Integrative descriptions and molecular phylogeny of two new needle nematodes of the genus *Longidorus* (Nematoda: Longidoridae) from Spain

Ruihang CAI^{1,2}, Antonio ARCHIDONA-YUSTE¹, Carolina CANTALAPIEDRA-NAVARRETE¹, Juan E. PALOMARES-RIUS¹, Pablo CASTILLO^{1,*}

¹*Institute for Sustainable Agriculture (IAS), Spanish National Research Council (CSIC), Avenida Menéndez Pidal s/n, 14004 Córdoba, Campus de Excelencia Internacional Agroalimentario, ceiA3, Spain*

²*Laboratory of Plant Nematology, Institute of Biotechnology, College of Agriculture & Biotechnology, Zhejiang University, Hangzhou 310058, Zhejiang, P.R. China*

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*Author for correspondence: Pablo Castillo

E-mail: p.castillo@csic.es

Short Title: New *Longidorus* from Spain

Abstract Needle nematodes have an economic importance by causing damage to a wide range of natural and cultivated plants not only by directly feeding on root cells, but also by transmitting plant nepoviruses. Two new *Longidorus* nematodes, *Longidorus oakcrassus* n. sp. and *Longidorus oakgracilis* n. sp., are described and illustrated from populations associated with the rhizosphere of Pyrenean oak (*Quercus pyrenaica* Wild.) in southern Spain. The taxonomic position of both new species within the genus was assigned using an integrative approach. Morphologically, *L. oakcrassus* n. sp. is characterized by a female with a large and robust body size (9.2-12.2 mm), lip region anteriorly flattened to slightly rounded and almost continuous or slightly offset by a depression with body contour, ca 25.5-32.0 μ m wide, amphidial fovea with slightly asymmetrical lobes, stylet composed by an odontostyle moderately long (110.0-133.5 μ m) and odontophore weakly developed, pharynx short ending in a terminal pharyngeal bulb with normal arrangement of pharyngeal glands, tail short almost hemispherical shape. *Longidorus oakgracilis* n. sp. is characterized by having a moderately long and thin female body (5.4-7.9 mm in length), a bluntly-rounded lip region, set off from body contour by a slight depression, amphidial fovea funnel-shaped without lobe, odontostyle moderately long (94.0-106.0 μ m), pharyngeal bulb with normal arrangement of pharyngeal glands, short tail, bluntly hemispherical. The presence of males is common in both species. Integrative diagnosis was based on molecular data using D2-D3 expansion domains of the 28S rRNA, 18S rRNA, ITS1 rRNA and partial *coxI* gene sequences and morphology. Although different gene markers show variations in the phylogenetic relationships, phylogeny indicated that *L. oakcrassus* n. sp. is phylogenetically related with several species described from the Iberian Peninsula, including *L. oakgracilis* n. sp., which is clustered with *L. cf. olegi*, *L. lusitanicus* and *L. silvestris*.

Keywords: 18S rDNA, 28S rDNA D2-D3, species description, *coxI*, ITS1, longidorids, phylogeny, taxonomy.

Introduction

Needle nematodes of the genus *Longidorus* Micoletzky, 1922 are cosmopolitan obligate migratory ectoparasites that are polyphagous and distributed almost worldwide. These nematodes spend their entire life cycle in the rhizosphere, using their needle stylet to feed on the apical root cells inducing galls in the tips and arresting root growth (Taylor & Brown 1997; Palomares-Rius *et al.* 2017a). *Longidorus* species have economic importance owing to their ability to cause serious damage to a wide range of crops by not only directly feeding on root cells but also transmitting plant nepoviruses (Taylor & Brown, 1997; Decraemer & Robbins, 2007). This genus constitutes a large group of approximately 170 species (Archidona-Yuste *et al.* 2019) and species delimitation is critical from a phytopathological, ecological and biogeographical point of view. The morphological convergence and the existence of cryptic species make the accurate identification of species considerably more difficult. (De Luca *et al.* 2004; Gutiérrez-Gutiérrez *et al.* 2013; Archidona-Yuste *et al.* 2016b, 2019). Consequently, morphological taxonomy could lead to under-estimation of the diversity in the genus *Longidorus* as reported in other genera of plant-parasitic nematodes (Palomares-Rius *et al.* 2014; Archidona-Yuste *et al.* 2016a; 2016c; Janssen *et al.* 2017).

The utility of DNA barcoding and molecular species delimitation approaches in species discovery and to uncover cryptic lineages into the genus *Longidorus* have been demonstrated by numerous studies (Ye *et al.* 2004; Pedram *et al.* 2012a; Gutiérrez-Gutiérrez *et al.* 2013; Palomares-Rius *et al.* 2017c; Archidona-Yuste *et al.* 2016b, 2019; Lazarova *et al.* 2019). Specifically, molecular methods using different fragments of nuclear ribosomal DNA (including 28S rRNA, 18S rRNA and ITS) and mitochondrial DNA (particularly the cytochrome c oxidase subunit I (*coxI*)) gene sequences have been used to provide precise identification of species and elucidate the phylogenetic relationships within the genus *Longidorus* (Ye *et al.* 2004; Neilson *et al.* 2004; Palomares-Rius *et al.* 2008; Gutiérrez-Gutiérrez *et al.* 2012; Archidona-Yuste *et al.* 2016b; 2019). In this sense, D2-D3 expansion segments of 28S rRNA and the partial *coxI* fragment have been proven as a better molecular markers for molecular species identification than partial 18S rRNA in *Longidorus* (Neilson *et al.* 2004; He *et al.* 2005; Pedram *et al.* 2012a, 2012b; Gutiérrez-Gutiérrez *et al.* 2013; Gutiérrez-Gutiérrez *et al.* 2018; Archidona-Yuste *et al.* 2016b; 2019). While, ITS1 rRNA region has been considered to be more appropriate for species delimitation rather than for phylogenetic analyses (Palomares-Rius *et al.* 2017b). Recently, mitochondrial genomes from *Longidorus vineacola* Sturhan & Weischer 1954 has been used to determine the

phylogenetic relationships with other genera (i.e., *Xiphinema americanum* Cobb 1913, *X. rivesi* Dalmaso 1969, *X. pachtaicum* (Tulaganov 1938) Kirjanova 1951, *Paralongidorus litoralis* Palomares-Rius *et al.* 2008) within Longidoridae (Palomares-Rius *et al.* 2017b).

In Spain, integrative taxonomy approaches have deciphered a large diversity of *Longidorus* spp. in recent years. In fact, 11 new species have been described including *L. andalusicus* Gutiérrez-Gutiérrez *et al.* 2013, *L. baeticus* Gutiérrez-Gutiérrez *et al.* 2013, *L. iliturgiensis* Archidona-Yuste *et al.* 2019, *L. indalus* Archidona-Yuste *et al.* 2016, *L. macrodorus* Archidona-Yuste *et al.* 2016, *L. oleae* Gutiérrez-Gutiérrez *et al.* 2013, *L. onubensis* Archidona-Yuste *et al.* 2016, *L. pacensis* Archidona-Yuste *et al.* 2019, *L. silvestris* Archidona-Yuste *et al.* 2016, *L. vallensis* Archidona-Yuste *et al.* 2016, *L. wicuoalea* Archidona-Yuste *et al.* 2016 (Gutiérrez-Gutiérrez *et al.* 2013; Archidona-Yuste *et al.* 2016b; 2019), and five new records were reported (*L. intermedius* Kozłowska & Seinhorst 1979, *L. lusitanicus* Macara 1985, *L. nevesi* Macara 1985, *L. cf. olegi* Kankina & Metlitskaya 1983 and *L. africanus* Merny 1966) (Archidona-Yuste *et al.* 2016b; 2019). Additional samplings carried out in natural areas in southern Spain revealed two populations of needle nematodes of the genus *Longidorus*. The preliminary studies of morphology and morphometry showed that these populations belonged to unknown species. Therefore, an integrative approach was conducted in order to describe these putative new species.

The objectives of this study were: (1) to describe two new species of the genus *Longidorus* through integrative diagnosis method, based on combination of morphological, morphometrical and molecular data; (2) to characterise molecularly the sampled *Longidorus* spp. populations using the D2-D3 expansion segments of the 28S rRNA gene, ITS1, partial 18S rRNA gene, and the partial mitochondrial *coxI* gene sequences; and (3) to study the phylogenetic relationships of the identified *Longidorus* species with available sequenced species.

Material and methods

Nematode population sampling, extraction and morphological identification

Specimens from the populations of the unidentified *Longidorus* species were collected during the spring season of 2019 in forest ecosystems in Andalusia, southern Spain (Table 1). Nematodes were isolated from sandy soil samples collected from the rhizosphere of Pyrenean oak (*Quercus pyrenaica* Wild.) at Cardeña, Córdoba province, Spain. Soil samples

were collected using a shovel, randomly selecting four to five cores, and considering the upper 5-50 cm depth of soil, always from 50-100 cm of the tree trunk. Nematodes were extracted from a 500-cm³ sub-sample of soil by centrifugal flotation and a modification of Cobb's decanting and sieving methods (Coolen 1979; Flegg 1967).

Specimens for study using light microscopy (LM) and morphometric studies 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) as modified by De Grisse (1969). Specimens were examined using a Zeiss III compound microscope with differential interference contrast at magnifications up to 1,000x. Photographs of nematodes were taken by a Nikon DM100 (Nikon, Barcelona, Spain). All measurements were expressed in micrometres (µm). For line drawings of the new species, light micrographs were imported to CorelDraw version X7 and redrawn. All other abbreviations used are as defined in Jairajpuri & Ahmad (1992).

Nematode molecular identification

To avoid mistakes in the case of mixed populations in the same sample, from three to four live nematodes from each population were temporarily mounted in a drop of 1M NaCl containing glass beads to ensure specimens are not damaged. 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 polymerase chain reaction (PCR) assays were performed as described by Subbotin *et al.* (2000). One nematode specimen of each sample was transferred to an Eppendorf tube containing 16 µl ddH₂O, 2 µl 10x 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 (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' TTGATTACGTCCCTGCCCTTT-3') (Vrain et al. 1992) and reverse primer rDNA1 (5'-ACGAGCCGAGTGATCCACCG-3') (Cherry et al. 1997). 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). 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' -131 CWACATAATAAGTATCATG-3').

PCR cycle conditions were: one cycle of 94 °C for 10 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 (Affimetrix, USB products), and used for direct sequencing in both directions using the primers referred 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 Table 1 and the phylogenetic trees.

Phylogenetic analyses

D2-D3 expansion segments of 28S rRNA, ITS1, and partial 18S rRNA sequences of *L. oakcrassus* n. sp. and *L. oakgracilis* n. sp. were obtained in this study. These sequences and from other species of *Longidorus* spp. 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). 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). 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, the partial 18S rRNA, and the partial *coxI*. These BI analyses were run separately per dataset using four chains for 2×10^6 generations for each molecular marker. 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 FigTree software V.1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Results and descriptions

*Longidorus oakcrassus*¹ n. sp. (Figs. 1-3, Table 2)

Female: Body very long and rather robust, slightly tapering towards anterior end, open C- to spiral shape when heat relaxed. Cuticle 6.5 ± 1.4 (4.0-8.5) μm thick at mid body, and 18.1 ± 3.0 (12.5-23.0) μm thick at tail hyaline part. Lip region anteriorly flattened to slightly rounded and continuous or slightly offset by a depression with body contour. Amphidial fovea pocket-shaped and symmetrically bilobed, one lobe is slightly shorter than the other one, and extending about 1/2 part of oral aperture-guiding ring distance. Guiding ring single, located approximately *ca.* 1.5 times the lip region width from anterior end. Odontostyle moderately long, 1.6-1.7 times as long as odontophore, straight or slightly arcuate. Odontophore weakly developed, with rather weak swollen base. Pharynx very short, 636.9 ± 43.4 (564.5-708.0) μm , extending to the terminal pharyngeal bulb with one dorsal nucleus (DN) and two subventral gland nuclei (SVN) separately located at 37.0 ± 1.9 (33.8-39.5) %, 48.9 ± 1.5 (47.0-50.7) % and 51.3 ± 1.7 (49.2-53.3) % of distance from anterior end of

¹ The species epithet refers to the compound name from the word oak (the host plant where the species was detected) and the Latin name *crassus* = fat/stout.

pharyngeal bulb. Glandularium 158.4 ± 12.0 (142.0-176.0) μm long. Reproductive system didelphic-amphidelphic, anterior branch 987.0 ± 121.2 (804.0-1109.0) μm long and posterior branch 1075.4 ± 260.0 (695.0-1333.0) μm long, each branch composed of a reflexed ovary 268-352 μm long. *Pars dilatata oviductus* and uterus of about equal length (227-433 μm long), separated by a strong and muscularized sphincter. Vulva slit-like, situated at 46.2-54.8% of body length. Vagina perpendicular to body axis, 57.3 ± 5.2 (52.0-66.5) μm long, occupying 37.7-38.4% of corresponding body diameter and surrounded by well-developed muscles; *pars distalis vaginae* 33.5-45.5 μm long, *pars proximalis vaginae* measuring 23.5-31.0 x 24.5-30.5 μm . Prerectum 556.3 ± 77.6 (461.0-644.0) μm long, rectum 49.3 ± 9.3 (43.0-60.0) μm long. Tail short and bluntly conoid, with between three and four pairs of caudal pores present on each side.

Male: Common. Morphologically similar to female except for genital system, close C- to spiral shape when heat relaxed. Male genital tract diorchic with testes opposed, containing multiple rows of spermatogonia. Tail conoid-rounded and slightly ventrally curved, with 23 to 25 mid-ventral supplements. Spicules ventrally curved and robust, lateral guiding piece almost straight or with curved proximal end.

Juveniles: Four developmental juvenile stages were distinguished based on body, odontostyle and replacement odontostyle length (Fig. 3). Morphologically similar to female, except for their size and sexual characteristics (Table 2). The first-stage juvenile was characterized by the replacement odontostyle inserted into odontophore base and tail conoid-rounded with a c' ratio 1.5-1.6.

Type habitat and locality

Rhizosphere of Pyrenean oak (*Quercus pyrenaica* Wild.) from Cardeña, Córdoba province, Spain (GPS coordinates: 38°12'32.1"E; 4°17'31.8"W) collected by R. Cai on February 19, 2019.

Type material and nomenclatural registration

Holotype female (slide CARF-02) and female and male paratypes (CARF-03-CARF-06) were deposited in the Nematode Collection of Institute for Sustainable Agriculture, IAS-

CSIC, Córdoba, Spain. One female and one male paratypes deposited at each of the following collections: Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (C.N.R.), Sezione di Bari, Bari, Italy (CARF07); USDA Nematode Collection (T-7222p); and Nematode Collection of Zhejiang University, Hangzhou, China (ZJU-21-1). Specific D2-D3, ITS1-rRNA, partial 18S, and partial *coxI* sequences deposited in GenBank with accession numbers MK941187-MK941190, MK941251-MK941252, MK941258-MK941259, and MK937584-MK937585, respectively.

The new species binomial has been registered in the ZooBank database (zoobank.org) under the identifier: urn:lsid:zoobank.org:act:BDC37C52-6FB9-41CE-9FA7-6A061FB84DE6. The LSID for the publication is: urn:lsid:zoobank.org:pub:F68FC5F4-0D19-4ECF-9BCB-9FF3EE44D0E7.

Diagnosis and relationships

Longidorus oakcrassus n. sp. is an amphimictic species characterized by a thick and long body (9.2-12.2 mm); lip region 25.5-32.0 μ m wide and continuous with body contour; amphidial fovea asymmetrically bilobed; relatively long odontostyle (110.0-133.5 μ m); guiding ring located at 38.0-48.5 μ m from anterior end; vulva located at 46.2-54.8% of body length; female tail short and bluntly conoid (42.5-64.0 μ m long, $c=159.3-231.4$, $c'=0.5-0.7$), with three or four pairs of caudal pores. Males with long spicules (127.0-143.0 μ m) and 23-25 ventromedian supplements. Four developmental juvenile stages were identified, the 1st-stage juvenile with conoid tail ($c'=1.5-1.6$). According to the polytomous key by Chen *et al.* 1997, supplement by Loof & Chen 1999 and the addition of some characters by Peneva *et al.* 2013, codes for the new species are (codes in parentheses are exceptions): A45-B5-C4(3)-D3-E3-F5-G1-H1-I2-J1-K23; and specific D2-D3 expansion segments of 28S rRNA, ITS1, partial 18S-rRNA and partial *coxI* sequences.

According to the body and odontostyle length, distance of guiding ring from anterior body end, lip region and tail shape, the new species is most similar to *L. magnus* Lamberti, Bleve-Zacheo & Arias 1982, *L. nevesi*, *L. paravineacola* Ye & Robbins 2003, *L. polya* Lazarova, Elshishka, Radoslavov, Lozanova, Hristov, Mladenov, Zheng, Fanelli, Luca & Peneva 2019, and *L. vinearum* Bravo & Roca 1995. The new species differs from *L. magnus* by its longer odontostyle (110.0-133.5 μ m *vs* 100.0-118.0 μ m), lower a value (59.5-76.1 *vs* 75.0-95.0), lip region shape (flatten to- slightly rounded *vs* subacute, rounded laterally and slightly flattened frontally conoid-rounded) and the presence *vs* absence of males. From *L.*

nevesi, it can be mainly separated by lower a ratio (59.5-76.1 vs 72.0-102.0), a shorter odontostyle (110.0-133.5 μm vs 133.0-152.0 μm), a wider lip region width (25.5-32.0 μm vs 14.0-18.0 μm) and a longer spicules length (127.0-143.0 μm vs 87.0-100.0 μm). *Longidorus oakcrassus* n. sp. mainly differs from *L. paravineacola* by lower a ratio (59.5-76.1 vs 105.2-161.3), a longer distance from oral aperture to guiding ring (38.0-48.5 μm vs 28.4-36.5 μm) and the lip region shape (flatten-slightly rounded vs rounded slightly expanded). From *L. polya* it differs in having lower a and d ratios (59.5-76.1 vs 95.7-119.5, 1.3-1.8 vs 2.4, 2.8; respectively), and a wider lip width (25.5-32.0 μm vs 14.0-15.5 μm). Finally, the new species can be differentiated from *L. vinearum* by lower a value (59.5-76.1 vs 70.7-101.3), a wider lip region width (25.5-32.0 μm vs 18.0-27.5 μm), and the J1 tail shape (conoid-rounded vs elongate-conoid with rounded digitate terminus).

***Longidorus oakgracilis*² n. sp.** (Figs. 4-6, Table 3)

Female: Body with medium length, thin, slightly tapering towards both ends, assuming a straight body upon fixation. Cuticle 4.2 ± 0.3 (4.0-4.5) μm thick at mid body but thicker (12.7 ± 1.2 (11.0-14.0) μm) at tail hyaline part. Lip region anteriorly rounded, separated from body contour by slight depression, 16.7 ± 1.0 (15.0-18.5) μm wide and 8.4 ± 0.7 (7.0-9.5) μm high. Amphidial fovea elongate, funnel-shaped, not lobed, occupying approximately 1/2 part of distance from oral aperture to guiding ring. Guiding ring single, located about 1.9-2.0 times the lip region width distance from anterior end. Odontostyle relatively long (99.1 ± 3.9 (94.0-106.0) μm), straight or slightly curved; odontophore typical of the genus, about 0.6 times as long as odontostyle length. Basal bulb long and cylindrical, 128.9 ± 13.5 (116.0-150.0) μm long and 27.9 ± 3.3 (24.5-33.0) μm in diam., DN and SVN gland nuclei situated at 34.2 ± 2.5 (32.0-37.8) % and 52.6 ± 4.6 (48.5-58.9) % of distance from anterior end of pharyngeal bulb, respectively. Glandularium 113.8 ± 12.5 (97.0-131.0) μm long. Reproductive system didelphic-amphidelphic, with both genital branches, occupying 10.1 ± 2.1 (8.6-13.6) % and 8.7 ± 2.0 (7.3-11.7) % of body length, respectively; each branch composed of a reflexed ovary (179.0-291.0 μm long). Uterus thick-walled, well-developed sphincter between uterus and *pars dilatata oviductus*, usually containing numerous sperm cells, tubular uterus 118.0-178.0 μm long. Vulva in form of a transverse slit, situated at 51.6

² The species epithet refers to the compound name from the word oak (the host plant where was detected) and the Latin name *gracilis* = slim/thin.

± 3.5 (46.5-58.6) % of body length. Vagina perpendicular to body axis, 38.0 ± 4.3 (34.5-45.5) μm long, occupying about 1/3 of corresponding body width; *pars distalis vaginae* 19.5-23.5 μm long, *pars proximalis vaginae* measuring 12.0-15.5 x 14.5-20.5 μm . Prerectum 345.8 ± 49.6 (306.0-428.0) μm long, rectum 25.3 ± 4.7 (18.5-30.0) μm long. Tail short, bluntly conoid to almost hemispherical, bearing three to four pairs of caudal pores on each sides.

Male: Common. Morphologically similar to female except for genital system and body anteriorly straight, ventrally curved in caudal part when heated relaxed. Male genital tract diorchic with testes opposed, containing multiple rows of spermatogonia. Tail short, bluntly conoid, ventrally concave with broad blunt terminus and thickened ventral outer cuticular layer. Spicules arcuate and moderate robust, 83.3 ± 5.2 (77.0-93.0) μm long, lateral guiding pieces straight or slightly curved. One pair of adanal supplements preceded by a single row of 11-13 ventromedian supplements.

Juveniles: Three juvenile stages (second-, third- and fourth- stage) were detected and morphologically similar to female, except for their smaller body size and sexual characteristics (Fig. 4; Table 3). The J2 has a conoid-rounded tail, and J3-J4 a shorter conoid-rounded tail. They can be distinguished by their relative body lengths, tail shape, odontostyle, and replacement odontostyle length.

Type habitat and locality

Rhizosphere of Pyrenean oak (*Quercus pyrenaica* Wild.) from Cardeña, Córdoba province, Spain (GPS coordinates: 38°12'36.6"E; 4°17'27.6"W) collected by R. Cai on February 19, 2019.

Type material and nomenclatural registration

Holotype female (slide CARS-02) and female and male paratypes (CARS-03-CARS-06) deposited in the Nematode Collection of Institute for Sustainable Agriculture, IAS-CSIC, Córdoba, Spain. One female and one male paratypes deposited at each of the following collections: Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (C.N.R.), Sezione di Bari, Bari, Italy (CARS07); USDA Nematode Collection (T-7223p); and Nematode Collection of Zhejiang University, Hangzhou, China (ZJU-22-1). Specific D2-D3, ITS1-rRNA, partial 18S, and partial *coxI* sequences deposited in GenBank

with accession numbers MK941191-MK941193, MK941253-MK941255, MK941260, and MK937586, respectively.

The new species binomial has been registered in the ZooBank database (zoobank.org) under the identifier: urn:lsid:zoobank.org:act:D9D9A20E-37CE-4254-9D5C-9DEAF1F7C375. The LSID for the publication is: urn:lsid:zoobank.org:pub:F68FC5F4-0D19-4ECF-9BCB-9FF3EE44D0E7.

Diagnosis and relationships

Longidorus oakgracilis n. sp. is an amphimictic species characterized by a moderately long body (5.2-7.5mm) when heated relaxed, females assume open C-shape, males anteriorly straight, curved ventrally in caudal end; lip region anteriorly rounded, 15.0-18.5 μ m wide and slightly set off by depression; amphidial fovea elongated, funnel-shaped, without lobes; guiding ring located 30.0-36.0 μ m from anterior end; moderate long odontostyle (94.0-106.0 μ m); female tail short, bluntly conoid to almost hemispherical (38.0-50.5 μ m long, $c'=0.6-0.8$). Males common, with long spicules (77.0-93.0 μ m) and 12-14 ventromedian supplements; According to the polytomous key by Chen et al. 1997, supplement by Loof and Chen 1999 and additional characters by Peneva et al. 2013, codes for the new species are (codes in parentheses are exceptions): A34-B3(2)-C3(2)-D2-E4-F3(4)-G1(2)-H1-I2-J1-K?. The DNA sequences of D2-D3, 18S rRNA, ITS1 rRNA and partial coxI deposited in GenBank under the accession numbers MK941191-MK941193, MK941260, MK941253-MK941255 and MK937586, respectively.

According to the body length, odontostyle length, distance of guiding ring from anterior body end, lip region shape and tail shape, *L. oakgracilis* n. sp. is closely related to *L. belloi* Andrés & Arias 1988, *L. caespiticola* Hooper 1961, *L. macrosoma* Hooper 1961, *L. poessneckensis* Altherr 1974, *L. pseudoelongatus* Altherr 1976, and *L. raskii* Lamberti & Agostinelli 1993. From *L. belloi* it differs mainly in having a wider lip region width (15.5-18.0 μ m vs 9.5 μ m), the lip region (anteriorly rounded vs anteriorly truncate or concave), and the amphidial fovea shape (funnel-shaped without lobes vs pouch-shaped with asymmetrically bilobed lobes). . From *L. caespiticola*, it mainly differs by higher c ratio (123.6-178.4 vs 76.0-125.0) and lower number of ventromedian supplement (12-14 vs 16-21). From *L. macrosoma*, it can be mainly differentiated by a shorter body (5.4-7.9 mm vs 7.1-11.9 mm) and odontostyle length (94.0-106.0 μ m vs 113.0-148.0 μ m). From *L. poessneckensis*, it differs in having higher a ratio (63.6-86.4 vs 91.0-131.0), shorter

odontostyle length (94.0-106.0 μm vs 122.0-142.0 μm) and the presence vs absence of males. From *L. pseudoelongatus*, it mainly differs in a shorter odontostyle (94.0-106.0 μm vs 115.0-125.0 μm). Finally, the new species mainly differs from *L. raskii* by the lip region (slightly set off with body contour vs continuous with body contour) and amphidial fovea shape (funnel-shaped without lobes vs pouch-shaped with bilobed lobes).

Molecular characterization and phylogenetic relationships of Longidorus oakcrassus n. sp. and Longidorus oakgracilis n. sp. within the genus Longidorus

The amplification of D2-D3 segments of 28S rRNA, ITS1, partial 18S rRNA, and partial *coxI* regions yielded single fragments of ca 800 bp, 1000 bp, 1700 bp, and 300bp, respectively, based on gel electrophoresis.

The D2-D3 sequence divergence was significant between *L. oakcrassus* n. sp. (MK941187-MK941190) with other congeneric species, showing 92% similarity with *L. magnus* (HM921361, 58 different nucleotides and 6 indels), *L. lusitanicus* (KT308869, 65 different nucleotides and 10 indels) and *L. vinearum* (KT308877, 62 different nucleotides, 5 indels). A low intraspecific variation was observed in this population, differing in 3 nucleotides and no indels. The ITS1 of *L. oakcrassus* n. sp. (MK941251, MK941252) showed 83% similarity with *L. cf. olegi* (MH429999, 149 different nucleotides and 79 indels) and *L. wicuoalea* (KT308889, 112 different nucleotides and 67 indels). The partial 18S rRNA sequences of *L. oakcrassus* n. sp. (MK941258, MK941259) closely matched with several species, such as *L. vineacola* (AY283169), *L. wicuoalea* (KT308900) and *L. cf. olegi* (MH430010). Finally, the partial *coxI* sequences of *L. oakcrassus* n. sp. (MK937584, MK937585) showed 80% similarity with *L. vineacola* (KY816709) and 78% similarity with *L. vinearum* (KY816713).

The D2-D3 segments of *L. oakgracilis* n. sp. (MK941191-MK941193) showed 95% and 94% similarity with *L. cf. olegi* (MH430026, 38 different nucleotides, 10 indels) and *L. crataegi* (JX445114, 48 different nucleotides, 13 indels), respectively. ITS1 of *L. oakgracilis* n. sp. (MK941253-MK941255) showed some similarity with *L. olegi* (MH429999, 83% similarity, 146 different nucleotides and 74 indels) and *L. wicuoalea* (KT308889, 83% similarity, 112 different nucleotides, 65 indels). The partial 18S rRNA of this new species (MK941260) was closely related to several species, showed 99% similarity with *L. wicuoalea* (KT308900, 8 different nucleotides, 1 indels), *L. olegi* (MH430010, 8 different nucleotides, 1 indel). Finally, the partial *coxI* sequence of *L. oakgracilis* n. sp.

(MK937586) showed 79% similarity with *L. vineacola* (KY816711) and 78% similarity with *L. magnus* (KY816687).

Phylogenetic relationships among *Longidorus* species inferred from analyses of D2-D3 expansion segments of 28S rRNA, partial 18S rRNA, and *coxI* gene sequences using BI are given in Figures 7, 8 and 9, respectively. Since scarce similarity was detected for ITS1 sequences from *L. oakcrassus* n. sp. and *L. oakgracilis* n. sp. with those deposited in GenBank, with a coverage ranging from 22 to 76 % with the closer species, no phylogenetic analyses were carried out on this molecular marker.

The D2-D3 expansion segments of 28S rRNA tree of *Longidorus* spp. based on a multiple edited alignment including 98 sequences and 754 total characters revealed five major clades (marked with roman numerals from I to V) (Fig. 7). Clade I is well-supported (PP=1.00) including 21 species, all these species shared a hemispherical to bluntly conoid tail (Fig 7) ($c' < 1.0$, except some species as *L. wicuoalea* and *L. andalusicus* with $c' 1.0-1.5$), and lip region anteriorly rounded, continuous or slightly depressed with body contour (Fig 7) (except *L. oleae* with an anteriorly concave lip region and *L. lusitanicus* with anteriorly flattened lip region). *Longidorus oakgracilis* n. sp. (MK941191-MK941193) and *L. oakcrassus* n. sp. (MK941187-MK941190) were placed in Clade I. *Longidorus oakgracilis* n. sp. was phylogenetically related to *L. cf. olegi* (MH430026), *L. silvestris* (KT308860), and *L. wicuoalea* (KT308865) with 1.00 PP value. On the other hand, *L. oakcrassus* n. sp. sequences formed a specific clade which was related to other *Longidorus* spp. as *L. crataegi* (JX445114), *L. lusitanicus* (KT308869), *L. onubensis* (KT308858), *L. goodeyi* (AY601581), *L. magnus* (HM921361), *L. vinearum* (KT308865) and *L. oakgracilis* n. sp. with related species in a supported clade (PP = 0.98).

For partial 18S rRNA gene sequences, the 50% majority rule consensus BI tree was based on a multiple sequence alignment containing 82 sequences and 1710 characters. *Longidorus oakgracilis* n. sp. (MK941260) clustered with *L. silvestris* (KT308898) and *L. lusitanicus* (KT308901) in a moderate-supported clade (PP = 0.94). In addition, *L. oakcrassus* n. sp. (MK941258, MK941259) did not form any supported clade with other species (Fig 8), but it was inside a major highly supported clade (PP = 1.00) including *L. oakgracilis* n. sp. and other species (i.e. *L. vinearum* (KT308903), *L. magnus* (HM921345, KT308902). Finally, for partial *coxI* mtDNA gene sequences, the 50% majority rule consensus BI tree of a multiple sequence alignment containing 56 sequences and 389 characters (Fig 9). *Longidorus oakcrassus* n. sp. (MK937484, MK937485) clustered with *L. caespiticola* (KJ567474) in a moderately high supported clade (PP = 0.96). In addition, *L.*

oakgracilis n. sp. (MK937486) clustered with several species *L. iuglandis* (KY816678), *L. onubensis* (KY81695), *L. lusitanicus* (KY81684) and *L. crataegi* (KY816668) in a low supported clade (PP = 0.78).

Discussion

The primary objective of this study was to describe two new species of the genus *Longidorus* associated with oak in southern Spain using an integrative approach and their phylogenetic relationships with other species of the genus *Longidorus*. Our results demonstrate that the use of morphological studies together with rDNA and mitochondrial molecular markers may unravel the specific biodiversity in this complex group of plant-parasitic nematodes. Thus, *L. oakcrassus* n. sp. and *L. oakgracilis* n. sp. are described and the phylogenetic relationships into the genus *Longidorus* based on nuclear rDNA and mitochondrial markers are showed. The phylogenetic relationships of *L. oakcrassus* n. sp. and *L. oakgracilis* n. sp. with the nuclear and mitochondrial markers showed a similar topology. It consisted of a major clade comprising the majority of *Longidorus* species reported in the Iberian Peninsula with a characteristic tail (hemispherical convex-conoid tail shape) as reported in previous studies (Gutiérrez-Gutiérrez et al. 2013; Archidona-Yuste et al. 2016b; 2019). Sequences of nuclear ribosomal RNA genes, particularly D2-D3 expansion segments of the 28S rRNA gene, ITS1 region, and the gene *CoxI*, have proven to be a powerful tool for providing accurate species identification of Longidoridae (Palomares-Rius et al. 2017b). However, the low nucleotide variability found in partial 18S rRNA makes it difficult to identify individuals to the species level as previously described in other studies (Gutiérrez-Gutiérrez et al. 2013; Archidona-Yuste et al. 2016b; 2019).

The description of *L. oakcrassus* n. sp. and *L. oakgracilis* n. sp. suggests that the biodiversity of these nematodes in Southern Europe is still not completely described, and requires further research. Interestingly, the phylogenetic relationships among Iberian Peninsula species could provide insight into the speciation of some of these species specifically to the Iberian Peninsula, additionally of other main centres of origin in other parts of the world, as suggested by Coomans (1985). However, this hypothesis regarding the evolutionary patterns in the genus *Longidorus* must be analysed using biogeographical models and a higher number of sequences from other *Longidorus* spp. given the increasing diversity of this genus in the samplings at the Iberian Peninsula (Archidona-Yuste et al. 2016b; 2019). This point has also been documented for other genera such as dagger nematodes of the genus *Xiphinema* in an extensive sampling and phylogenetic study in

Southern Spain (Archidona-Yuste *et al.* 2016a, 2016c, 2016d). These results enlarge the diversity of *Longidorus* in Spain and agree with previous data obtained for the phylogeny and biogeography of the genus *Longidorus* in the Mediterranean Basin (Navas *et al.* 1990; Navas *et al.* 1993; Gutiérrez-Gutiérrez *et al.* 2011; Gutiérrez-Gutiérrez *et al.* 2013; Archidona-Yuste *et al.* 2016b).

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

References

- Altherr, E. (1974). Nematodes de la nappe phreatique du réseau fluvial de la Saale (Thuringe). II. *Limnologica*, 9, 81-132.
- Altherr, E. (1976). La faune des eaux profondes interstitiales de la region de Wiesbaden. *Bulletin Société vaudoise of Sciences naturelles*, 73, 97-116.
- Andrés, M.F., & Arias, M. (1988). *Longidorus belloi* n. sp. (Nematoda: Longidoridae) from Spain. *Revue de Nématologie*, 11, 415-421.
- Archidona-Yuste, A., Navas-Cortés, J.A., Cantalapiedra-Navarrete, C., Palomares-Rius, J.E., & Castillo, P. (2016a). Cryptic diversity and species delimitation in the *Xiphinema americanum*-group complex (Nematoda: Longidoridae) as inferred from morphometrics and molecular markers. *Zoological Journal of the Linnean Society*, 176, 231-265.
- Archidona-Yuste, A., Navas-Cortés, J.A., Cantalapiedra-Navarrete, C., Palomares-Rius, J.E., & Castillo, P. (2016b). Unravelling the biodiversity and molecular phylogeny of needle nematodes of the genus *Longidorus* (Nematoda: Longidoridae) in olive and a description of six new species. *PloS One*, 11, e0147689. doi:10.1371/journal.pone.0147689.
- Archidona-Yuste, A., Navas-Cortés, J.A., Cantalapiedra-Navarrete, C., Palomares-Rius, J.E., & Castillo P. (2016c). Remarkable diversity and prevalence of dagger nematodes

- 553 of the genus *Xiphinema* Cobb, 1913 (Nematoda: Longidoridae) in olives revealed by
554 integrative approaches. *PLoS ONE*, 11, e0165412.
- 555 Archidona-Yuste, A., Navas-Cortés, J.A., Cantalapiedra-Navarrete, C., Palomares-Rius,
556 J.E., & Castillo, P. (2016d). Molecular phylogenetic analysis and comparative
557 morphology resolve two new species of olive-tree soil related dagger nematodes of the
558 genus *Xiphinema* (Dorylaimida: Longidoridae) from Spain. *Invertebrate Systematics*,
559 30, 547-565.
- 560 Archidona-Yuste, A., Cantalapiedra-Navarrete, C., Castillo, P. & Palomares-Rius, J.E.
561 (2019). Molecular phylogenetic analysis and comparative morphology reveals the
562 diversity and distribution of needle nematodes of the genus *Longidorus* (Dorylaimida:
563 Longidoridae) from Spain. *Contributions to Zoology*, 88, 1-41.
- 564 Bravo, M.A., & Roca, F. (1995). Observations on *Longidorus africanus* Merny from
565 Portugal with description of *L. vinearum* n. sp. (Nematoda: Longidoridae).
566 *Fundamental and Applied Nematology*, 18, 87-94.
- 567 Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use
568 in phylogenetic analysis. *Molecular Biology and Evolution*, 17, 540-552.
- 569 Chen, Q.W., Hooper, D.J., Loof, P.A.A., & Xu, J. (1997). A revised polytomous key for the
570 identification of species of the genus *Longidorus* Micoletzky, 1922 (Nematoda:
571 Dorylaimoidea). *Fundamental and Applied Nematology*, 20, 15-28.
- 572 Cherry, T., Szalanski, A.L., Todd, T.C., & Powers, T.O. (1997). The internal transcribed
573 spacer region of *Belonolaimus* (Nemata: Belonolaimidae). *Journal of Nematology*, 29,
574 23-29.
- 575 Cobb, N.A. (1913). Helminthology. - New nematode genera found inhabiting fresh water
576 and non-brackish soils. *Journal of the Washington Academy of Science*, 3, 432-444.
- 577 Coolen, W.A. (1979). Methods for extraction of *Meloidogyne* spp. and other nematodes
578 from roots and soil. In: Root-knot nematodes (*Meloidogyne* species). Systematics,

- 579 biology and control. Lamberti F., Taylor C.E. (Editors), Academic Press, New York,
580 USA, pp. 317-329.
- 581 Coomans, A. (1985). A phylogenetic approach to the classification of the Longidoridae
582 (Nematoda: Dorylaimida). *Agriculture, Ecosystems and Environment*, 12, 335-354.
- 583 Coomans, A., Huys, R., Heyns, J., & Luc, M. (2001). Character analysis, phylogeny, and
584 biogeography of the genus *Xiphinema* Cobb, 1973 (Nematoda, Longidoridae). Vol.
585 287, *Annales du Musée Royal de l'Afrique Centrale (Zoologie)*, Tervuren, Belgique.
- 586 Dalmasso, A. (1969). Etude anatomique et taxonomique des genres *Xiphinema*, *Longidorus*
587 et *Paralongidorus* (Nematoda: Dorylaimidae). *Memoires du Museum National*
588 *d'Histoire Naturelle Nouvelle serie, Serie A Zoologie*, 61, 82 pp
- 589 Darriba, D., Taboada, G.L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models,
590 new heuristics and parallel computing. *Nature methods*, 9, 772.
- 591 De Grisse, A.T. (1969). Redescription ou modifications de quelques techniques utilisées
592 dans l'étude de nématodes phytoparasitaires. *Mededelingen Rijksfaculteit*
593 *Landbouwwetenschappen Gent*, 34, 315-59.
- 594 De Ley, P., Félix, M.A., Frisse, L.M., Nadler, S.A., Sternberg, P.W., & Thomas, K.W.
595 (1999). Molecular and morphological characterisation of two reproductively isolated
596 species with mirror-image anatomy (Nematoda: Cephalobidae). *Nematology*, 1, 591-
597 612.
- 598 De Luca, F., Reyes, A., Grunder, J., Kunz, P., Agostinelli, A., De Giorgi, C., & Lamberti, F.
599 (2004). Characterization and sequence variation in the rDNA region of six nematode
600 species of the genus *Longidorus* (Nematoda). *Journal of Nematology*, 36, 147-152.
- 601 Decraemer, W., & Robbins, R.T. (2007). The who, what and where of Longidoridae and
602 Trichodoridae. *Journal of Nematology*, 39, 295-297.

- 603 Flegg, J.J.M. (1967). Extraction of *Xiphinema* and *Longidorus* species from soil by a
604 modification of Cobb's decanting and sieving technique. *Annals of Applied Biology*,
605 60, 429-437.
- 606 Gutiérrez-Gutiérrez, C., Cantalapiedra-Navarrete, C., Decraemer, W., Vovlas, N., Prior, T.,
607 Rius, J., & Castillo, P. (2012). Phylogeny, diversity, and species delimitation in some
608 species of the *Xiphinema americanum*-group complex (Nematoda: Longidoridae), as
609 inferred from nuclear and mitochondrial DNA sequences and morphology. *European*
610 *Journal of Plant Pathology* 134, 561-597.
- 611 Gutiérrez-Gutiérrez, C., Cantalapiedra-Navarrete, C., Montes-Borrego, M., Palomares-Rius,
612 J. E., & Castillo, P. (2013). Molecular phylogeny of the nematode genus *Longidorus*
613 (Nematoda: Longidoridae) with description of three new species. *Zoological Journal of*
614 *the Linnean Society*, 167, 473-500.
- 615 Gutiérrez-Gutiérrez, C., Mota, M., Castillo, P., Santos, M.T., Palomares-Rius, J.E. (2018).
616 Description and molecular phylogeny of one new and one known needle nematode of
617 the genus *Paralongidorus* (Nematoda: Longidoridae) from grapevine in Portugal using
618 integrative approach. *European Journal of Plant Pathology*, 151, 155-172.
- 619 Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis
620 program for windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95-98.
- 621 He, Y., Subbotin, S., Rubtsova, T.V., Lamberti, F., Brown, D.J. F., & Moens, M. (2005). A
622 molecular phylogenetic approach to Longidoridae (Nematoda: Dorylaimida).
623 *Nematology*, 7, 111-124.
- 624 Heyns, J. (1988). Description of two new *Longidoroides* species (Nematoda: Dorylaimida)
625 from South Africa, with note on *L. strelitziae* (Heyns, 1966). *Fundamental and Applied*
626 *Nematology*, 21, 491-499.
- 627 Holterman, M., Van Der Wurff, A., Van Den Elsen, S., Van Megen, H., Bongers, T.,
628 Holovachov, O., Bakker, J., & Helder, J. (2006). Phylum-wide analysis of SSU rDNA

- 629 reveals deep phylogenetic relationships among nematodes and accelerated evolution
630 toward crown clades. *Molecular Phylogenetics and Evolution*, 23, 1792-1800.
- 631 Hooper, D.J. (1961). A redescription of *Longidorus elongatus* (De Man, 1876) Thorne &
632 Swanger 1936 (Nematoda: Dorylaimidae) and descriptions of five new species of
633 *Longidorus* from Great Britain. *Nematologica*, 6, 237-257.
- 634 Jairajpuri, M.S., & Ahmad, W. (1992). *Dorylaimida. Freelifving, predaceous and plant-*
635 *parasitic nematodes*. New Delhi, India, Oxford & IBH Publishing Co., 458 pp.
- 636 Janssen, J., Karssen, G., Orlando, V., Subbotin, S.A. & Bert, W. (2017). Molecular and
637 species delimiting of plant-parasitic nematodes of the genus *Pratylenchus* from the
638 *penetrans* group (Nematoda: Pratylenchidae). *Molecular Phylogenetics and Evolution*,
639 117, 30-48.
- 640 Kankina, V.K., & Metlitskaya, K.V. (1983). Parasitic nematodes of the family Longidoridae
641 (Thorne, 1935) Meyl, 1961, on currant and raspberry. *Izvestiya Akademii Nauk*
642 *Tadzhikskoi SSR Otdelenie Biologicheskikh Nauk*, 4, 50-52.
- 643 Katoh, K., & Standley, D.M. (2013). MAFFT multiple sequence alignment 542 software
644 version 7: improvements in performance and usability. *Molecular Biology and*
645 *Evolution*, 30, 772-780.
- 646 Kijrjanova, E.S. (1951). Soil nematodes found in cotton fields and in virgin soil of
647 Golodnaya Steppe (Uzbekistan). (In Russian. *Trudy Zoo I. Inst. Akad. Nauk SSSR*, 9,
648 625-657.
- 649 Kozłowska, J., & Seinhorst, J.W. (1979). *Longidorus elongatus* and closely related species
650 in the Netherlands and lower Saxony (Germany), with the description of two new
651 species, *L. cylindricaudatus* and *L. intermedius* (Nematoda: Dorylaimida).
652 *Nematologica*, 25: 42-53.
- 653 Lamberti, F., & Agostinelli, A. (1993). *Longidorus raskii* sp. n. (Nematoda: Dorylaimida)
654 from Switzerland. *Nematologia Mediterranea*, 21, 243-246.

- 655 Lamberti, F, Bleve-Zacheo, T., & Arias, M. (1982). The Longidoridae of the Maltese Islands
656 with the description of *Longidorus magnus* sp. n. and *Xiphinema melitense* sp. n.
657 *Nematologia Mediterranea*, 10, 183-200.
- 658 Lazarova, S.S., Malloch, G., Oliveira C.M.G., Hübschen, J., & Neilson, R. (2006).
659 Ribosomal and mitochondrial DNA analyses of *Xiphinema americanum*-group
660 populations. *Journal of Nematology*, 38, 404- 410.
- 661 Lazarova, S.S., Elshishka, M., Radoslavov, G., Lozanova, L., Hristov, P., Mladenov, A.,
662 Zheng, J., Fanelli, E., De Luca, F., Peneva, V.K. (2019). Molecular and morphological
663 characterisation of *Longidorus polyae* sp. n. and *L. pisi* Edward, Misra & Singh, 1964
664 (Dorylaimida, Longidoridae) from Bulgaria. *ZooKeys*, 830, 75-98.
- 665 Loof, P.A.A., & Coomans, A. (1972). The oesophageal gland nuclei of Longidoridae
666 (Dorylaimida). *Nematologica*, 18, 213-233.
- 667 Loof, P.A.A., & Chen, Q. (1999). A revised polytomous key for the identification of the
668 species of the genus *Longidorus* Micoletzky, 1922 (Nematoda: Dorylaimoidea).
669 Supplement 1. *Nematology*, 1, 55-59.
- 670 Macara, A. (1985). Two new species of *Longidorus* (Nematoda: Longidoridae) associated
671 with forest plants in Portugal. *Nematologica*, 3, 410-423.
- 672 Merny, G. (1966). Nematodes from tropical Africa: A new *Paratylenchus* (Criconematidae)
673 two new *Longidorus* and observations on *Longidorus laevicapitatus* Williams, 1959
674 (Dorylaimidae). *Nematologica*, 12, 385-395.
- 675 Micoletzky, H. (1922). Die freilebenden Erd-Nematoden. *Archiv für Naturgeschichte Berlin*
676 *Abt.* 87, 1-650.
- 677 Navas, A., Andres, M.F., & Arias, M. (1990). Biogeography of Longidoridae in the
678 Euromediterranea area. *Nematologia Mediterranea*, 18, 103-112.
- 679 Navas, A., Baldwin, J.G., Barrios, L., N & Nombela, G. (1993). Phylogeny and
680 biogeography of *Longidorus* (Nematoda: Longidoridae) in Euromediterranea. *Nematol*
681 *Nematologia Mediterranea*, 21, 71-88.

- 682 Neilson, R., Ye, W., Oliveira, C.M.G., Hübschen, J., Robbins, R.T., Brown, D.J.F., &
683 Szalanski, A.L. (2004). Phylogenetic relationships of Longidoridae species (Nematoda:
684 Dorylaimida) from North America inferred from 18S rDNA sequence data.
685 *Helminthologia*, 41, 209-215.
- 686 Page, R.D. (1996). TreeView: an application to display phylogenetic trees on personal
687 computers. *Computer Applications in the Biosciences*, 12, 357-358.
- 688 Palomares-Rius, J.E., Subbotin, Sergei A., Landa, B.B., Vovlas, N., & Castillo, P. (2008).
689 Description and molecular characterisation of *Paralongidorus litoralis* sp. n. and *P.*
690 *paramaximus* Heyns, 1965 (Nematoda: Longidoridae) from Spain. *Nematology*, 10,
691 87-101.
- 692 Palomares-Rius, J.E., Cantalapiedra-Navarrete, C., & Castillo, P. (2014). Cryptic species in
693 plant-parasitic nematodes. *Nematology*, 16, 1105-1118.
- 694 Palomares-Rius, J.E., Escobar, C., Cabrera, J., Vovlas, A., & Castillo, P. (2017a).
695 Anatomical alterations in plant tissues induced by plant-parasitic Nematodes. *Frontiers*
696 *in Plant Sciences*, 8:1987. doi: 10.3389/fpls.2017.01987
- 697 Palomares-Rius, J.E., Cantalapiedra-Navarrete, C., Archidona-Yuste, A., Blok, V.C., &
698 Castillo, P. (2017b). Mitochondrial genome diversity in dagger and needle nematodes
699 (Nematoda: Longidoridae). *Scientific Reports*, 7, 41813.
- 700 Palomares-Rius, J.E., Cantalapiedra-Navarrete, C., Archidona-Yuste, A., Subbotin, S.A., &
701 Castillo, P. (2017c). The utility of mtDNA and rDNA for barcoding and phylogeny of
702 plant-parasitic nematodes from Longidoridae (Nematoda, Enoplea). *Scientific Reports*,
703 7, 10905.
- 704 Pedram, M., Pourjam, E. G., Palomares-Rius, G. R., Cantalapiedra-Navarrete, C., &
705 Castillo, P. (2012a). Molecular and morphological characterisation of *Xiphinema*
706 *granatum* n. sp. and *Longidorus pisi* Edward, Misra and Singh, 1964 (Dorylaimida:
707 Longidoridae) from Iran. *Nematology*, 14, 949–960.

- 708 Pedram, M., Pourjam, E., Namjou, S., Atighi, M. R., Cantalapiedra-Navarrete, C., Liébanas,
709 G., Palomares-Rius, J. E., & Castillo, P. (2012b). Molecular and morphological
710 characterisation of *Paralongidorus iranicus* n. sp. and *Paralongidorus bikanerensis*
711 (Lal & Mathur, 1987) Siddiqi, Baujard & Mounport, 1993 (Nematoda: Longidoridae)
712 from Iran. *Nematology*, 14, 427-443.
- 713 Peneva, V.K., Lazarova, S.S., De Luca F., Brown, D.J.F. (2013). Description of *Longidorus*
714 *cholevae* n. sp. (Nematoda, Dorylaimida) from a riparian habitat in the Rila Mountains,
715 Bulgaria. *ZooKeys*, 330, 1-26.
- 716 Ronquist, F., & Huelsenbeck, J.P. (2003). MRBAYES 3: Bayesian phylogenetic inference
717 under mixed models. *Bioinformatics*, 19, 1572-1574.
- 718 Seinhorst, J.W. (1966). Killing nematodes for taxonomic study with hot f.a. 4:1.
719 *Nematologica*, 12, 178.
- 720 Sturhan, D., & Weischer, B. (1954). *Longidorus vineacola* n. sp. (Nematoda: Dorylaimidae).
721 *Nematologica*, 10: 335-341.
- 722 Subbotin, S., Waeyenberge, A.L., & Moens, M. (2000). Identification of cyst forming
723 nematodes of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal
724 DNA-RFLPs. *Nematology*, 2, 153-164.
- 725 Subbotin, S.A., Rogozhin, E.A., & Chizhov, V.N. (2014). Molecular characterisation and
726 diagnostics of some *Longidorus* species (Nematoda: Dorylaimida) from Russia and
727 other countries using rRNA genes. *European Journal of Plant Pathology*, 138, 377-
728 390.
- 729 Taylor, C.E., & Brown, D.J.F. (1997). Nematode vectors of plant viruses. CABI,
730 Wallingford, UK, 296 pp.
- 731 Tulaganov A.T. (1938). The fauna of nematodes of cotton and surrounding soil in Katta-
732 Kurgan district of the Uzbek SSR. *Trans. Uzbeksk. Gas. Univ.*, 12, 1-25.

- 733 Vrain, T.C., Wakarchuk, D. A., Levesque, A.C., & Hamilton, R.I. (1992). Intraspecific
734 rDNA Restriction Fragment Length Polymorphism in the *Xiphinema americanum*
735 group. *Fundamental and Applied Nematology*, 15, 563-573.
- 736 Ye, W., & Robbins, R.T. (2003). *Longidorus paravineacola* n. sp. (Nematoda:
737 Longidoridae), a new species from Arkansas. *Journal of Nematology*, 35, 388-394.
- 738 Ye, W., Szalanski, A.L., & Robbins, R.T. (2004). Phylogenetic relationships and genetic
739 variation in *Longidorus* and *Xiphinema* species (Nematoda: Longidoridae) using ITS1
740 sequences of nuclear ribosomal DNA. *Journal of Nematology*, 36, 14-19.

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Figure legend

Fig. 1. Line drawings of *Longidorus oakcrassus* n. sp. paratypes from the rhizosphere of oak (*Quercus robur* L. from Cardeña, Córdoba, Spain (A-G). A: female neck region; B,C: female lip region; D,E: female tail region; F: male tail region; G: tail of first-stage juvenile (J1).

Fig. 2. Light micrographs of *Longidorus oakcrassus* n. sp. female paratypes from the rhizosphere of oak (*Quercus pyrenaica* Wild. from Cardeña, Córdoba, Spain (A-V). A-E. Female anterior regions; F,G. Lip regions showing amphidial fovea (arrowed); H: Detail of basal bulb; I: Detail of sperm in uterus; J: Vulva region; K-O; Female tail regions; P,Q: Male tail region; R: First-stage juvenile anterior region; S-V: Tail of J1, J2, J3 and J4, respectively. Abbreviations: a = anus; af = amphidial fovea; dn = dorsal nucleus; gr = guiding ring; Rost = replacement odontostyle; sp = spicules; spm = sperm; svn = subventral nucleus; V = vulva. (Scale bars: A-G & R-V = 25µm; H-Q = 50µm)

Fig. 3. Relationship of body length to length of functional and replacement odontostyle (▲ = Odontostyle and ● = Replacement odontostyle) length in all developmental stages from first-stage juveniles (J1) to mature females of *Longidorus oakcrassus* n. sp.

Fig. 4. Line drawings of *Longidorus oakgracilis* n. sp. paratypes from the rhizosphere of oak (*Quercus robur* L. from Cardeña, Córdoba, Spain (A-F). A: female neck region; B,C: female lip region; D,E: female tail region; F: male tail region.

Fig. 5. Light micrographs of *Longidorus oakgracilis* n. sp. female paratypes from the rhizosphere of oak (*Quercus pyrenaica* Wild. from Cardeña, Córdoba, Spain (A-T). A-E: Female anterior regions; F, G: Detail of basal bulb; H-M: Tail region; N-P: Tail region of 2nd, 3rd and 4th stage juveniles; Q: Male tail region. Abbreviations: a = anus; af = amphidial fovea; dn = dorsal nucleus; gr = guiding ring; sp = spicule; spl = ventromedian supplements; svn = subventral nucleus. (Scale bars A = 30µm; B-Q = 25µm)

Fig. 6. Relationship of body length to length of functional and replacement odontostyle (▲ = Odontostyle and ● = Replacement odontostyle) length in all developmental stages from second-stage juveniles (J2) to mature females of *Longidorus oakgracilis* n. sp.

777

778 **Fig. 7.** Phylogenetic relationships of *Longidorus oakcrassus* n. sp. and *Longidorus*
779 *oakgracilis* n. sp. within the genus *Longidorus*. Bayesian 50% majority rule consensus trees
780 as inferred from D2-D3 expansion segments of 28S rRNA sequences alignments under the
781 GTR + I + G model. Posterior probabilities more than 70% are given for appropriate clades.
782 Newly obtained sequences in this study are in bold.

783

784 **Fig. 8.** Phylogenetic relationships of *Longidorus oakcrassus* n. sp. and *Longidorus*
785 *oakgracilis* n. sp. within the genus *Longidorus*. Bayesian 50% majority rule consensus trees
786 as inferred from 18S rRNA sequences alignments under the GTR + I + G model. Posterior
787 probabilities more than 70% are given for appropriate clades. Newly obtained sequences in
788 this study are in bold.

789

790 **Fig. 9.** Phylogenetic relationships of *Longidorus oakcrassus* n. sp. and *Longidorus*
791 *oakgracilis* n. sp. within the genus *Longidorus*. Bayesian 50% majority rule consensus trees
792 as inferred from *coxI* mtDNA sequences alignments under the GTR + I + G model. Posterior
793 probabilities more than 70% are given for appropriate clades. Newly obtained sequences in
794 this study are in bold letters.

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

Species	Sample code	Host-plant, locality, province	<i>D2-D3</i>	<i>ITS1</i>	partial <i>18S</i>	<i>coxI</i>
<i>Longidorus oakcrassus</i> n. sp.	CARF	Pyrenean oak, Cardeña, Córdoba	MK941187- MK941190	MK941251- MK941252	MK941258- MK941259	MK937584- MK937585
<i>Longidorus oakgracilis</i> n. sp.	CARS	Pyrenean oak, Cardeña, Córdoba	MK941191- MK941193	MK941253- MK941255	MK941260	MK937586

Table 2. Morphometrics of *Longidorus oakcrassus* n. sp. from Cardeña (Córdoba, Spain)^a.

Characters/ratios ^b	Holotype	Paratypes					
		Females	Males	J1	J2	J3	J4
N	1	19	9	3	6	7	10
L (mm)	10.9	10.5±0.75 (9.2-12.2)	9.7±0.62 (8.7-10.5)	2.2±0.12 (2.1-2.3)	4.4±0.46 (3.5-4.9)	5.7±0.47 (5.0-6.2)	8.2±0.55 (7.5-9.5)
a	71.7	67.5±5.2 (59.5-76.1)	65.0±5.4 (54.8-71.5)	47.8±4.8 (45.0-53.3)	46.5±7.0 (40.5-60.4)	50.8±2.8 (48.0-55.9)	57.1±2.9 (53.4-63.7)
b	15.3	16.6±1.6 (14.1-19.2)	15.9±1.8 (12.9-19.8)	7.0±0.8 (6.2-7.9)	10.2±1.6 (8.0-11.7)	11.3±0.6 (10.7-12.2)	14.5±2.2 (12.5-19.2)
c	199.3	202.4±22.2 (159.3-231.4)	157.2±26.3 (115.5-191.7)	42.3±4.6 (37.0-45.0)	84.3±7.0 (70.5-90.0)	102.9±13.5 (89.8-121.7)	153.9±13.2 (134.0-178.4)
c'	0.6	0.6±0.1 (0.5-0.7)	0.8±0.05 (0.8-0.9)	1.5±0.1 (1.5-1.6)	0.8±0.1 (0.8-0.9)	0.8±0.1 (0.6-0.9)	0.6±0.1 (0.6-0.8)
d	1.7	1.6±0.2 (1.3-1.8)	1.7±0.1 (1.5-1.8)	1.6±0.2 (1.5-1.8)	1.5±0.2 (1.3-1.7)	1.6±0.1 (1.5-1.7)	1.5±0.2 (1.2-1.7)
d'	1.8	1.8±0.1 (1.5-2.0)	1.8±0.1 (1.6-1.9)	1.6±0.04 (1.5-1.6)	1.7±0.2 (1.4-1.8)	1.8±0.1 (1.7-2.0)	1.7±0.1 (1.6-1.9)
V or T	49.4	50.2±2.3 (46.2-54.8)	53.7±4.7 (48.3-59.6)	-	-	-	-
Odontostyle length	124.5	123.6±6.1 (110.0-133.5)	121.7±3.7 (115.0-126.0)	74.0±4.4 (69.0-77.0)	82.8±3.1 (79.0-86.0)	95.3±3.8 (90.0-101.0)	110.5±3.0 (107.5-117.0)
Replacement odontostyle length	-	-	-	82.3±2.3 (81.0-85.0)	95.9±1.9 (94.0-99.5)	107.2±4.9 (102.5-113.0)	119.9±4.1 (115.5-126.5)
Odontophore length	79.5	74.3±5.0 (67.0-83.0)	74.3±5.6 (69.0-86.0)	37.3±2.8 (34.5-40.0)	46.1±2.9 (43.0-49.5)	54.7±5.4 (45.0-61.0)	53.4±6.5 (46.0-64.0)
Lip region width	27.5	27.7±1.8 (25.5-32.0)	28.1±1.6 (26.5-30.5)	15.7±0.3 (15.5-16.0)	21.1±2.1 (19.5-25.0)	23.5±1.4 (21.5-25.0)	26.8±1.8 (23.5-28.5)
Oral aperture-guiding ring	47.5	44.6±2.9 (38.0-48.5)	46.6±1.8 (44.5-48.5)	25.0±3.0 (23.0-28.5)	31.7±1.7 (29.5-33.5)	37.1±1.4 (34.5-38.5)	39.1±2.5 (35.0-42.0)
Tail length	54.5	52.4±6.5 (42.5-64.0)	63.1±7.7 (54.0-75.0)	52.8±3.2 (50.5-56.5)	51.7±2.5 (49.0-56.0)	56.0±8.3 (46.5-65.5)	53.5±4.0 (46.5-58.5)
Spicules	-	-	134.9±5.1 (127.0-143.0)	-	-	-	-

Lateral accessory piece	-	-	36.9±2.0 (34.0-39.0)	-	-	-	-
J	17.5	18.1±3.0 (12.5-23.0)	16.4±2.6 (11.0-21.0)	13.7±0.6 (13.0-14.0)	15.4±1.2 (13.5-16.5)	16.3±2.7 (12.5-19.0)	16.6±2.8 (12.5-20.0)

^a Measurements are in μm and in the form: mean $\pm$ standard deviation (range).

^b a = body length/maximum body width; b = body length/pharyngeal length; c = body length/tail length; c' = tail length/body width at anus; d = anterior to guiding ring/body diameter at lip region; d' = body diameter at guiding ring/body diameter at lip region; V = (distance from anterior end to vulva/body length) x 100; T = ((distance from cloacal aperture to anterior end of testis/body length) x 100); J = hyaline tail region length.

Table 3. Morphometrics of *Longidorus oakgracilis* n. sp. from Cardena (Córdoba, Spain)^a.

Characters/ratios ^b	Holotype	Paratypes				
		Females	Males	J2	J3	J4
n	1	14	10	4	3	7
L (mm)	6.2	6.6±0.7 (5.4-7.9)	6.1±0.7 (5.2-7.5)	2.7±0.2 (2.4-3.0)	3.7±0.3 (3.3-4.0)	5.2±0.4 (4.6-5.8)
a	63.9	71.6±6.1 (63.6-86.4)	73.8±6.1 (62.3-83.6)	55.9±4.9 (51.2-60.8)	58.0±4.7 (53.6-63.0)	64.2±6.7 (53.4-71.6)
b	13.6	15.2±2.5 (12.4-21.5)	14.1±2.0 (11.3-17.2)	10.9±0.4 (10.4-11.2)	11.4±2.0 (9.7-13.6)	13.7±1.4 (11.7-15.9)
c	151.9	152.9±18.2 (123.6-178.4)	137.6±20.7 (110.7-183.5)	66.3±6.5 (61.6-75.8)	81.8±8.1 (74.2-90.4)	126.0±14.8 (102.7-147.3)
c'	0.7	0.7±0.1 (0.6-0.8)	0.9±0.1 (0.8-1.1)	1.4±0.1 (1.2-1.5)	1.1±0.2 (0.9-1.3)	0.8±0.03 (0.7-0.8)
d	1.8	2.0±0.2 (1.6-2.4)	2.0±0.2 (1.7-2.3)	2.1±0.1 (2.0-2.3)	2.0±0.1 (1.9-2.1)	2.0±0.2 (1.6-2.2)
d'	1.6	1.8±0.1 (1.6-2.0)	1.8±0.1 (1.7-1.8)	1.8±0.1 (1.7-2.0)	1.6±0.1 (1.6-1.7)	1.8±0.2 (1.5-2.0)
V or T	48.2	51.6±3.5 (46.5-58.6)	48.5±4.7 (43.9-55.3)	-	-	-
Odontostyle length	100.0	99.1±3.9 (94.0-106.0)	101.1±3.6 (95.5-106.0)	60.3±1.6 (58.0-61.5)	73.8±1.4 (73.0-75.5)	87.5±4.8 (80.0-92.0)
Replacement odontostyle length	-	-	-	74.5±4.3 (70.5-78.5)	80.8±0.8 (80.0-81.5)	97.5±2.5 (94.5-101.0)
Odontophore length	63.0	59.9±2.7 (54.0-64.0)	50.1±5.5 (40.5-57.0)	34.3±1.7 (32.0-36.0)	39.8±0.6 (39.5-40.5)	50.0±2.1 (47.0-52.0)
Lip region width	17.5	16.7±1.0 (15.0-18.5)	17.0±0.9 (15.5-18.0)	10.5±0.7 (10.0-11.5)	13.7±0.3 (13.5-14.0)	14.9±0.5 (14.5-15.5)

Oral aperture-guiding ring	32.0	32.9±2.0 (30.0-36.0)	34.2±3.0 (30.0-38.5)	22.1±1.3 (20.5-23.5)	27.7±1.0 (26.5-28.5)	29.2±3.2 (24.5-33.5)
Tail length	41.0	43.5±4.2 (38.0-50.5)	44.6±3.0 (40.5-49.5)	41.8±5.2 (36.0-48.0)	44.8±0.3 (44.5-45.0)	41.4±3.2 (36.0-44.5)
Spicules	-	-	83.3±5.2 (77.0-93.0)	-	-	-
Lateral accessory piece	-	-	19.6±1.3 (18.5-21.5)	-	-	-
J	15.5	12.7±1.8 (10.5-15.5)	12.7±1.2 (11.0-14.0)	6.9±1.5 (5.0-8.5)	11.0±0.7 (10.5-11.5)	10.6±1.6 (9.0-13.5)

^a Measurements are in μm and in the form: mean $\pm$ standard deviation (range).

^b a = body length/maximum body width; b = body length/pharyngeal length; c = body length/tail length; c' = tail length/body width at anus; d = anterior to guiding ring/body diameter at lip region; d' = body diameter at guiding ring/body diameter at lip region; V = (distance from anterior end to vulva/body length) x 100; T = ((distance from cloacal aperture to anterior end of testis/body length) x 100); J = hyaline tail region length.