

Description and molecular phylogeny of one new and one known needle nematode of the genus *Paralongidorus* (Nematoda: Longidoridae) from grapevine in Portugal using integrative approach

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Abstract A new and a known longidorid nematode, *Paralongidorus lusitanicus* n. sp. and *Paralongidorus plesioepimikis*, are described and illustrated from populations extracted from soil associated with grapevine (*Vitis vinifera* L.) from Escaroupim and Pó (central-Western Portugal), respectively. The new needle nematode *P. lusitanicus* n. sp. is characterised by a very large body size (8072–12,022 μm), an expanded and rounded lip region, ca 30 μm wide, with a clear constriction followed by a depression posterior to the amphidial aperture, amphidial fovea very large (11.0–19.0 μm), stirrup-shaped, with conspicuous slit-like aperture as shown in

scanning electron microscopy studies, a very long and flexible odontostyle (180.0–223.0 μm), guiding ring located at 28.0–41.5 μm from anterior end, vulva anterior to the mid-body (34–41%), a dorsally convex-conoid tail with rounded terminus (29–42 μm long), bearing two or three pairs of caudal pores and males common (ratio 1:1.6 females) with spicules ca 80 μm long. Morphological and morphometric traits for *P. plesioepimikis* fit well with the original description, and is reported for the first time in Portugal. Integrative diagnosis of both species was completed with molecular data obtained using D2-D3 expansion segments of 28S rDNA, ITS1-rDNA and partial 18S-rDNA. The phylogenetic relationships of these species with other *Paralongidorus* spp. using these three molecular markers indicated that *P. lusitanicus* n. sp. clustered together with other *Paralongidorus* spp. forming a sister clade with *P. plesioepimikis*, both of them sharing a large body, long odontostyle, an anteriorly located vulva and an expanded and rounded lip region with a clear constriction followed by a depression posterior to the amphidial aperture.

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Introduction

Longidoridae is one of the largest families of soil nematodes in term of species diversity given that they

comprise a total of seven genera and more than 750 species (Decraemer & Robbins 2007). The taxonomic and systematic position of the genus *Paralongidorus* Siddiqi, Hooper & Klan, 1963 within the family Longidoridae (Thorne, 1935) Meyl, 1961 appears to be accepted by the scientific community, however the species composition are continuously being subject of debate (Hunt 1993, Siddiqi et al. 1993, Coomans 1996, Escuer & Arias 1997, Decraemer & Robbins 2007). This genus, member of the commonly known needle nematodes, is quite diverse with about 90 valid species of migratory ectoparasites that parasitise a wide range of agronomic crops, ornamentals, and forest trees (Taylor & Brown 1997, Decraemer & Robbins 2007, Palomares-Rius et al. 2013, Kornobis et al. 2015, Barsi & De Luca 2017). This group of phytopathogenic species are of global interest because they cause directly damage on the roots of the host plant attributable to their ectoparasitic feeding and one species is able to transmit damaging nepoviruses (Taylor & Brown 1997, Decraemer & Robbins 2007). *Paralongidorus maximus* (Bütschli 1874) Siddiqi 1964 has important phytopathological implications because it is a vector of *Raspberry ringspot virus* (RpRSV) (Jones et al. 1994, Taylor & Brown 1997). This species is considered as a major pest in Europe and other many parts of the world (Taylor & Brown 1997), and for this reason is included in the A2 list of pest recommended regulation of the European and Mediterranean Plant Protection Organization (EPPO, www.eppo.int/QUARANTINE/).

An accurate and detailed description of needle nematodes is essential to establish unequivocal diagnosis in order to discern virus vectors and/or quarantine pathogen species, select appropriate management strategies for preventing the spread of them and establish efficient control measures. Morphometric and morphological identification within this genus at species level is mainly based on characteristics of adult females (Escuer & Arias 1997). However, the high intraspecific variability of some diagnostic features and the great diversity in phenotypic plasticity make species identification based on gross morphology and internal anatomical features a technically difficult task even for experts. Recently, the sequencing of RNA-based markers is an increasingly powerful approach for the molecular diagnostics and for understanding their inter- and intra-genetic variability (Palomares-Rius et al. 2013, Kornobis et al. 2015, Barsi & De Luca 2017). Several ribosomal RNA (rRNA) genes are used for molecular characterizations of these

nematodes: partial 28S rRNA gene (He et al. 2005, Palomares-Rius et al. 2008, 2013, Pedram et al. 2012, Kornobis et al. 2015, Barsi & De Luca 2017), 18S rRNA gene (Palomares-Rius et al. 2008, 2013, Pedram et al. 2012, Kornobis et al. 2015) and internal transcribed spacer (ITS) region of the rRNA genes (Palomares-Rius et al. 2008, 2013, Pedram et al. 2012, Kornobis et al. 2015, Barsi & De Luca 2017). Kornobis et al. (2015) designed a PCR–RFLP of the D2D3 region of 28S rRNA gene with five restriction enzymes to establish diagnostic profiles from *P. rex* Andrassy, 1986. Recently, Barsi & De Luca (2017) designed a PCR–RFLP of ITS region based on six restriction enzymes for genotyping of species-specific variations for *P. francolambertii* Barsi & De Luca, 2017. D2–D3 expansion segments of 28S rRNA and ITS rRNA have proved to be more effective in species identification when compared to partial 18S rRNA, as both these markers display more molecular variability than partial 18S rRNA (He et al. 2005, Palomares-Rius et al. 2008, 2013, Pedram et al. 2012, Kornobis et al. 2015, Barsi & De Luca 2017). Additionally, the mitochondrial DNA (mtDNA) markers have been used for species identification, phylogeny and phylogeography in a large number of species inside Longidoridae (Lazarova et al. 2006, 2016, Gutiérrez-Gutiérrez et al. 2011, 2013a,b, Kumari 2014, Subbotin et al. 2014, 2015), however there is a lack of the mitochondrial molecular data on *Paralongidorus* spp. Only a small fraction of *Paralongidorus* species (nine identified species) has so far sequences in the GenBank database. These sequences should be obtained by using voucher specimens from the described species in order to perform molecular species comparison and to differentiate molecularly cryptic species (Palomares-Rius et al. 2014). Based on our current knowledge, eleven valid *Paralongidorus* species have been reported in several cultivated and wild plants in Europe and in the Mediterranean Basin (Bravo & Lemos 1997, Escuer & Arias 1997, Taylor & Brown 1997, Lišková & Brown 2003, Barsi & De Luca 2017): i) *P. maximus* is the most common *Paralongidorus* spp. in Europe and it is frequently found parasitizing a large number of forest trees, grapevine, chestnut tree (*Castanea* spp.), walnut trees (*Juglans regia* L.) and a wide range of vegetable crops in Europe (Heyns, 1975; Lamberti et al., 1983; Roca et al. 1988; Macara 1994; Lišková, 1995; Bravo & Lemos 1997; Taylor & Brown 1997; Lišková & Brown, 1998, 1999, 2003; Gutiérrez-Gutiérrez et al. 2016), ii) *P. iberis*

Escuer & Arias, 1997 and *P. monegrensis* Escuer & Arias, 1997 in the rhizosphere of plants in the *Rhamno-Cocciferelum* ecosystem in Spain (Escuer & Arias 1997), iii) *P. litoralis* Palomares-Rius, Subbotin, Landa, Vovlas & Castillo 2008, in the rhizosphere of mastic tree (*Pistacia lentiscus* L.) in Spain (Palomares-Rius et al. 2008), iv) *P. paramaximus* Heyns, 1965 on soil around plants in citrus orchards (*Citrus aurantium* L.) in Spain (Palomares-Rius et al. 2008), v) *P. epimikis* Dalmasso, 1969 in grapevine in Algeria (Dalmasso 1969), vi) *P. plesioepimikis* Palomares-Rius, Cantalapedra-Navarrete, Gutiérrez-Gutiérrez, Liébanas & Castillo, 2013 in the rhizosphere of stone pine (*Pinus pinea* L.) in Spain (Palomares-Rius et al. 2013), vii) *P. georgiensis* (Tulaganov, 1937) Luc & Doucet 1984, in a vegetable garden soil in Egypt (Tulaganov 1937), viii) *P. remeyi* Altherr 1963 in an unknown host in France (Altherr, 1963), ix) *P. rex* is commonly found infesting grapevine and oak forest in Hungary (Andrássy 1986, Barsi et al. 2007), forest tree species and grasses in Poland and Ukraine (Kornobis et al. 2015), and x) *P. francolambertii* in the rhizosphere of silver lime trees (*Tilia tomentosa* Moench) and juniper trees (*Juniperus communis* L.) in a forest in Serbia (Barsi & De Luca 2017). In Europe the low species diversity of *Paralongidorus* and *Longidorus* and their current geographical distribution can be attributed to their center of origin, which is supposed to be located in South African-Indian division when it was only one supercontinent (Pangea), before the separation of major plate tectonics (Coomans, 1985).

In order to establish the species diversity of the genus *Paralongidorus* in Portugal and its distribution, we sampled several vineyards in the Lisbon and Tejo wine regions from Spring to Autumn of 2016 in central-Western Portugal, and as a result, a population of an unknown *Paralongidorus* species was found in the rhizosphere of grapevine (*Vitis vinifera* L.) from an old vineyard at Escaroupim (Salvaterra de Magos, Ribatejo province) and another population resembling *P. plesioepimikis* was found in a new vineyard at Pó (Bombarral, Estremadura province). One of the unidentified populations (Escaroupim) was visually difficult to identify using the polytomous identification key proposed by Escuer & Arias (1997) because shared a large number of diagnostic characters with a group of *Paralongidorus* species characterized by a large body and very long odontostyle, and an expanded lip region, set off from the rest of the body by a clear constriction

followed by a usually slight depression posterior to the amphidial aperture (Palomares-Rius et al. 2013). These traits prompted us to undertake a detailed morphological and molecular sequence-based comparative study with previously described *Paralongidorus* species. These observations indicated that one of the detected species appeared to be morphologically and morphometrically unrelated to nominal *Paralongidorus* species and did not fit with their descriptions. Detailed studies using light and scanning electron microscopy (SEM) and molecular analyses indicated that this population should be assigned to a new species and is described herein as *Paralongidorus lusitanicus* n. sp.

The objectives of the present study were: (i) to characterise two Portuguese populations of *Paralongidorus* morphologically and genetically using the D2-D3 expansion segments of 28S rRNA, ITS1 rRNA and the partial 18S rRNA gene sequences; and (ii) to explore the phylogenetic relationships of these needle nematode populations within *Paralongidorus* spp.

Material and methods

Nematode population sampling, extraction and morphological identification

Specimens of the unidentified *Paralongidorus* species detected in this study were isolated from infested sandy soil samples collected from the rhizosphere of grapevine (*Vitis* spp.) from an old vineyard at Escaroupim (Salvaterra de Magos, Ribaltejo province, Portugal) and a new vineyard at Pó (Bombarral, Estremadura province, Portugal). Nematodes were extracted from soil by a modification of Cobb's decanting and sieving method (Flegg 1967).

Specimens for light microscopy (LM) study were killed and fixed in an aqueous solution of 4% formaldehyde +1% glycerol, dehydrated using alcohol-saturated chamber and processed to pure glycerine using Seinhorst's method (Seinhorst 1966). Specimens were examined using an Olympus BX50 light microscope with differential interference contrast at magnifications up to 1,000× magnification. Photographs of nematodes were taken by an Olympus DP70 camera and Cell software (Olympus Software Imaging for Life Sciences). All measurements were expressed in micrometers (µm). For line drawings of the new species, light micrographs were imported to CorelDraw ver. X6 and

redrawn. All other abbreviations used are as defined in Jairajpuri and Ahmad (1992).

For SEM studies, fixed specimens were dehydrated in a graded ethanol series, critical point dried, mounted on SEM stubs, sputter-coated with a thin layer of gold (Abolafia et al. 2002), and observed with a Hitachi S-3700 N microscope coupled with a Bruker XFlash5010 SDD detector.

Nematode molecular identification

For molecular analyses and in order to avoid mistakes in the case of mixed populations in the same sample, two live nematodes from each population were temporarily mounted in a drop of 1 M NaCl containing glass beads to ensure specimens conformed to the unidentified populations of *Paralongidorus*. Morphometric measurements and photomicrographs recorded during this initial study were not used as part of the morphological study and morphometric analyses. Following morphological confirmation, the slides were dismantled and DNA extracted. Nematode DNA was extracted from single female individuals and PCR assays were conducted as described by Castillo et al. (2003). One nematode specimen of each sample was transferred to an Eppendorf tube containing 16 µl ddH₂O, 2 µl 10× PCR buffer and 2 µl proteinase K (600 µg/ml) (Promega, Benelux, The Netherlands) and crushed during 2 min with a micro-homogeniser, Vibro Mixer (Zürich, Switzerland). The tubes were frozen at –80 °C for (15 min) and incubated at 65 °C (1 h), then at 95 °C (10 min). One µl of extracted DNA was transferred to an Eppendorf tube containing: 2.5 µl 10X NH₄ reaction buffer, 0.75 µl MgCl₂ (50 mM), 0.25 µl dNTPs mixture (10 mM each), 0.75 µl of each primer (10 mM), 0.2 µl BIOTAQ DNA Polymerase (BIOLINE, UK) and ddH₂O to a final volume of 25 µl. The D2-D3 expansion segments of 28S rRNA was amplified using the D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (De Ley et al. 1999). The ITS1 region was amplified using forward primer 18S (5'TTGATTACGTCCTGCCCTT-3') (Vrain et al. 1992) and reverse primer rDNA1 (5'-ACGAGCCGAGTGATCCACCG-3') (Cherry et al. 1997). Finally, the portion of 18S rRNA was amplified using primers 988F (5'-CTC AAA GAT TAA GCC ATG C-3'), 1912R (5'-TTT ACGGTC AGA ACT AGG G-30), 1813F (5'-CTG CGT GAG

AGGTGA AAT-3') and 2646R (50-GCT ACC TTG TTA CGA CTT TT-3') (Holterman et al. 2006).

PCR cycle conditions were: one cycle of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, annealing temperature of 55 °C for 45 s, 72 °C for 3 min, and finally 72 °C for 10 min. PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB products), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct sequencing in both directions using the primers referred to above. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyser; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA), at the Stab Vida sequencing facilities (Caparica, Portugal). The newly obtained sequences were submitted to the GenBank database under accession numbers indicated on the phylogenetic trees (KY750560-KY750569).

Phylogenetic analyses

D2-D3 expansion segments of 28S rRNA, ITS1 region, and partial 18S rRNA sequences of different *Longidorus* spp. and *Paralongidorus* spp. from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen following previous published studies (He et al. 2005, Holterman et al. 2006, Gutiérrez-Gutiérrez et al. 2013b, Palomares-Rius et al. 2013, Archidona-Yuste et al. 2016a). Multiple sequence alignments of the different genes were made using the Q-INS-i algorithm of MAFFT V.7.205 (Kato & Standley 2013), which accounts for secondary RNA structure. Sequence alignments were visualised using BioEdit (Hall 1999) and edited by Gblocks ver. 0.91b (Castresana 2000) in Castresana Laboratory server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) using options for a less stringent selection (minimum number of sequences for a conserved or a flanking position: 50% of the number of sequences +1; maximum number of contiguous non-conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half). Percentage similarity between sequences was calculated using a sequence identity matrix using BioEdit. For that, the score for each pair of sequences was compared directly and all gap or placeholder characters were treated as a gap. When the same position for both sequences had a gap it was not treated

as a difference. Phylogenetic analyses of the sequence datasets were based on Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). The best-fit model of DNA evolution was obtained using JModelTest V.2.1.7 (Darriba et al. 2012) with the Akaike Information Criterion (AIC). The best-fit model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then given to MrBayes for the phylogenetic analyses. Unlinked general time-reversible model with invariable sites and a gamma-shaped distribution (GTR + I + G) for the D2-D3 expansion segments of 28S rRNA, and for the ITS1 region, and the partial 18S rRNA under a transitional model with invariable sites and a gamma-shaped distribution (TIM2 + I + G). These BI analyses were run separately per dataset using four chains for 2×10^6 generations for the D2-D3, and 1×10^6 generations for the rest of molecular markers. The Markov Chains were sampled at intervals of 100 generations. Two runs were conducted for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority-rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees from all analyses were visualised using TreeView (Page 1996) and FigTree software V.1.42 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Results

*Paralongidorus lusitanicus*¹ n. sp. (Figs. 1, 2, 3 and 4, Table 1)

Female: Body very long and rather robust, slightly tapering towards posterior end, usually assuming an open close C to spiral shape, always more coiled in the posterior half, when heat relaxed. Cuticle usually smooth under low magnifications, 4.6 ± 0.8 (3.5–6.5) μm thick at mid-body, but thicker 11.7 ± 0.7 (8.5–16.5) μm at tail end. A variable number of body pores in the

pharyngeal region; 5–7 lateral, 1–2 ventral and 1–2 dorsal pores. Lip region wide expanded, anteriorly slightly convex, almost flat at the end and widely rounded in lateral view, clearly set off by a constriction followed by a depression as shown by SEM, 29.9 ± 1.5 (27.0–33.0) μm wide and 13.1 ± 1.1 (13.5–14.5) μm high. Amphidial fovea very large, stirrup-shaped, with conspicuous aperture *ca* two-third as wide as lip region as shown by SEM. Twelve cephalic papillae appearing as small apertures, an inner line and other line outer, both lines composed by six labial papillae, each outer labial papillae located just anterior to a distinct cephalic lobe 3.1 ± 0.7 (2.0–4.5) μm long (Figs. 1, 2, and 3, Table 1). A single guide ring, 6.0–8.5 μm wide, located 0.9 ± 0.1 (0.8–1.1) times the lip region diam. From anterior end. Lateral chord 16.0 (11.0–26.0) μm wide, *ca* 24.4% of corresponding body diam. at mid-body. Odontostyle very long and flexible, 2.0 (1.3–3.1) times as long as odontophore which is weakly developed, with rather faint basal swellings (Figs. 1–2, Table 1). Nerve ring encircling part of pharynx near odontophore base, at 355.6 (236–461) μm from the anterior end. Basal bulb long and cylindrical, 149.5 ± 16.8 (104.0–177.0) μm long or *ca* 4.1 times the total pharynx length, and 26.3 ± 4.9 (22.0–38.0) μm in diam. Glandularium 129.7 ± 13.3 (111.0–140.0) μm long. Dorsal pharyngeal gland nucleus (DN) located at 18.1 ± 3.2 (13.8–25.9) % and ventrosublateral nuclei (SVN) near middle of bulb, 45.9 ± 7.5 (30.7–54.8) % of distance from anterior end of pharyngeal bulb. Nucleoli of DN one-fourth times larger than nucleolus of SVN. Cardia conoid-rounded, 15.1 (8.0–25.0) μm long (Figs. 1–2, Table 1). Reproductive system typical of genus with both genital branches equally developed; anterior branch 383–1559 μm long and posterior branch 382–1346 μm long. Ovaries paired and reflexed, usually equally developed and with a single row of oocytes, variable in length (183–251 μm long), vulva in form of a transverse slit, located anteriorly to mid-body (33.0–41.0% of towards the front of the body), vagina perpendicular to body axis, 40–58 μm long, *ca* 65% of corresponding body diam., surrounded by well-developed muscles. Anterior and posterior oviduct of similar size. Uteri 248–499 μm long, with presence of sperm cells in some females; conspicuous muscle sphincter between uterus and oviduct. Prerectum variable in length, 531.3 ± 180.5 (408.0–701.0) μm long or *ca* 6.1–10.6.5 times anal body diameter, and rectum *ca* 0.7–1.2 times as long as anal body diameter. Tail short,

¹ The species epithet refers to Lusitania, the ancient roman province which included present day Portugal.

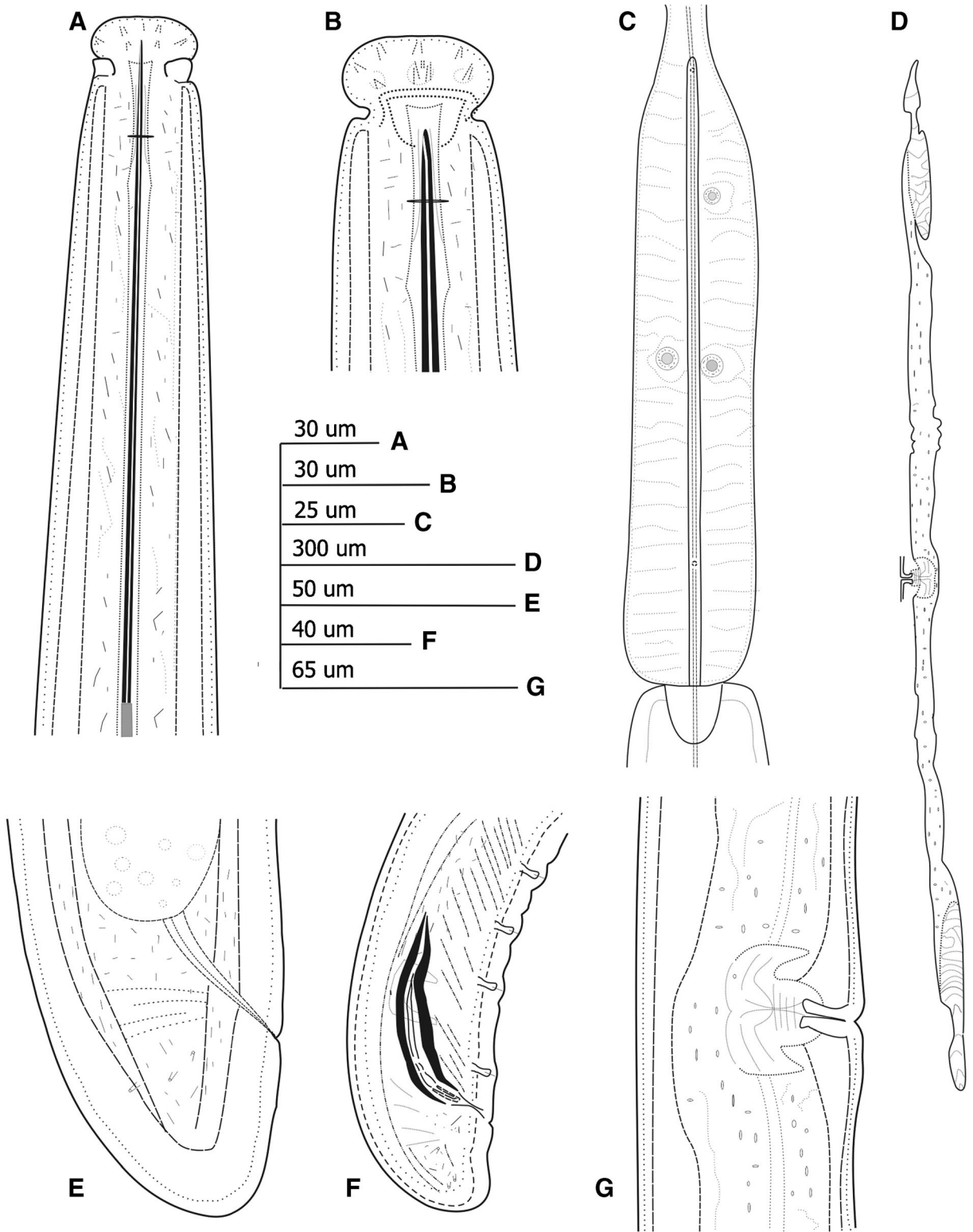


Fig. 1 Line drawings of *Paralongidorus lusitanicus* n. sp. paratypes from the rhizosphere of grapevine (*Vitis* from Escaroupin, Portugal (A-F). A: female neck region; B: female lip

region; C: detail of basal pharyngeal bulb; D: Detail of genital branches; E: female tail region; F: male tail region; G: vulva region

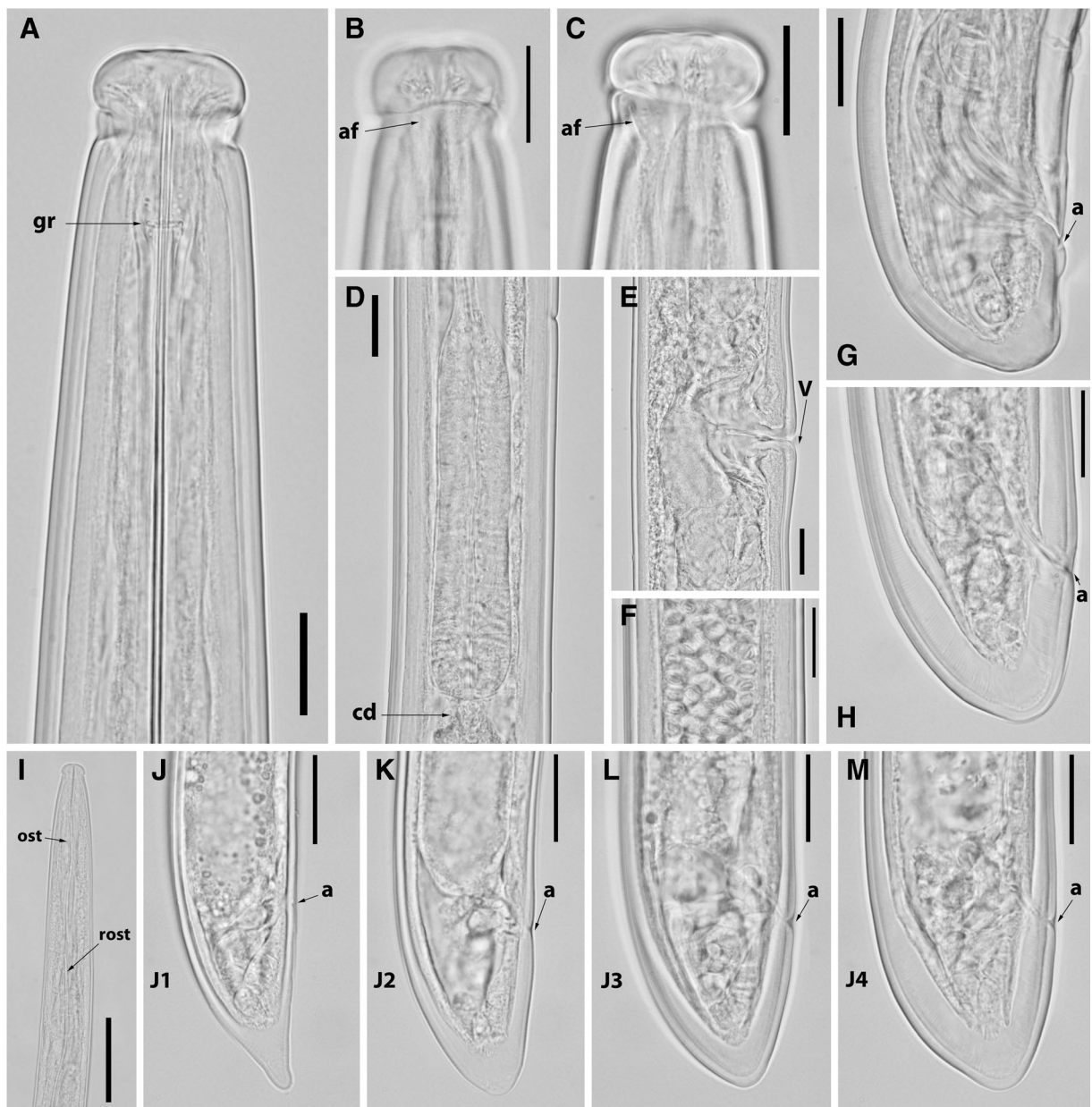


Fig. 2 Light micrographs of *Paralongidorus lusitanicus* n. sp. female paratypes from the rhizosphere of grapevine (*Vitis* sp.) from Escaroupim, Portugal (A–M). A. Female anterior regions; B–C. Lip regions showing amphidial fovea at different focus; D: Detail of basal bulb; E: Vulva region; F: sperm cells; G: Male tail region; H: Female tail region; I: First-stage juvenile anterior region; J–M:

Tail of J1, J2, J3 and J4, respectively. Abbreviations: a = anus; af = amphidial fovea; cd = cardia; gr = guiding ring; ost = odontostyle; ov = ovary; rost = replacement odontostyle; V = vulva. (Scale bars: A, G, L = 20 μ m; B, C, F, H, M = 22.5 μ m; D–E: 17 μ m; I = 50 μ m; J: 25 μ m; K: 30 μ m)

convex-conoid with a broadly rounded terminus (Figs. 1, 2 and 3, Table 1).

Male Common (ratio 1:1.6 females). Morphologically similar to female except for genital system, but with

posterior region strongly curved ventrally when heat relaxed. Genital tract diorchic with testes opposed, occupying ca 51.6% of body length containing multiple rows of different stages of spermatogonia. Tail conoid-rounded, with broad blunt terminus and thickened outer

Fig. 3 SEM micrographs of *Paralongidorus lusitanicus* n. sp. A-D: Female anterior end in lateral, ventro-lateral and frontal view showing cephalic lobe (cl), amphidial aperture (af), inner labial papillae (ip) and outer labial papillae (op); E-F: Female tail, oblique and lateral view showing caudal pores (cp) and anus (a). (Scale bars: A-D = 30 μ m; B-D = 35 μ m.)

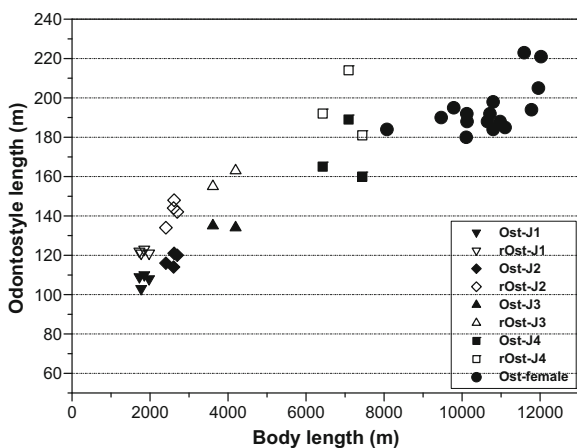
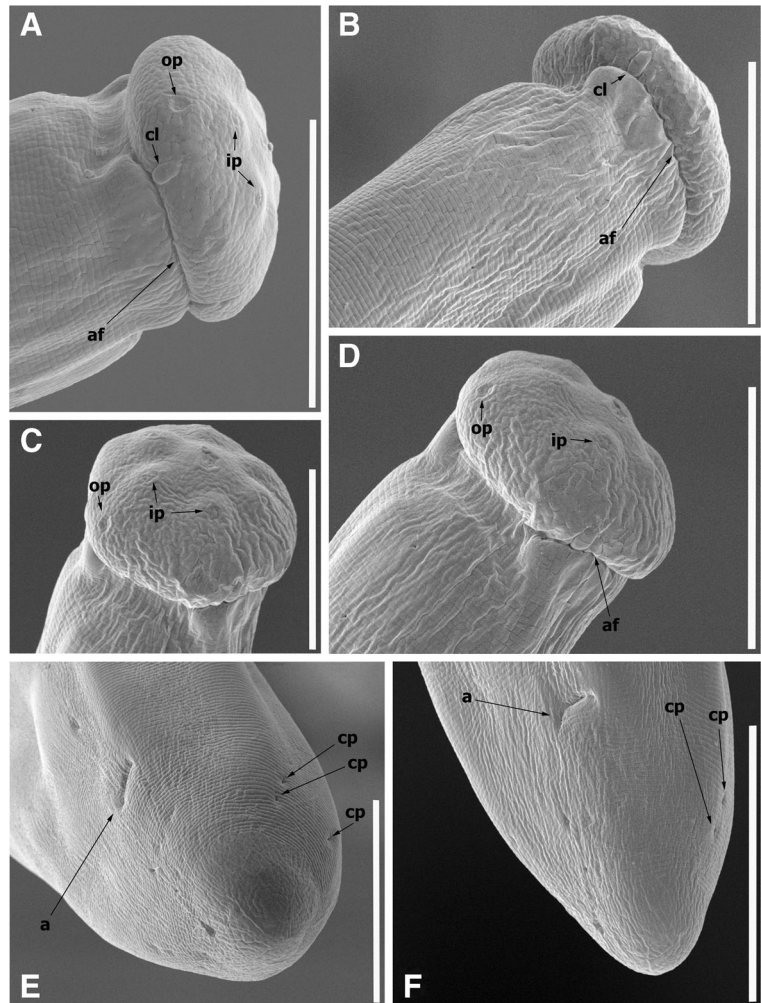


Fig. 4 Relationship of body length to length of functional and replacement odontostyle (Ost and rOst, respectively) length in all developmental stages from second-stage juveniles (J2) to mature females of *Paralongidorus lusitanicus* n. sp.

cuticular layer. Spicules arcuate, robust, 77–85 μ m long; lateral guiding pieces 17–24 μ m long, more or less straight or with curved proximal end. One pair of adcloacal supplements and a variable number of midventral supplements (11–13) (Figs. 1–2, Table 1).

Juveniles All four juvenile stages were found and distinguished by relative lengths of body, functional and replacement odontostyle (Table 1, Fig. 4; Robbins et al. 1995, 1996) and genital primordium. Body usually assuming a J-shape, a C-shape to spiral C-shape in J2 and J3 and similar to adults in J4 when heat relaxed. Lip region of all juvenile stages similar to that of adults. First-stage juveniles (J1) characterised by a bluntly conoid-rounded tail with ventral peg with a c' ratio 1.6–1.8, odontostyle length 103–110 μ m long, and slightly shorter distance from anterior end to stylet

Table 1 Morphometrics of *Paralongidorus lusitanicus* n. sp. from the rhizosphere of grapevine (*Vitis vinifera* L.) from Escaroupim, Portugal. All measurements in μm and in the format: mean \pm s.d. (range)*

Character	Holotype	Paratype Females	Paratype Males	J1	J2	J3	J4
n	1	16	9	4	4	2	7
L	10,649	10,627 \pm 1024 (8072–12,022)	9357 \pm 1022 (7809–10,663)	1833 \pm 109 (1729–1977)	2583 \pm 123 (2409–2700)	3614, 4196	6362 \pm 672 (5575–7442)
a	162.0	163.0 \pm 22.7 (116.5–192.4)	165.4 \pm 23.1 (123.0–198.9)	54.9 \pm 4.0 (50.6–60.2)	61.7 \pm 4.3 (56.5–66.9)	73.8, 88.6	116.3 \pm 19.1 (97.4–143.1)
b	18.9	18.0 \pm 3.9 (13.8–31.1)	15.5 \pm 1.6 (12.8–18.0)	5.9 \pm 4.0 (5.6–7.9)	7.2 \pm 0.6 (6.7–7.9)	9.7, 9.8	11.0 \pm 1.3 (9.6–13.4)
c	317.7	302.9 \pm 34.5 (240.3–369.4)	252.4 \pm 37.3 (192.2–327.4)	42.7 \pm 2.1 (41.1–45.6)	67.4 \pm 2.1 (65.8–70.5)	86.0, 108.4	176.1 \pm 20.4 (156.6–216.6)
c'	0.7	0.8 \pm 0.1 (0.7–0.9)	0.9 \pm 0.1 (0.7–1.2)	1.6 \pm 0.1 (1.6–1.8)	1.2 \pm 0.02 (1.1–1.2)	1.0, 1.1	0.8 \pm 0.1 (0.7–0.9)
V or T	39.3	37.6 \pm 2.5 (33.0–40.9)	51.6 \pm 2.3 (48.4–55.0)	–	–	–	–
G ₁	5.5	6.6 \pm 2.8 (3.2–13.4)	–	–	–	–	–
G ₂	4.4	6.0 \pm 2.2 (3.2–12.4)	–	–	–	–	–
Odontostyle length	188.5	194.2 \pm 12.4 (180.0–223.0)	180.4 \pm 26.8 (117.0–206.0)	107.7 \pm 3.2 (103.0–110.0)	117.8 \pm 3.4 (114.0–121.0)	133.0, 135.0	166.1 \pm 13.6 (159.0–189.0)
Replacement odontostyle length	–	–	–	121.7 \pm 1.2 (120.5–123.0)	142.0 \pm 5.8 (134.0–148.0)	155.0, 163.0	189.9 \pm 15.3 (181.0–214.0)
Odontophore length	97.0	102.9 \pm 18.6 (72.0–140.0)	95.1 \pm 10.4 (74.0–107.0)	59.6 \pm 2.8 (57.0–63.0)	68.4 \pm 12.9 (50.0–80.0)	64.0, 84.5	91.0 \pm 14.6 (65.0–98.0)
Lip region width	29.0	29.9 \pm 1.5 (27.0–33.0)	29.5 \pm 2.9 (29.0–31.0)	14.9 \pm 0.7 (14.0–16.0)	19.5 \pm 1.5 (18.0–21.0)	24.0, 24.5	26.8 \pm 2.2 (25.0–29.0)
Oral aperture-guiding ring	33.0	34.8 \pm 3.9 (28.0–41.5)	34.9 \pm 2.5 (31.9–43.8)	21.2 \pm 0.7 (20.4–21.9)	24.0 \pm 1.3 (22.0–25.0)	26.5, 28.0	30.2 \pm 1.5 (29.0–33.0)
Tail length	33.5	35.3 \pm 3.5 (29.0–42.0)	37.4 \pm 4.2 (32.0–44.0)	42.9 \pm 1.7 (41.1–45.0)	38.3 \pm 2.2 (36.0–41.0)	38.7, 42.0	36.2 \pm 3.3 (32.0–39.0)
Spicules	–	–	80.3 \pm 2.7 (77.0–85.0)	–	–	–	–
Lateral accessory piece	–	–	21.0 \pm 2.3 (18.0–24.0)	–	–	–	–
J	111.0	11.7 \pm 2.5 (8.6–16.5)	12.3 \pm 2.4 (8.5–16.0)	13.0 \pm 1.5 (11.0–14.0)	9.5 \pm 0.9 (9.0–11.0)	10.0, 11.0	11.6 \pm 1.0 (9.5–14.0)

* Abbreviations are defined in Jairajpuri & Ahmad (1992)

guiding ring to the next juvenile stages. Tail shape of J2 conoid-rounded. J3 and J4 tail shape barely dorsally convex-conoid, but more elongate than that of female, shorter body length, shorter odontostyle length, and shorter distance from anterior end to guiding ring (Fig. 2, Table 1).

Type habitat and locality

Rhizosphere of grapevine (*Vitis* sp. at Escaroupim, locality of Salvaterra de Magos, Ribatejo province,

Portugal (GPS coordinates: N 39° 4' 35.922" E 8° 43' 58.471").

Type material

Holotype female (slide LLP001) and seven female paratypes (LLP002–LLP008) together with six male paratypes (LLP009–LLP014) deposited in the Nematode Collection of the Nematology Lab, Institute for Mediterranean Agricultural and Environment Sciences,

ICAAM, University of Évora, Évora, Portugal, and three female (LLP015–LLP017) and one male paratypes (LLP018) in the Institute for Sustainable Agriculture, IAS-CSIC, Córdoba, Spain. Two female and one male paratypes deposited at each of the following collections: Royal Belgian Institute of Natural Sciences, Brussels, Belgium, and Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (C.N.R.), Sezione di Bari, Bari, Italy. Specific D2-D3, ITS1-rRNA, and partial 18S rDNA sequences were deposited in GenBank with accession numbers KY750560–KY750562, KY750565–KY750566, and KY750569, respectively.

Diagnosis and relationships

Paralongidorus lusitanicus n. sp. is characterised by a large body size (8.07–12.02 mm), an expanded and rounded lip region, ca 30 µm wide, with a clear constriction followed by a depression posterior to the amphidial aperture, amphidial fovea large, stirrup-shaped, with conspicuous slit-like aperture, a very long and flexible odontostyle (180.0–223.0 µm), a guiding ring located at 28.0–41.5 µm from anterior end, normal arrangement of the pharyngeal gland nuclei, dorsal pharyngeal gland nucleus in anterior part of bulb, a pair of ventrosublateral pharyngeal glands near the middle of bulb, vulva anterior to the mid-body (33–41%), and a dorsally convex-conoid tail with a rounded terminus (29–42 µm long), from 0.7 to 0.9 times longer than width, bearing two or three pairs of caudal pores and male common; and specific D2-D3, ITS1, and partial 18S-rRNA sequences. According to the polytomous key of Escuer & Arias (1997), the new species has the following codes (codes in parentheses are exceptions): A1, B1, C3, D2, E2, F6, G7, H23, I2(3), J1(2), K56, L(3)4, M(2)3, N2, O2.

On the basis of amphidial fovea, lip region, body length, odontostyle length, ratios a, c, and c', distance from oral aperture to guiding ring, bulb length, tail length and shape, spicules length, number of supplements in male tail and tail morphology of the J1, *P. lusitanicus* n. sp. is close to *P. epimikis*, *P. deborae* (Jacobs & Heyns 1982) Luc & Doucet, 1984, *P. iranicus* Pedram, Pourjam, Namjou, Reza Atighi, Cantalapiedra-Navarrete, Liébanas, Palomares-Rius & Castillo 2012, *P. francolambertii*, *P. litoralis*, *P. maximus*, *P. rex* and *P. plesioepimikis*. Morphologically and morphometrically, *P. lusitanicus* n. sp. can be distinguished from these

species by several features as indicated below. From *P. epimikis* it differs by a longer lip region diam. (27.0–33.0 vs 23.0–24.0), a different shape of amphidial fovea (stirrup vs funnel), a slightly smaller a ratio (116.5–192.4 vs 184–211), a vulva more anteriorly located (33.0–41.0 vs 44–46%), a different tail shape (dorsally convex-conoid vs conoid-rounded) and abundance of males (frequent vs rare) having usually a longer spicules (77.0–85.0 vs 57–58 µm) (Dalmasso 1969). First-fourth juvenile stages of the new species and *P. epimikis* show morphologically a high similarity; however, the J1 stages can be differentiated by a lower c' ratio (1.6–1.8 vs 2.22–2.23) and a shorter tail length (41.0–45.0 vs 49.0–50.0 µm). On the other hand, *P. lusitanicus* n. sp. differs from *P. deborae* in having lower V ratio (33.0–41.0 vs 42–48%), a longer lip region diam. (27.0–33.0 vs 25.0–27.0 µm), a longer odontostyle (180.0–223.0 vs 156.0–166.0 µm), a different female tail shape (dorsally convex-conoid vs conoid-rounded) and longer spicules length (77.0–85.0 vs 63–72 µm) (Jacobs & Heyns 1982). From *P. iranicus* it differs in a, c, c' ratios (116.5–192.4 vs 101.9–137.7, 240.3–369.4 vs 221.3–314.8, 0.7–0.9 vs 0.5–0.7, respectively), V ratio (33–41 vs 37–44%), lip region diam. (27.0–33.0 vs 25.0–30.0 µm), type of depression between lip region and body contour (less marked in the new species), odontostyle length (180.0–223.0 vs 155.0–184.0 µm), odontophore length (72.0–139.6 vs 82.0–100.0 µm), and tail length and shape (29.0–42.0 vs 25–37 µm, dorsally convex-conoid vs broadly rounded) (Pedram et al. 2012). From *P. francolambertii* it can be differentiated mainly in a, c, and V ratios (116.5–192.4 vs 143.0–197.0, 116.5–192.4 vs 227.0–344.0, 33.0–41.0 vs 42.0–49.0%, respectively), lip region diam. (27.0–33.0 vs 18.0–20.0 µm), female tail shape and length (dorsally convex-conoid vs convex-conoid with a terminal rounded point and thickened outer cuticular layer, 29.0–42.0 vs 22–29), type of depression between lip region and body contour (less marked in the new species), body length (8.0–12.0 vs 5.9–8.3 mm), odontostyle length (180.0–223.0 vs 131.0–149.0 µm) and spicules length (77.0–85.0 vs 49.0–58.0 µm) (Barsi et al. 2017). First juvenile stages of the new species and *P. francolambertii* are highly similar morphologically, in spite they can be differentiated by the tail shape (bluntly conoid-rounded tail with ventral peg vs elongate-conoid without peg). From *P. litoralis* it can be differentiated mainly by means of a and V ratios (116.5–192.4 vs 113.7–164.4, 33.0–41.0 vs 37.0–44.0%, respectively), lip region

diam. (27.0–33.0 vs 25.0–30.0 μm), female tail shape (dorsally convex-conoid vs bluntly rounded) and spicules length (77.0–85.0 vs 63.0–72.0 μm) (Palomares-Rius et al. 2008). All juvenile stages of the new species and *P. litoralis* are highly similar morphologically, in fact the J1 stages of them are almost identical, only having small anatomical differences such as tail shape (bluntly conoid-rounded tail with ventral peg vs conoid-rounded with a central peg). From *P. maximus* it differs in a, c, c' ratios (116.5–192.4 vs 77.0–133.0, 240.3–369.4 vs 178.0–320.0, 0.7–0.9 vs 0.44–0.60, respectively), lip region diam. (27.0–33.0 vs 34.0–39.0 μm), odontostyle length (180.0–223.0 vs 152.0–187.0 μm), distance from oral aperture to guiding ring (28.0–41.5 vs 37–47 μm), tail shape (dorsally convex-conoid vs bluntly rounded), abundance of males (frequent vs rare) and spicules length (77.0–85.0 vs 63.0–72.0 μm) (Heyns 1975). All juvenile stages of the new species and *P. maximus* are highly similar morphologically, in spite of that the J1 stage can be differentiated by a bigger c' ratio (1.6–1.8 vs 1.06–1.25). From *P. rex* it differs mainly in a, c, c' ratio (116.5–192.4 vs 106.0–111.0, 240.3–369.4 vs 221.3–314.8, 0.7–0.9 vs 0.5–0.6 respectively), V ratio (33–41 vs 47%), amphidial fovea shape (stirrup vs funnel), odontostyle length (180.0–223.0 vs 178.0–180.0 μm), female tail length and shape (29–42.0 vs 40.0–45.0 μm , convex-conoid vs conoid-rounded) and abundance of males (frequent vs not found) (Andrassy 1986). All juvenile stages of the new species and *P. rex*

showed anatomically and morphologically a high similarity with *P. lusitanicus* n. sp., however the J1 stages can be differentiated by a higher c' ratio (1.6–1.8 vs 1.1–1.3) and a longer tail length (41.0–45.0 vs 29.0–36.0 μm). From *P. plesioepimikis* it differs only in the odontophore length (72.0–140.0 vs 66.0–87.0 μm), abundance of males (frequent vs not found) (Palomares-Rius et al. 2013). All juvenile stages of the new species and *P. plesioepimikis* are almost indistinguishable, however the J1 stages can be differentiated by a bigger c' ratio than the new species (1.6–1.8 vs 1.3–1.6). Furthermore, the new species can be clearly separated from all other sequenced *Paralongidorus* spp. and similar morphologically and morphometrically (*P. iranicus*, *P. francolambertii*, *P. litoralis*, *P. paramaximus*, *P. maximus*, *P. plesioepimikis*, *P. bikanerensis* (Lal & Mathur, 1987) Siddiqi, Baujard & Mounport, 1993 and *P. rex*) by using the three molecular markers of D2-D3 expansion segments of 28S rDNA, ITS1 rRNA and partial 18S rRNA (Figs. 6, 7, and 8).

***Paralongidorus plesioepimikis* Palomares-Rius et al. 2013** (Fig. 5, Table 2)

The Portuguese population of this species from the rhizosphere of grapevine (*V. vinifera* L.) in the locality of Pó (Bombarral, Estremadura province) (Table 2) was characterised by the absence of males; females exhibited

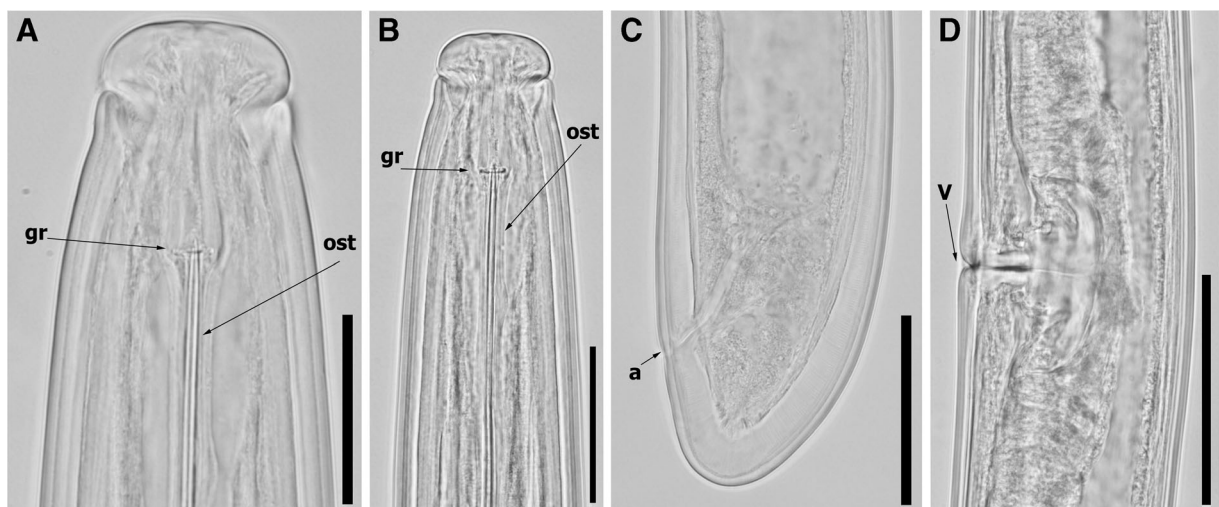


Fig. 5 Light micrographs of *Paralongidorus plesioepimikis* Palomares-Rius et al. 2013 females from the rhizosphere of grapevine (*Vitis vinifera* L.) from Pó, Bombarral, Portugal (A–D). A: Lip regions showing amphidial fovea at different focus; B: Female

anterior regions; C: Female tail region; D: Vulva region. Abbreviations: a = anus; gr = guiding ring; ost = odontostyle; V = vulva. (Scale bars: A–B = 20 μm ; C = 40 μm ; D = 75 μm)

Table 2 Morphometrics of *Paralongidorus plesioepimikis* Palomares-Rius et al. 2013 from the rhizosphere of grapevine (*Vitis vinifera*) from Pó, Portugal. All measurements in μm and in the format: mean \pm s.d. (range)*

Character	Females
n	2
L	10,353, 11,948
a	141.5, 152.7
b	16.4, 19.8
c	169.8, 287.8
c'	0.7, 0.7
V or T	(34.2, 36.8)
Odontostyle length	215.0, 220.0
Odontophore length	132.0, 135.0
Lip region width	(29.0, 33.0)
Oral aperture-guiding ring	(36.0, 40.0)
Tail length	(38.0, 41.5)
J	(15.0, 15.5)

* Abbreviations are defined in Jairajpuri & Ahmad (1992)

a robust and very large body length (10.3–11.9 mm), lip region rather expanded, 29.0–33.0 μm wide and 14.0–14.5 μm high, oval-rounded in lateral view and clearly set off by a clear constriction followed by a depression posterior to amphidial aperture, *ca* 19.0 μm long. Amphidial fovea *ca* two-third as wide as lip region, stirrup-shaped, with conspicuous slit-like aperture. Odontostyle very long *ca* 1.6 times as long as odontophore, which is weakly developed, with rather weak basal swellings (Fig. 5, Table 2). Cuticle of mid-body 5.0–6.0 μm thick, 15.0–15.5 μm thick at tail tip, marked by very fine superficial transverse striae. Basal bulb cylindrical occupying 169.0–183.5 μm long or 22.5 (21.5–23.5) % of total pharynx length. Dorsal pharyngeal gland nucleus in anterior part of bulb, one subventral pair of nuclei near middle of basal bulb. Glandularium 147.2 ± 2.1 (146.0–149.0) μm long. Cardia clearly visible, elongate and conoid-rounded, 21.1 ± 1.6 (20.0–22.0) μm long (Fig. 5, Table 2). Genital tracts typical of the genus, with both genital branches equally developed, anterior branch 410–548 μm long and posterior branch 382–480 μm long, ovaries paired and reflexed, usually equally developed occupying 37.4–56.4% of the total branch length, vulva in form of a transverse slit, usually located anteriorly to mid-body (34.2–37.6%), vagina perpendicular to body axis, 40.5–49.0 μm long, *ca* 59% of corresponding body

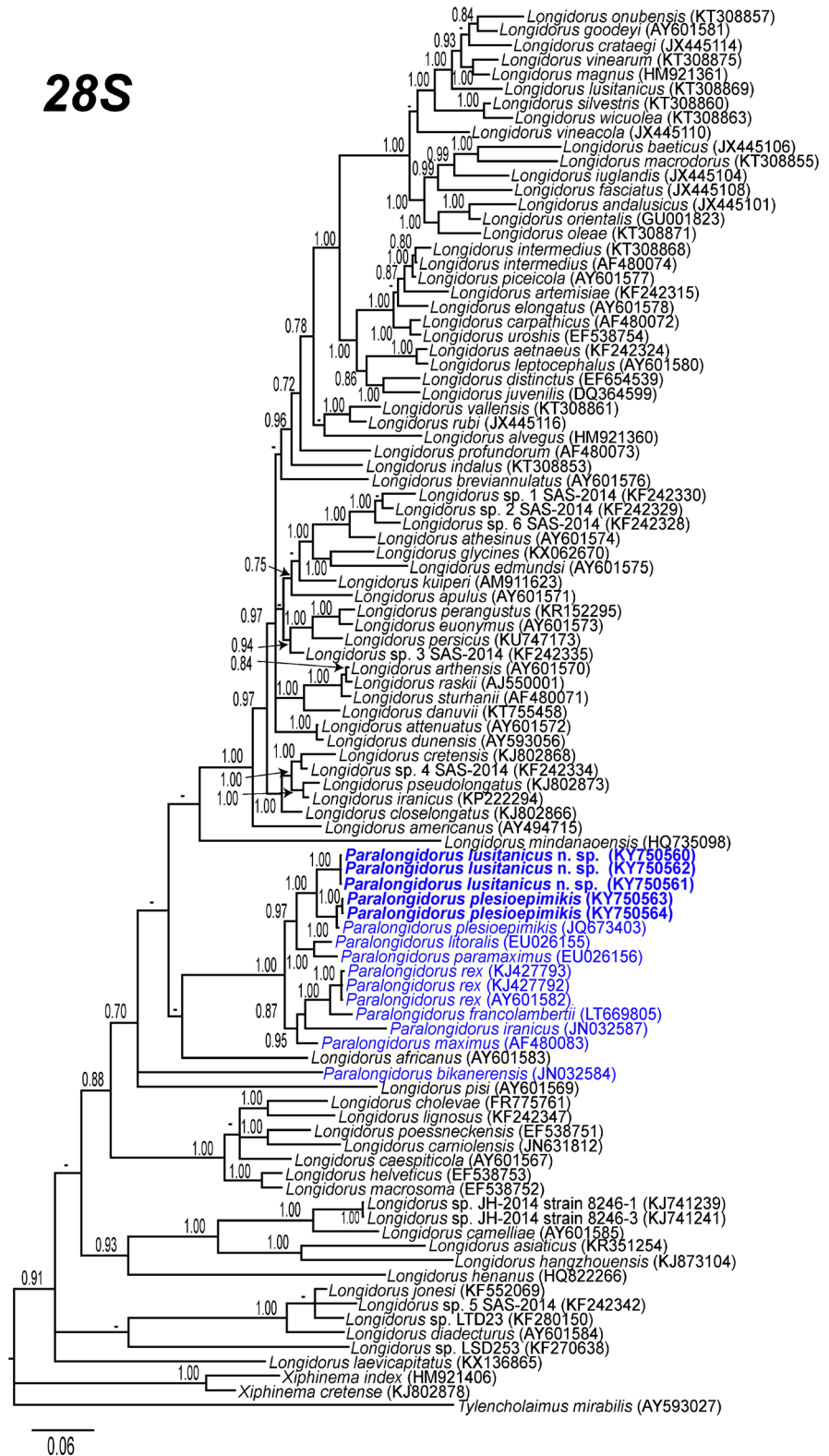
diam., surrounded by visible well-developed muscles. Anterior and posterior oviduct of similar size, separated of uterus by a conspicuous muscle sphincter. Uteri large, 146–176 μm long, where spermatic cells can usually be seen. Prerectum 846 (830–862) μm long and rectum 56.4 (55.0–58.0) μm long. Tail dorsally convex-conoid, broadly rounded terminus, with two or three pores each side of the tail and characterised by 39.0–41.5 μm long and *c'* *ca* 0.7 (Fig. 5, Table 2). Morphological and morphometric traits of this population agree with the original description of this species by Palomares-Rius et al. (2013). In fact, morphological features and morphometric measurements of females from the Portuguese population were within the range of the original description from Palomares-Rius et al. (2013), except by some slight differences in tail length, *c'* ratio and lip region diameter, which may be due to geographical intraspecific variability, such as reported in others species of *Paralongidorus* (Kornobis et al. 2015). This is the first report of this species for Portugal and confirms a wider distribution of this specie in the Iberian Peninsula and Mediterranean Basin since it has only been reported from Spain (Palomares-Rius et al. 2013). The alphanumeric codes for *P. plesioepimikis* to be applied to the polytomic identification key for the *Paralongidorus* species by Escuer & Arias (1997): A1, B1, C3, D2, E1, F6, G7, H2, I23, J1, K56, L34, M3, N-, O-.

Phylogenetic relationships of Paralongidorus lusitanicus n. sp. and P. plesioepimikis within the genera Longidorus and Paralongidorus

Amplification of D2-D3 expansion segment of 28S rRNA, ITS1 rRNA, and the partial 18S rRNA from *Paralongidorus lusitanicus n. sp.* and *P. plesioepimikis* yielded a single fragment of *ca* 800, 1100, and 1700 bp, respectively. Sequences from *P. lusitanicus n. sp.* matched well with the *Paralongidorus* spp. sequences deposited in GenBank being clearly different from all of them. Four new D2-D3 of 28S rRNA gene sequences were obtained in the present study. D2-D3 expansion segments of 28S rRNA sequences of *P. lusitanicus n. sp.* (KY750560-KY750562) showed a 93–97% similarity values (differed in a range from 21 to 54 nucleotides) with several *Paralongidorus* spp. such as *P. plesioepimikis* (JQ673403), *P. maximus* (KF412826), *P. paramaximus* (EU026156), *P. litoralis* (EU026155), *P. rex* (KJ427793), *P. iranicus* (JN032587) and *P. francolambertii* (LT669805). ITS1

Fig. 6 Phylogenetic relationships of *Paralongidorus lusitanicus* n. sp. and *P. plesioepimikis* Palomares-Rius et al. 2013 within the genus *Longidorus* and *Paralongidorus*. Bayesian 50% majority rule consensus trees as inferred from D2-D3 expansion segments of 28S rRNA sequences alignments under the GTR + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences in this study are in bold

28S



showed some moderate similarity, 83% with *P. plesioepimikis* (JQ673407), 81%, with *P. paramaximus* (JQ673410), and 80%, with *P. litoralis* (JQ673409), differing in a range from 120 to 136 nucleotides. The partial 18S rRNA sequence for *P. lusitanicus* n. sp. (KY750569) showed high similarity (99% similar) with several *Paralongidorus* spp., such as *P. plesioepimikis* (JQ673405), *P. litoralis* (EU026158), *P. paramaximus* (EU026157), *P. maximus* (AJ875152), differing in a range from 3 to 8 nucleotides. Intraspecific sequence diversity (uncorrected p-distance) of ribosomal markers among the studied specimens for *P. lusitanicus* n. sp. was small. In fact, D2-D3 showed no intraspecific sequence diversity and ITS1 region only varied 1% (3 bp, 3 indels). Finally, the Portuguese population of *P. plesioepimikis* showed some high similarity for D2-D3 and ITS1 region (99% (5 nucleotides) and 97% (34 nucleotides), respectively) with *P. plesioepimikis* from Spain (JQ673403, JQ673407).

Phylogenetic trees reconstructed by the BI method for the three rRNA markers (D2-D3 expansion regions

of 28S rRNA gene, ITS1 region and the partial 18S rRNA) are presented in Figs. 6, 7 and 8. The D2-D3 domains of 28S rRNA gene tree based on a multiple edited alignment (97 sequences) of 765 total characters revealed a major clade for the majority of the *Paralongidorus* species, including *P. lusitanicus* n. sp. (Fig. 6). This tree is similar to the most recent phylogenetic analysis showed by Palomares-Rius et al. (2013). *Paralongidorus lusitanicus* n. sp. is well-related phylogenetically to other species described and/or cited in the Iberian Peninsula such as *P. plesioepimikis* (JQ673403), *P. litoralis* (EU026155), *P. maximus* (AF480083) and *P. paramaximus* (EU026156). However, as in other studies (Palomares-Rius et al. 2008, 2013, Barsi and De Luca 2017), *P. bikanerensis* (JN032584) showed a position outside of the main clade for *Paralongidorus*.

Similarly, the 50% majority rule consensus BI tree of a multiple alignment including 15 18S rRNA sequences with a 1654 bp alignment length (Fig. 7); and 17 ITS1 region sequences with a 984 bp alignment length (Fig. 8) showed a clear phylogenetic relationship of

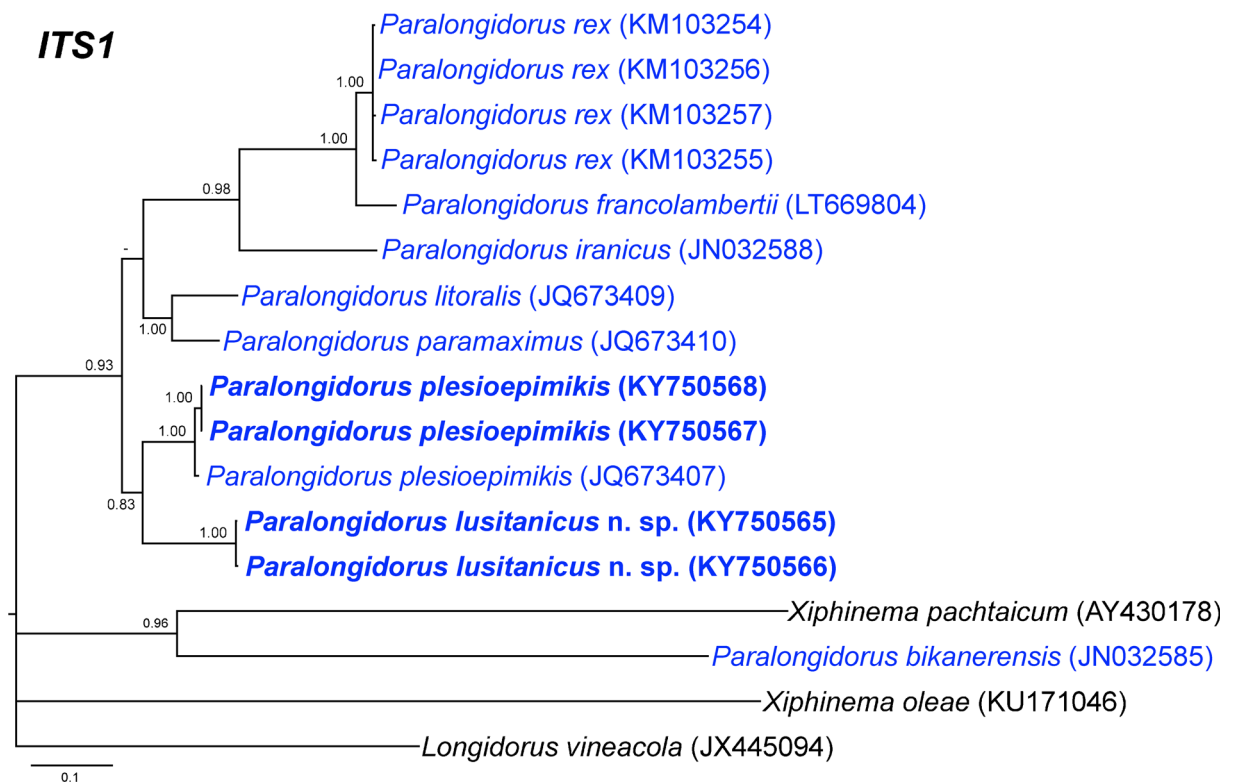


Fig. 7 Phylogenetic relationships of *Paralongidorus lusitanicus* n. sp. and *P. plesioepimikis* Palomares-Rius et al. 2013 within the genus *Paralongidorus*. Bayesian 50% majority rule consensus trees as inferred from ITS1 rRNA sequences alignments under

the GTR + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences in this study are in bold

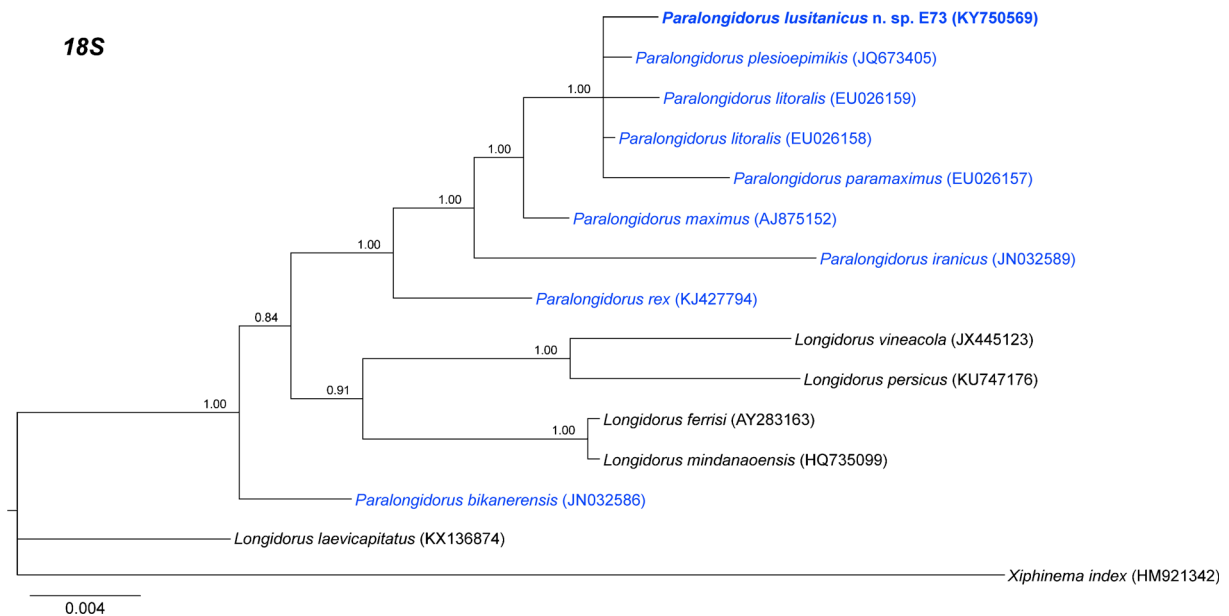


Fig. 8 Phylogenetic relationships of *Paralongidorus lusitanicus* n. sp. and *P. plesioepimikis* Palomares-Rius et al. 2013 within the genus *Longidorus* and *Paralongidorus*. Bayesian 50% majority rule consensus trees as inferred from 18S rRNA sequences

P. lusitanicus n. sp. with *P. plesioepimikis* in both datasets and also with *P. litoralis* and *P. paramaximus* in the partial 18S rRNA.

The description of *P. lusitanicus* n. sp. in the rhizosphere of grapevine suggests that the diversity of these nematodes in Southern Europe is still not fully clarified, and requires further study. Interestingly, the phylogenetic relationship among Iberian Peninsula species could provide insight into the speciation of some of these species, additionally of the main centre of origin in other parts of the world as suggested by Coomans (1985). However, this idea must be studied with more sequences from other species of *Paralongidorus*, because extensive sampling for Longidoridae in Southern Spain showed a scarce presence of this genus (Archidona-Yuste et al. 2016a, b). This point has also been documented for several species of *Longidorus*, but not for *Xiphinema* in an extensive sampling and phylogenetic study in Southern Spain (Archidona-Yuste et al. 2016a, b).

In summary, the present study extends our knowledge on the species diversity of the genus *Paralongidorus* by integrating morphological and molecular characterizations and elucidates phylogenetic relationships with other *Paralongidorus* spp. The molecular markers obtained could be used for precise and unequivocal diagnosis of this species which may help for effective quarantine

alignments under the TIM2 + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences in this study are in bold letters

inspection and appropriate application of exclusion principles.

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Compliance with Ethical Standards

Conflict of interest All authors certify that 1) they do not have any actual or potential conflict of interest, 2) the study described is original and has not been published previously, and is not under consideration for publication elsewhere, 3) all prevailing local, national and international regulations and conventions, and normal scientific ethical practices, have been respected. We also certify

that all authors have reviewed the manuscript and approved the final version of manuscript before submission.

Research involving Human Participants and/or Animals No specific permits were required for the described fieldwork studies. Permission for sampling the vineyards was granted by the landowner. The sites are not protected in any way.

Informed Consent All the authors certify that the work carried out in this research followed the principles of ethical and professional conduct have been followed. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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