ± 1.0 (14.0–17.7) |
| Median bulb length | 22.6 ± 1.5 (19.9–25.7) | ----- |
| Median bulb diameter | 17.2 ± 1.6 (15.2–21.7) | ----- |
| Anterior end to end of median bulb (AEMB) | 70.9 ± 5.9 (56.3–78.6) | ----- |
| Tail length (TL) | 60.1 ± 4.7 (47.4–65.2) | ----- |
| Body width at anus (BWA) | 12.0 ± 0.9 (10.7–13.8) | ----- |
| Anterior end to vulva | 499.0 ± 40.4 (432.2–583.9) | |
| Vulva to anus | 153.9 ± 17.2 (131.2–191.1) | ----- |
| *Ratio* | | |
| a = L/GBW | 29.2 ± 1.7 (25.0–31.7) | 33.9 ± 1.5 (31.2–35.7) |
| $b_1$=L/AEMB | 10.1 ± 1.2 (8.4–12.4) | ----- |
| c=L/TL | 11.9 ± 0.8 (10.6–13.6) | 15.0 ± 1.7 (13.6–19.1) |
| c'=TL/BWA | 5.0 ± 0.5 (3.9–5.6) | 4.8 ± 0.4 (4.2–5.4) |
| *Percentage* | | |
| V = Distance anterior end to vulva x 100/L | 70.4 ± 1.3 (68.5–73.0) | 72.3 ± 1.0 (70.8–73.7) |

**Table 2.** Morphometric data of *Bursaphelenchus arthuri* males from Portugal (BaPt1) and Taiwan. Values are means ± SD, and those in parentheses are minima and maxima.

| Character | Males | |
|---|---|---|
| | Portugal (n = 15) (BaPt1, this study) | Taiwan (n = 15) (Burgermeister *et al.*, 2005) |
| Linear (µm) | | |
| Body length (L) | 671.4 ± 63.3 (564.2–784.7) | 922.8 ± 35.3 (882–980) |
| Greatest body width (GBW) | 24.0 ± 2.6 (20.0–29.8) | ----- |
| Stylet length | 14.2 ± 0.8 (12.7–15.7) | 15.7 ± 0.8 (15.1–17.4) |
| Median bulb length | 20.4 ± 1.9 (17.9–24.7) | ----- |
| Median bulb diameter | 16.0 ± 1.9 (13.5–19.8) | ----- |
| Anterior end to end of median bulb (AEMB) | 69.0 ± 5.8 (56.3–79.6) | ----- |
| Tail length (TL) | 34.4 ± 3.2 (29.9–40.4) | ----- |
| Body width at anus (BWA) | 14.0 ± 1.4 (12.4–17.7) | ----- |
| Spicule length | 14.4 ± 1.0** (12.9–15.8) | 19.4± 1.4* (16.0–20.9) |
| Ratio | | |
| a=L/GBW | 28.1 ± 1.8 (24.7–30.8) | 31.9 ± 3.0 (27.0–36.0) |
| $b_1$=L/DAEMB | 9.8 ± 1.1 (8.5–11.3) | ----- |
| c=L/TL | 19.6 ± 1.6 (17.7–23.1) | 27.3 ± 1.2 (25.9–29.8) |
| c'=TL/BWA | 2.5 ± 0.3 (1.8–2.9) | 2.2 ± 0.1 (2.1–2.3) |

* Measured as bow.
** Measured by curved median line.

### *Molecular identification*

#### Restriction fragment length polymorphism analysis

The BaPt1 ITS rDNA regions PCR amplification resulted in a product with length approx. 950 bp. The restriction patterns obtained through digestion with the five endonucleases (Figure 2), corresponded with those previously described for *B. arthuri* (Burgermeister *et al.*, 2009; EPPO, 2023).

#### *Sequencing and phylogenetic analysis*

The sequence of BaPt1 D2-D3 LSU rDNA was deposited in the GenBank database under the accession number PQ304253. Phylogenetic analysis revealed that the corresponding BaPt1 D2-D3 LSU rDNA clusters together with other *B. arthuri* isolate submitted in the GenBank and formed a clear separate group (Figure 3). This analysis confirms the identity of isolate BaPt1 as *B. arthuri*.

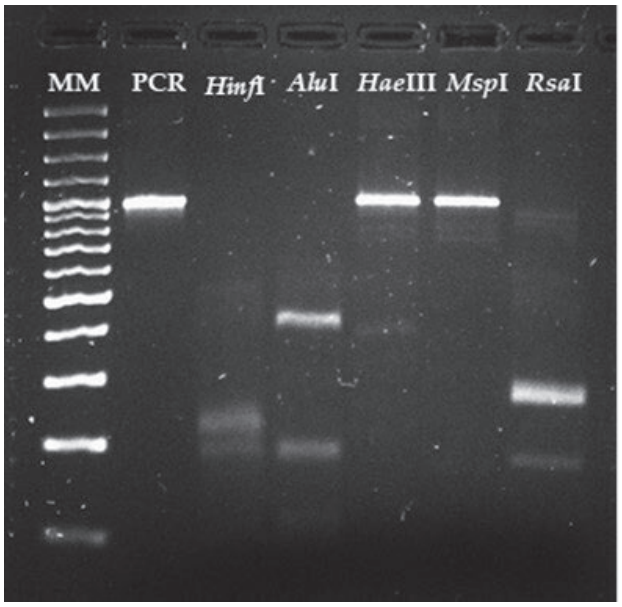


**Figure 2.** ITS-RFLP patterns of the Portuguese *Bursaphelenchus arthuri* (BaPt1) with the restriction endonucleases *Hinf*I, *Alu*I, *Hae*III, *Msp*I and *Rsa*I. MM—DNA Molecular Marker (Gene Ruller™ 100 bp Plus DNA Ladder).

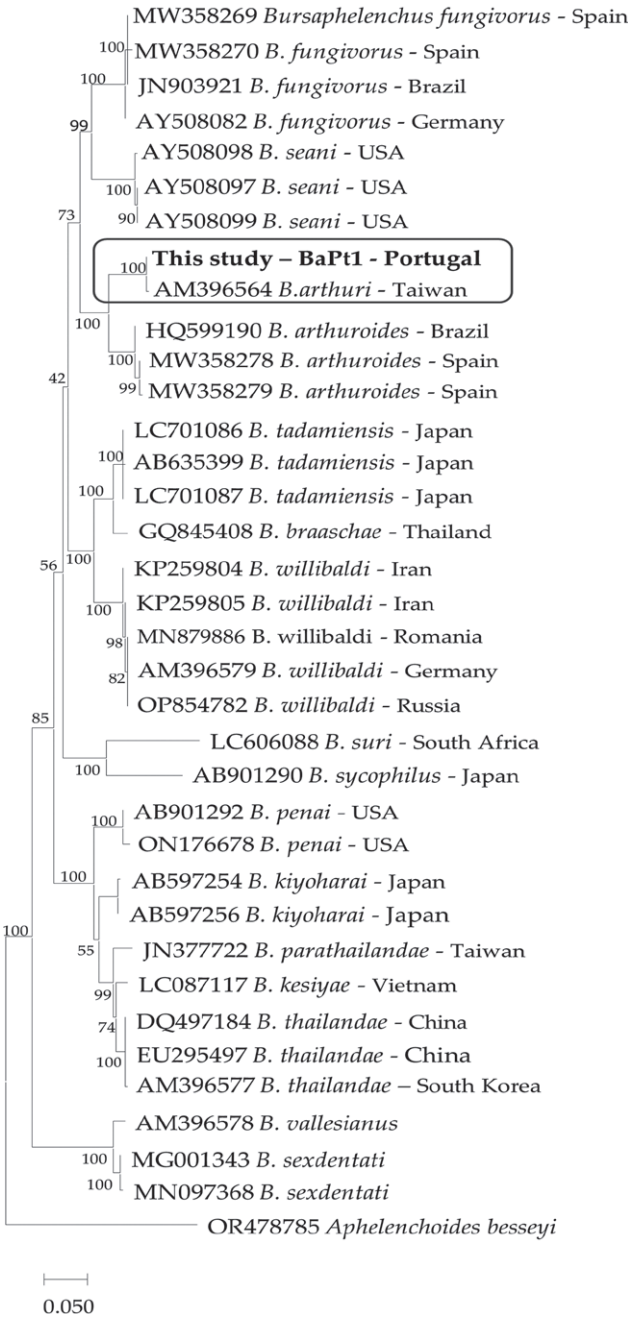


**Figure 3.** Phylogenetic tree generated by the Neighbor-Joining method using the multiple sequence alignment between D2-D3 LSU rDNA sequences. A *Aphelenchoides besseyi* sequence was used as the outgroup. Bootstrap values are shown at branch points, and the scale bar indicates 0.050 substitutions per site.

## DISCUSSION

To date, *B. arthuri* has only been detected in Ningbo Port, China, from coniferous packaging wood imported from Taiwan, South Korea, USA, China and Japan (Burgermeister *et al.,* 2005; Gu *et al.,* 2006; 2008). In the present study, *B. arthuri* was detected, for the first time, associated with a *P. pinea* tree. In addition, from this tree, other *Aphelenchoididae* nematodes were found co-existing, including *Potensaphelenchus stammeri* (Silva *et al*., 2023), demonstrating that wilting coniferous trees can be reservoirs of different nematode species.

The morphology of the Portuguese *B. arthuri* BaPt1 isolate, is consistent with the diagnostic morphological characters outlined in the original description of this nematode (Burgermeister *et al.,* 2005), and most of the morphometric data of females and males are in accordance with the original description (Burgermeister *et al.,* 2005). The morphometric differences found in total body length and *a* and *c* ratios could be because nematodes measured in the present study were previously maintained in fungus cultures for a few weeks. Previous nematode morphometric studies have demonstrated correlations between some morphometric characters and environmental variables (Amin, 2007; Grzelak *et al.,* 2020; Nguyen *et al.,* 2023).

*Bursaphelenchus arthuri* morphological and morphometric differentiation is difficult within the Fungivorus group (Burgermeister *et al*., 2005), making molecular methods (ITS-RFLP analysis and rDNA region sequencing) useful for the species diagnosis. ITS-RFLP analysis, through digestion with *Hinf*I, *Alu*I, *Hae*III, *Msp*I, and *Rsa*I endonucleases, matched with the previ-

ously described pattern for *B. arthuri* species (Burgermeister *et al.,* 2009; EPPO, 2023), confirmed the species identification. Furthermore, the phylogenetic analysis, using the BaPt1 D2-D3 rDNA sequence, showed that the Portuguese isolate clustered together with other *B. arthuri* isolate with D2-D3 sequences available in the databases, making a separate phylogenetic group from those formed by other isolates closely related to nematodes of the Fungivorus group. This clearly identified the BxPta1 isolate as belonging to *B. arthuri*.

Kanzaki *et al.* (2016) divided the Fungivorus group into four subgroups based on morphological characteristics, and placed *B. arthuri* in the same subgroup as *B. hunti*, *B. gonzalezi*, *B. fungivorus*, *B. seani*, and *B. arthuroides*. In the present study, phylogenetic analysis placed together, in the same phylogenetic branch, the species *B. fungivorus*, *B. seani*, *B. arthuroides*, and *B. arthuri* available in GenBank, supporting the previously made subgroup division (Gu *et al.,* 2006). Among these species, phylogenetic analysis showed that *B. arthuroides* is most closely related to *B. arthuri*. Morphologically, these two species are also very similar, with small differences in the shapes of the female tail and in the spicule (Gu *et al.,* 2012).

The present study is the first report of the presence of *B. arthuri* in Portugal and in Europe. Species identification was based on species-specific morphological diagnostic characters and was confirmed by PCR-ITS-RFLP analysis and D2-D3 LSU rDNA sequencing. In addition, this study identifies *P. pinea* as a new host for *B. arthuri* adding new information to the occurrence of *Bursaphelenchus* species in Europe.

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