Zoosyst. Evol. 96 (1) 2020, 175–193 | DOI 10.3897/zse.96.49022

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Description of *Longidorus bordonensis* sp. nov. from Portugal, with systematics and molecular phylogeny of the genus (Nematoda, Longidoridae)

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http://zoobank.org/16388413-BF9F-4339-AF96-1179BB8CED1D

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Academic editor: A. Schmidt-Rhaesa ♦ **Received** 13 December 2019 ♦ **Accepted** 27 March 2020 ♦ **Published** 5 May 2020

Abstract

The genus *Longidorus* currently comprises 176 species of polyphagous plant ectoparasites, including eight species that vector nepoviruses. *Longidorus* is one of the most difficult genera to accurately identify species because of the similar morphology and overlapping measurements and ratios among species. Sequences of ribosomal RNA (rRNA)-genes are a powerful level-species diagnostic tool for the genus *Longidorus*. From 2015 to 2019, a nematode survey was conducted in vineyards and agro-forest environments in Portugal. The populations of *Longidorus* spp. were characterized through an integrative approach based on morphological data and molecular phylogenetic analysis from rRNA genes (D2-D3 expansion segments of the 28S, ITS1, and partial 18S), including the topotype of *L. vinearum*. *Longidorus bordonensis* **sp. nov.**, a didelphic species recovered from the rhizosphere of grasses, is described and illustrated. *Longidorus vineacola*, with cork oak and wild olive as hosts, is also characterized. This is the first time that *L. wicuolea*, from cork oak, is reported for Portugal. Bayesian inference (BI) phylogenetic trees for these three molecular markers established phylogenetic relationships among the new species with other *Longidorus* spp. Phylogenetic trees indicated that i) *L. bordonensis* **sp. nov.** is clustered together with other *Longidorus* spp. and forms a sister clade with *L. pini* and *L. carpetanensis*, sharing a short body and odontostyle length, and elongate to conical female tail, and ii) all the other species described and illustrated are phylogenetically associated, including the topotype isolate of *L. vinearum*.

Key Words

Bayesian inference, D2–D3 expansion segments of large ribosomal subunit 28S, genomic data, needle nematodes, internal transcribed spacer 1, partial small ribosomal subunit

Introduction

Dorylaimida Pearse, 1942 is one of the most diverse orders in terms of number of species within the phylum Nematoda (Jairajpuri and Ahmad 1992). Members of the genus *Longidorus* Micoletzky, 1922, commonly known as needle nematodes, belong to the subfamily Longidorinae within the family Longidoridae Thorne, 1935 (order Dorylaimida) and includes a large group of metazoan ectoparasites of herbaceous plants, bushes, and trees (Coomans 1996; Taylor and Brown 1997; Decraemer and Robbins 2007). *Longidorus* is one of the most species-rich genera, taking into account the species described by Lazarova et al. (2019) and Cai et al. (2019); it contains approximately 176 valid species. These nematodes drill into the root by moving their needle-like stylet

through root cells during feeding, which causes severe negative effects on the architecture of the root (Taylor and Brown 1997). In the field, the presence of this group is associated with poor plant growth, including reduced root systems which are characterized by severely stunted lateral and tap roots (Taylor and Brown 1997; Archidona-Yuste et al. 2016). However, the major pest status of *Longidorus* spp. is earned because *Longidorus* is a vector of the subgroup B Nepoviruses (genus *Nepovirus*, subfamily Comovirinae, family Secoviridae) (Taylor and Brown 1997; Decraemer and Robbins 2007). In spite of being an extensive genus, only 4.6% of their members, i.e. eight species (*L. apulus* Lamberti & Bleve-Zacheo, 1977, *L. arthensis* Brown, Grunder, Hooper, Klinger & Kunz, 1994, *L. attenuatus* Hooper, 1961, *L. diadecturus* Eveleigh, 1982, *L. elongatus* (de Man, 1876) Micoletzky, 1922, *L. fasciatus* Roca & Lamberti, 1981, *L. macrosoma* Hooper, 1961, and *L. martini* Merny, 1966), transmit 21% of the known Nepoviruses (Taylor and Brown 1997; Decraemer and Robbins 2007), which emphasize the need for establishing a selective strategy based on the correct identification of species. According to data collected from EU Directive 2000/29/EC and/or the European and Mediterranean Plant Protection Organization (EPPO) quarantine lists, some species are recommended for regulation as quarantine pests.

Most *Longidorus* species have a restricted geographical distribution. However, this genus is a cosmopolitan group, with South America being the less diverse region, followed in increasing order by Oceania, China, South Africa, North America, India, and Europe (Decraemer and Robbins 2007; Cai et al. 2019; Xu and Zhao 2019). Fifteen species of *Longidorus* have been reported in several cultivated and wild plants including angiosperms and gymnosperms in Portugal (Bravo and Lemos 1997; Gutiérrez-Gutiérrez et al. 2016): *L. africanus* Merny, 1966, *L. alvegus* Roca, Pereira & Lamberti, 1989, *L. belloi* Andres & Arias, 1988, *L. carpetanensis* Arias, Andres & Navas, 1986, *L. crataegi* Roca & Bravo, 1996, *L. goodeyi* Hopper, 1961, *L. juvenilis* Dalmasso, 1969, *L. lusitanicus* Macara 1985, *L. macrosoma*, *L. nevesi* Macara, 1986, *L. profundorum* Hopper, 1996, *L. reisi* Roca & Bravo, 1993, *L. unedoi* Arias, Andres & Navas, 1986, *L. vineacola* Sturhan & Weischer, 1964, and *L. vinearum* Bravo & Roca, 1995. Most of these species appear to have an endemic distribution limited to the Iberian Peninsula (Andres and Arias 1982; Roca et al. 1989; Bravo and Lemos 1997; Peña-Santiago al. 2006; Gutiérrez -Gutiérrez et al. 2016; Archidona-Yuste et al. 2019), while some have a wider geographical distribution (Navas et al. 1990, 1993; Taylor and Brown 1997; Bravo and Lemos 1997; Gutiérrez-Gutiérrez et al. 2016), such as *L. juvenilis* (Taylor and Brown 1997; Barsi and Lamberti 2004; Sirca and Urek 2009; Gutiérrez-Gutiérrez et al. 2016), *L. goodeyi* (Dalmasso 1969; Seinhorst and van Hoof 1981; Taylor and Brown 1997; Bravo and Lemos 1997; Gutiérrez -Gutiérrez et al. 2016), *L. africanus* (Cohn 1969; Jacobs and Heyns 1987; Vadivelu and Muthukrishnan 1987; Zeidan and Coomans 1991; Bravo and Roca 1995; Lamberti et al. 1996; Fadaei Tehrani and Kheiri 2005; Anwar and McKenry 2012; Subbotin et al. 2014; Guesmi-Mzoughi et al. 2017; Archidona-Yuste et al. 2019), *L. macrosoma* (Barsi 1989; Taylor and Brown 1997; Bravo and Lemos 1997; Lamberti et al. 2001; Gutiérrez-Gutiérrez et al. 2016), and *L. profundorum* (Hooper 1965; Klingler et al. 1983; Andres and Bello 1984; Topham and Alphey 1985; Prikhodko 1988; Romanenko 1994; Bravo and Lemos 1997; Taylor and Brown 1997; Lamberti et al. 2001; Lišková 2012; Gutiérrez-Gutiérrez et al. 2016).

According to the morphological features and morphometric measurements of adult (mainly females) and that of juveniles [mainly first-stage juveniles (J1)], each *Longidorus* species is defined by 11 matrix codes (A–K) in the polytomous key published by Chen et al. (1997) and two additional supplements published by Loof and Chen (1999) and Peneva et al. (2013). However, the high intraspecific variability of some diagnostic features and the great diversity in phenotypic plasticity makes species identification based on metric and morphological data of external morphology and internal anatomy difficult and sometimes unreliable. Sequencing of RNA-based markers is a powerful molecular diagnostic approach within this group (Archidona-Yuste et al. 2016, 2019; Palomares-Rius et al. 2017; Peraza-Padilla et al. 2017; Tzortzakakis et al. 2017; Xu et al. 2018). Recently, several studies (Archidona-Yuste et al. 2016, 2019; Roshan-Bakhsh et al. 2016; Esmaeili et al. 2017; Tzortzakakis et al. 2017; Xu et al. 2017, 2018; Barsalote et al. 2018; Cai et al. 2019; Lazarova et al. 2019) have shown the usefulness of a pair of molecular markers based on ribosomal RNA (rRNA) (D2–D3 domains of 28S gene and the ITS regions, particularly ITS1) for a fast and precise diagnosis of *Longidorus* species, even in extreme situations such as sibling and cryptic species. Subbotin et al. (2015) and Barsi and De Luca (2008) designed a PCR–RFLP of the D2–D3 segments of the 28S rRNA and ITS. Both assays were based on five restriction enzymes for genotyping of species-specific variations: the first from *L. orientalis* Loof, 1982, and the other from *L. pius* Barsi & Lamberti, 2001 (Barsi and De Luca 2008; Subbotin et al. 2015). Additionally, studies have revealed that the mitochondrial marker gene, *COI*, is useful for the delineation of closely related species within Longidoridae (Palomares-Rius et al. 2017; Archidona-Yuste et al. 2019; Cai et al. 2019). Thus far, more than half of the valid *Longidorus* species have molecular markers deposited in the GenBank database; however, only a small number belong to topotypes. Genomic data of topotypes are very useful for confirming identifications and clarifying the composition of species complexes within the Longidoridae (Gutiérrez-Gutiérrez et al. 2010, 2013; Kornobis et al. 2017; Archidona-Yuste et al. 2019; Fouladvand et al. 2019).

Members of the genus *Longidorus* have not been studied in detail during the past 18 years in Portugal, and updated information on the present occurrence and distribution is lacking, as well as molecular data (Gutiér-

rez-Gutiérrez et al. 2016). This prompted us to carry out surveys in vineyards and agro-forestry systems in Portugal, from 2015 to 2019. The objectives of the present work are: 1) to characterise 11 populations of *Longidorus* species through an integrative approach based on morphological, morphometric, and molecular data, including topotypes of *L. vinearum* and of *L. bordonensis* sp. nov.; 2) to establish phylogenetic relationships of the identified *Longidorus* species from the surveys with available sequences of the known species.

Methods

Nematode population sampling, extraction, and morphological characterization

Nematode surveys were conducted in spring and autumn from 2015 to 2019 in vineyards (*Vitis* sp.) and agro-forestry soils and included several host plants (Table 1). A total of 65 and 85 sampling sites of vineyards and agro-forestry areas, respectively, were arbitrarily chosen in Portugal. Field samples were taken in a zigzag pattern according to EPPO diagnostic protocols (OEPP/EPPO 2009). Each sample was collected using a drill from the upper 60 cm of the rhizosphere of 10–20 plants (sub-samples) from each field. Nematodes were extracted from 250 cm^3 of soil by a modification of Cobb's decanting and sieving method (Flegg 1967). Additional soil was collected to guarantee enough specimens for morphological and/or molecular analyses.

Nematodes were placed in a drop of water, killed in a hot fixative solution (4% formaldehyde + 1% glycerol + 85% distilled water), maintained for 48–72 h at room temperature (25 °C), and processed into pure glycerine by a modification of Seinhorst's method (Seinhorst 1966). Specimens were examined using an Olympus BX50 light microscope with differential interference contrast (DIC) up to 1,000× magnification. Photographs were taken with an Olympus DP70 camera. Cell software (Olympus Software Imaging for Life Sciences) was used for image analysis and measurements. All measurements were expressed in micrometers (µm). For line drawings of the new species, light micrographs were imported to CorelDraw v. X6 and the main features were outlined. All abbreviations are as defined in Jairajpuri and Ahmad (1992). According to metric (such as lip region length and width, body length, odontostyle length, maximum body width, guiding ring position, vulva position, pharyngeal length, and tail length and width) and non-metric (e.g., tail shape, size, and position of amphidial fovea, vulva size and shape, and lip region shape) morphological data of adult specimens and juveniles (J1–J4), each species was defined by the matrix code for the polytomous key (Chen et al. 1997; Loof and Chen 1999; Peneva et al. 2013). In addition, specimens of *L. vinearum* from its type locality, Dois Portos, Torres Vedras, Portugal, were collected. After verifying that their morphology was consistent with that of the original description, they were processed for their genotypic and phenotypic characterizations, and included as one of the 11 populations (Table 1).

DNA extraction, PCR, and sequencing

After nematodes were extracted from the soil, specimens were examined by light microscopy (LM) on temporary glass slide mounts and digital images were recorded. These photomicrographs were used to match each phenotype with its associated genotype. Temporary slides were dismantled and individual nematodes were placed in a 2 µl drop of sterile water on the cover of a PCR tube, and the specimen was cut into six small pieces with a surface-sterilized scalpel. Subsequently, they were centrifuged in 18 µl of solution containing 10 µl ddH_2O, 6 µl 10× PCR buffer, and 2 µl of proteinase K (20 mg/ml) (Nalgene), and frozen at −80 °C (15 min). Samples were mixed for 15 sec and PCR assays were conducted as described by Gutiérrez-Gutiérrez et al. (2018). The tubes were incubated at 57 °C (1 h), 65 °C (1 h), and 95 °C (15 min). Half of one µl of extracted DNA was transferred to an Eppendorf tube containing reaction mixtures of 25 ul NZYTaq 2× Green Master Mix (2.5 mM $MgCl_2$, 200 mM dNTPs, 0.2 U/µl DNA Polymerase) (NYZTech, Portugal), 0.4 µl of each primer (25 mM), and ddH_2O was added to make a final volume of 50 µl. The D2–D3 expansion segments of 28S rRNA gene, the ITS1 region of rRNA, and a partial potion of 18S rRNA gene were amplified using several primer pairs (Suppl. material 3: Table S1).

PCR cycle conditions for markers of ribosomal DNA included one cycle of 95 °C for 3 min; followed by 30 cycles of 94 °C for 30 s; an annealing temperature of 54 °C (D2A/D3B or 28LeX/ D3B), 53 °C (rDNA1/18S), and 50 °C (988F/1912R, 1813F/2646R) for 30 s, 72 °C for 15–45 s; and one cycle of 72 °C for 7 min. PCR products were purified after amplification using NZYGelpure (NZYTech Genes & Enzymes, Portugal) following the manufacturer's instructions and used as template for direct sequencing at Eurofins Genomic (Germany) using the primers listed (Suppl. material 3: Table S1). The sequences were deposited in the GenBank database under accession numbers (Table 1) and used for constructing phylogenetic trees (Figs 3–5) were inferred using the methods descripted in the following section.

Phylogenetic analyses

D2–D3 expansion segments of 28S rRNA, partial 18S rRNA, and ITS1 rRNA sequences from *L. bordonensis* sp. nov. and all other known *Longidorus* spp. found in the survey (Table 1), together with additional accessions belonging to *Longidorus* spp. available in GenBank were used for phylogenetic reconstructions. Outgroup taxa for each dataset were chosen according to previously published data (Archidona-Yuste et al. 2016, 2019; Xu et al.

Table 1. Taxa sampled for *Longidorus* species, and sequences used in this study.

Species	Sample code	Locality	Host	Genbank accessions		
				D2D3	ITS1	18S
Longidorus sp. 3	ST	Carregueira, Santarém	cork oak tree	MN082424	–	–
L. bordonensis sp. nov.	70-08-18	Bordonhos, S.Pedro Sul	grass	MN082421 MN082422	MN150062	MN1297570
**L. vinearum*	LISB-03-04	Dois Portos, Torres Vedras	grapevine	MN082431 MN082432	MN150065 MN150067 MN150068	–
L. vinearum	**LISB-22	Picanceira, Mafra	grapevine	MN082434	–	–
L. vinearum	LISB-13	Macheia, Ordasqueira, Torres Vedras	grapevine	MN082433	–	–
L. vinearum	M3-OLV	Valverde, Évora	wild olive	MN082430	MN150066	–
L. vineacola	119/015	Q. da Amoreirinhas da Cima , Montemor-o-Novo	cork oak tree	MN082425	–	–
L. vineacola	120/015	Q. da Amoreirinhas da Cima, Montemor-o-Novo	cork oak tree	MN082426 MN082427	–	–
L. vineacola	129/015	Q. da Amoreirinhas da Cima, Montemor-o-Novo	cork oak tree	MN082428	MN150064	MN129758
L. vineacola	M3-OLV	Herdade da Mitra,Valverde, Évora	wild olive	MN082429	–	–
L. wicuolea	**Beja-16	Linhares, Beja	cork oak tree	MN082423	MN150063	–

* Topotype specimens ** Only one juvenile specimen was detected in this sample (–) Not obtained or not performed.

2018; Cai et al. 2019). The sequences were deposited in Genbank for each gene studied and they were aligned using an online version of MAFFT v. 7 (Katoh and Standley 2013) with default parameters. Sequence alignments were visualised with ClustalX2 (Thompson et al. 1997) and edited by Gblocks v. 0.91b (Castresana 2000) in Castresana Lab server (http://molevol.cmima.csic.es/castresana/Gblocks_ server.html) using options for a relaxed selection of blocks (minimum number of sequences for a conserved position: 68 (D2–D3), 56 (18S) and 59 (ITS1); minimum number of sequences for a flanking position: 68 (D2–D3), 56 (18S) and 59 (ITS1); maximum number of contiguous non-conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half). Homogeneities of base frequencies and optional substitution models for 28S rRNA, 18S rRNA, and ITS1 datasets were tested with Kakusan4 (Tanabe 2011). The homogeneity test indicated that the base composition of each dataset was significantly homogeneous. Bayesian inference (BI) trees of all molecular data were constructed with MrBayes v. 3.2.1 (Ronquist and Huelsenbeck 2003). For BI analysis, the substitution model was tested by the Bayesian Information Criterion and SYM model with a gamma-shaped distribution was selected for the three molecular regions studied. BI analysis was run for 5,000,000 generations, sampling every 100th tree and discarding 'burn in' first 25% of the sampled tree.

Results

Measurements and morphological images are presented here in the description of species. Molecular data also helped to discriminate species within the genus *Longidorus* in this study. For the description of the new species *L. bordonensis* sp. nov., DNA data were added to the previously established morphological and morphometric analysis. Here, we provided metrical and non-metrical morphological data as well as molecular phylogenetic analyses of two known species previously reported from Portugal, *L. vinearum* and *L. vineacola*. Unfortunately, only one specimen was found in both *L. wicuolea* Archidona-Yuste, Navas Cortés, Cantalapiedra-Navarrete, Palomares-Rius, & Castillo, 2016 and one unidentified *Longidorus* species (*Longidorus* sp. 3 isolate ST), which were used for sequencing of the D2–D3 expansion domains of 28S rRNA or/and ITS1. In this study, in addition to the description of the new species, the molecular characterization of *L. wicuolea* comprises the first report of this species for Portugal. For both *L. vineacola* and *L. vinearum*, a brief description and comparison with the original description and other previous records are provided; however, for the description of the second species, topotype material was included too. Paratypes of *L. vinearum* were provided by Maria L. Inácio (INIAV, Oeiras, Lisbon, Portugal); however, they were not incorporated in this study because the specimens were in poor condition.

Longidorus bordonensis sp. nov.

http://zoobank.org/D753E7C6-512E-4A69-B9FE-326B9783D74C

Figs 1(1–7), 2(1–10), Table 2, Suppl. material 4: Table S2)

Holotype. Slide PLB001.

Paratypes. 6 females and 6 males (slides PLB002-PLB 013) mounted on glass slides.

Type repositories. The holotype (PLB001) and 8 paratypes (4 females and 4 males) (slides PLB002–PLB005 and PLB008–PLB011) are deposited in the Nematode Collection of the Nematology Lab, Institute for Mediter-

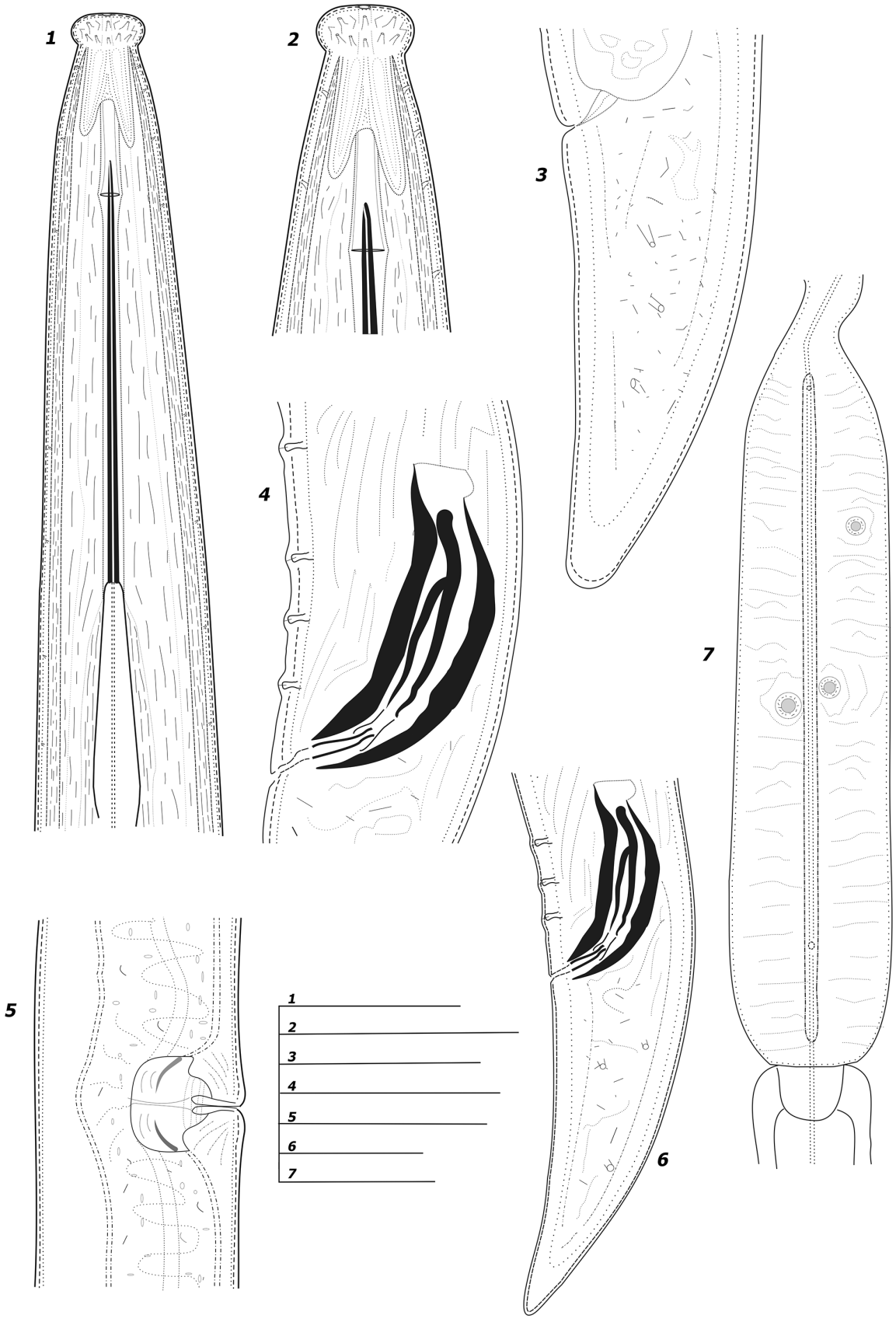

Figure 1. Line drawings of *Longidorus bordonensis* sp. nov. paratypes from the rhizosphere of grass (unknown species) at São Pedro do Sul, Viseu district, northern Portugal (1–7). **1.** Female anterior end. **2.** Female lip region. **3.** Female tail region. **4.** Spicule and lateral guiding piece of gubernaculum. **5.** Vulva region. **6.** Male tail region. **7.** Detail of basal pharyngeal bulb. Scale bars: 23 μm (**1–3**); 24 μm (**4, 6**); 29 μm (**5**); 15 μm (**7**).

ranean Agricultural and Environment Sciences, ICAAM, University of Évora, Évora; 2 paratypes (1 female and 1 male) (slides PLB006 and PLB012) in the Royal Belgian Institute of Natural Sciences, Brussels, Belgium; 2 paratypes (1 female and 1 male) (slides PLB007 and PLB013) in the Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (C.N.R.), Sezione di Bari, Bari, Italy.

Type locality. Holotype and paratype specimens were extracted from a soil sample collected from the rhizosphere of an unidentified grass species at Bordonhos, São Pedro do Sul, Viseu district, Beira Alta province, northern Portugal (40°45'53"N, 8°5'12"W) (Table 1)

Etymology. The specific epithet of this species refers to the region of the type locality (Bordonhos) where the new species was found.

Description of female. Short and slender body, slightly tapering at both ends, more pronounced in the tail. Curved in open J- or C-shaped relaxed by heat. Cuticle thin, appearing smooth under low magnifications, 1.8 ± 0.3 (1.3–2.2) µm thick at mid body, but thicker (9.1 ± 0.7 (8.1–9.8) µm) in hyaline region located at the end of tail region (Figs 1(3), 2(6, 7); Table 2). Lateral chord ca 11.1 µm wide at mid-body or ca 34% of corresponding body diam. Lip region anteriorly flattened, expanded and rounded laterally, 10.1 ± 0.4 (9.6–10.7) µm wide and 4.1 ± 0.5 (3.6–5.0) µm high, set-off from body contour by a constriction (Figs 1(1, 2), 2(1, 2), Table 2). Amphidial fovea large asymmetrically bilobed pouch occupying from 2/3 to 3/4 of the distance from oral aperture to guiding ring (Figs 1(1, 2), 2(3), Table 2). Stylet guiding-ring single and posteriorly situated, 2.7–2.3 times lip region diameter from anterior end. Moderate and straight odontostyle, 1.5 ± 0.1 (1.3–1.7) times as long as odontophore; weakly developed, with rather weak basal swellings (Figs 1(1), 2(4) Table 2). Nerve ring surrounding the tubular portion of the pharynx behind the odontophore base at 149.9 ± 7.7 (138.1–157.7) µm from anterior end. Anterior slender part of pharynx usually coiled in its posterior region. Basal bulb short and cylindrical, 79.9 ± 6.2 (68.0–87.2) µm long and 13.5 ± 1.0 (12.1–14.7) µm in diameter. Glandularium 71.9 ± 3.0 (67.0–74.3) µm long. Dorsal pharyngeal gland nucleus (DN) and ventro-sublateral nuclei (SVN) located at 33.1 ± 3.2 (29.4–35.3)% and 53.1 ± 0.7 (52.6–53.9)% of distance from anterior end of pharyngeal bulb, respectively. Cardia conoid to rounded, 8.6 ± 2.7 (6.3–13.1) µm long (Figs 1(7), 2(5), Table 2). Reproductive system with both genital branches equally developed, 8.4 ± 0.5 (7.5–8.7) or 8.0 ± 1.2 (6.7–9.5)% of body length (Table 2). Ovaries reflexed, variable in length, anterior ovary 71.4 ± 14.6 (52.0–85.8 µm long) and posterior ovary 82.3 ± 13.6 (68.0–99.0 µm long) (Table 2). Oviducts slightly longer than ovaries. Uteri cylindrical, quite variable in length, anterior uteri 283.9 ± 94.0 (209.0–418.0 µm long) and posterior uteri 251.3 ± 43.7 (194.0–295.0 µm long); sphincter usually well developed, delimiting uterus and oviduct. Sperm commonly found in the uteri of female reproductive tract. Vulva transverse, located slightly anterior to the middle of the body, vagina perpendicular to body axis, 21.8 ± 1.9 (19.0–24.6) µm long (Figs 1(5), 2(9), Table 2). Prerectum visible, variable in length, 547.5 ± 503.7 (162.0–1201.0) µm long, and short rectum 20.0 ± 2.9 (15.0– 23.0) µm long or 1.2 (0.9–1.6) times anal body width. Tail long, bluntly conoid, slightly ventrally curved with rounded terminus, bearing three pairs of caudal pores (Figs 1(3), 2(6, 7); Table 2).

Description of male. Males are as common as females. Appearance of body similar to female, except for reproductive organs. Male diorchic with testes paired and opposed. Tail conoid, more convex-curved ventrally than that of the female, with rounded terminus at the end of tail (Figs 1(6), 2(8), Table 2). Spicules short, moderately developed, and quite curved ventrally; lateral guiding pieces more or less straight, sometimes with slightly curved proximal ends (Figs 1(4, 6), 2(8, 10), Table 2). Large number of visible supplements, one pair of adanal and 9–11 mid-ventral supplements (Figs 1(3), 2(8)).

Differential diagnosis. *Longidorus bordonensis* sp. nov. is characterized by a short body within the genus *Longidorus* (average = 4443 µm and 4560 µm in females and males, respectively), short odontostyle within the genus *Longidorus* (average = 70.0 µm and 69.5 µm in females and males, respectively), lip region anteriorly flattened, expanded (average = 10.0 µm in both females and males) and rounded laterally, set-off from body contour by a constriction, asymmetrically bilobed amphidial pouches with lobes occupying from 2/3 to 3/4 part of the distance from oral aperture to guiding ring, tail long (average = 51.0 µm and 55.0 µm in females and males, respectively), bluntly conoid, slightly ventrally curved with rounded terminus, short to medium spicules (average = 37.0 µm) with one pair of adanal and 9–11 mid-ventral supplements (Figs 1(1–7), 2(1–10), Table 2, Suppl. material 4: Table S2). According to the polytomous key of Chen et al. (1997) and two subsequent supplements (Loof and Chen 1999; Peneva et al. 2013), *L. bordonensis* sp. nov. has the following codes (codes in parentheses are exceptions): A2, B1, C2, D4, E3, F2(3), G3, H(5)6, I2, J?, K?. On the basis of the diagnostic characters (body length, odontostyle length, lip region width, shape of anterior region, shape of amphidial pouch, oral aperture-guiding ring distance, tail length, spicule length, tail shape, a and c' ratios, and frequency of males) used in the polytomous key by Chen et al. (1997), and supplements by Loof and Chen (1999) and Peneva et al. (2013), *L. bordonensis* sp. nov. is grouped with *L. indalus* Archidona-Yuste, Navas-Cortés, Cantalapiedra-Navarrete, Palomares-Rius & Castillo, 2016, *L. carpetanensis* Arias, Andrés & Navas, 1986 , *L. unedoi* Arias, Andrés & Navas, 1986, *L. juvenilis*, *L . pini* Jacobs & Heyns, 1987, *L. pisi* Edward,

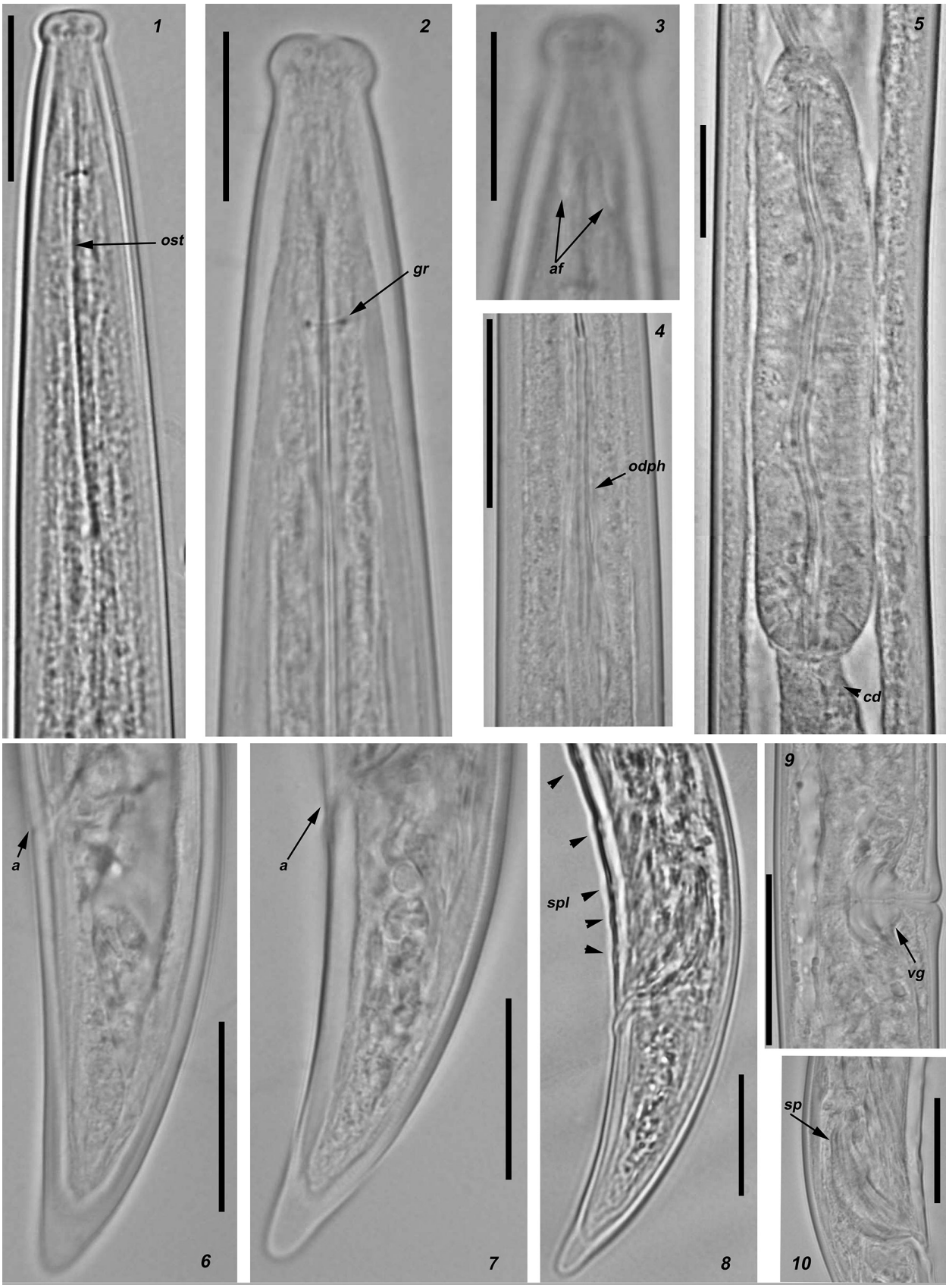

Figure 2. Light micrographs of *Longidorus bordonensis* sp. nov. paratypes from the rhizosphere of grass (unknown species) at São Pedro do Sul, Viseu district, northern Portugal (1–10). **1.** Anterior region. **2.** Odontostyle region. **3.** Lip region showing amphidial fovea. **4.** Odontophore region. **5.** Detail of basal bulb. **6, 7.** Female tail region. **8.** Male tail region. **9.** vulva region. **10.** Detail of spicule region. Abbreviations: **a** anus, **af** amphidial fovea, **cd** cardia, **gr** guiding ring, **ost** odontostyle, **odph** odontophore, **sp** spicules, **spl** ventromedian supplements, **V** vulva, **vg** vagina. Scale bars: 23 µm (**1, 4**); 15 µm (**2, 3, 5**); 50 µm (**6, 7**); 25 µm (**8, 10**); 30 µm (**9**).

Table 2. Morphometrics of females and males of *Longidorus bordonensis* sp. nov. from the rhizosphere of grass (unknown) at Bordonhos, São Pedro do Sul, Viseu district, Beira Alta province, northern Portugal.

Characters/ratios	Holotype	Females	Males
n	1	7	6
L	4115.8	4443.0 ± 593.1 (3671.4–5396.2)	4560.0 ± 357.4 (4188.6–5201.0)
a	126.7	135.8 ± 13.1 (115.4–153.6)	155.3 ± 11.5 (133.4–164.7)
b	12.8	13.8 ± 1.8 (10.7–15.9)	13.9 ± 1.1 (12.3–15.7)
c	78.6	88.0 ± 13.7 (67.7–110.1)	83.4 ± 9.7 (72.4–95.0)
c'	2.3	2.3 ± 0.4(1.9–3.1)	2.3 ± 0.2(1.9–2.5)
V or T	45.6	46.7 ± 2.0 (44.4–50.2)	35.3 ± 8.7 (24.5–42.0)
G_1 (%)	7.5	8.4 ± 0.5 (7.5–8.7)	–
G_2 (%)	8.0	8.0 ± 1.2 (6.7–9.5)	–
Odontostyle length	67.1	69.8 ± 2.4 (67.1–73.5)	69.5 ± 2.3 (67.1–73.4)
Odontophore length	45.6	47.0 ± 3.7 (42.4–52.2)	45.5 ± 7.1 (37.0–54.6)
Lip region width	9.7	10.1 ± 0.4 (9.6–10.7)	9.9 ± 0.7 (8.8–10.8)
Oral aperture-guiding ring	23.0	24.7 ± 1.4 (23.0–26.7)	25.9 ± 1.0 (24.8–27.6)
Tail length	52.3	51.0 ± 6.8 (43.1–63.8)	55.0 ± 4.6 (46.9–59.5)
Spicules	–	–	37.0 ± 3.4 (32.6–41.8)
Lateral accessory piece	–	–	8.4 ± 0.5 (8.1–8.7) (n = 2)
J	8.0	9.2 ± 0.7 (8.1–9.8)	9.1 ± 0.6 (8.2–9.8)

*Abbreviations are defined in Jairajpuri and Ahmad 1992. (–) Not obtained or not performed.

Misra & Singh, 1964, and *L. distintus* Lamberti, Choleva & Agostinelli, 1983. Morphological and morphometric characters of the new species are compared with its closely related species (Suppl. material 4: Table S2). *Longidorus bordonensis* sp. nov. differs from paratypes of *L. pini* by small differences in the distance from the guiding ring to the anterior end (23.0–26.7 µm vs 26–27 µm), amphidial pouch shapes (asymmetrically bilobed with lobes occupying from 2/3 to 3/4 of the oa–gr distance vs symmetrically bilobed with lobes occupying from 1/3 to 2/3 of the oa–gr distance), tail shape (bluntly conoid, slightly ventrally curved with round terminus vs tail long, conical dorsally convex and ventrally concave, with the round terminus slightly subdigitate) and the frequency of males (common vs absent). Also, the new species differs from some previously cited species in measurements and ratios, including L, c' and a ratios, odontostyle length, lip region diameter and shape, the distance from guiding ring to anterior, and tail length and shape (Suppl. material 4: Table S2).

Longidorus vinearum Bravo & Roca, 1995

Suppl. material 1: Fig. S1(1–9), Suppl. material 5: Table S3

Remarks. *Longidorus vinearum* was originally described from around roots of grapevine (*Vitis* L.) in Dois Portos, Torres Vedras, Portugal (Bravo and Roca 1995). Subsequently, Bravo and Roca (1998) reported it from the rhizosphere of olive trees (*Olea europaea* L.) in Matela, Vimioso, Portugal. Recently, Archidona-Yuste et al. (2016) found four populations of this species associated with wild olive trees in Andalusia (Spain) and characterized these populations molecularly. Three populations resembling this *Longidorus* species were detected parasitizing grapevine roots at Dois Portos, Torres Vedras (type locality of *L. vinearum*), Ordasqueira, Torres Vedras, and Picanceira, Mafra, and another population from around the roots of wild olive trees at Valverde, Évora, all in Portugal (Table 1, Suppl. material 5: Table S3). These populations prompted us to characterize them genotypically and phenotypically, including the topotype specimens, in order to confirm their identification. Unfortunately, only one specimen was found at Picanceira, Mafra (Table 1) and used to complete the molecular analysis. These findings represent the third and fourth records of this species for Portugal and the Iberian Peninsula, respectively. We confirm a wider geographical distribution of this species in this geographical region.

Longidorus vinearum populations are characterized by a lip region, which is broadly rounded frontally, and more so laterally, and almost totally continuous with the outline of the body; a vulva near mid-body; the amphidial fovea large and clearly asymmetrically bilobed; the odontostyle long and robust; short tail characterized by having a bluntly rounded to hemispherical shape, dorsal side quite more convex than ventral side with rounded terminus; males characterized by large-sized spicules (average = 112.0 µm) and a large number of supplements, one pair of adanal and 18 or 19 mid-ventral supplements (Suppl. material 1: Fig. S1(1–9); Suppl. material 5: Table S3). Morphological and morphometrical traits of the topotype population from Dois Portos, Torres Vedras (Suppl. material 5: Table S3) agree very well with the original description (Bravo and Roca 1995). Morphometric measurements of adult specimens of the topotype population are coincident with those provided in the original description (Bravo and Roca 1995) (Suppl. material 5: Table S3) except for minor differences in a and c' ratios (69.2–79.8 vs 70.7–101.3; 0.6–0.7 vs 0.5–0.8), lip region diameter (21.9–23.4 vs 18.0–27.5), length from the oral aperture to guiding ring (34.5–43.2 vs 36.0–47.0), tail length (45.3–61.8 vs 38.0–57.0), odontostyle length (113.8–126.4 vs 105.5–132.0), and odontophore length (65.3–82.5 vs 58.0–85.0) for the females (Suppl. material 5: Table S3), which may be due

to intraspecific variability, as reported by Archidona-Yuste et al. (2016). Also, the topotype population shows similarity to four populations from Córdoba province, southern Spain (Archidona-Yuste et al. 2016); however, minor differences were detected in females such as L, a and c' ratios, lip region diameter length, odontostyle and odontophore lengths, distance from oral aperture to guiding ring, and, in males, spicule length. In addition, the topotype population agrees closely with the morphological features and morphometric measurements of all Portuguese populations examined (Suppl. material 5: Table S3), except for a higher V ratio (47.1–50.1 vs 45.8–51.4, 44.0), a longer odontostyle (113.8–126.4 vs 112.9–121.7, 116.8) µm and a smaller J length (11.7–17.4 vs 13.1–21.3, 19.7) µm (Suppl. material 5: Table S3). Nevertheless, these differences further expand the intraspecific variation of the species and should be regarded as geographical intraspecific variation. According to the polytomous key by Chen et al. (1997) and its supplements (Loof and Chen 1999; Peneva et al. 2013), the topotypes and other studied Portuguese populations of this species have the following codes: A45, B45, C34, D2, E3, F45, G1, H1, I12, J?, K?. Unfortunately, we did not detect the first juvenile-stage. However, this stage was characterized in the original description (Bravo and Roca 1995) and later by Archidona-Yuste et al. (2016), who also characterized this species molecularly.

Longidorus vineacola Sturhan & Weischer, 1964

Suppl.material 2: Fig. S2(1–11), Suppl.material 6: Table S4

Remarks. Three populations of *L. vineacola* from cork oak (*Quercus suber* L.) trees at Amoreirinhas da Cima, Montemor-o-Novo, Portugal and one population from wild olive (*Olea europaea* L. var. *sylvestris*) at Valverde, Évora, Portugal, are characterized morphometrically and morphologically: body medium-length to long (6.9–9.6 mm in females and 6.4–8.2 mm in males); odontostyle long (87.0–99.5 µm in females and 91.5–100.9 µm in males); lip region slightly set off from body contour by a depression; amphidial pouches asymmetrically bilobed; two equally developed female genital branches; females with broadly rounded tail usually as long as anal diameter; vulva posterior to mid-body; males with spicules well developed (69.9–79.9 µm long), and supplements consisting of an adanal pair and 14 or 15 ventromedians (Suppl. material 2: Fig. S2(1–11); Suppl. material 6: Table S4). The morphological and metrical traits closely agree with the original description of the species (Sturhan and Weischer 1964) and subsequent records (Boag and Brown 1987; Brown and Taylor 1987; Andrés et al. 1991; Roca and Bravo 1996; Bravo and Lemos 1997; Brown et al. 1997; Gutiérrez-Gutiérrez et al. 2013, 2016; Archidona-Yuste et al. 2016), except for minor intraspecific variations in a and c ratios and length of odontostyle and spicules (Suppl. material 6: Table S4). This species was originally described parasitizing grapevine roots in Germany (Sturhan and Weischer 1964) and has since been reported in a large number of records from various Euro-Mediterranean countries from a wide range of herbaceous and woody hosts (Bravo and Lemos 1997; Brown et al. 1997; Taylor and Brown 1997; Gutiérrez-Gutiérrez et al. 2013, 2016; Archidona-Yuste et al. 2016). The alphanumeric codes for these populations of *L. vineacola* were applied in the diagnostic identification key for *Longidorus* spp. by Chen et al. (1997) and successive supplements by Loof and Chen (1999) and Peneva et al. (2013); they are (codes in parentheses are exceptions): A3(4), B3(4), C(2)3 ,D2, E3, F45, G23, H1, I2, J?, K?. Unfortunately, we detected no juvenile stages in our surveys; however, four juvenile stages were described by Sturhan and Weischer (1964) and by Roca and Bravo (1996). Additionally, Gutiérrez-Gutiérrez et al. (2013) using an integrative strategy characterized females, males, and first-stage juveniles (J1) of several populations of southern Spain and assigned molecular markers for this species.

Molecular results and phylogenetic relationships of *Longidorus bordonensis* sp. nov. and other *Longidorus* spp.

Polymerase chain reaction (PCR) was used to amplify the D2–D3 expansion segments of 28S rRNA, ITS1 rRNA, and partial 18S rRNA from *L. bordonensis* sp. nov. and four other *Longidorus* spp. For each of the species studied, these three genes had an approximate size of 700–800, 900–1000, and 1600 bp, respectively, based on visualization of the band on the electrophoresis gel and the subsequent direct sequencing.

D2–D3 sequences of *L. bordonensis* sp. nov. (MN082421–MN082422) matched well with the *Longidorus* spp. deposited in GenBank. Both D2–D3 sequences of *L. bordonensis* sp. nov. (MN082421 and MN082422) were almost identical, with a 99.31 % of sequence similarity. D2–D3 sequences of the new species were 95, 95, and 87%, similar to *L. pini* (MH430028, Spain), *L. carpetanensis* (MH430019–MH430020, Spain), and *Longidorus* sp. 1 FG-2018 isolate (MG765547, Iran) , respectively, and differed in 27, 28–30, and 75 nucleotides, respectively. ITS1 sequence of *L. bordonensis* sp. nov. (MN150062) appropriately matched with other *Longidorus* spp. deposited in GenBank. This ITS1 sequence was 83–82, and 83% similar to *L. carpetanenesis* (MH429991–MH429993, Spain) and *L. pini* (MH430001, Spain), respectively. The variations among the ITS1 sequences of these species were from 143 to 157 nucleotides. The partial 18S rRNA gene sequences of *L. bordonensis* sp. nov. (MN129757) showed a high homology (more than 99% similarity) with two sequences deposited in GenBank belonging to *L. carpetanensis* (MH430006, Spain) and *L. pini* (MH430011, Spain). The variations among the 18S sequences of these species were from 8 to 15 nucleotides.

The D2–D3 expansion segments of 28S rRNA, ITS1 rRNA, and the partial 18S rRNA gene sequences obtained in this study for *L. vinearum*, *L. vineacola*, and

L. wicuolea matched well with sequences from the same species previously deposited in GenBank, increasing knowledge of the genotypic diversity in *Longidorus* (Table 1). For the species of *Longidorus* studied here, there were multiple failed attempts to sequence the ITS1 region and a partial portion of 18S rRNA gene before our study was concluded (Table 1). The D2–D3 expansion segments of 28S rRNA gene sequences from *L. vinearum* (MN082430–MN082434) matched closely (99% similarity) to sequences of Spanish populations of this species in GenBank (KT308874, KT308876–KT308877); and the variations among these D2–D3 sequences ranged from 2 to 5 nucleotides. Intra-specific variation of D2–D3 detected among the populations of *L. vinearum* (three from grapevine and one from wild olive) (Table 1) was from 0 to 2 nucleotides (99% similarity and 0–1 indels). For *L. vinearum*, three ITS1 sequences (MN150065, MN150067, MN150068) from Dois Portos (LISB-03-04, grapevine) and an ITS1 sequence (MN150066) from Evora (M3-OLV, wild olive) were sequenced and showed a high similarity (99%), with some minor intra-specific variations among them (2–14 nucleotides and 0–1 indels). Our ITS1 sequences (MN150065–MN150068) had 98% similarity to others deposited in GenBank for *L. vinearum* (KT308892–KT308893, Spain); and the variations among them ranged from 17–23 nucleotides and 1–3 indels. The D2–D3 expansion segments of 28S rRNA gene sequences from *L. vineacola* (MN082425–MN082429) also had 99% similarity to several sequences of Spanish populations of this species in GenBank (JX445109–JX445111; KT308872, KT308873); and the variations among them ranged from 2 to 9 nucleotides. Intra-specific variation of the D2–D3 region among the four populations of *L. vineacola* (three from cork oak and one from wild olive) (Table 1) was low, varying from 0 to 6 nucleotides (99–100% similarity and 0 indels). For *L. vineacola*, the ITS1 region and the partial portion of 18S gene sequenced agree with results obtained from the D2–D3 fragments. The partial 18S rRNA gene sequence of *L. vineacola* (MN129758) was identical (100% similarity) to several *L. vineacola* sequences deposited in GenBank (AY283169, UK; JX445123, Spain), and 99% similar to *L. onubensis* Archidona-Yuste, Navas Cortés, Cantalapiedra-Navarrete, Palomares-Rius & Castillo, 2016 (KT308897, Spain), *L. nevesi* (MH430009, Spain), *L. wicuolea* (KT308900, Spain), *L. fasciatus* (MH430008, Spain; JX445122, Spain), and *L. pacensis* Archidona-Yuste, Cantalapiedra-Navarrete, Castillo & Palomare-Rius, 2019 (MH430004–MH430005, Spain). The variations among the partial 18S rRNA gene sequences of these species varied from 8 to 16 nucleotides. Our ITS1 sequence of *L. vineacola* (MN150064) showed a variable and low sequence homology with other sequences of *L. vineacola* in Genbank; the homology ranged from 97% (JX445094, Spain) to 94% (JX445096, Spain). The variations among the ITS1 sequences of these three sequences ranged from 18 to 51 nucleotides. The D2–D3 expansion segments of 28S rRNA gene sequence from *L. wicuolea* (MN082423) were 99% similar to three sequences of Spanish populations of this same species in GenBank (KT308863–KT308865, Spain). The variations among these four D2–D3 sequences ranged from 1 to 5 nucleotides. For *L. wicuolea*, the ITS1 sequences agree with results from the D2–D3 region. Our ITS1 sequence of *L. wicuolea* (MN150063) was 100% identical to other two sequences of this species in Genbank (KT308887 and KT308889, Spain) and clearly different (90%–88% similarity) from *L. silvestris* Archidona-Yuste, Navas Cortés, Cantalapiedra-Navarrete, Palomares-Rius & Castillo, 2016 (KT308884, Spain), and *L.* cf. *olegi* Kankina & Metlitskaya, 1983 (MH429999, MH430000, Spain); and the variations among these ITS1 sequences ranged from 18 to 51 nucleotides. The D2–D3 expansion segments of 28S rRNA gene sequence from *Longidorus* sp. 3 isolate ST (MN082424) matched well with several *Longidorus* spp. deposited in GenBank, including *L. lusitanicus* (KT308869, Spain) as the closest with 98.55% similarity, followed by *L. magnus* Lamberti, Bleve-Zacheo & Arias, 1982 (JX445113 and KT308870, Spain), *L. crataegi* Roca & Bravo, 1996 (JX445114, Spain), *L. goodeyi* Hooper, 1961 (AY601581), and *L. vinearum* (KT308876, Spain) with 94–95% similarity; and the sequence variations among the D2–D3 sequences of these species were from 11–42 nucleotides and 1–12 indels .

Using Bayesian inference (BI), we compared the phylogenetic position of *L. bordonensis* sp. nov. and other *Longidorus* spp. by using the D2–D3 expansion segments of 28S rRNA, the ITS1 region, and the partial 18S rRNA gene sequences (Figs 3–5). The BI tree (50% majority rule consensus tree) of the D2–D3 domains of 28S rRNA gene (Fig. 3) was based on a multiple-edited alignment (135 total sequences) of 722 total characters and revealed a major clade containing the majority of these species, including *L. bordonensis* sp. nov. and the remaining Iberian populations of *Longidorus* spp. (Fig. 3). The generated phylogenetic tree, using sequences of these D2–D3 fragments (Fig. 3), showed a clearly congruent position of *L. bordonensis* sp. nov. (MN082421, MN082422). The clade, including *L. bordonensis* sp. nov., grouped morphologically related species characterized by a short body and odontostyle and elongate to conical female tail, such as in *L. pini* (MH430028, Spain) and *L. carpetanensis* (MH430019, MH430020, Spain). The D2–D3 tree showed a consistent position for *L. vinearum* (MN082430– MN082434), which was placed within a well-supported clade of available GenBank entries belonging to *L. vinearum* (KT308874, KT308876, Spain) and clearly separated from the new species and other morphologically related species, such as *L. magnus* (KT308870, KX445113, Spain), *L. crataegi* (JX445114, Spain), *L. goodeyi* (AY601581), *L. onubensis* (KT308857, KT308858, Spain), *L. oakcrassus* Cai, Archidona-Yuste, Cantalapiedra-Navarrete, Palomares-Rius & Pablo Castillo, 2019 (MK941187–MK941190), *L. oakgracilis* Cai, Archidona-Yuste, Cantalapiedra-Navarrete, Palomares-Rius & Pablo Castillo, 2019 (MK941191–MK941193), *L. wicuolea* (KT308863–KT308865,

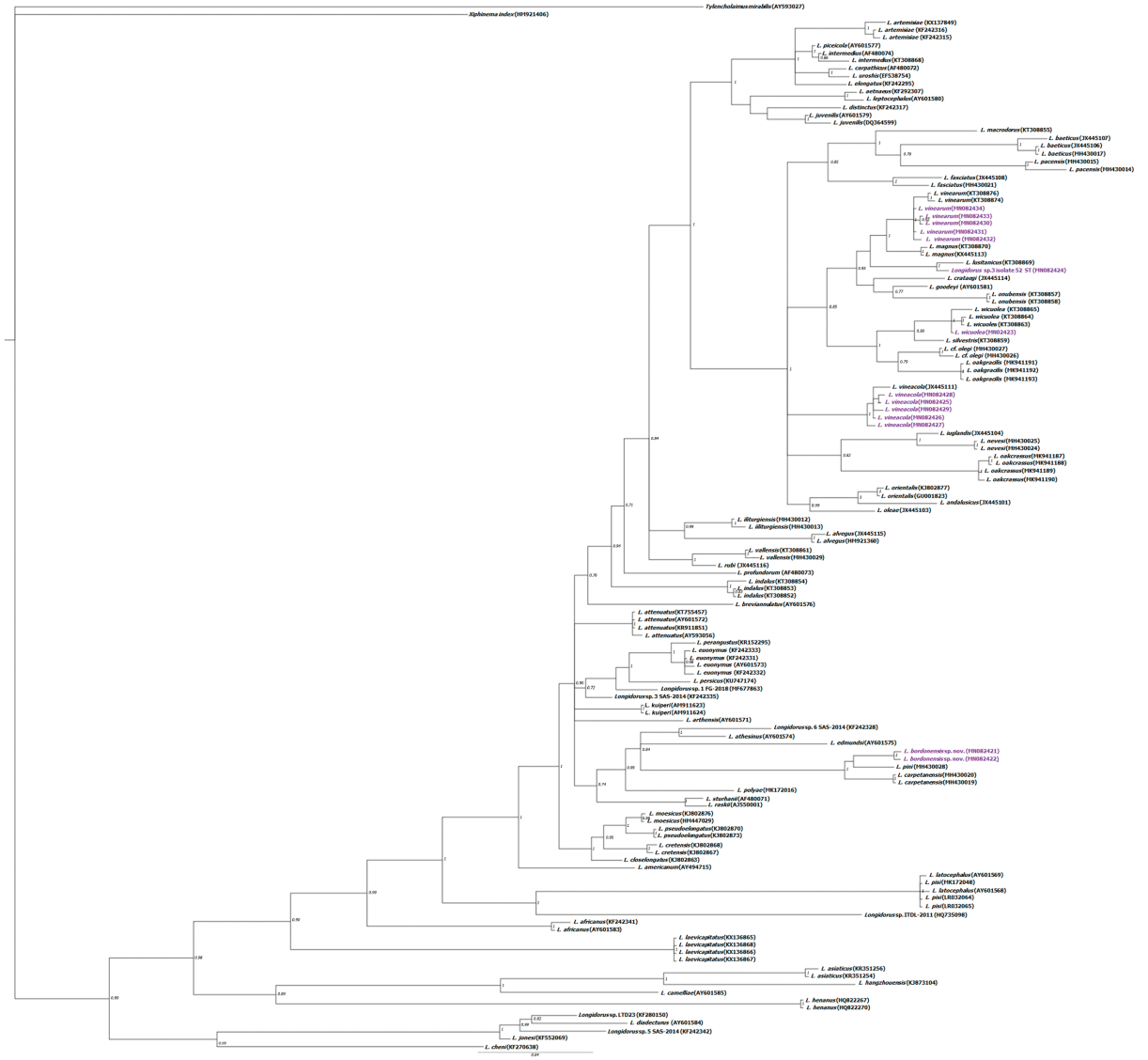

Figure 3. Phylogenetic relationships of *Longidorus bordonensis* sp. nov., *L. wicuolea* Archidona-Yuste, Navas-Cortés, Cantalapiedra-Navarrete, Palomares-Rius & Castillo, *L. lusitanicus* Macara, 1986, *L. vinearum* Bravo & Roca and *L. vineacola* Sturhan & Weischer, 1964 within the genus *Longidorus*. Bayesian 50% majority rule consensus trees as inferred from D2–D3 expansion segments of 28S rRNA sequences alignments under the SYM model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences in this study are coloured in purple. Scale bar: expected changes per site.

Spain; MN022423, Portugal), *L. andalusicus* (JX445101, Spain), and *L. vineacola* (JX445111, Spain; MN082425–MN082429 Portugal). Likewise, this D2–D3 tree also showed congruence for the phylogenetic positions of *L. vineacola* sequences obtained here (MN082425–MN082429), as it was positioned within a well-supported clade together an available sequence in Genbank belonging to *L. vineacola* (JX445111, Spain). This clade, including all *L. vineacola* sequences, were separated from the new species and other phenotypically similar species, such as *L.* cf. *olegi* (MH430026–MH430027, Spain), *L. silvestris* (KT308859, Spain), *L. lusitanicus* (KT308869, Spain), *L. oakcrassus* (MK941187–MK941190), and *L. wicuolea* (KT308863–KT308865, Spain; MN022423, Portugal). In addition, *Longidorus* sp. 3 isolate ST (MN082424) was placed in a separated position within a well-supported subclade, clustering together to *L. lusitanicus* (KT308889, Spain) and *L. crataegi* (JX445114, Spain).

Similarly, the BI tree (50% majority rule consensus tree) of a multiple-edited alignment, including 116 18S rRNA sequences and 1690 total characters (Fig. 4) and 116 ITS1 sequences and 570 total characters (Fig. 5), showed a topology similar to that of the D2–D3 fragments of the 28S gene. Both the partial 18S and ITS1 trees using BI (Figs 4, 5) showed a close phylogenetic relationship of *L. bordonensis* sp. nov. (18S, MN129757; ITS1, MN150062) with *L. pini* (18S, MH430011, Spain; ITS1, MH430001, Spain)

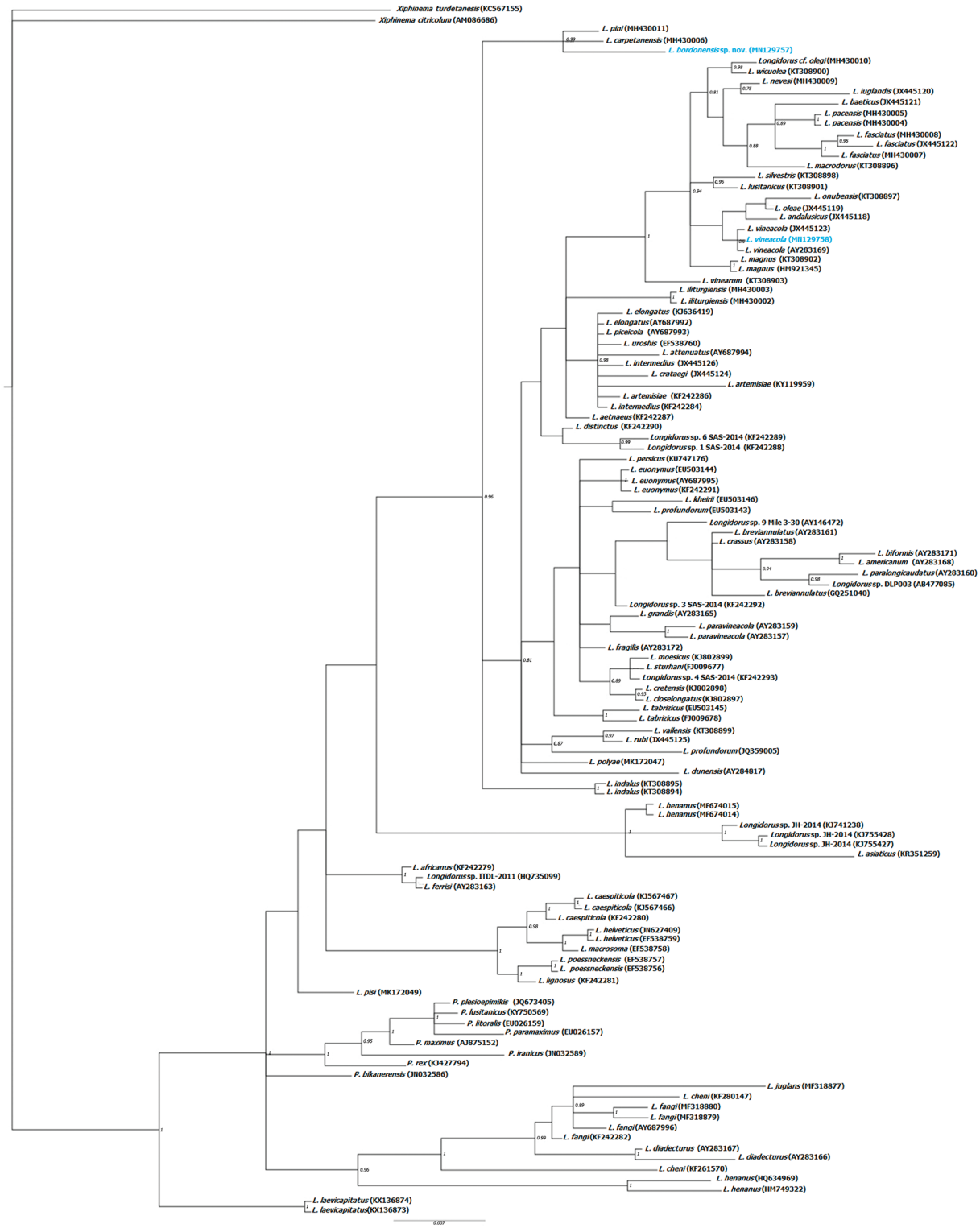

Figure 4. Phylogenetic relationships of *Longidorus bordonensis* sp. nov. and *L. vineacola* Sturhan & Weischer, 1964 within the genera *Longidorus* and *Paralongidorus*. Bayesian 50% majority rule consensus trees as inferred from 18S rRNA sequences alignments under the SYM model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences in this study are coloured in light blue. Scale bar: expected changes per site.

and *L. carpetanensis* (18S, MH430006 Spain; ITS1, MH429991–MH429993, Spain). Both 18S and ITS1 trees showed a congruent position for all known species found in this study. For 18S and ITS1 trees, our *L. vineacola* sequences (18S, MN129758; ITS1, MN150064) were grouped in a well-supported clade also contain-

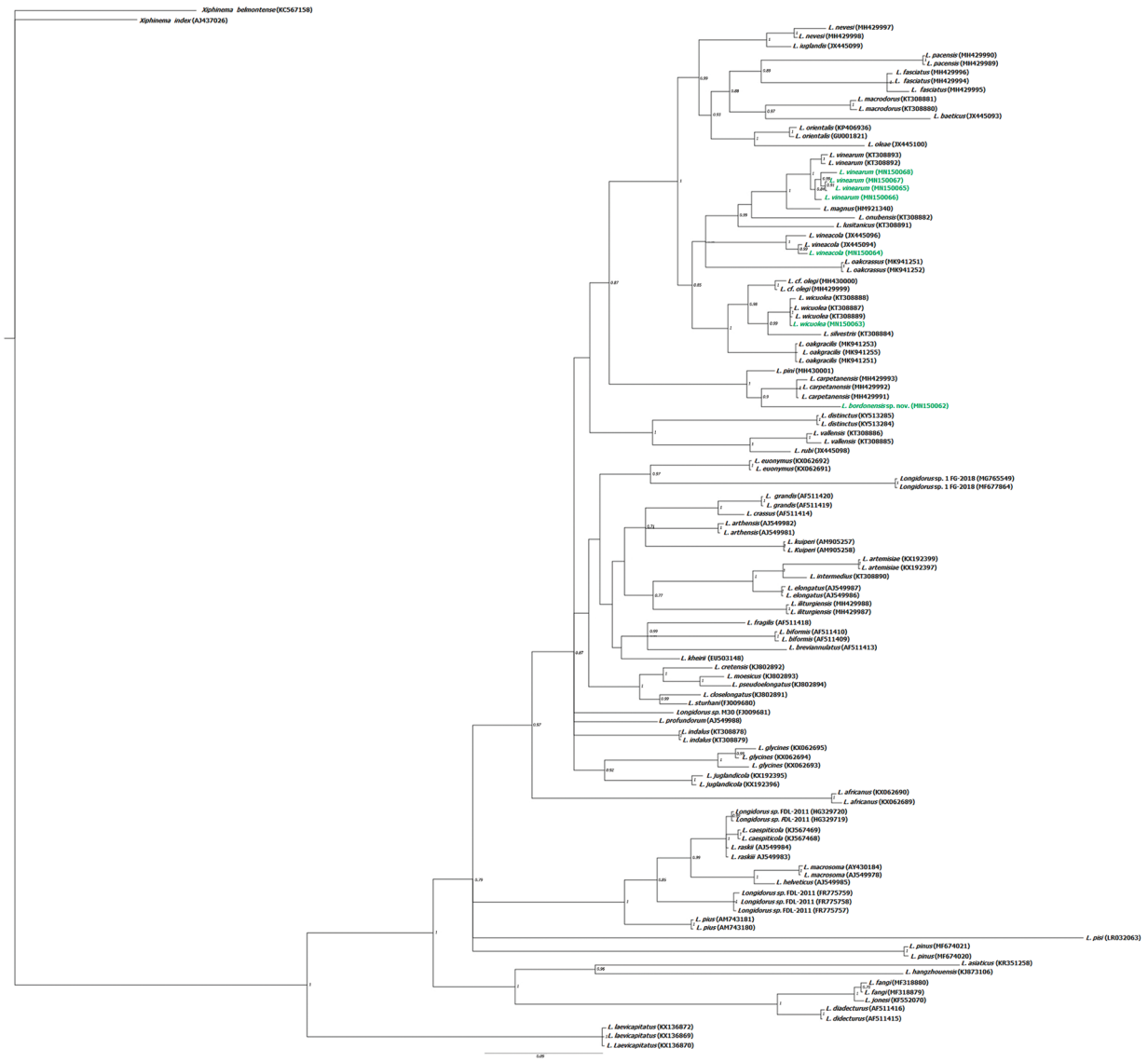

Figure 5. Phylogenetic relationships of *Longidorus bordonensis* sp. nov., *L. wicuolea* Archidona-Yuste, Navas-Cortés, Cantalapiedra-Navarrete, Palomares-Rius & Castillo, *L. vinearum* Bravo & Roca and *L. vineacola* Sturhan & Weischer, 1964 within the genus *Longidorus*. Bayesian 50% majority rule consensus trees as inferred from ITS1 rRNA sequences alignments under the SYM model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences in this study are coloured in green. Scale bar: expected changes per site.

ing GenBank entries belonging to *L. vineacola* (18S, JX445111, Spain and AY283169; ITS1, JX445094, JX445096, Spain). In the ITS1 tree, all sequences from *L. vinearum*, including the accessions from our sequences (MN082425–MN082429) and GenBank accessions (KT308892–KT308893, Spain) clustered in the same well-supported sub-clade; however, they also clustered together with *L. magnus* (HM921340, Spain). Similarly, the tree generated using the ITS1 dataset, sequences from *L. wicuolea*, including the accession from our population (MN150063) and other populations in GenBank (KT308886–KT308888, Spain), clustered in the same well-supported sub-clade; however, this sub-clade also included *L. silvestris* (KT308884, Spain).

Discussion

The main goal of our study was to identify and describe, morphologically and molecularly, *Longidorus* spp. parasitizing herbaceous and woody plants in vineyards and agro-forestry systems in Portugal. This was conducted in a nematological survey that included 150 sampling sites, with 43% of them in vineyards and the rest in agro-forestry areas. Eleven soil samples, each infested with only

one needle nematode population, were selected for this study (Table 1). Our results confirmed the usefulness of developing an integrative approach based on the combination of morphometric and morphological characteristics and genotyping rRNA markers to correctly discriminate among *Longidorus* species. We described one new species (*L. bordonensis* sp. nov.) and identified several populations by integrating morphological analyses, morphometric measurements, and molecular data based on rRNA sequences to elucidate their phylogenetic relationships within *Longidorus*. New molecular markers were described for the new species, and the molecular diversity of three species (*L. vinearum*, *L. vineacola*, and *L. wicuolea*) was evaluated.

The comparative morphological taxonomic study of the 11 Portuguese populations of *Longidorus* spp. confirmed that the identification of species from phenotypic features including morphometric and morphometrical data is not easy due to inter- and intra- variability, overlapping of measurements and de Man ratios between species, and ambiguity caused by the presence of hundreds of species. As for previous biogeographic studies (Navas et al. 1993; Taylor and Brown 1997; Archidona-Yuste et al. 2019), our study has revealed a new species (*L. bordonensis* sp. nov.), a first report of *Longidorus* spp. for Portugal (*L. wicuolea*), and new geographic records for other species, such as *L. vinearum* and *L. vineacola*. Our findings confirm that *Longidorus* is widespread in Southern Europe. This is in agreement with previous studies in Europe and around the Mediterranean Basin (Navas et al. 1993; Taylor and Brown 1997; Archidona-Yuste et al. 2019), in which a dispersalist model was the main hypothesis to explain the large number of *Longidorus* spp. in Iberian Peninsula (Navas et al. 1993; Taylor and Brown 1997; Archidona-Yuste et al. 2019). However, Navas et al. (1993) proposed vicariant speciation as an alternative explanation for the current distribution of some *Longidorus* species; Navas et al. (1993) proposed that species survived the Pleistocene glaciation in refugia in the southern European peninsulas of Iberia, Italy, and the Balkans. We suggest that further molecular and phylogenetic studies are needed before assigning the correct model of speciation to explain the current biodiversity and distribution patterns of longidorids in Europe and the Mediterranean Basin. The biodiversity of *Longidorus* spp. in Portugal is considerable, with approximately 17 species reported (Bravo and Lemos 1997; Gutiérrez-Gutiérrez et al. 2016), including *L. bordonensis* sp. nov. and newly reported *L. wicuolea*. A major goal of our study was to generate DNA barcode markers that are useful tools for identifying new species and distinguishing among species within this genus. We update the geographical distribution and occurrence of *Longidorus* spp. in Portugal, which is important to the understanding of their current dispersion, the risks of spreading, the recognition of endemics and invasive species, and the diagnosis of quarantine pests.

Twenty sequences belonging to three nuclear rRNA markers were used in this study: 11 D2–D3, seven ITS1, and two partial 18S sequences (Table 1; Figs 3–5). The results corroborate previous studies (Gutiérrez-Gutiérrez et al. 2013; Esmaeili et al. 2017; Kornobis et al. 2017; Xu et al. 2017, 2018; Archidona-Yuste et al. 2016, 2019; Lazarova et al. 2019; Cai et al. 2019) in finding the utility of this integrative approach for species discrimination in *Longidorus*. Our findings, according to the results of recent studies (Archidona-Yuste et al. 2016, 2019; Lazarova et al. 2019), show that two molecular markers based on rRNA, the D2–D3 fragments of the 28S gene, and the ITS1 region were the most decisive, precise, and reliable for discriminating among species and diagnosis new taxa. For example, in *L. bordonensis* sp. nov., the sequence of the partial 18S (MN129758) was compared to other sequences belonging to known *Longidorus* species (MH430006, Spain; MH430011, Spain), and was greater than 99% similar with fewer than 15 nucleotides differences, whereas sequences of both the D2–D3 fragments of 28S rRNA and the ITS1 region (D2–D3, MN082421–MN082422; ITS1, MN150062) had a low homology with maximum similarity values of 95% and 83%, respectively, to *L. pini* (D2–D3, MH430028, Spain; ITS1, MH430001, Spain) and *L. carpetanensis* (D2–D3, MH430019, MH430020, Spain; ITS1, MH429991, Spain). Likewise, the partial 18S rRNA sequence of *L. vineacola* (MN129758) was more closely related to two sequences belonging to *L. vineacola* (JX445123, Spain; AY283169) with a 100% similarity, however it was also close (with a sequence homology greater than 99%) to *L. onubensis* (KT308897, Spain), *L. nevesi* (MH430009, Spain), and *L. magnus* (KT308902, Spain). However, the D2–D3 marker of *L. vineacola* (MN082428) was identical (100%) to several sequences from the same species in Genbank (JX445109–JX445111, Spain; KT308872, KT308873 Spain) and clearly distinguishable (with a sequence similarity of 92%) from three sequences belonging to *L. lusitanicus* (KT308869, Spain), *L. vinearum* (JX445112, Spain), and *L. magnus* (KT308876, Spain). These results agree with Barsalote et al. (2018), Lazarova et al. (2019), and Cai et al. (2019), who established the lowest dissimilarity values among the closest species for the partial 18S rRNA gene. In addition, the ITS1 region and D2–D3 of 28S rRNA gene show higher dissimilarity value among *Longidorus* species to the closest species than with a partial 18S rRNA gene (Barsalote et al. 2018; Archidona-Yuste et al. 2019; Cai et al. 2019; Lazarova et al. 2019). However, recently Palomares et al. (2017) reviewed the main rRNA and mRNA molecular markers used for taxonomic evaluation of longidorid nematodes and highlighted that the partial 18S rRNA gene showed a potential value to discriminate species.

Phylogenetic trees reconstructed by the BI approach using new sequences and others from Genbank inferred similar patterns. For all trees generated, *L. bordonensis* sp. nov. was clearly grouped with *L. pini* and *L. carpetanensis*, two species sharing a similar morphology characterized by a short body and odontostyle, with an elongate to conical female tail. However, as is common

in phylogenetic studies, other morphologically similar species to *L. bordonensis* sp. nov. were outside this sub-clade, such as *L. distintus* (KF242317, Italy), *L. iliturgiensis* Archidona-Yuste, Cantalapiedra-Navarrete, Castillo & Palomares-Rius, 2019 (MH430012, MH430013, Spain), *L. indalus* (KT308852–KT308854, Spain), *L. juvenilis* (AY601579, DQ364599, Slovenia), and *L. pisi* (MK172048, Bulgaria; LR032064–LR032065, Italy). The sub-clade composed of *L. bordonensis* sp. nov., *L. pini*, and *L. carpetanensis* shared a similar outward appearance characterized by the same code for most of five characters in the polytomous key (A2, B1, C2, H56, I2), and clustered with other members of the sub-clade composed of several other *Longidorus* species (i.e. for the D2–D3 tree: *L. athesinus* Lamberti, Coiro & Agostinelli, 1991, *L. edmundsi* Hunt & Siddiqi, 1977, *L. polyae*, *L. sturhani* Rubtsova, Subbotin, Brown & Moens, 2001, *L. raskii* Lamberti & Agostinelli, 1993), sharing a common ancestor. Our results agree with Navas et al. (1993) in finding that *L. carpetanensis* clustered together to other two pleomorphic species, *L. bordonensis* sp. nov. and *L. pini*. They occupy basal positions in three trees, which shows their ancestral position in relation to the majority of the Iberian species (i.e. *L. vineacola*, *L. oleae*, *L. andalusicus*, *L. orientalis*, *L. fasciatus*, *L. nevesi*, *L. paciencis*, *L. macrodorus* Archidona-Yuste, Navas-Cortés, Cantalapiedra-Navarrete, Palomares-Rius & Castillo, 2016, *L. silvestris*, *L. iuglandis*, *L. baeticus*, *L.* cf. *olegi*, *L. wicuolea*, *L. onubensis*, *L. crataegi*, *L. lusitanicus*, *L. magnus*, and *L. vinearum*). These three species shared an outward appearance and have some ecological similarities; in fact, they are characterized by having low population densities, a filiform aspect, thinner and longer bodies than other plant nematodes, and a longer odontostyle than other plant nematodes. This allows them to parasitize of wide range of herbaceous and woody plants commonly found in nutrient-poor and dry soils around the Mediterranean region.

Conclusion

Our work contributes greater understanding of the biodiversity within the genus *Longidorus*, describes *Longidorus* spp. by utilizing both morphological and molecular data, and establishes these species' phylogenetic relationships within the genus. Our study also establishes the value of using rRNA molecular markers, especially from topotype specimens, for the identification of *Longidorus* spp., when other methods are difficult and inconclusive. In addition, we establish molecular markers for precise and unequivocal diagnosis of a new species, *L. bordonensis* sp. nov., and show that molecular markers are useful to differentiate this species from other species that are virus vectors. Additionally, these markers were used to characterize the topotype of *L. vinearum*. To our knowledge, this is also the first time that *L. wicuolea* is reported for Portugal.

Acknowledgements

This research was financially supported by National Funds through Foundation for Science and Technology under the project UID/AGR/00115/2019 (Portugal). We thank M.I. Ferreira and G. Albarrán from Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade of Évora for their excellent technical assistance.

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Supplementary material 1

Figure S1

Authors: Carlos Gutiérrez-Gutiérrez, Margarida Teixeira Santos, Maria Lurdes Inácio, Jonathan D. Eisenback, Manuel Mota

Data type: TIF file

Explanation note: Light micrographs of female topotypes *Longidorus vinearum* Bravo & Roca, 1995 (1–9) infesting the grapevine (*Vitis* sp.) rhizosphere from Portugal. **1, 2.** Female anterior ends. **3.** Lip region showing amphidial fovea at different level of focus. **4.** Vulva region. **5.** Male tail region. **6.** Female tail regions. **7–9.** Juvenile tails (J2, J3, and J4, respectively). Abbreviations: **a** anus, **af** amphidial fovea, **gr** guiding ring, **ost** odontostyle, **sp** spicules, **V** vulva. Scale bars: (S1.1–3) 40 µm; (S1.4) 130 µm; (S1.5–6) 50 µm; (S1.7) 50 µm; (S1.8) 55 µm; (S1.9) 45 µm.

Link: https://doi.org/10.3897/zse.96.49022.suppl1

Supplementary material 2

Figure S2

Authors: Carlos Gutiérrez-Gutiérrez, Margarida Teixeira Santos, Maria Lurdes Inácio, Jonathan D. Eisenback, Manuel Mota

Data type: TIF file

Explanation note: Light micrographs of *Longidorus vineacola* Sturhan & Weischer, 1964 (1–11) infesting the rhizosphere from cork oak (*Quercus suber* L.) tree and wild olive (*Olea europaea* var. *sylvestris* L.) from Portugal. **1–3.** Female anterior ends. **4.** Lip region showing amphidial fovea at different focus. **5.** Odontophore region. **6.** Male tail, ventromedian supplements arrowed. **7.** Vulva region. **8–10.** Female tail regions. **11.** Male tail with detail of spicules. Abbreviations: **a** anus, **af** amphidial fovea, **gr** guiding ring, ost odontostyle, **odph** odontophore, **sp** spicules, **V** vulva. Scale bars: (S2.1–2) 18 µm; (S2.3–4) 30 µm; (S2.5, S2.7) 75 µm; (S2.8–10) 50 µm; (S2.11, S2.6) 50 µm.

Link: https://doi.org/10.3897/zse.96.49022.suppl2

Supplementary material 3

Table S1

Authors: Carlos Gutiérrez-Gutiérrez, Margarida Teixeira Santos, Maria Lurdes Inácio, Jonathan D. Eisenback, Manuel Mota

Data type: DOCX file

Explanation note: List of primers used in this study.

Link: https://doi.org/10.3897/zse.96.49022.suppl3

Supplementary material 4

Table S2

Authors: Carlos Gutiérrez-Gutiérrez, Margarida Teixeira Santos, Maria Lurdes Inácio, Jonathan D. Eisenback, Manuel Mota

Data type: DOCX file

Explanation note: Comparison of the type population of the eight closest species to *L. bordonensis* sp. nov. for the most important diagnostic features.

Link: https://doi.org/10.3897/zse.96.49022.suppl4

Supplementary material 5

Table S3

Authors: Carlos Gutiérrez-Gutiérrez, Margarida Teixeira Santos, Maria Lurdes Inácio, Jonathan D. Eisenback, Manuel Mota

Data type: DOCX file

Explanation note: Morphometrics of *Longidorus vinearum* Sturhan & Weischer, 1964 from the rhizosphere of grapevine (*Vitis* sp.) in vineyards and wild olive (*Olea europaea* L. var. *sylvestris*) in agro-forestry systems in Portugal. All measurements in µm and in the format: mean ± s.d. (range)*.

Link: https://doi.org/10.3897/zse.96.49022.suppl5

Supplementary material 6

Table S4

Authors: Carlos Gutiérrez-Gutiérrez, Margarida Teixeira Santos, Maria Lurdes Inácio, Jonathan D. Eisenback, Manuel Mota

Data type: DOCX file

Explanation note: Morphometrics of *Longidorus vineacola* Sturhan & Weischer, 1964 from the rhizosphere of uncultivated plants in agro-forestry systems from Portugal. All measurements in µm and in the format: mean ± s.d. (range)*.

Link: https://doi.org/10.3897/zse.96.49022.suppl6