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A new needle nematode, *Longidorus maginicus* n. sp. (Nematoda: Longidoridae) from southern Spain

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Research Paper

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Abstract

During nematode surveys in natural vegetation in Sierra Mágina, Jaén province, southern Spain, a *Longidorus* species closely resembling *Longidorus carpetanensis* was found, but application of integrative taxonomic approaches clearly demonstrated that it is a new species described herein as *Longidorus maginicus* n. sp. The new species is amphimictic, characterized by a moderately long body (4.2–5.2 mm); lip region anteriorly flattened, slightly separated from the rest of body by a depression, 9.0–11.0 µm wide and 3.5–6.0 µm high; amphidial fovea not lobed; relatively short odontostyle (61.0–70.5 µm); guiding ring located 23.5–27.0 µm from anterior end; vulva located at 42.0%–51.3% of body length; female tail 39.0–61.0 µm long, conoid, dorsally convex with rounded terminus ($c' = 1.3–2.1$), with two or three pairs of caudal pores; and males common (1:2 ratio males:females), with moderately long spicules (39.0–48.5 µm) and 1 + 6–9 ventromedian supplements and three juvenile developmental stages. According to the polytomous key, codes for the new species are (codes in parentheses are exceptions): A2-B1-C2-D2-E1-F2(3)-G2-H5(4)-I2-J1-K6. The results of molecular analysis of D2–D3 28S, internal transcribed spacer region, partial 18S rDNA, and cytochrome oxidase c subunit 1 (*coxI*) gene sequences further characterized the new species status, and separated it from *L. carpetanensis* and other related species.

Introduction

Longidorus Micoletzky, 1922 comprises a large and complex genus of the family Longidoridae Thorne, 1935 with approximately 180 species of plant-ectoparasitic nematodes that are polyphagous and distributed almost worldwide (Peneva *et al.*, 2013; Subbotin *et al.*, 2014; Trisciuzzi *et al.*, 2015; Archidona-Yuste *et al.*, 2019; Amrei *et al.*, 2020; Cai *et al.*, 2020a, b; Clavero-Camacho *et al.*, 2021a, b). In Spain, 41 species of *Longidorus* have been reported, from which 31 were molecularly characterized, and 19 of them were described as new species of this genus (Arias *et al.*, 1986; Andrés & Arias, 1987; Gutiérrez-Gutiérrez *et al.*, 2013; Archidona-Yuste *et al.*, 2016, 2019; Cai *et al.*, 2020a, b; Clavero-Camacho *et al.*, 2021a, b). The importance of these nematodes is based not only in their polyphagy and cosmopolitan distribution but also some species are vectors of plant viruses that cause significant damage to a wide range of agricultural crops (Decraemer & Robbins, 2007; Cai *et al.*, 2020a). Species discrimination in *Longidorus* is difficult because of the large number of species and because the conserved morphology and morphometric characters often overlap, leading to potential misidentification. Consequently, an accurate *Longidorus* species identification is essential and has significant implications in food security, quarantine measures and agronomic management in the field (Palomares-Rius *et al.*, 2014; Cai *et al.*, 2020a).

For these reasons, the development of molecular methods using different fragments of nuclear ribosomal and mitochondrial DNA gene sequences to be used in DNA barcoding during the last years led to an accurate species diagnosis, to clarify phylogenetic relationships and species delimitation under the genus *Longidorus* (Neilson *et al.*, 2004; Ye *et al.*, 2004; Kumari & Subbotin, 2012; Gutiérrez-Gutiérrez *et al.*, 2013; Subbotin *et al.*, 2014; Archidona-Yuste *et al.*, 2016, 2019; Amrei *et al.*, 2020; Cai *et al.*, 2020a, b; Clavero-Camacho *et al.*, 2021a, b). In addition, the use of these molecular markers in species identification of *Longidorus* over the last decade has indicated that some species actually comprise multiple genetically divergent and morphologically similar cryptic species (Archidona-Yuste *et al.*, 2016, 2019; Gutiérrez-Gutiérrez *et al.*, 2020; Cai *et al.*, 2020a, b).

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A species of needle nematode of the genus *Longidorus* morphologically resembling *Longidorus carpetanensis* Arias *et al.*, 1986, was detected during nematological surveys conducted in 2019 and 2020 in the natural vegetation of a mountain in southern Spain (Sierra Mágina, province of Jaén, Spain). Recently, however, topotypes of this species were recently morphologically and molecularly characterized (Archidona-Yuste *et al.*, 2019) which prompted us to study this population under an integrative taxonomic approach based on morphological and molecular, as well as a comprehensive, scanning electron microscope (SEM) analyses.

Therefore, the main objectives of this study were: (i) to characterize morphologically and morphometrically the new Spanish population of *Longidorus* and compare it with *L. carpetanensis* and related species; (ii) to characterize molecularly the new *Longidorus* population using the D2–D3 28S rRNA, internal transcribed spacer region (ITS) rRNA, partial 18S rRNA and cytochrome oxidase c subunit 1 (*coxI*) gene sequences; and (iii) to study the phylogenetic relationships of the identified *Longidorus* species with available sequenced species of the genus.

Material and methods

Nematode population and morphological characterization

In the spring of 2019 and 2020, seven soil samples were collected, with a shovel, from the upper 40-cm of soil in the rhizosphere of spiny madwort (*Ptilotrichum spinosum* (L.) Boiss.) in Albanchez de Mágina, Jaén province, Spain, and in three of them, low to moderate population densities of a new *Longidorus* population were detected. Subsequently, nematodes were extracted from a 500 cm^3 subsample of soil by centrifugal flotation and a modification of Cobb's decanting and sieving methods (Flegg, 1967; Coolen, 1979).

Specimens for study using light microscopy (LM) and morphometric studies were killed and fixed in a heat aqueous solution of 4% formaldehyde + 1% glycerine, dehydrated using an alcohol-saturated chamber and processed to pure glycerine using Seinhorst's method (Seinhorst, 1966) as modified by De Grisse (1969). Light micrographs and measurements of the nematode population, including important diagnostic characteristics (i.e. de Man indices, body length, odontostyle length, lip region, tail shape, amphid shape and oral aperture-guiding ring) (Jairajpuri & Ahmad, 1992) were done using a Leica DM6 compound microscope with a Leica DFC7000T digital camera (Leica, Wetzlar, Germany). The raw photographs were edited using Adobe Photoshop v. 22.5.2 (San Francisco, CA, USA).

For SEM, fixed specimens were dehydrated in a gradient series of ethanol, critical-point dried, sputter-coated with gold according to the protocol by Abolafia *et al.* (2002) and observed with a Zeiss Merlin Scanning Electron Microscope (5 kV; Zeiss, Oberkochen, Germany).

Molecular characterization and phylogenetic analyses

For molecular analyses, and in order to avoid mistakes in case of mixed populations in the sample, single specimens were temporarily mounted in a drop of 1 M sodium chloride containing glass beads (to avoid nematode crushing/damaging specimens) to ensure specimens conformed with the target population. All necessary morphological and morphometric data were recorded. This was followed by DNA extraction from single individuals as described by Archidona-Yuste *et al.* (2016). The D2–D3 segments were amplified using the D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) primers (De Ley *et al.*, 1999). The ITS was amplified using forward primer 18S (5′-TTGATTACGTCCCTGCCCTTT-3′) and reverse primer 26S (5′-TTTCACTCGCCGTTACTAAGG-3′) (Vrain *et al.*, 1992). The portion of 18S rRNA was amplified using primers 988F (5′-CTCAAAGATTAAGCCATGC-3′), 1912R (5′-TTTACGGTC AGAACTAGGG-3′), 1813F (5′-CTGCGTGAGAGGTGAAAT-3′) and 2646R (5′-GCTACCTTGTTACGACTTTT-3′) (Holterman *et al.*, 2006). Finally, the portion of the *coxI* gene was amplified as described by Lazarova *et al.* (2006) using the primers COIF (5′-GATTTTTTGGKCATCCWGARG-3′) and COIR (5′- CWAC ATAATAAGTATCATG-3′).

All polymerase chain reaction (PCR) assays were done according to the conditions described by Archidona-Yuste *et al.* (2016). Then, the amplified PCR products were purified using ExoSAP-IT (Affimetrix, USB products) and used for direct sequencing 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 StabVida sequencing facilities (Costa da Caparica, Portugal). The newly obtained sequences were submitted to the GenBank database under the accession numbers indicated on the phylogenetic trees.

Phylogenetic analyses

The D2–D3 expansion segments of 28S, ITS and 18S rDNA, and *coxI* mtDNA sequences, of the recently recovered unidentified *Longidorus* species were obtained in this study. These sequences, and other sequences from species of *Longidorus* from GenBank, were used for phylogenetic analyses. Outgroup taxa for each dataset were chosen following previously published studies (He *et al.*, 2005; Holterman *et al.*, 2006; Gutiérrez-Gutiérrez *et al.*, 2013; Archidona-Yuste *et al.*, 2019; Cai *et al.*, 2020a). Multiple sequence alignments of the different genes were made using the FFT-NS-2 algorithm of MAFFT V.7.450 (Katoh *et al.*, 2019). Sequence alignments were manually visualized using BioEdit (Hall, 1999) and edited by Gblocks ver. 0.91b (Castresana, 2000) in the 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). 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, the gamma distribution shape parameters and substitution rates in the AIC were then used in MrBayes for the phylogenetic analyses. BI analyses were performed under a general time-reversible model with invariable sites and a gamma-shaped distribution (GTR + I + G) for the D2–D3 segments of 28S, ITS, and the partial 18S rDNA, and Tamura–Nei model with invariable sites and a gamma-shaped distribution (TRN + I + G) for the partial *coxI* gene. All Bayesian analyses were run separately per dataset with four chains for 4×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were conducted for each analysis. After discarding burn-in samples of 30% and

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evaluating convergence, the remaining samples were retained for in-depth analyses. The topologies were used to generate a 50% majority-rule consensus tree. Posterior probabilities (PP) were given on appropriate clades. Trees from all analyses were visualized using FigTree software version v.1.42 (Rambaut, 2014). A combined analysis of the three ribosomal genes was not undertaken due to some sequences not being available for all species.

Results

Low to moderate densities (5–10 nematodes/500 cm^3 of soil) of the presently studied population of *Longidorus* were detected in three out of seven soil samples collected from the rhizosphere of spiny madwort (*P. spinosum* (L.) Boiss.) in Albanchez de Mágina, Jaén province, Spain. Detailed morphological, morphometrical and molecular information about this species is provided below, confirming its identity as a new species of the genus described herein.

Taxonomy

Phylum: Nematoda Rudolphi, 1808
Class: Enoplea Inglis, 1983
Order: Dorylaimida Pearse, 1942
Suborder: Dorylaimina Pearse, 1936
Superfamily: Longidoroidea Khan and Ahmad, 1975
Family: Longidoridae Thorne, 1935
Genus: *Longidorus* Micoletzky, 1922
***Longidorus maginicus* n. sp.**
ZooBank: urn:lsid:zoobank.org:act: CFEEC5B2-A34F-440E-B92C-029818AFC9E1

Description

(Figures 1–4, table 1)

Females. Body G-shaped after being relaxed by gentle heat (fig. 2a), gradually tapering in both ends and with greater curvature in posterior end. Cuticle apparently composed of two layers (2.5–3.5 µm at mid-body), thickened in tail region. Lateral body pores observed in pharyngeal region (fig. 3). Lip region anteriorly flattened, slightly separated from the rest of body by a depression. Amphidial fovea pouchlike, not lobed. SEM observations showed a slit-like oral aperture surrounded by six labial papillae in *en face* view, and a pore-like aperture of amphidial fovea (fig. 4). Odontostyle thin and moderately long, narrow, 1.5 (1.3–1.8) times as long as odontophore, straight or flexible (figs 1 and 2), odontophore moderately developed surrounding muscles at base. Pharynx with three visible nuclei, the dorsal gland's nucleus at approximately 15.1–19.4%, and two smaller ventrosublateral nucei (S1N) at almost the same level, 48.6–55.3% of pharyngeal bulb length (according to Loof & Coomans, 1972). Nerve ring surrounding anterior part of isthmus. Glandularium 74.0 (64.0–82.0) µm long. Cardia small, conoid-rounded. Reproductive system didelphic amphidelphic with genital branches almost equally developed with reflexed ovaries, 448.5 (310.0–623.0) µm, 423.0 (308.0–505.0) µm long, respectively. Vulva a transverse slit in ventral view, situated about 50% of body length and vagina 10.1 (8.0–12.0) µm long, perpendicular to body axis at approximately 20% of corresponding body width long, surrounded by constrictor muscles. Sperm cells present in the genital branches of some specimens. Rectum 25.6 (22.0–28.0) µm long. Tail moderately long, conoid, dorsally convex with rounded terminus, with two or three pairs of caudal pores on each side.

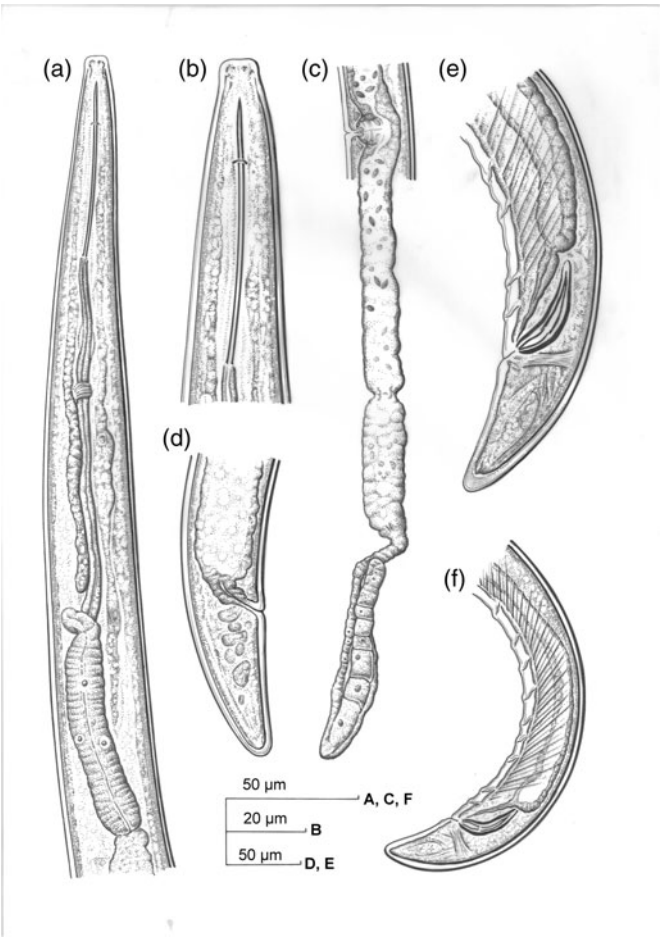

Fig. 1. *Longidorus maginicus* n. sp. (drawings). (a) female neck region; (b) female anterior region; (c) detail of posterior genital branch; (d) female tail; and (e, f) male tail.

Males. Common, but less frequent than female (1:2 ratio males:females). Morphologically similar to female except for genital system and secondary sexual features, and posterior region more strongly curved ventrally. Genital tract diorchic with testes opposed, containing multiple rows of spermatogonia. Tail conoid, dorsally convex with rounded terminus and thickened outer cuticular layer. Spicules dorylaimoid, moderately developed and curved ventrally, lateral guiding piece more or less straight or with curved proximal end. Supplements comprising one cloacal pair 12–17 µm from cloacal aperture and a midventral row of 6–9.

Juveniles. Three developmental juvenile stages were detected and distinguished by body length, odontostyle length and replacement odontostyle length (Robbins *et al.*, 1995, 1996). Morphology resembling female except in slight variations in c' ratio, body size and sexual characteristics (table 1, fig. 2). The first-stage juvenile was characterized by a bluntly conoid tail ($c' = 2.6$; fig. 2).

Diagnosis and relationships

Longidorus maginicus n. sp. is an amphimictic species characterized by a moderately long body (4.2–5.2 mm); lip region anteriorly flattened, slightly separated from the rest of body by depression, 9–11 µm wide and 3.5–6.0 µm high; amphidial fovea not lobed; relatively short odontostyle (61.0–70.5 µm);

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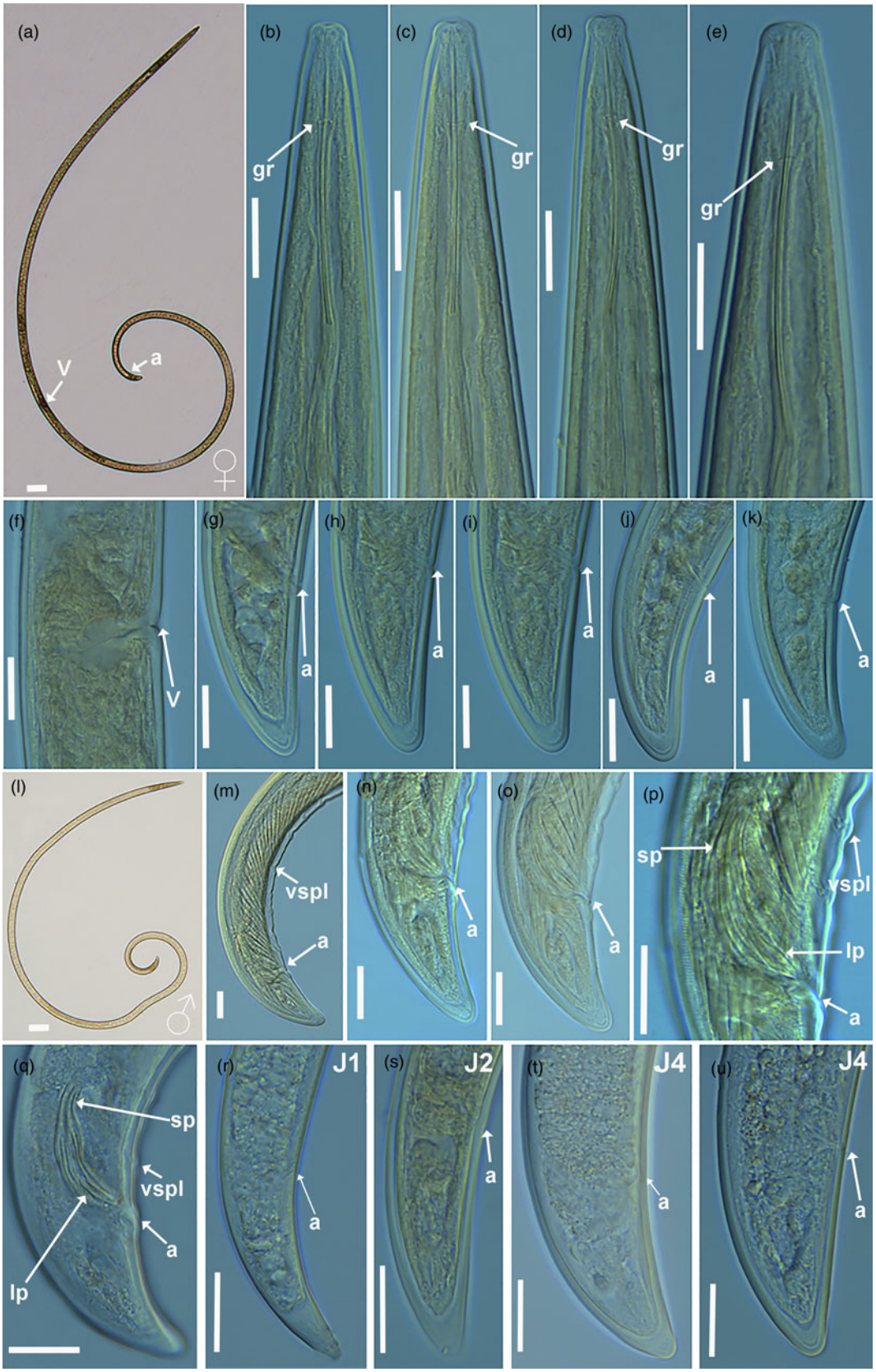

Fig. 2. Light micrographs of *Longidorus maginicus* n. sp. (a) entire female; (b–e) female anterior end showing guiding ring (arrowed); (f) vulval region; (g–k) female tail; (l) entire male; (m–q) male tail with details of spicules, lateral guiding piece and ventromedian supplements (arrowed); and (r–u) tail region of 1st, 2nd and 4th stage juveniles (J1, J2 and J4). Abbreviations: a = anus; gr = guiding ring; lp = lateral guiding piece; sp = spicule; V = vulva; vspl = ventromedian supplement. (Scale bars: a, l = 100 μm; b–k, m–u = 20 μm).

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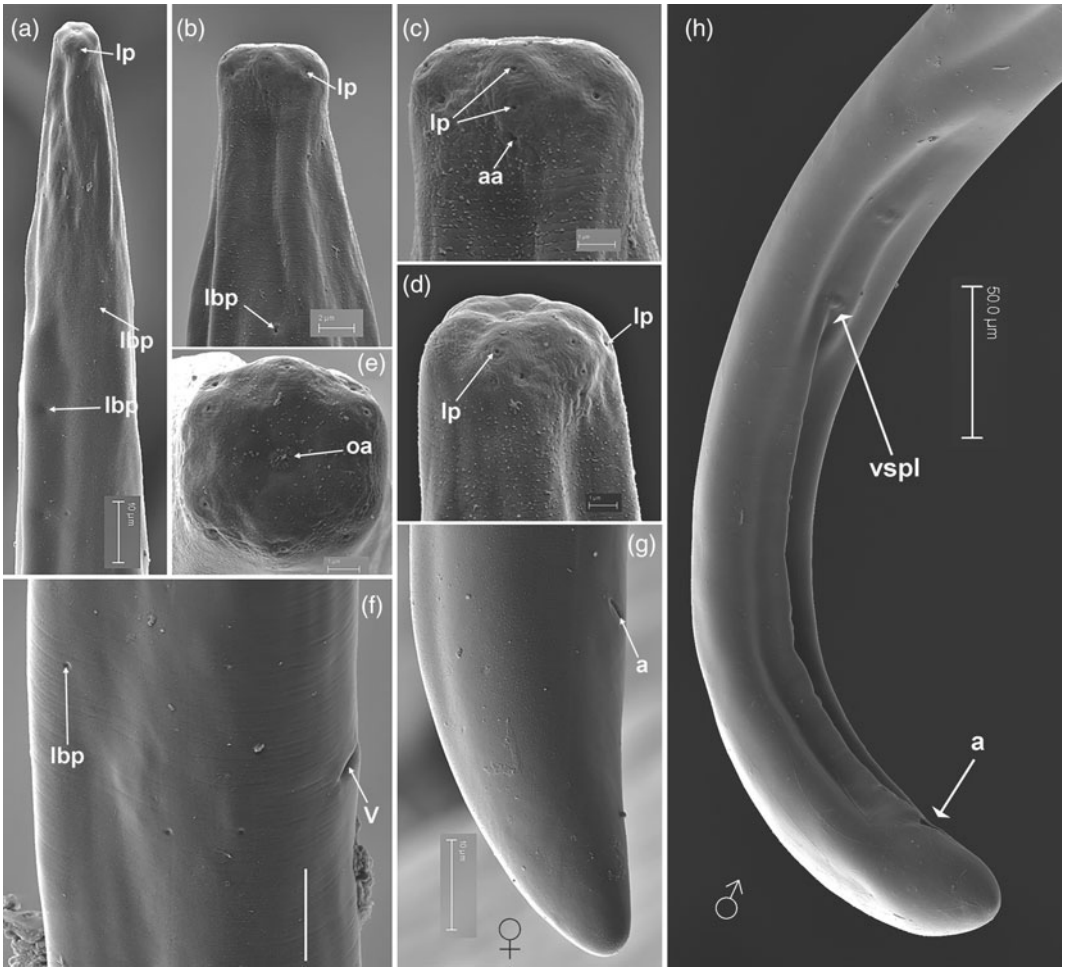

Fig. 3. Scanning electron microscope micrographs of *Longidorus maginicus* n. sp. (a, b) female anterior end in lateral view showing outer labial papillae (lp) and lateral body pore (lbp); (c, d) female lip region showing labial papillae (lp) and amphidial aperture (aa) arrowed; (e) *en face* view showing oral aperture (oa) arrowed; (f) vulval region showing vulva (V) and lateral body pore (lbp); (g) female tail showing anus (a); and (h): male posterior body region showing anus and ventromedian supplements. Abbreviations: a = anus; aa = amphidial aperture; lbp = lateral body pore; lp = labial papilla; oa = oral aperture; V = vulva; vspl = ventromedian supplement. (Scale bars: a, f, g = 10 µm; b = 2 µm; c–e = 1 µm; h = 50 µm).

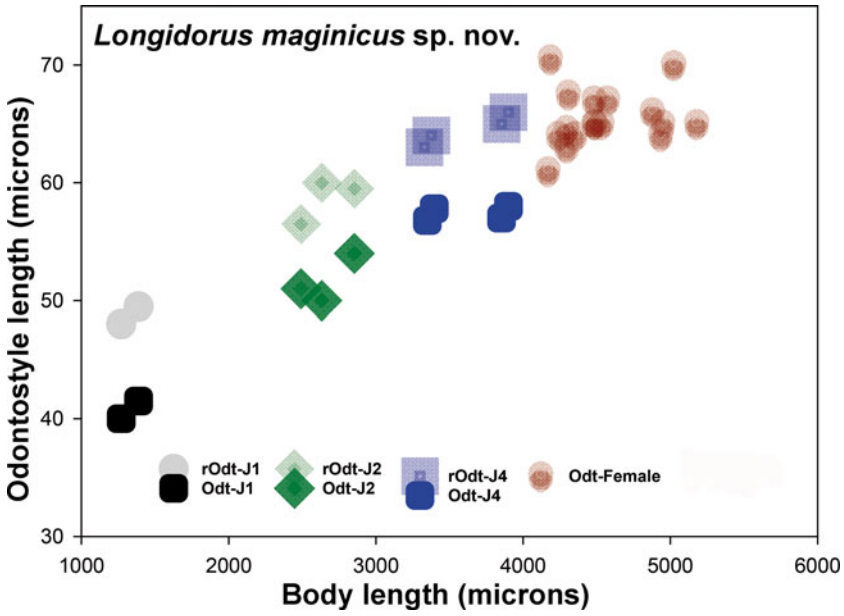

Fig. 4. Relationship of body length to length of functional and replacement odontostyle (Odt and rOdt, respectively) length in all developmental stages from first-, second and fourth-stage juveniles (J1, J2 and J4) and mature females of *Longidorus maginicus* n. sp.

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Table 1. Morphometrics of *Longidorus maginicus* n. sp. from of spiny madwort (*Ptilotrichum spinosum* (L.) Boiss.) in Albanchez de Mágina, Jaén province, Spain). All measurements are in μm and in the form: mean ± standard deviation (range).

		Paratypes				
Character [a]	Holotype	Female	Male	J1	J2	J4
n		19	9	2	3	4
L	4.351	4.554 ± 0.296	4.222 ± 0.215	(1.270, 1.388)	2.658 ± 0.183	3.792 ± 0.575
		(4.168–5.177)	(3.939–4.560)		(2491–2853)	(3.306–4.560)
a	106.1	99.4 ± 11.5	95.2 ± 10.1	(57.7, 60.3)	75.7 ± 2.7	116.8 ± 18.6
		(80.2–120.4)	(82.1–108.1)		(73.2–78.5)	(103.1–142.5)
b	13.9	14.9 ± 1.3	13.9 ± 1.0	(7.2, 7.6)	11.2 ± 1.2	14.6 ± 1.0
		(11.7–18.1)	(12.4–15.5)		(9.9–12.0)	(13.1–15.5)
c	85.3	88.6 ± 10.4	79.7 ± 4.4	(25.4, 27.2)	50.3 ± 1.1	67.5 ± 8.3
		(76.5–114.9)	(73.1–86.5)		(49.6–51.6)	(60.7–79.3)
c′	1.8	1.8 ± 0.2	1.6 ± 0.1	(2.6, 2.6)	2.4 ± 0.1	1.7 ± 0.1
		(1.3–2.1)	(1.6–1.7)		(2.3–2.4)	(1.6–1.7)
d	2.5	2.5 ± 0.2	2.7 ± 0.1	(2.6, 2.6)	1.9 ± 0.4	2.3 ± 0.1
		(2.3–2.9)	(2.5–2.9)		(1.6–2.3)	(2.2–2.4)
d′	1.7	1.7 ± 0.1	1.75 ± 0.1	(1.9, 1.9)	2.1 ± 0.5	1.6 ± 0.1
		(1.5–1.9)	(1.6–1.8)		(1.6–2.4)	(1.5–1.7)
V or *T*	49.1	49.4 ± 1.3	50.0 ± 7.2	–	–	–
		(46.4–51.3)	(43.1–61.6)			
G_1	10.7	9.9 ± 1.7 (6.9–12.8)	–	–	–	–
G_2	9.5	9.3 ± 1.4 (6.8–11.3)	–	–	–	–
odontostyle	64.0	66.1 ± 2.6	67.7 ± 1.4	(40.0, 41.5)	51.7 ± 2.1	58.0 ± 0.8
		(61.0–70.5)	(66–70)		(50–54)	(57–59)
odontophore	42.0	43.9 ± 3.0	45.6 ± 3.0	(25.0, 26.0)	36.5 ± 2.3	42.0 ± 0.8
		(38–48)	(39–49)		(34.0–38.5)	(41–43)
total stylet	106.0	110.0 ± 3.5	113.3 ± 2.9	(65.0, 67.5)	86.8 ± 3.9	100.0 ± 1.6
		(104–117)	(109–117)		(84.0–89.5)	(98–102)
replacement odontostyle	–	–	–	(48.0, 49.5)	58.7 ± 1.9	64.1 ± 1.7
					(56.5–60.0)	(62.5–66.0)
lip region diameter	9.5	10.0 ± 0.7	9.8 ± 0.5	(6.5, 7.0)	9.2 ± 0.1	10.4 ± 0.5
		(9–11)	(9.0–10.5)		(9.0–9.5)	(10–11)

oral aperture to guiding ring	24.0	25.1 ± 1.0	26.8 ± 1.4	(17, 18)	16.7 ± 3.8	23.5 ± 1.3
		(23.5–27.0)	(25–29)		(14–22)	(22–25)
maximum body diameter	41.0	46.2 ± 4.3	45 ± 4	(22, 23)	35.2 ± 3.3	32.5 ± 0.6
		(39–55)	(39–49)		(33–39)	(32–33)
tail length	51.0	51.9 ± 5.4	53.8 ± 3.3	(50, 51)	52.8 ± 4.1	56 ± 2
		(39–61)	(48.0–59.0)		(50.0–57.5)	(54–58)
J	7.5	8.3 ± 0.7	8.5 ± 0.8	(15, 16)	8.5 ± 0.5	8.8 ± 0.3
		(7.5–10.0)	(7.0–9.5)		(8–9)	(8.5–9.0)
spicules length	–	–	44.1 ± 3.1	–	–	–
			(39.0–48.5)			
lateral guiding piece length	–	–	12.0 ± 0.9	–	–	–
			(11–13)			
ventromedian supplements	–	–	9 ± 1	–	–	–
			(7–10)			

[a]*d* = anterior to guiding ring/body diameter at lip region; *d′* = body diameter at guiding ring/body diameter at lip region; and *J* = hyaline tail region length.

guiding ring located 23.5–27.0 µm from anterior end; vulva located at 46.4%–51.3% of body length; female tail 39–61 µm long, conoid, dorsally convex with rounded terminus (*c′* = 1.3–2.1), with two or three pairs of caudal pores; and males common (1:2 ratio males:females), with medium size spicules (39.0–-48.5 µm) and 1 pair + 6–9 ventromedian supplements. According to the polytomous key by Chen *et al.* (1997) and the new code by Peneva *et al.* (2013), codes for the new species are (codes in parentheses are exceptions): A2-B1-C2-D2-E1-F2(3)-G2-H5(4)-I2-J1-K6.

Conforming to the polytomous key by Chen *et al.* (1997), and on the basis of sorting on matrix codes A (odontostyle length), B (lip region width), C (distance of guiding-ring from anterior body end), D (lip region shape) and F (body length), *L. maginicus* n. sp. is closely related to *Longidorus pini* Andrés & Arias, 1987, *L. carpetanensis* Arias *et al.*, 1986, *Longidorus unedoi* Arias *et al.*, 1986, *Longidorus indalus* Archidona-Yuste *et al.*, 2016, and *Longidorus bordonensis* Gutiérrez-Gutiérrez *et al.*, 2020, from which it can be differentiated by a combination of characters discussed below. *Longidorus maginicus*. n. sp. differs from *L. carpetanensis* by a slightly longer odontostyle (61.0–70.5 vs. 54.0–65.0 µm), longer body (4.2–5.2 vs. 3.5–4.4 mm), longer spicules (39.0–48.5 vs. 34.0–38.5 µm), and amphidial fovea (not lobed vs. bilobed symmetrically) (Arias *et al.*, 1986). From *L. pini* by lip region shape (anteriorly flattened, slightly separated from the rest of body by a depression vs. offset and slightly expanded lip region), and amphidial fovea (not lobed vs. bilobed symmetrically) (Andrés & Arias, 1987). From *L. unedoi* by a slightly longer odontostyle (61.0–70.5 vs. 52.0–64.0 µm), a shorter body length (4.2–5.2 vs. 5.0–6.0 mm), larger spicules (39.0–48.5 vs. 35.0 µm) and amphidial fovea (not lobed vs. bilobed asymmetrically) (Arias *et al.*, 1986). From *L. indalus* by a longer odontostyle (61.0–70.5 vs. 54.0–59.5 µm) and lip region shape (anteriorly flattened, slightly separated from the rest of body by a depression vs. expanded distinctly set off from body contour) (Archidona-Yuste *et al.*, 2016). From *L. bordonensis* by lip region shape (anteriorly flattened, slightly separated from the rest of body by a depression vs. offset and slightly expanded lip region), amphidial fovea (not lobed vs. bilobed asymmetrically) and *c′* ratio (1.3–2.1 vs. 1.9–3.1) (Gutiérrez-Gutiérrez *et al.*, 2020). In addition, *L. maginicus* n. sp. can be separated from these species by ribosomal and mitochondrial molecular markers (see below).

Etymology

The species epithet refers to the mountain where the species was detected (Sierra Mágina, Jaén province, Spain).

Type host and locality

The new species was recovered from the rhizosphere of spiny madwort (*P. spinosum* (L.) Boiss.) in Albanchez de Mágina, Jaén province, Spain (coordinates 37°43′58.7″N 3°28′11.4″W).

Type material

Holotype female and 17 female and seven male paratypes deposited at the Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (slide numbers SM7-02-SM7-11); one female and one male at the Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (C.N.R.), Sezione di Bari, Bari, Italy (SM7-012); and

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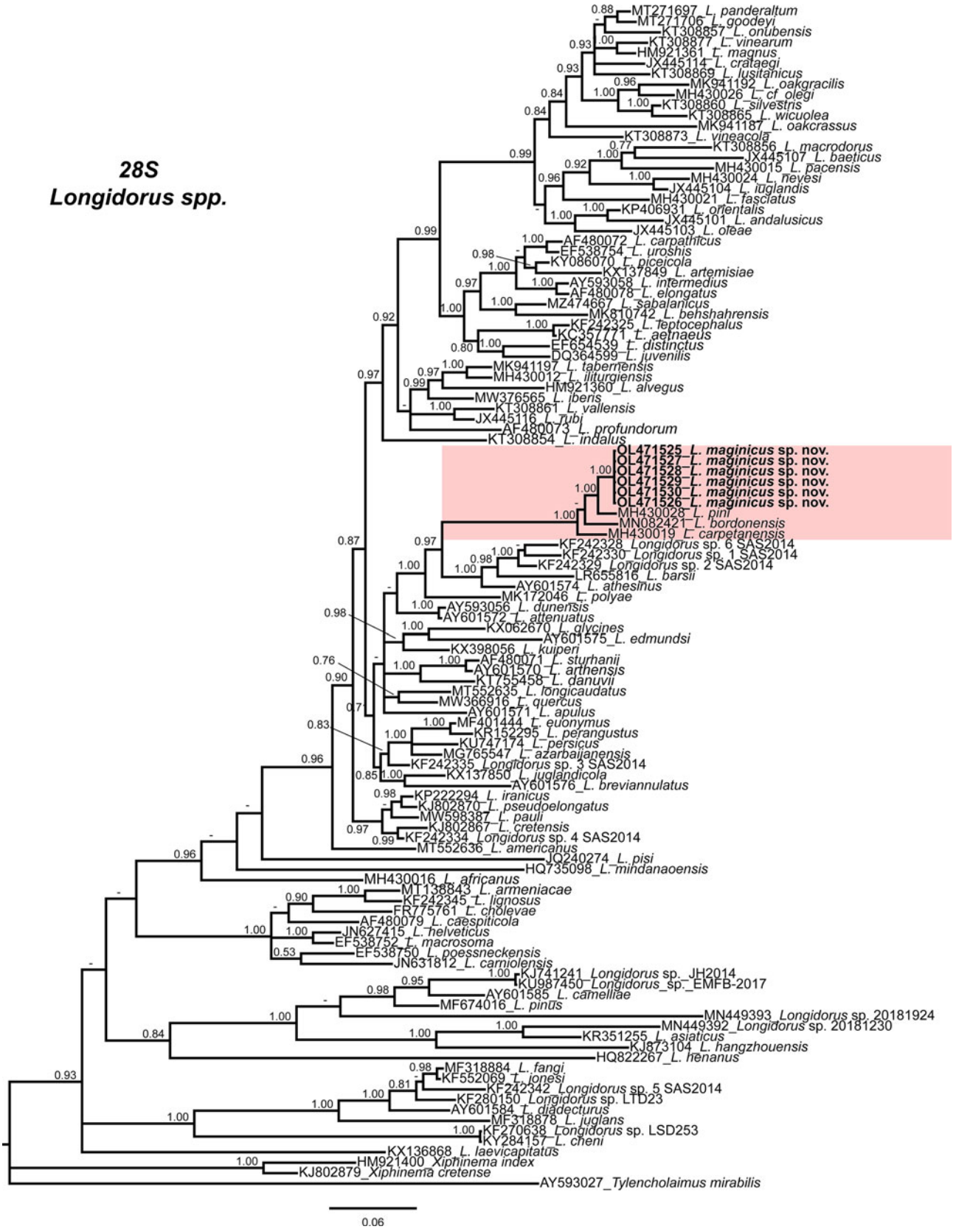

Fig. 5. Phylogenetic relationships of *Longidorus maginicus* n. sp. with species of *Longidorus*. Bayesian 50% majority rule consensus tree as inferred from D2 and D3 expansion domains of 28S rDNA sequence alignment under the GTR + I+ G model (–lnL = 16,234.1335; Akaike information criterion = 32,936.2669; freqA = 0.2265; freqC = 0.2250; freqG = 0.2913; freqT = 0.2571; R(a) = 0.9207; R(b) = 2.7156; R(c) = 1.6004; R(d) = 0.4273; R(e) = 5.2793; R(f) = 1.0000; Pinva = 0.3230; and shape = 0.8070). Posterior probabilities more than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in boldface type, and coloured box indicates clade association of the new species. Scale bar = expected changes per site.

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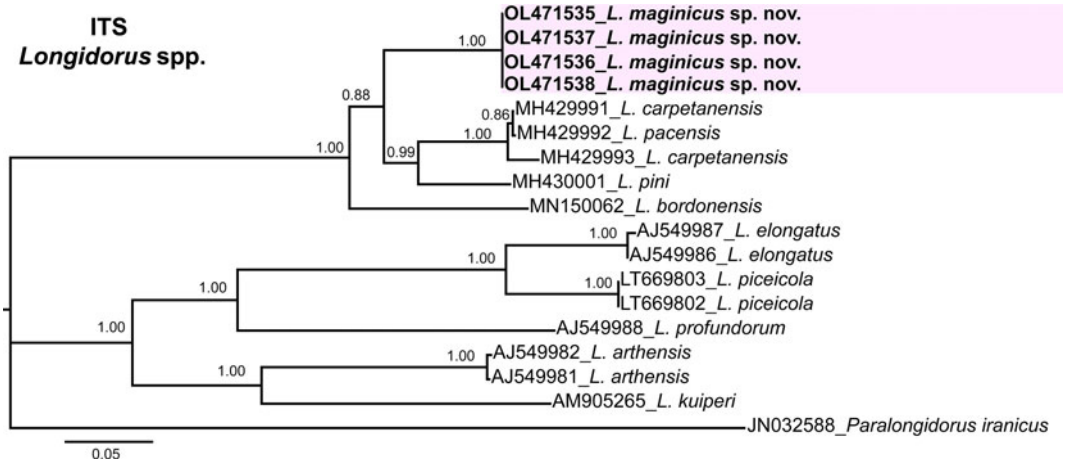

Fig. 6. Phylogenetic relationships of *Longidorus maginicus* n. sp. with species of *Longidorus*. Bayesian 50% majority rule consensus tree as inferred from ITS rDNA sequence alignment under the GTR + I+ G model (−lnL = 9666.2264; Akaike information criterion = 19,420.4529; freqA = 0.2695; freqC = 0.1995; freqG = 0.2704; freqT = 0.2606; R(a) = 0.9112; R(b) = 2.6397; R(c) = 1.4133; R(d) = 0.7800; R(e) = 4.0411; R(f) = 1.0000; Pinva = 0.2010; and shape = 1.2680). Posterior probabilities more than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in boldface type, and coloured box indicates clade association of the new species. Scale bar = expected changes per site.

one female and one male at the United States Department of Agriculture Nematode Collection (T-7607p).

Molecular characterization

Longidorus maginicus n. sp. was molecularly characterized by the sequences of three ribosomal genes, D2–D3 segment of 28S, ITS and partial 18S rDNA, and the mitochondrial gene *coxI*. The amplification of these regions yielded single fragments of approximately 900, 1600, 1600 and 400 base pairs (bp), respectively, based on gel electrophoresis. Six D2–D3 of 28S rDNA sequences from 716 to 749 bp (OL471525–OL471530), four ITS rDNA sequences from 1504 to 1522 bp (OL471535–OL471538), four 18S rDNA sequences from 1719 to 1733bp (OL471531–OL471534) and three *coxI* sequences of 365 bp (OL471046–OL471048) were generated for this new species without intraspecific sequence variations. D2–D3 segment of 28S rDNA of *L. maginicus* n. sp. (OL471525–OL471530) was 97% identical to *L. carpetanensis* (MH430019) and *L. pini* (MH430028) and 96% identical to *L. bordonensis* (MN082422). ITS of *L. maginicus* n. sp. (OL471535–OL471538) was 86% identical to *L. pini* (MH430001), 84% identical to *L. carpetanensis* (MH429991), *L. pacensis* Archidona-Yuste *et al.*, 2019 (MH429992) and 83% similar to *L. bordonensis* (MN150062). Partial 18S of *L. maginicus* n. sp. (OL471531–OL471534) was 99% identical to several *Longidorus* species such as *Longidorus vineacola* (AY283169), *Longidorus elongatus* de Man 1876 (EU503141), *L. pini* (MH430011) and *Longidorus tabernensis* Cai *et al.*, 2020a, b (MK941261). Finally, *coxI* of *L. maginicus* n. sp. (OL471046–OL471048) was 80% identical to *L. carpetanensis* (MH454068), and 79% to *L. pini* (MH454070) and *Longidorus iliturgiensis* Archidona-Yuste *et al.*, 2019 (MH454065).

Phylogenetic relationships of L. maginicus *n. sp. with other* Longidorus *spp.*

Phylogenetic relationships among *Longidorus* species, as inferred from analyses of D2–D3 expansion domains of 28S, ITS, the partial 18S rDNA and the partial *coxI* mtDNA gene sequences using BI, are shown in figs 5–8, respectively. The phylogenetic trees generated with the ribosomal and mitochondrial DNA markers included 113, 18, 89 and 60 sequences, and their alignment had 741, 1709, 1675 and 383 characters, respectively. The presently resolved D2–D3 tree of *Longidorus* spp., *L. maginus* n. sp. (OL471525–OL471530), *L. pini* (MH430028), *L. bordonensis* (MN082421) and *L. carpetanensis* (MH430019) formed a well-supported subclade (PP = 1.00), including while other morphologically related species (*viz. L. indalus* and *L. iliturgiensis*) occupied a separate clade (PP = 0.97) (fig. 5).

Difficulties were detected in finding suitable sequences for the phylogenetic reconstruction of ITS sequences due to the low coverage and identity with other sequences of *Longidorus*. Consequently, only related sequences were chosen for ITS phylogeny and ambiguously aligned regions were discarded from the alignment (fig. 6). In ITS phylogeny, the new species occupied a placement inside a well-supported clade (PP = 1.00). However, the clade of the new species (OL471535–OL471538) with *L. carpetanensis* (MH429991 and MH429993), *L. pacensis* (MH429992) and *L. pini* (MH430001) received a 0.88 PP. Other morphologically related species (*viz. L. indalus* and *L. iliturgiensis*) showed very low sequence identity and were excluded from the analysis.

In a 50% majority rule consensus 18S rDNA BI tree, *L. maginicus* n. sp. (OL471531–OL471534), *L. carpetanensis* (MH430006) and *L. pini* (MH430011) formed a well-supported clade (PP = 1.00), while *L. indalus* (KT308894) and *L. iliturgiensis* (MH430002) clustered separately (fig. 7).

Finally, the phylogenetic relationships of *L. maginicus* n. sp. (OL471046–OL471048) with other species, using *coxI* gene sequences, were not resolved due to polytomy (fig. 8).

Discussion

Accurate species identification in the genus *Longidorus* is often problematic due to a huge number of valid species, presence of cryptic species and many potential diagnostic features that must be considered. The main objective of this study was to identify and describe, morphologically (including LM and SEM analyses) and molecularly, a new population of *Longidorus* detected in a natural environment in Albanchez de Mágina, Jaén province, southern Spain, as well as clarify the phylogenetic relationships

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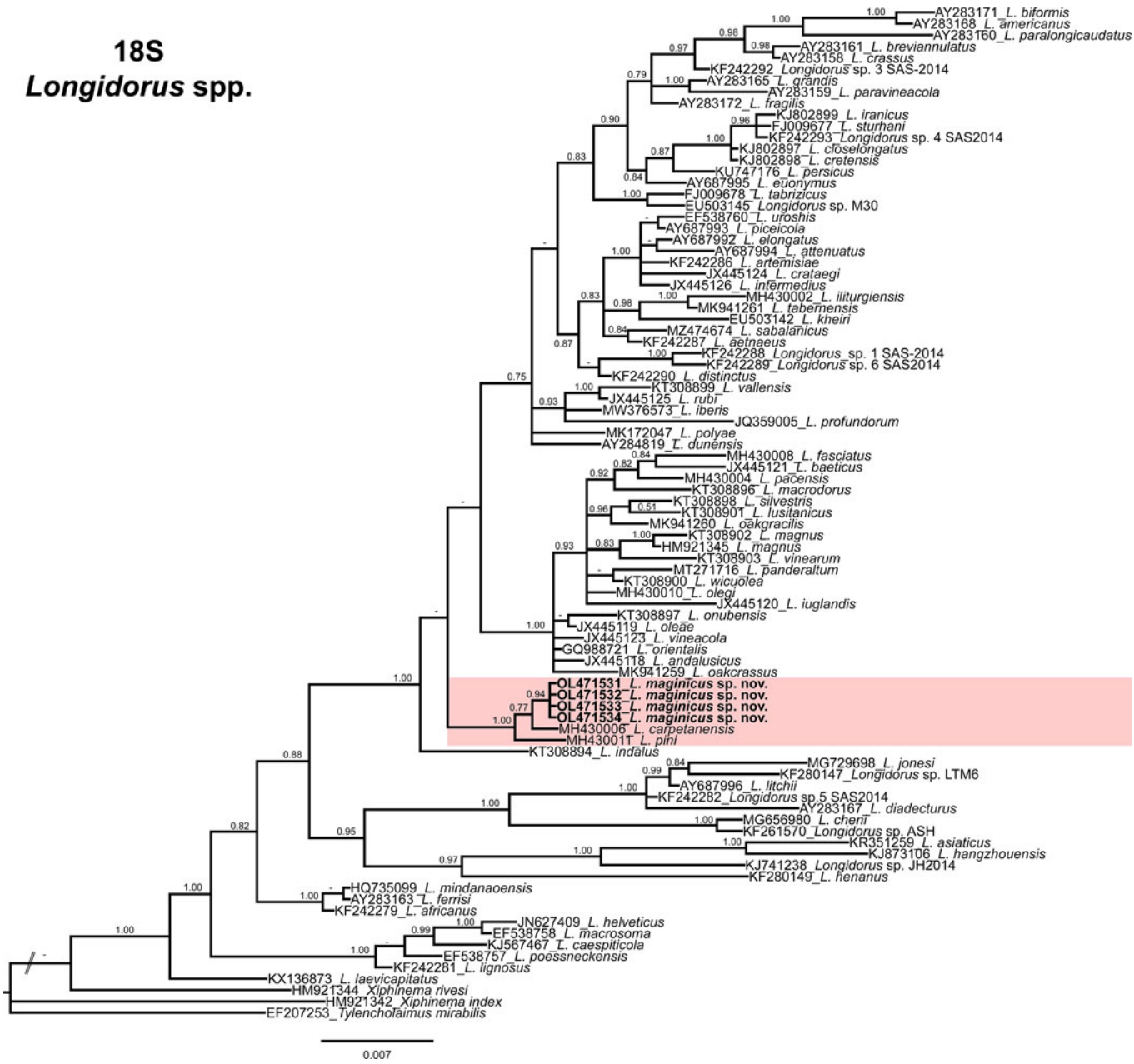

Fig. 7. Phylogenetic relationships of *Longidorus maginicus* n. sp. with species of *Longidorus*. Bayesian 50% majority rule consensus tree as inferred from 18S rDNA sequence alignment under the GTR + I+ G model (−lnL = 6419.3228; Akaike information criterion = 13,210.6455; freqA = 0.2726; freqC = 0.2111; freqG = 0.2616; freqT = 0.2547; R(a) = 1.2039; R(b) = 3.4138; R(c) = 1.7952; R(d) = 0.4804; R(e) = 7.2334; R(f) = 1.0000; Pinva = 0.7480; and shape = 0.5440). Posterior probabilities more than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in boldface type, and coloured box indicates clade association of the new species. Scale bar = expected changes per site.

within the genus *Longidorus*. All the provided results confirmed that the unknown *Longidorus* population is morphologically and morphometrically very close to *L. carpetanensis* described from central Spain, except for slight differences in odontostyle length and shape of the amphidial fovea. Nevertheless, all the molecular markers clearly separated both taxa confirming that the new population is a new valid species of the genus. Only a few species of *Longidorus* and *Paralongidorus* have been described with complete SEM observations (Roca, 2006; Cai *et al.*, 2018; Clavero-Camacho *et al.*, 2021a, b). Our SEM results provide clear evidence of pore-like amphidial apertures dissipating doubts about the generic placement as recently established for *Longidorus iberis* (Clavero-Camacho *et al.*, 2021a, b). Interestingly, although more studies are needed for confirming this hypothesis, these results suggest that native deciduous leguminous shrubs (brooms) of the family Fabaceae may be a common host-plant of these nematodes, since the latter species was also described from common broom (*Cytisus scoparius* (L.) Link). Likewise, these results increase the already high biodiversity of this genus in the Iberian Peninsula (45 species reported so far) and support that species richness in natural environments is higher than in cultivated areas (Archidona-Yuste *et al.*, 2016, 2019; Gutiérrez-Gutiérrez *et al.*, 2016, 2020; Cai *et al.*, 2020a, b; Clavero-Camacho *et al.*, 2021a, b). This fact can be easily

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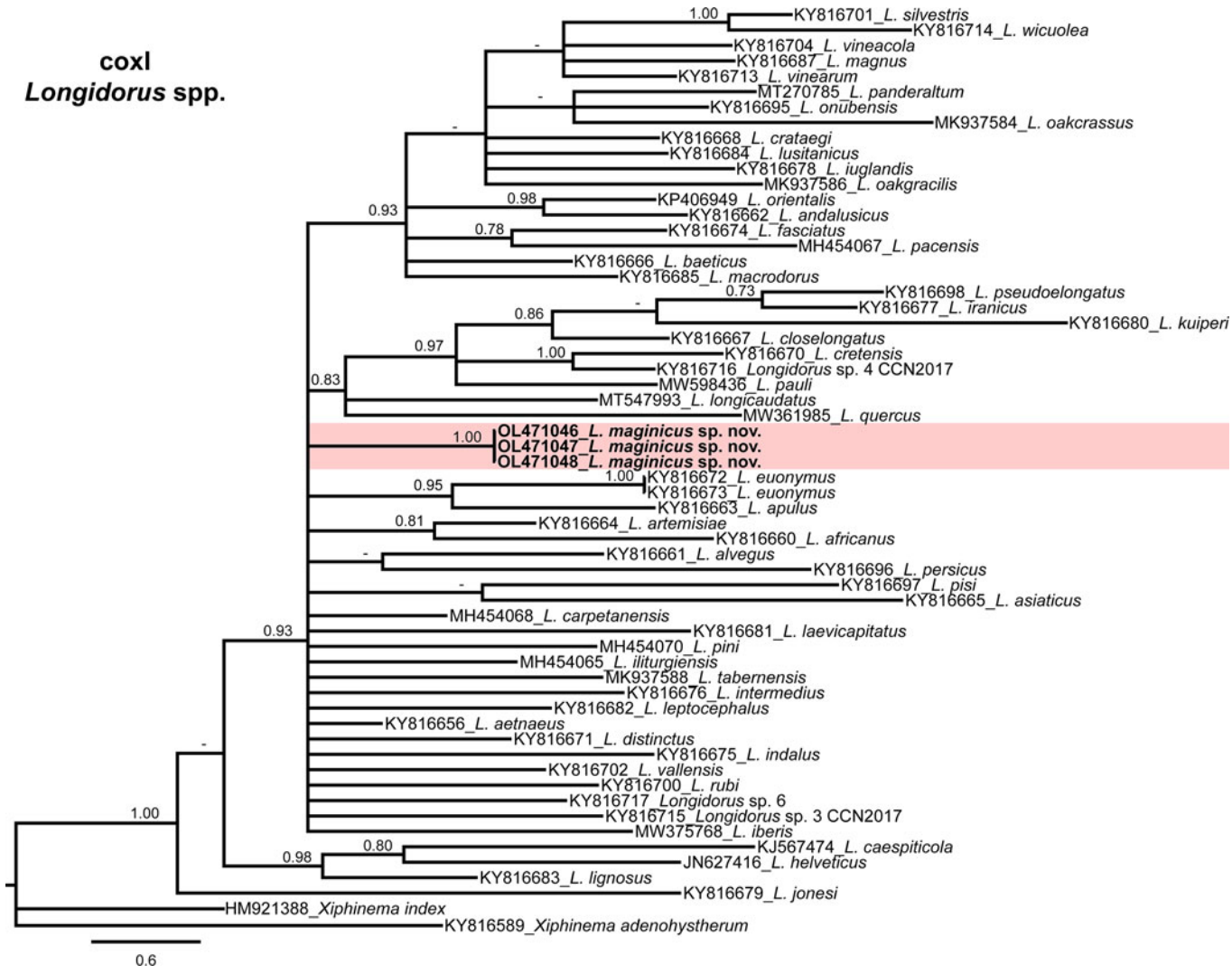

Fig. 8. Phylogenetic relationships of *Longidorus maginicus* n. sp. with species of *Longidorus*. Bayesian 50% majority-rule consensus trees as inferred from cytochrome c oxidase subunit I (*coxI*) mtDNA gene sequence alignments under the TRN + I + G model (−lnL = 9999.6926; Akaike information criterion = 20,249.3853; freqA = 0.3063; freqC = 0.1721; freqG = 0.1875; freqT = 0.3341; R(a) = 1.0000; R(b) = 7.1246; R(c) = 1.0000; R(d) = 1.0000; R(e) = 47.8802; R(f) = 1.0000; Pinva = 0.3720; and shape = 0.327). Posterior probabilities more than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in boldface type, and coloured box indicates clade association of the new species. Scale bar = expected changes per site.

understood from the consideration that the larger nematodes (i.e. *Longidorus* spp.) seem to be mainly affected by the short-term consequences of the establishment of agricultural systems (Archidona-Yuste *et al.*, 2021). Notably, the description of this new species increases the data on the biodiversity of the genus *Longidorus* in the Euro-Mediterranean region (Navas *et al.*, 1993).

Again, ribosomal and mitochondrial markers (D2–D3 expansion domains of the 28S, ITS rDNA, and the mtDNA gene *coxI*) are important tools for accurate identification of *Longidorus* and remain essential for accurate diagnosis of needle nematodes (Archidona-Yuste *et al.*, 2019; Cai *et al.*, 2020a, b; Clavero-Camacho *et al.*, 2021a, b). However, the low nucleotide variability found in partial 18S rRNA makes it difficult to identify individuals to the species level. Phylogenetic analyses based on ribosomal genes resulted in a consensus of species' phylogenetic positions for the majority of species and this was congruent with those given by previous phylogenetic analyses (Peneva *et al.*, 2013; Subbotin *et al.*, 2014; Trisciuzzi *et al.*, 2015; Archidona-Yuste *et al.*, 2019; Amrei *et al.*, 2020; Cai *et al.*, 2020a, b; Clavero-Camacho *et al.*, 2021a, b). In particular, in all three ribosomal genes trees, *L. maginicus* n. sp. clustered with all the morphologically related species, including *L. carpetanensis*, *L. pini* and *L. bordonensis* (figs 5–7). The close phylogenetic relationships of species of *Longidorus* have been already documented (Gharibzadeh *et al.*, 2018). However, the resolution of the phylogenetic relationship for this genus with *coxI* was very low due to polytomy (fig. 8), probably because mtDNA evolves faster than ribosomal DNA and also by the relatively shorter size of the region sequenced (Lazarova *et al.*, 2006; Kumari *et al.*, 2010; Palomares-Rius *et al.*, 2017). However, *coxI* is an excellent molecular marker for species separation and identification (Gutiérrez-Gutiérrez *et al.*, 2012; Cai *et al.*, 2020a; Clavero-Camacho *et al.*, 2021a, b).

A brief review of the available literature shows that nematological efforts during the last decades on longidorid species in southern Spain have been higher than those in other parts of our country; however, the current distribution of the genus *Longidorus* in Spain suggests that this part of the country can

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be considered as a potential hotspot of biodiversity. Nevertheless, further research is needed to definitely confirm this hypothesis.

In summary, the present study confirms the usefulness of application of an integrative approach based on the combination of morphometric and morphological traits and genotyping rRNA and mtDNA markers for correct species discriminating among *Longidorus* species, and suggesting the need for continuing nematode surveys in natural environments in order to complete the unexplored biodiversity of this genus in this region and all over the world.

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Conflicts of interest. None.

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Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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