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First report of *Laimaphelenchus heidelbergi* (Nematoda: Aphelenchoididae) in Europe

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Summary

A survey of nematodes associated with branches of cork oak, *Quercus suber*, a species in decline since the second half of the 20th century, was conducted on two farms located in Alentejo, Portugal. Using specific morphological characters, some nematodes were identified as belonging to the genus *Laimaphelenchus* and one of the isolates being identified as *L. heidelbergi.* This research aimed to characterize the Portuguese *L. heidelbergi* isolate using morphobiometrical and molecular analyses and to analyze its phylogenetic relationship to other *Laimaphelenchus* spp. Morphometric and morphological characteristics of *L. heidelbergi* females and males were similar to the original description. For molecular analyses, the mitochondrial DNA region from the cytochrome oxidase subunit I and the D2/D3 expansion segments of the large subunit of rDNA were amplified and sequenced. In phylogenetic analyses, sequences of the Portuguese *L. heidelbergi* isolate clustered with sequences from the Australian isolate. *Laimaphelenchus heidelbergi* was originally described from wood of *Pinus radiata* growing in Australia and is here reported for the first time in Europe and Portugal. Cork oak is a new host record for *L. heidelbergi*.

1 Introduction

Plant-parasitic nematodes have been reported to be associated with oak trees, and species of the family Aphelenchoididae have been collected from damaged or dead oak trees. For example, *Bursaphelenchus parvispicularis* Kanzaki and Futai, 2005 was isolated from *Quercus mongolica* and *B. eremus* Rühm, 1956, from *Q. robur*, *Q. cerris* and *Q. suber* (Kanzaki and Futai 2005; Marianelli et al. 2011). The aphelenchid genus *Laimaphelenchus* Fuchs, 1937 comprises non-pathogenic species found mostly associated with moss, algae and lichen on conifers and with galleries of bark beetle larvae. *Laimaphelenchus* spp. frequently cohabit with other genera such as *Bursaphelenchus* Fuchs, 1937 (Yeates et al. 1993; Zhao et al. 2006a). The *Laimaphelenchus* genus comprises 15 valid species plus the following five species with doubtful identity that require further investigation: *L. corticilis* Truskova and Eroshenko, 1977; *L. exilis* Truskova and Eroshenko, 1977; *L. sapinus* Truskova and Eroshenko, 1977; *L. tenarius* Truskova and Eroshenko, 1977 and *L. vescus* Truskova and Eroshenko, 1977 (Asghari et al. 2012; Table 1). According to Hunt (2008), *L. vescus* is a synonym of *L. pini* Baujard, 1981. It has been suggested that *Laimaphelenchus* species can be divided into two groups: one has females with a distinct vulvar flap that is easily observed using light microscopy. Females in the other group do not have vulvar flap (Hunt 1993). Given that species having females without vulvar flaps were transferred by Hirling (1986) to the genus *Aphelenchoides* and phylogenetic analyses reveal that the genus *Laimaphelenchus* is paraphyletic, it is clearly difficult to know what features actually characterize the *Laimaphelenchus* genus, which is morphologically similar to *Aphelenchoides* (Zhao et al. 2008).

Laimaphelenchus heidelbergi Zhao, Davies, Riley and Nobbs, 2007, originally described from wood of the exotic pine, *Pinus radiata*, from Victoria, Australia (Zhao et al. 2007), is here reported for the first time in Europe and Portugal, and the cork oak is a new host record for this species. The purposes of this research were to characterize the Portuguese isolate of *L. heidelbergi* by morphobiometrical and molecular analyses and to analyze its phylogenetic relationship to other *Laimaphelenchus* spp. using available mitochondrial DNA (mtDNA) region from cytochrome oxidase subunit I (COI) and D2/D3 expansion segments of the large subunit (LSU) of rDNA sequences.

2 Material and Methods

2.1 Nematode Isolate

As part of a study to evaluate the decline of the cork oak in agro-silvo-pastoral ecosystems in Portugal, a survey of nematodes on cork oak was conducted on two farms located in Alentejo. A total of 40 branch samples were collected from the crowns of both healthy (20) and declining trees (20), the latter showing visible symptoms such as defoliation. Using the modified Baermann funnel method (Abrantes et al. 1976), nematodes were extracted from the wood and bark tissues of 30 g of branches/sample. After 48 h, nematodes extracted from wood and bark tissues were collected separately and identified as belonging to the genus *Laimaphelenchus* on the basis of specific morphological characteristics. Individuals belonging to this genus were then hand-picked, washed several times with sterilized tap water and transferred to cultures of *Botrytis cinerea* grown on malt extract agar. Cultures containing nematodes were subsequently maintained in a growth chamber at 25°C. After 2 months, one of the nematode isolates, identified as *L. heidelbergi,* was established and transferred, every month, to new cultures of *B. cinerea*, using either 15 nematodes alone or small plugs of agar containing nematodes and *B. cinerea*. Nematodes obtained from these cultures were used for the morphobiometrical, molecular and phylogenetic

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Table 1. List of valid *Laimaphelenchus* species and their host, distribution and reference.

Species	Host	Distribution	References
L. australis	*Pinus pinaster* *P. radiata* (bark)	Australia	Zhao et al. (2006a)
L. cocuccii	*P. elliottii*	Argentina	Doucet (1992)
L. deconincki	Sand and soil around *Ligustrum* sp.	France	Elmiligy and Geraert (1971)
L. heidelbergi	*P. radiata* (wood)	Australia	Zhao et al. (2007)
L. helicosoma	Soil around roots of *Deschampsia antarctica*	Antarctica	Peneva and Chipev (1999)
L. pannocaudus	*Dendroctonus adjunctus* *P. pinaster*	Mexico	Massey (1966)
		France	Baujard (1981)
L. patulus	*P. pinaster*	South Africa	Swart (1997)
L. penardi	*Chlorella* sp.	Switzerland	Baujard (1981)
	P. pinaster	Germany	
	P. insignis	USA	
	Scolytes	Canada	
	Saperda populae galleries	India	
	Grimmia pulvinata	France	
L. pensobrinus	*Dendroctonus adjunctus*	Mexico	Massey (1966)
L. persicus	*P. sylvestris* (bark)	Iran	Asghari et al. (2012)
L. phloesini	Bark beetles	USA	Massey (1974)
L. pini = L. vescus	*P. pinaster*	France	Baujard (1981)
	G. pulvinata		Truskova and Eroshenko (1977) Hunt (2008)
L. preissii	*Callitris preissii* (bark)	Australia	Zhao et al. (2006b)
L. simlaensis	*P. roxburghii*	India	Sapna et al. (2009)
L. unituberculus	Insect frass	India	Bajaj and Walia (2000)

analyses. Maintenance of other isolates of *Laimaphelenchus* that we collected was not possible in our laboratory, so we were unable to identify them at the species level.

2.2 Morphobiometrical Analysis

Morphological and morphometric studies were conducted on at least 20 males and 20 females, using light microscopy (LM) and scanning electron microscopy (SEM). For LM studies, the specimens were transferred to a slide containing a drop of water, killed by heating for 4–6 s using a small flame and measured immediately with the help of a drawing tube. Some samples mounted under a LeitzDialux 20 bright field LM (Leitz, Oberkochen, Germany) were photographed using a Leica DFC450 digital camera (Leica, Wetzlar, Germany). No nematode slides were permanently mounted. Male and female nematodes were processed for SEM as described in Abrantes and Santos (1991). Briefly, the specimens were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), post-fixed in 2% osmium tetroxide and dehydrated. Subsequently, the specimens were 'critical-point'dried with carbon dioxide. The dried specimens were mounted on stubs, coated with gold (200Å), observed and photographed with an EDAX PEGASUS X4M (Materials Centre of the University of Porto, Portugal).

2.3 Molecular Analysis

The mitochondrial cytochrome oxidase subunit I (mtCOI) gene and D2/D3 expansion segments of LSU were selected for the molecular characterization of the isolate of *L. heidelbergi*. After hand-picking a mixture or single specimens of *L. heidelbergi* males and females from a culture plate, total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Manchester, UK). The primers for the mtCOI amplification were the forward primer COI-F1 (5′-CCT ACT ATG ATT GGT GGT TTT GGT AAT TG-3′) and the reverse primer COI-R2 (5′-GTA GCA GCA GTA AAA TAA GCA CG-3′; Kanzaki and Futai 2002). Primers for amplification of the D2/D3 expansion segments of LSU were the forward primer D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and reverse primer D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′; Castillo et al. 2009).

The PCR [25 µl containing 1× buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 1.5 U Taq DNA polymerase (Bioline, London, UK) and 10 µl of DNA] and the thermal cycling program for mtCOI were performed according to Zhao et al. (2008) in a MJ Mini – Personal Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). For LSU, although the PCR reactions were the same as for mtCOI, the amplification was carried out with the following conditions: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 42°C for 1 min and extension at 72°C for 2 min, and a final extension for 10 min at 72°C. The PCR products were analysed on a 1% agarose gel in 1× TBE buffer stained with GreenSafe (NZYTech, Lisbon, Portugal).

The amplified products were purified with the QIAquick PCR Purification Kit (QIAGEN, Manchester, UK) and quantified using the NanoDrop 2000C Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), according to the manufacturer's instructions. Amplified DNA was sequenced in both directions with the same amplification primers using standard procedures at Macrogen, Inc. (Seoul, Korea). Chromatograms were checked and corrected manually using BioEdit software (Hall 1999).

2.4 Phylogenetic Analysis

The mtCOI and LSU sequences of *Laimaphelenchus* spp. included in the phylogenetic analysis were obtained from the GenBank nucleotide database; *Bursaphelenchus xylophilus* (Steiner and Bührer, 1934) Nickle, 1970 was included as an out-group. Sequences from the *L. heidelbergi* LSU and mtCOI regions were aligned using Muscle (Edgar 2004) with homologous *Laimaphelenchus* spp. and *B. xylophilus* sequences. Alignment was manually adjusted when necessary. Phylogenetic relationships were reconstructed using neighbor-joining (NJ) (Jukes and Cantor 1969) and maximum likelihood (ML) (Saitou and Nei 1987) methods. The NJ analyses were performed using the maximum composite likelihood model (Tamura et al. 2004) with complete deletion. One thousand bootstrap replicates were performed to test the support of each node on the trees (Felsenstein 1985). The ML analyses were based on the Jukes–Cantor model (Jukes and Cantor 1969). All positions containing gaps and missing data were eliminated. Alignment and evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

3 Results

3.1 Morphobiometrical Analysis

Laimaphelenchus spp. nematodes were detected in wood and bark tissues of cork oak branches, the largest proportion of them (97.6%) being associated with bark. All *L. heidelbergi* specimens were associated with only the bark of cork oak branches. Morphometrics of females and males of the Portuguese *L. heidelbergi* isolate are reported in Table 2; range values of the morphometric characteristics either overlap or are within the expected range according to the original description.

Females slender with length ranging from 513 to 660 μm; habitus slightly ventrally arcuate. The anterior end rounded, offset, cephalic region annulated forming six lobes and a distinct labial disc (Fig. 1a,b). Amphids pore-like. Lateral fields with three incisures, not areolated, extending to tail tip. Vulva without flap, 358.3–447.1 μm from anterior end (Fig. 1f,g). Posterior end conoid with a single tubercle, visible with LM, covered by tiny finger-like appendages, only observed in detail with SEM (Fig. 1c).

Males 523–658 μm long, anterior and posterior ends and lateral fields similar to females (Fig. 1a–c). Spicules paired, 12.1–16.6 μm long (Fig. 1d). Five caudal papillae: one pair at level of the spicule rostrum, one at approximately half distance between cloaca and tail tip, and a single papilla mid-ventral on the posterior end, anterior to tubercle (Fig. 1e).

Table 2. Morphometric comparison of females and males of the Portuguese isolate of *Laimaphelenchus heidelbergi* with the original description (Zhao et al. 2007)[1].

	Females		Males	
Characteristic	Portuguese isolate (N = 20)	Zhao et al. 2007 (N = 12)	Portuguese isolate (N = 20)	Zhao et al. 2007 (N = 10)
Linear (μm)				
Body length	593 ± 38.0 (513–660)	750 ± 77.3 (533–895)	591 ± 33.0 (523–658)	672 ± 69.4 (567–752)
Greatest body width	18.6 ± 0.9 (15.5–20.0)	–	17.1 ± 1.7 (12.4–19.5)	–
Anterior end to posterior end of oesophageal glands	125.5 ± 13.2 (101.4–148.6)	–	121.5 ± 11.6 (99.3–145.7)	–
Head region height	2.8 ± 0.2 (2.4–3.2)	2.3 ± 0.4 (2.0–3.0)	2.7 ± 0.2 (2.1–3.2)	2.1 ± 0.3 (1.5–2.0)
Head region width	5.8 ± 0.3 (5.3–6.3)	5.8 ± 0.3 (5.0–6.0)	5.6 ± 0.2 (5.5–6.1)	5.5 ± 0.4 (5.0–6.0)
Stylet length	10.9 ± 0.4 (10.3–12.1)	10.7 ± 0.8 (10.0–13.0)	10.9 ± 0.4 (10.0–11.6)	10.5 ± 0.5 (9.0–11.0)
Anterior end to valves of medium bulb	53.0 ± 2.5 (48.4–57.4)	56.1 ± 2.4 (52.0–60.0)	54.0 ± 1.4 (51.3–55.8)	55.0 ± 6.5 (43.0–64.0)
Tail length	–	41.1 ± 6.5 (33.0–48.0)	35.8 ± 2.1 (32.1–39.2)	36.2 ± 4.4 (29.0–43.0)
Body width at anus	–	–	11.8 ± 0.5 (10.8–12.6)	–
Anterior end to vulva	408.4 ± 25.8 (358.3–447.1)	522.6 ± 48.3 (433.0–609.0)	–	
Vulva width	18.1 ± 1.0 (15.5–20.0)	–	–	–
Spicule length	–	–	14.5 ± 1.3 (12.1–16.6)	– (15.0–16.0)
Ratio				
a = Body length/ body width	31.8 ± 1.8 (28.3–35.9)	39.9 ± 2.4 (28.6–40.5)	34.7 ± 2.6 (31.1–42.3)	39.0 ± 2.7 (34.3–44.7)
b' = Body length/ oesophageal glands	4.8 ± 0.5 (3.9–5.7)	5.3 ± 0.5 (4.7–6.5)	4.9 ± 0.5 (4.3–6.1)	5.1 ± 0.6 (4.5–6.1)
c = Body length/ tail length	–	18.6 ± 2.7 (10.7–22.3)	16.6 ± 0.9 (14.8–17.7)	19.0 ± 2.7 (15.2–24.2)
c' = Tail/body width at anus	–	–	3.0 ± 0.2 (2.8–3.5)	2.9 ± 0.5 (2.2–3.6)

[1]Values are mean ± standard deviation (range).

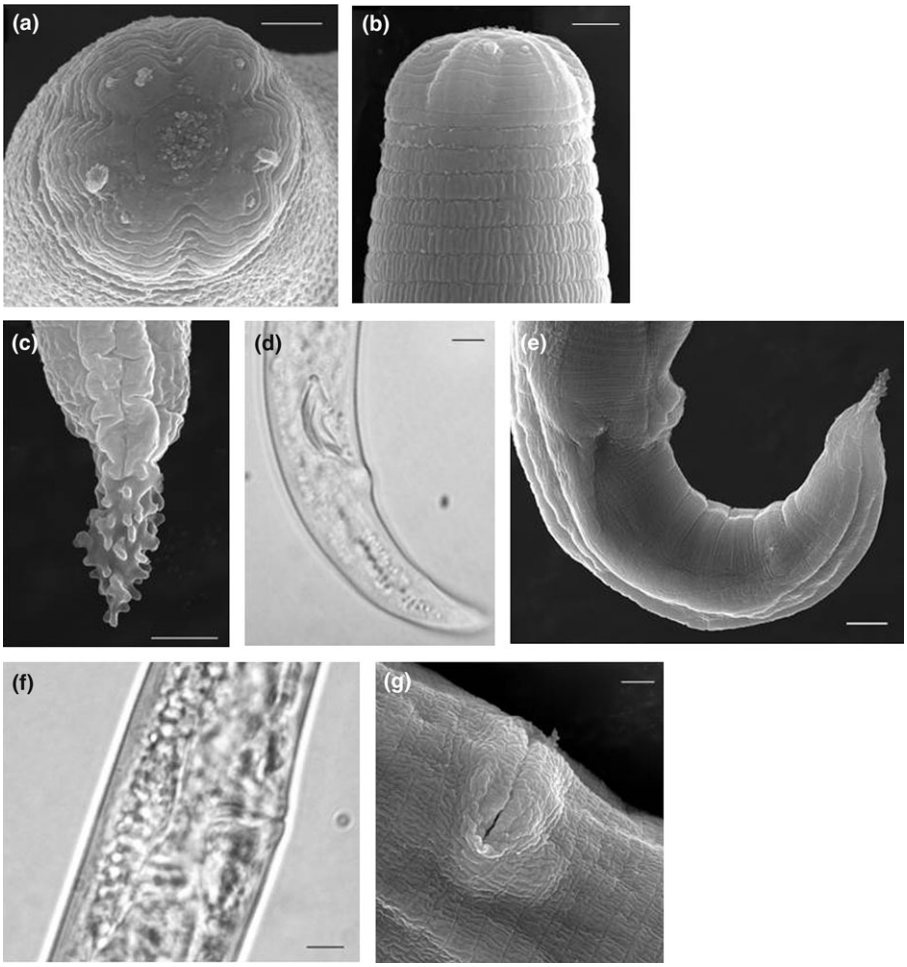

Fig. 1. Scanning electron (a, b, c, e and g) and light microscope (d and f) photographs of *Laimaphelenchus heidelbergi*. (a and b) Anterior region; (c) posterior end; (d and e) male tail; and (f and g) female vulval region. Scale bars: 1 μm (a–c, e and g), and 5 μm (d and f).

3.2 Molecular and Phylogenetic Analyses

The mtCOI and D2/D3 expansion segments of LSU regions, amplified with the primer sets COI-F1/COI-R2 and D2A/D3B from purified genomic DNA, yielded single fragments of approximately 700 and 800 bp, respectively (data not shown). To obtain useful information for species discrimination and characterization, the PCR products were sequenced, the mtDNA and LSU sequences deposited in the GenBank as KJ564292 and KJ564293, respectively, compared *w*ith the equivalent sequences of *L. heidelbergi* available in GenBank. The size of mtCOI and D2/D3 sequences from the Portuguese isolate of *L. heidelbergi*, although not complete, was 597 and 741 bp, respectively. The sequences were compared *with* the corresponding fragments of *L. heidelbergi* from GenBank (Accession Nos. EU287592.1 and EU287595.1). They had 97.8 and 99.8% homology for mtCOI and D2/D3, respectively, with only 13 and 1 differences in alignment (data not shown).

The phylogenetic relationship of *L. heidelbergi* to other *Laimaphelenchus* spp., using available mtCOI and D2/D3 sequences, was also analysed (Fig. 2). The Portuguese isolate of *L. heidelbergi* formed a close cluster with *L. heidelbergi* from Australia for mtCOI, with high bootstrap support (100%), and clustered separately from *L. preissii* Zhao, Davies, Riley and Nobbs, 2006 (Fig. 2a). With the D2/D3 region, the two isolates of *L. heidelbergi* formed a supported clade (99%) and were a sister group to *Laimaphelenchus* sp. isolate from Louisiana, USA (96%). *Laimaphelenchus australis* Zhao, Davies, Riley and Nobbs, 2006, and *L. preissii* the most divergent species, formed a clade with 99% (Fig. 2b).

4 Discussion

The original description of *L. heidelbergi* was made from specimens isolated from wood of the exotic pine, *P. radiata*, from Victoria, Australia (Zhao et al. 2007). To our knowledge, this is the first report of *L. heidelbergi* in Portugal and Europe. *Quercus suber* represents a new host for this species. It is likely that *L. heidelbergi* is associated with lichens, algae or mosses growing on the bark of cork oak trees; an association with fungi is also possible given that the nematode successfully fed on

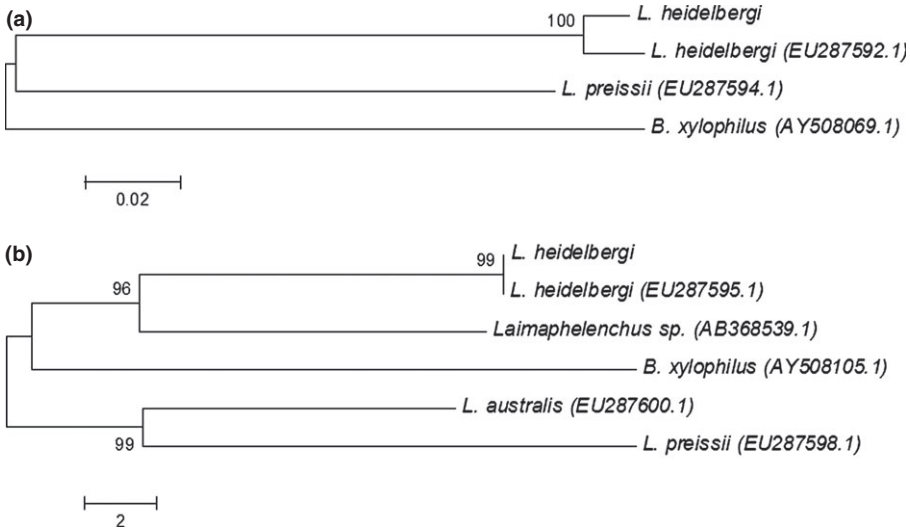

Fig. 2. Neighbor-joining trees based on mtCOI (a) and LSU (b) partial sequences of *Laimaphelenchus* spp. The percentage of replicate trees in which the *Laimaphelenchus* spp. clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary distances were computed using the maximum composite likelihood method and all positions containing gaps and missing data were eliminated. *Bursaphelenchus xylophilus* was included as an out-group.

B. cinerea in the laboratory. Beetle activities were not observed on the cork oaks we sample, so associations between these insects and *L. heidelbergi* are not known.

Ranges of the morphometric characteristics for females and males of the Portuguese isolate either overlap or are within the values of the original description. The morphologies of the diagnostic characters (LM and SEM observations) are also similar to the original description (Zhao et al. 2007). *Laimaphelenchus heidelbergi* belongs to the group of species with females without a vulvar flap and can be easily differentiated from other *Laimaphelenchus* species by the combination of tail shape, spicule shape, number of incisures in the lateral field, number of caudal papillae and presence/absence of a vulvar flap (Zhao et al. 2007). However, some of the characters such as tail shape and number of caudal papillae are only visible in detail with SEM. Therefore, the morphobiometrical diagnostic characters need to be sustained by other characters, such as molecular markers. The molecular data obtained in this study confirm the identification of the Portuguese *Laimaphelenchus* isolate, based on the morphological and morphometric characters, as *L. heidelbergi*. The NJ analysis of the mtCOI and D2/D3 expansion segments of LSU also revealed that the Portuguese *L. heidelbergi* isolate forms a clade with high bootstrap support with the Australian isolate and that it is distinct from the other *Laimaphelenchus* spp., for which sequences are available.

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