Molecular phylogenetic analysis and comparative morphology reveals the diversity and distribution of needle nematodes of the genus *Longidorus* (Dorylaimida: Longidoridae) from Spain

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**Abstract**

The genus *Longidorus* constitutes a large group of approximately 170 species of plant-ectoparasitic nematodes that are polyphagous and distributed almost worldwide. Some of the species of this genus are vectors of plant viruses. Species discrimination in *Longidorus* is difficult because the morphology is very conservative, and morphometric characters often overlap, leading to potential misidentification. Integrative taxonomy, based on the combination of molecular analyses with morphology, is a useful and necessary approach in *Longidorus* species identification. In Spain from 2014 to 2017, we conducted nematode surveys among cultivated and wild plants, from which we identified 13 populations of *Longidorus*, two of which appeared to represent new species and are described herein as *L. iliturgiensis* sp. nov. and *L. pacensis* sp. nov., and 11 populations belonging to eight known species: *L. africanus*, *L. baeticus*, *L. carpetanensis*, *L. fasciatus*, *L. nevesi, L. cf. olegi*, *L. pini*, and *L. vallensis*. Three species are new geographical records for Spain (*L. nevesi, L. cf. olegi*, and *L. africanus*). We report molecular data for *L. nevesi, L. cf. olegi*, and *L. africanus*.
L. carpetanensis and L. pini for the first time. Additionally, we describe the males of L. pini and the juveniles of L. cf. olegi.

Keywords

downground diversity – Bayesian inference – CoxI – D2–D3 expansion domains of 28S rRNA gene – integrative taxonomy

Introduction

Nematodes include some of the most abundant metazoans on earth with a global distribution and an estimated ~100,000 species (Boucher & Lambshead, 1995; Blaxter et al., 1998; Coomans, 2000). In addition, among soil fauna, nematodes are the most common and diverse multicellular animals, being found in many environments and representing one of the most ubiquitous animal phyla in the soil (Ferris et al., 2001). Nematodes occupy all trophic levels within the soil food web, which leads them to play a central role in numerous soil functions (Ferris et al., 2001).

The needle nematodes of the genus Longidorus Micoletzky, 1922 belong to the family Longidoridae Thorne, 1935 comprising a wide and diverse group of plant migratory ectoparasitic nematode species (Coomans, 1996). Longidorus is considered cosmopolitan and contains approximately 170 species (Coomans, 1996; Archidona-Yuste et al., 2016a). Damage by Longidorus spp. to host plants is caused by direct feeding on root cells as well as by transmitting nepoviruses (genus Nepovirus, subfamily Comovirinae) (Taylor & Brown, 1997). In addition, 8 species of this genus (6.5%) (L. apulus Lamberti & Bleve-Zacheo, 1977, L. arthensis Brown, Grunder, Hooper, Klinger & Kunz, 1994, L. attenuatus Hooper, 1961, L. diadecturus Eveleigh, 1982, L. elongatus (de Man, 1876) Micoletzky, 1922, L. fasciatus Roca & Lamberti, 1981, L. macrosoma Hooper, 1961, and L. martini Merny, 1966) are specific vectors of seven out of 38 known nepoviruses (Taylor & Brown, 1997; Decraemer & Robbins, 2007) of the Longidorus species vector nepoviruses, including artichoke Italian latent virus (AILV), carnation ringspot virus (CRSV), cherry rosette disease virus (CRDV), mulberry ringspot virus (MRSV), peach rosette mosaic virus (PRMV), raspberry ringspot virus (RpRSV), and tomato black ring virus (TBRV) (Taylor & Brown, 1997; Decraemer & Robbins, 2007). These transmissions showed a marked specificity between plant viruses and their specific Longidorus vector species, except for L. elongatus transmitting tomato black ring virus (TomBRV) and raspberry ringspot virus (RRSV) (Taylor & Brown, 1997), which confirms the need for accurate identification of Longidorus species.

Due to the highly conserved morphology, with similar anatomical characteristics and high inter- and intraspecific morphometric variability, species delimitation in Longidorus is a very complex and time-consuming task (Coomans et al., 2001; Archidona-Yuste et al., 2016a). Accurate identification of needle nematodes is essential to establish an unequivocal diagnosis to discriminate virus vector species, select appropriate management strategies for preventing their spread and establish efficient control measures. Recent studies using an integrative approach for identification highlighted the difficulty of correct identification at the species level within the genus Longidorus, as well as cryptic species separation (Subbotin et al., 2015; Archidona-Yuste et al., 2016a; Peraza-Padilla et al., 2017).
Xu et al., 2017, 2018). These studies provide molecular markers based on ribosomal RNA (rRNA) (D2–D3 expansion domains of 28S rRNA, ITS and 18S rRNA) and mitochondrial DNA (mtDNA) genes (cytochrome c oxidase subunit I or CoxI) for precise and unequivocal diagnoses of some species of *Longidorus*, demonstrating that this genus is a complex and taxonomically important group of plant-parasitic nematodes.

Approximately forty species of *Longidorus* have been reported from the Iberian Peninsula, including the recent descriptions of fifteen new species (Peña Santiago et al., 2003; Gutiérrez-Gutiérrez et al., 2013; Archidona-Yuste et al., 2016a). A survey of nematodes from cultivated and wild plants in Spain revealed the presence of thirteen unidentified populations of needle nematodes belonging to the genus *Longidorus*. Preliminary morphological observations indicated that two of these populations appeared to be morphologically different from all other species described in the genus, while the other populations were assigned to eight known species of *Longidorus*. Detailed observations using light microscopy and molecular characterisation indicated that these two populations should be assigned to two new species. In the present study, we describe the two new species, *Longidorus iliturgiensis* sp. nov. and *Longidorus pacensis* sp. nov. and present phylogenetic data that confirm their species status.

The objectives of this study were (1) to morphologically and morphometrically characterise the two new species belonging to the genus *Longidorus* and to compare them with previous records; (2) to characterise molecularly the sampled *Longidorus* spp. populations using the D2–D3 expansion domains 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.

**Materials and methods**

*Nematode populations and morphological studies*

Nematode surveys were conducted from the spring of 2014 to 2017 in the soil around cultivated and wild plants in Spain (supplementary fig. S1). Soil samples were collected with a shovel from the upper 50 cm of soil surrounding four or five plants arbitrarily chosen in each locality. Nematodes were extracted from 500 cm$^3$ of soil by centrifugal flotation (Coolen, 1979) and a modification of Cobb’s decanting and sieving (Flegg, 1967) methods. In some cases, additional soil samples were collected from the same locality for additional specimens for morphological and/or molecular identification.

Specimens for light microscopy were killed by hot fixative using a solution of 4% formaldehyde + 1% propionic acid and embedded in pure glycerine using Seinhorst’s (1966) method. Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast at powers up to 1,000x magnification. The morphometric study of each nematode population included morphology-based diagnostic features in *Longidoridae* (i.e., de Man body ratios (Jairajpuri & Ahmad, 1992)), lip region and amphid shape, oral aperture-guiding-ring, odontostyle length and female tail shape) (Jairajpuri & Ahmad, 1992). For line drawings of the new species, light micrographs were imported to CorelDraw ver. X7 and redrawn. In addition, a comparative morphological study on the type specimens of one species was conducted with specimens kindly provided by Dr. A. Navas from the Nematode Collection of the Spanish National Museum of Natural Sciences-CSIC, Madrid, Spain (viz. *L. pini* Andrés & Arias, 1988).

Topotype specimens of *L. carpetanensis* Arias, Andrés & Navas, 1986 were used for morphological and molecular studies after verifying that their morphology was congruent with
that of the original description. Nematode populations of known *Longidorus* species were analysed morphologically and molecularly in this study and proposed as standard and reference populations for each species until topotype specimens become available and were molecularly characterised.

**DNA extraction, PCR and sequencing**

To avoid mistakes caused by mixed populations in the same sample, two live nematodes from each sample were temporarily mounted in a drop of 1 M NaCl containing glass beads and were measured and identified to ensure specimens belong to the unidentified populations of *Longidorus*. Morphometrics and photomicrographs recorded during this initial study were not used as part of the morphological study. Following morphological confirmation of the species, the specimens were removed from the slides, and DNA was extracted. Nematode DNA was extracted from single individuals as described by Subbotin et al. (2000). The D2–D3 expansion domains of 28S rRNA were amplified using the D2A (5′-ACAAGTACCCTGGAGGGAAGTTT-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) primers (Nunn, 1992). The ITS1 region was amplified using forward primer 18S rRNA (5′-TTATACGTCCCTGCCCTTT-3′) (Vrain et al., 1992) and reverse primer rDNA1 (5′-ACGAGCGAGTGATCCACCG-3′) (Cherry et al., 1997). Finally, the portion of the 18S rRNA was amplified using primers 988F (5′-CTCTAAGATTAAGCCATGC-3′), 1912R (5′-TTTACCGTGCAACTAGGG-3′), 1813F (5′-CTCGGTGAGGTGAAT-3′) and 2646R (5′-GCTACCTTGTACGACTT-3′) (Holterman et al., 2006). Finally, the portion of the Cox1 gene was amplified as described by Lazarova et al. (2006) using the primers COIF (5′-GATTTTTGGKCATCWWGARG-3′) and COIR (5′-131 CWACATAAAAGTATCAG-3′).

PCR cycle conditions were one cycle of 94 °C for 2 min., followed by 35 cycles of 94 °C for 30 s., annealing temperature of 55 °C for 45 s; 72 °C for 3 min; and finally one cycle of 72 °C for 10 min. PCR products were purified after amplification using ExoSAP-IT (Affimetrix, USB products, High Wycombe, UK), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct sequencing in both directions using the primers noted above. The resulting products were purified and run on a DNA multipipillary 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 in table 1 and on the phylogenetic trees.

**Phylogenetic analysis**

D2–D3 expansion domains of 28S rRNA, ITS1, partial 18S rDNA and the mtDNA gene and Cox1 sequences of different *Longidorus* spp. from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen based on previous published studies (He et al., 2005; Holterman et al., 2006; Gutiérrez-Gutiérrez et al., 2013; Subbotin et al., 2015; Archidona-Yuste et al., 2016a; Xu et al., 2017). Multiple sequence alignments of the different genes were made using the Q-INS-i algorithm of MAFFT v. 7.205 (Katoh & 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 Lab 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 no conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half).
Phylogenetic analyses of the sequence data sets 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 Akaike-supported model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then used in phylogenetic analyses. A symmetrical model with invariable sites and a gamma-shaped distribution (SYM + I + G) for the D2–D3 expansion domains of 28S rRNA, a 3-parameter model with invariable sites and a gamma-shaped distribution (TPM3 μf + I + G) for the ITS1 region, a transitional model with invariable sites and a gamma correction (TIM2 + I + G) for the partial 18S rRNA, and a general time-reversible model with invariable sites and a gamma-shaped distribution (GTR + I + G) model for the partial CoxI gene were run with four chains for $2 \times 10^6$ generations. The Markov chains were sampled at intervals of 100 generations. Two runs were conducted for each analysis. After discarding burn-in samples 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) were given on appropriate clades. Trees from all analyses were visualised using FigTree software version v.1.42 (http://tree.bio.ed.ac.uk/software/figtree/).

**Results**

**Species identification and delimitation**

According to the polytomous key by Chen et al. (1997) and the supplement by Loof & Chen (1999), matrix codes A (odontostyle length), B (lip region width), C (distance of guiding-ring from anterior body end), D (lip region shape), E (shape of amphidial fovea), F (body length), G (index “a”), H (tail shape), and I (presence/absence of males) were used for species identification and delimitation. Additionally, classical diagnostic features in Longidoridae (i.e., de Man body ratios (Jairajpuri & Ahmad, 1992)), lip region and amphid shape, distance from oral aperture to guiding-ring, odontostyle length and female tail shape (Jairajpuri & Ahmad, 1992) were used for species delimitation and presented in species descriptions. Molecular data were also considered to differentiate species within the genus. Based on an integrative taxonomy, we described two new species: *L. iliturgiensis* sp. nov. and *L. pacensis* sp. nov. We found additional species for which morphological and morphometric data as well as molecular data were provided for *L. africanus* Merny, 1966, *L. carpetanensis*, *L. nevesi* Macara, 1985, *L. cf. olegi* Kankina & Metlitskaya, 1983, and *L. pini*. For other previously studied species, such as *L. baeticus* Gutiérrez-Gutiérrez, Cantalapiedra-Navarrete, Montes-Borrego, Palomares-Rius & Castillo, 2013, *L. fasciatus*, and *L. vallensis* Archidona-Yuste, Navas-Cortés, Cantalapiedra-Navarrete, Palomares-Rius & Castillo, 2016a, only the D2–D3 expansion domains of 28S rRNA or ITS1 sequences have been provided. Paratype materials of *L. pini* were used for measurements and morphological studies to compare with our specimens. For *L. africanus*, *L. carpetanensis*, *L. nevesi*, *L. cf. olegi*, and *L. pini*, a brief description and comparison with previous records are provided in the Appendix because these species records represent the first molecular characterisation and/or new records in Spain.

**Molecular characterisation**

The amplification of D2–D3 expansion domains of 28S rRNA, ITS1, partial 18S rRNA, and partial CoxI genes yielded single fragments of ~900 bp, 1,100 bp, 1,800 bp, and 500 bp, respectively, based on gel electrophoresis. *Longidorus carpetanensis*, *L. nevesi*, *L. cf. olegi*, and *L. pini* have been molecularly characterised for the first time in this study. Eighteen
| Species                     | Sample code | Host plant, locality, province | $D_2$–$D_3$  | $r_{rsi}$  | partial $r_{18S}$ | CoxI   |
|-----------------------------|-------------|-------------------------------|--------------|------------|-------------------|--------|
| *Longidorus iliturgiensis*, sp. nov. | ALANU       | Black alder, Andújar, Jaén    | MH430012     | MH429987   | MH430002          | MH454065 |
| *Longidorus iliturgiensis*, sp. nov. | ALANU       | Black alder, Andújar, Jaén    | MH430013     | MH429988   | MH430003          | –       |
| *Longidorus pacensis*, sp. nov. | INB32       | Fallow, Badajoz, Badajoz      | MH430014     | MH429989   | MH430004          | MH454066 |
| *Longidorus pacensis*, sp. nov. | INB32       | Fallow, Badajoz, Badajoz      | MH430015     | MH429990   | MH430005          | MH454067 |
| *Longidorus africanus*       | ALORA       | Lemon tree, Álora, Málaga     | MH430016     | –          | –                 | –       |
| *Longidorus baeticus*        | MONTI       | Cultivated olive, La Montiela, Córdoba | MH430017 | –          | –                 | –       |
| *Longidorus carpetanensis*<sup>a</sup> | PNAVA     | Common broom, Navalmoral, Ávila | MH430018     | MH429991   | MH430006          | MH454068 |
| *Longidorus carpetanensis*<sup>a</sup> | PNAVA     | Common broom, Navalmoral, Ávila | MH430019     | MH429992   | –                 | –       |
| *Longidorus carpetanensis*   | SANAB       | Common oak, Puebla de Sanabria, Zamora | MH430020 | MH429993   | –                 | –       |
| *Longidorus fasciatus*       | PECOS       | Cultivated olive, Écija, Sevilla | MH430021     | MH429994   | MH430007          | MH454069 |
| *Longidorus fasciatus*       | M0110       | Grapevine, Montilla, Córdoba  | MH430022     | MH429995   | MH430008          | –       |
| *Longidorus fasciatus*       | J0130       | Grapevine, Jerez de la Frontera, Cádiz | MH430023 | MH429996   | –                 | –       |
| *Longidorus nevesi*          | SANOL       | Grapevine, Santa Olalla, Toledo | MH430024     | MH429997   | MH430009          | –       |
| *Longidorus nevesi*          | SANOL       | Grapevine, Santa Olalla, Toledo | MH430025     | MH429998   | –                 | –       |
| *Longidorus cf. olegi*       | CAZQU       | Portuguese oak, Arroyo Frío, Jaén | MH430026     | MH429999   | MH430010          | –       |
| *Longidorus cf. olegi*       | CAZQU       | Portuguese oak, Arroyo Frío, Jaén | MH430027     | MH430000   | –                 | –       |
| *Longidorus pini*            | CASAR       | Pyrenean oak, Nava de Francia, Salamanca | MH430028 | MH430001   | MH430011          | MH454070 |
| *Longidorus vallensis*       | EMONT       | Wheat, Córdoba, Córdoba        | MH430029     | –          | –                 | –       |

<sup>a</sup> Type locality (topotype specimens).
–, not obtained or not performed.
new D2–D3 of 28S rRNA gene sequences from ten different *Longidorus* spp. were obtained in the present study. D2–D3 expansion domains of 28S rRNA sequences of *L. illiturgiensis* sp. nov. (MH430012-MH430013) were related by sequence similarity (95–94%) with *L. closelongatus* (KJ802863, KJ802866; 40 bp different nucleotides, 0 indels), *Longidorus* sp. 3 SAS-2014 (KF242335, 38 bp different nucleotides, 2 indels), *L. pseudelongatus* (KJ802873, 47 bp different nucleotides, 2 indels), *L. rubi* (JX445116, 47 bp different nucleotides, 4 indels), *L. dunensis* (AY593057, 48 bp, 8 indels) and *L. alvegus* (KT308867, 57 bp different nucleotides, 7 indels). Intraspecific sequence diversity (uncorrected p-distance) of specimens from *L. illiturgiensis* sp. nov. (MH430012-MH430013) studied in the type locality ranged from 0 to 0.4% (from 0 to 3 substitutions). On the other hand, *Longidorus pacensis* sp. nov. showed similarity values of 90% with several accessions from GenBank, such as *L. macrorhizus* (KT308856), *L. vinearum* (KT308877), *L. magnus* (HM921361) and *L. lusitanicus* (KT308869), with substitutions ranging from 66 to 68 nucleotides and from 18 to 25 indels. The most closely related species to *L. carp tantrum* (MH430018-MH430020) for the 28S RNA gene was *L. pini* (MH430028), which was 96% similar (28 different nucleotides and 6 indels). *Longidorus nevesi* (MH430024-MH430025) showed a similarity value of 97% with *L. iuglandis* (JX445105) (19 different nucleotides and no indels); finally, *L. cf. olegi* (MH430026-MH430027) showed a similarity value of 94% with several accessions deposited in GenBank, such as *L. vinearum* (KT308875), *L. magnus* (HM921361), *L. wicuoeula* (KT308864) and *L. silvestris* (KT308860) (from 42 to 46 substitutions and from 7 to 19 indels). No intraspecific variability was found for *L. carp tantrum* (MH430018-MH430020) and *L. nevesi* (MH430024-MH430025), and only 1 variable position was found for *L. cf. olegi* (MH430026-MH430027). The D2–D3 expansion domains of 28S rRNA from *L. africana* (MH430016), *L. baeticus* (MH430017) and *L. vallensis* (MH430029) matched well with accessions from the same species deposited in GenBank, being 98–99% similar and increasing the molecular variability of these species.

The high variability of the ITS1 region makes it difficult to determine similarity values among the different *Longidorus* spp., and they also show low coverage values in the sequence pairwise BLAST comparisons. Intrapopulation variability of this region was low for *L. nevesi* (MH429997-MH429998), with 21 variable nucleotides, *L. carp tantrum* (MH429991-MH429993), from 1 to 20 variable nucleotides, *L. cf. olegi* (MH429999-MH430000), with 11 nucleotides, and finally only one different position for *L. illiturgiensis* sp. nov., and no variability for *L. pacensis* sp. nov. However, *L. fasciatus* (MH429994-MH429996) showed a much higher intraspecific variability for the ITS1 region, being 96–97% similar (from 31 to 51 variable nucleotides) among the 3 studied populations in this study and 88-89% similar with *L. fasciatus* (JX445097, Gutierrez-Gutierrez et al., 2013) (31–133 variable nucleotides).

For the 18S rRNA, ten new sequences were obtained in this study, and all of them showed very high similarity values with the other accessions from *Longidorus* spp. deposited in GenBank, being 98–99% similar. Finally, six new CoxI sequences were deposited in GenBank in this study. This gene showed an interspecific variability very similar among all *Longidorus* spp. deposited in GenBank (similarity values ranging from 77 to 80% among all the accessions with CoxI sequences available).

**Phylogenetic relationships**

Phylogenetic relationships among *Longidorus* species inferred from analyses of D2–D3 expansion domains of 28S rRNA, ITS1, the partial 18S rRNA and the partial CoxI mtDNA gene sequences, using BI are shown in figs.
Figure 1. Phylogenetic relationships within the genus *Longidorus*. Bayesian 50% majority rule consensus tree as inferred from D2 and D3 expansion domains of 28S rRNA sequence alignment under an SYM model with invariable sites and a gamma-shaped distribution (SYM + I + G). Posterior probabilities greater than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold. Scale bar = expected changes per site. ** = Branches collapsed, indicating clustered *Longidorus* species. For a more specific detail of collapsed clades, see supplementary fig. S1.
Figure 2. Phylogenetic relationships within the genus Longidorus. Bayesian 50% majority rule consensus tree as inferred from ITS1 rRNA sequence alignment under a 3-parameter model with invariable sites and a gamma-shaped distribution (TPM3uf + I + G). Posterior probabilities greater than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold. Scale bar = expected changes per site.
Figure 3  Phylogenetic relationships within the genus *Longidorus*. Bayesian 50% majority rule consensus tree as inferred from 18S rRNA gene sequence alignment under a transitional model with invariable sites and a gamma correction (TIM2 + I + G). Posterior probabilities greater than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold. Scale bar = expected changes per site.
1, 2, 3, and 4, respectively. The phylogenetic trees generated with the nuclear and mitochondrial markers included 156, 107, 106 and 67 sequences, with 735, 864, 1550 and 317 positions in length, respectively (figs. 1–4). To facilitate discussion, in trees of nuclear markers, clades that were well supported or taxonomically well founded were labelled in Roman numerals from I to VI. Poorly supported lineages were not explicitly labelled. These trees showed a very similar topology, which consisted of a major clade that clustered the majority of Longidorus spp. (approximately 75% of species with molecular data available).

Clade I from the 50% majority rule consensus 28S rRNA gene BI tree (PP = 1.00) comprised 19 species, all of them reported in the Iberian Peninsula with a characteristic tail (hemispherical convex-conoid tail shape), and included L. pacensis sp. nov. (fig. 1). Longidorus pacensis sp. nov. was phylogenetically related to L. baeticus and L. macrodorus in a well-supported clade (fig. 1). Clade II (PP = 1.00) comprised 12 species, mostly central European (fig. 1). Clade III (PP = 1.00) comprised 8 species with a similar distance from the anterior end to the guiding-ring and amphids with bilobed basal lobes including two newly sequenced species from Spain, viz. L. carpetanensis and L. pini (fig. 1). Clade IV (PP = 1.00) comprised five species from Crete and Iran, including nematodes with similar sizes and males absent or rare. Clade V (PP = 1.00) comprised seven species from...
Western Europe with a lip region continuous with body contour and short hemispherical tail (c’ < 1). Finally, Clade VI (PP = 1.00) comprised five species from Asia characterised by a long distance between the guiding-ring and anterior end (fig. 1). The second new species described here, L. iliturgiensis sp. nov., clustered in a clade with L. alveagus tree (fig. 1).

The 50% majority rule consensus ITS1 BI tree showed three major clades (I to III), although Clade II was moderately supported. Clade I (PP = 1.00) comprised 43 species with different geographical origins and morphologies and included the two new and five known species studied here, clustering with the same species as in the 28S rRNA gene tree but with lower supports (fig. 2). Clade II (PP = 0.81) comprised seven species mostly central European and Clade III (PP = 0.99) comprised seven species mostly from Asia (fig. 2).

The 50% majority rule consensus 18S rRNA gene BI tree contains three major clades. Clade I (PP = 0.99) included the majority of the species of the genus, comprising the new and known species studied here. Longidorus iliturgiensis sp. nov. clustered with L. leptocephalus in a not well-supported clade (fig. 3). Unfortunately, no data were available for the partial 18S rRNA of L. alveagus. In addition to the 28S rRNA gene and ITS1 trees, L. pacensis sp. nov. clustered with some species described from the Iberian Peninsula; however, these relationships were not well supported. Finally, L. carpetanensis and L. pini seem to be phylogenetically related since they appear together within a well-supported clade (PP = 1.00) (fig. 3). In addition, Clade II (PP = 0.90) included nine Asian species (fig. 3). The principal differences among the three rRNA trees were the support for some clades in partial 18S rRNA and ITS1.

The 50% majority rule consensus CoxI gene BI tree (fig. 4) again showed a major, not well-supported clade (PP = 0.73). Similar to the phylogenies based on ribosomal genes, L. pacensis sp. nov. (MH430066-MH430067) and L. fasciatus (MH430069) appeared within a well-supported subclade (PP = 1.00) with many other species from the Iberian Peninsula (fig. 4). Finally, L. iliturgiensis sp. nov. (MH430065), L. carpetanensis (MH430068) and L. pini (MH430070) did not form subclades with any Longidorus spp. Unfortunately, no CoxI sequences from L. nevesi and L. cf. olegi were obtained.

Discussion

The primary objective of this study was to identify and molecularly characterise species belonging to the genus Longidorus associated with different plant hosts and environments in Spain. Our results demonstrate that the use of morphological-morphometrical studies integrated with rRNA and mtDNA molecular markers deciphered the important diversity in this difficult group of nematodes. We described two new species and broadened the knowledge on the distribution and molecular and biological data of eight known species of the genus Longidorus based on integrative taxonomy and the phylogenetic relationships among the new and known species of the genus Longidorus (see Appendix).

The results (including new and known species) increased data on the biodiversity of Longidorus in the Iberian Peninsula and agree with previous results obtained for the phylogeny and biogeography of the genus Longidorus in the Euro-Mediterranean region (Navas et al., 1993; Archidona-Yuste et al., 2016a). The diversity of this genus in Spain is remarkable, with approximately 40 species reported including the species described in this article (Arias et al., 1986; Andrés & Arias, 1988; Andrés et al., 1991; Gutiérrez-Gutiérrez et al., 2011; Gutiérrez-Gutiérrez et al., 2013; Archidona-Yuste et al., 2016a). This study, in addition to the description of two new...
species, provides three first reports for Spain (L. africanus, L. nevesi and L. cf. olegi), and new geographic records for other species, such as L. baeticus, L. vallensis, L. carpetanensis and L. pini. The species distribution knowledge is important to understand nematode expansion, phytopathological risks and how some species are native to the Iberian Peninsula (i.e., L. baeticus, L. macrodorus, L. pacensis sp. nov.) while others are distributed in the Mediterranean region (i.e., L. iuglandis and L. fasciatus). Additionally, the biological knowledge of L. pini and L. cf. olegi is extended by presenting data for males in the case of L. pini and describing juveniles for L. cf. olegi. Males and juveniles are important for the diagnosis of Longidorus (Robbins et al., 1996; Peneva et al., 2013).

Sequences of nuclear ribosomal RNA genes, particularly D2–D3 expansion domains of the 28S rRNA gene, ITS1 region, and the mtDNA gene CoxI, have proven to be a powerful tool for providing accurate species identification of Longidoridae (Palomares-Rius et al., 2017). However, the low nucleotide variability found in partial 18S rRNA makes it difficult to identify individuals to the species level. New molecular markers were provided for L. nevesi, L. cf. olegi, L. carpetanensis and L. pini, in addition to the newly described species (L. iliturgiensis sp. nov. and L. pacensis sp. nov.). For other species, we increased their molecular diversity (L. africanus, L. baeticus, L. fasciatus, and L. vallensis). This latter point is important for the use of barcoding techniques for species identification in this genus (Palomares-Rius et al., 2017).

Phylogenetic analyses based on three rDNA molecular markers (D2–D3 expansion domains of 28S rRNA gene, ITS1 region and the partial 18S rRNA) resulted in a general consensus of species phylogenetic positions for the majority of species and were generally congruent with those given by previous phylogenetic analysis (Subbotin et al., 2014; Archidona-Yuste et al., 2016a; Esmaeili et al., 2016; Palomares-Rius et al., 2017). Only one previous work on the phylogeny of the CoxI gene in the family Longidoridae has been published to date (Palomares-Rius et al., 2017). However, this recent work, including all the genera with molecular data available within the family, did not resolve the phylogenetic relationships among the major different groups of Longidorus species, so it was difficult to compare both results. Base saturation (third nucleotide position in each codon), the short fragment used in this study, different mutation rates in the mitochondrial genome and the wide evolutionary differences within these studied groups could complicate the phylogeny for the CoxI marker (Palomares-Rius et al., 2017). As mentioned before, the phylogenetic position of all species sequenced in this study was coincident; L. pacensis sp. nov., L. nevesi, L. cf. olegi, L. baeticus and L. fasciatus were grouped in a clade with other species from the Iberian Peninsula in all analysed markers, although the relationships within this clade varied depending on which marker was used. Longidorus iliturgiensis sp. nov. clustered with L. alvegus in D2–D3 expansion region domains of 28S rRNA and ITS1 phylogenetic trees; however, it was not possible to study this relationship for the partial 18S rRNA because there were no 18S rRNA sequences from L. alvegus in GenBank. Longidorus carpetanensis and L. pini seem to be strongly phylogenetically related using all nuclear markers studied and only when mtDNA was analysed did these species appear separately, probably because mtDNA evolves faster than ribosomal DNA (Lazarova et al., 2006; Kumari et al., 2010; Palomares-Rius et al., 2017). Some clades were strongly supported and clustered species of a geographical area, as was the case for Asian species. This clade usually occupies basal positions showing their ancestral position in the genus, suggesting this area as the possible origin of the genus Longidorus, but
this hypothesis needs to be confirmed with additional studies.

In summary, the present study updates the biodiversity within the genus *Longidorus*, showing the plasticity of these nematodes and the importance of describing new species by integrating morphomterical and molecular approaches. The description of the two new species also expands the distribution of these nematodes in the Iberian Peninsula, specifically in Spain. New reports and molecular data of these nematodes with descriptions of juveniles in some species provide new data for the identification, biology and ecology of these plant-parasitic nematodes in the field.

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APPENDIX

Morphology and morphometry of *Longidorus* species

Systematic description

*Longidorus iliturgiensis* sp. nov.

http://zoobank.org/urn:lsid:zoobank.org:act:253C46DF-D3F4-42BB-B675-6E8EC0A7EF7A (figs. 5, 6 and 7, table 2)

**Material examined.** Holotype Adult female, collected from the rhizosphere of black alder (*Alnus glutinosa* L.) by J. Martin Barbarroja, March 9, 2014; mounted in pure glycerine and deposited in the nematode collection at Institute for Sustainable Agriculture (ias) of Spanish National Research Council (csic), Córdoba, Spain (collection number ALAN-09).

Paratypes. Female, male and juvenile paratypes extracted from soil samples were mounted in pure glycerine and deposited in the following nematode collections: Institute for Sustainable Agriculture (ias) of Spanish National Research Council (csic), Córdoba, Spain (collection numbers ALAN-01-ALAN-15); two females at Istituto per la Protezione Sostenibile delle Piante (ipsp), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (ALAN-16); and two females and one male at USDA Nematode Collection, Beltsville, MD, USA (T-6984p); collected by J. Martin Barbarroja, March 9, 2014.

**Type locality.** Andújar, Jaen province, Spain: 38° 9’7.45"N, 4° 0’54.35"W; 267 m above sea level (a.s.l.).

**Etymology.** The species name is derived from “iliturgi” the Roman name of Andújar, where the type specimens were found.

**Diagnosis.** *Longidorus iliturgiensis* sp. nov. is an amphimictic species characterised by a moderately long body (4.1–6.1 mm), assuming an open C-shaped body when heat relaxed; lip region expanded distinctly set off from body contour, 8.5–10.5 μm wide and 4.0–5.0 μm high; guiding-ring located 21.5–24.5 μm from anterior end; relatively short odontostyle 57.0–69.0 μm; amphidial fovea slightly asymmetrically bilobed; vulva almost equatorial; female tail long, dorsally convex-conoid, and bearing three pairs of caudal pores; c’ ratio (1.8–2.6); males frequent (2:3 ratio), with short spicules (30.5–37.0 μm) and 7–11 ventro-median supplements. According to the polymorphic key by Chen et al. (1997), the supplement by Loof & Chen (1999), and additional codes (Peneva et al., 2013; Archidona et al., 2016), new species has the following code (codes in parentheses are exceptions): A2(1)-B1-C2-D4-E2-F23-G3-H6(5)-I21-J1–K6. Specific D2–D3 region, ITS1 rRNA, partial 18S rRNA and CoxI mtDNA gene sequences (GenBank accession numbers MH430002-MH430013, MH429987-MH429988, MH430002-MH430003, and MH454065, respectively)

**Description.** Female. Body relatively long and thin, slightly tapering towards both ends. When heat relaxed, body ventrally curved in open C-shape. Cuticle thin appearing smooth under low magnifications, 1.7 ± 0.3 (1.5–2.0) μm thick at mid-body but thicker (9.0 ± 1.0 (7.0–10.0) μm) and marked by very fine superficial transverse striate mainly in tail region. Lateral chord approximately 9.0 μm wide at mid-body or approximately 29% of corresponding body diameter. Lip region expanded distinctly set off from body contour, anteriorly flattened, 9.6 ± 0.5 (8.5–10.0) μm wide and 4.4 ± 0.3 (4.0–5.0) μm high. Amphidial fovea slightly asymmetrically bilobed with lobes occupying approximately 1/2 of the distance between oral aperture and guiding-ring. Guiding-ring single, located 2.4 ± 0.2 (2.1–2.7) times lip region diameter from anterior end. Odontostyle 1.7 ± 0.2 (1.2–2.2) times as long as odontophore, straight or slightly arcuate; odontophore weakly developed, with rather weak...
basal swellings. Nerve ring surrounding odontophore base at 94.3 ± 6.8 (89.0–102.0) μm from anterior end. Anterior slender part of pharynx usually coiled in its posterior region. Basal bulb short and cylindrical, 82.3 ± 6.6 (68.5–92.0) μm long and 13.5 ± 1.7 (11.5–16.5) μm in diam. Glandularium 72.4 ± 6.6 (59.0–85.5) μm long. Dorsal pharyngeal gland nucleus (DN) and ventrosublateral nuclei (SVN) located at 23.9% ± 3.8 (18.1–28.4%) and 56.4% ± 3.5 (48.9–59.3%) of distance from anterior end of pharyngeal bulb, respectively. Nucleolus of DN larger than nucleoli of two SVN (3.5–4.0 vs 2.5–3.0 μm). Cardia conoid-rounded, 7.3 ± 1.2 (6.0–8.0) μm long. Prerectum very variable in length, 598.3 ± 163.7 (347.0–783.0) μm long, and rectum 21.7 ± 3.2 (16.5–26.0) μm long ending in anus as a small rounded slit. Reproductive system with both genital branches almost equally developed, each branch 236–578 μm long, with reflexed ovaries. Vulva in form of a transverse slit, located almost equatorial, vagina perpendicular to body axis, 13.9 ± 1.8 (11.0–17.5) μm long or 37–57% of corresponding body width, surrounded by well-developed muscles. Genital branches equally developed, (G1) 7.2 ± 1.4 (5.0–10.0), (G2) 6.9% ± 1.2 (4.9–8.7%) of body length, respectively. Uteri with sperm cells in all female specimens examined; sphincter well-developed, between uterus and oviduct. Eggs measuring 205.0 ± 13.1 (191.0–217.0) μm long and 30.7 ± 1.6 (29.5–32.5) μm wide. Anterior and posterior oviduct of similar size. Tail moderately long, dorsally convex-conoid, with rounded terminus, bearing three pairs of caudal pores.

**Male.** Almost as frequent as female. Morphologically similar to female except for genital system and posterior region slightly curved ventrally. Male genital tract doricch with testes opposed, containing multiple rows of spermatogonia in the germinal zone. Tail conoid, dorsally conoid and ventrally concave with acute terminus and thickened outer cuticular layer. Spicules very short, moderately developed and slightly curved ventrally, approximately 0.7–0.9 times shorter than tail length; lateral guiding pieces straight with curved proximal end. Moderate number of supplements, one pair of adanal and from 6 to 10 mid-ventral supplements.

**Juveniles.** All four juvenile stages (first-, second-, third- and fourth-stage) were identified using morphological characters such as body length, length of replacement and functional odontostyle (Robbins et al., 1996). Juveniles were similar to adults apart from developed reproductive system, shorter body length, tail shape and presence of replacement odontostyle (figs. 5, 6–7). Tail becomes progressively shorter and stouter in each moult (figs. 5, 6–7 and table 2). As for other longidorids, first-juvenile stage was characterised by the replacement odontostyle tip close to base of functional odontostyle and located at level of odontophore. In J2–J4, replacement odontostyle located at some distance from odontophore base. J1s were characterised by a bluntly rounded to cylindrical tail with a c’ ratio >3.0 (figs. 5–6 and table 2).

**Remarks.** According to the updated polytomous key by Chen et al. (1997) and the supplement by Loof & Chen (1999), the specific matrix code for this species was A2(1)-B1-C2-D4-E2-F23-G3-H6(5)-I21-J1–K6. Based on sorting of matrix codes A (odontostyle length), B (lip region width), C (distance of guiding-ring from anterior body end), D (lip region shape), and E (shape of amphidial fovea), *L. iliturgiensis* sp. nov. is closely related to several species described from Spain: *L. indalus* Archidona-Yuste, Navas-Cortés, Cantalapiedra-Navarrete, Palomares-Rius & & Castillo (2016a), *L. carpetanensis* and *L. unedoi* Arias, Andrés & Navas (1986), from which it can be differentiated by a combination of the characters discussed below. *Longidorus iliturgiensis* sp. nov. differs from *L. indalus* by a longer odontostyle (av. 61.9 (57.0–69.0) vs av. 56.7 (54.0–59.5) μm long), and males (frequent vs very rare) (Archidona-Yuste et al., 2016a). From *L. carpetanensis*, it differs by a longer body length (4.1–6.1 vs 3.5–4.4 mm), higher a ratio (138.6–179.4 vs 96.0–118.0), and higher c and c'.
Figure 5  Line drawings of *Longidorus iliturgiensis*, sp. nov. paratypes. (A) Female neck region. (B) and (C) Female lip regions. (D) and (E) Female tails. (F) Male tail. (G) First-stage juvenile tail.
Figure 6  Light micrographs of *Longidorus iliturgiensis*, sp. nov. (A)–(D) Anterior regions. (E) Vulval region. (F)–(I) Female tails. (J)–(M) First-, second-, third-, and fourth-stage juvenile (J1–J4) tails, respectively. (N)–(O) Male tail. Abbreviations: a = anus; af = amphidial fovea; spl = ventromedian supplements; v = vulva. Scale bars (A)–(C), (E)–(O) = 20 μm; (D) = 10 μm
Longidorus pacensis sp. nov.
http://zoobank.org/urn:lsid:zoobank.org:act:10E0F15B-7C7B-4B1D-B063-1A041F77EFFB (figs. 7, 8 and 9, table 3)

Material examined. Holotype. Adult female, collected from fallow by P. Castillo, May 6, 2016; mounted in pure glycerine and deposited in the nematode collection at Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number INB32-03).

Paratypes. Female, male and juvenile paratypes extracted from soil samples collected from the same locality as the holotype; mounted in pure glycerine and deposited in the following nematode collections: Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection numbers INB32-01-INB32-12); two females at Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy (INB32-14); and one female and one male at USDA Nematode Collection, Beltsville, MD, USA (T-6985p); collected by P. Castillo, May 6, 2016.

Type locality. Badajoz, Badajoz province, Spain: 38° 49′24.85″N, 7°1′42.36″W; 187 m above sea level (a.s.l.).

Etymology. The species name is derived from the Latin word “Pacensis” the Roman name of Badajoz, where the type specimens were found.

Diagnosis. Longidorus pacensis sp. nov. is an amphimictic species characterised by a moderately long body (6.7–8.3 mm), assuming a ventrally curved open C-shape upon fixation; lip region conoid-narrowed continuous with body contour, 11.0–12.5 μm long; guiding-ring located 36.5–40.5 μm from anterior end; moderately long odontostyle (115.0–129.0 μm) amphidal fovea pocket-shaped, asymmetrically bilobed; vulva almost equatorial; female tail short, bluntly conoid, and bearing between ratio (87.4–139.6 vs 77.0–96.0 and 1.8–2.6 vs 1.6–2.2, respectively), males presence (extremely rare vs frequent) (Arias, Andrés & Navas, 1986). From L. unedoi, it differs in that it has lower c and V ratio (87.4–139.6 vs 122.0–156.0 and 46.0–52.0 vs 52.0–58.0; respectively) (Arias et al., 1986). Finally, L. iliturgiensis sp. nov. can be related to L. alvegus Roca, Pereira & Lamberti, 1989 from which it can be differentiated by a shorter body length (4.1–6.1 vs 6.3–7.8 mm), a shorter odontostyle (57.0–69.0 vs 82.5–92.5 μm long), and shorter distance from anterior end to guiding-ring (21.5–24.5 vs 25.6–33.1 μm) (Roca et al., 1989). Nevertheless, it can be clearly separated by specific 28S rRNA, 18S rRNA and CoxI sequences.
two and three pairs of caudal pores; \( c' \) ratio (0.7–0.9); males less frequent than females, with long spicules (62.0–79.0 μm) and 15–20 ventromedian supplements; According to the polytomous key by Chen et al. (1997), the supplement by Loof & Chen (1999), and additional codes (Peneva et al., 2013; Archidona et al., 2016), the new species has the following code: A4(5)-Bi-C3-D1-E3-F4-G1-H1-I2-J1–K1. Specific D2–D3 region, ITS1 rRNA, partial 18S rRNA and partial CoxI sequences (GenBank accession numbers MH430014-MH430015, MH429989-MH429990, MH430004-MH430005, and MH430066-MH430067, respectively).

**Description. Female.** Body moderately long, cylindrical, tapering towards anterior end, and assuming a ventrally curved open C-shape upon fixation. Cuticle thin appearing smooth under low magnifications, 3.6 ± 0.7 (3.0–4.5) μm thick at mid body, but thicker (19.3 ± 2.4 (16.5–24.0) μm) and marked by very fine superficial transverse striae mainly in tail region. Lateral chord 18.0 ± 1.5 (16.5–19.5) μm wide at mid-body or 23–25% of corresponding body diameter. Lip region conoid-narrowed, anteriorly rounded, and continuous with body contour. Amphidial fovea pocket-shaped asymmetrically bilobed with lobes extending approximately 3/4 of distance between oral aperture and guiding-ring, openings obscure appearing as minute pores (not illustrated), not slit-like. Stylet guiding-ring single, located 3.3 ± 0.2 (2.9–3.7) times lip region diameter from anterior end. Odontostyle moderately long, straight or slightly arcuate, 3.2 ± 0.2 (2.9–3.5) times as long as distance between anterior end to guiding-ring, odontophore approximately 1/2 part of the odontostyle length, weakly developed. Nerve ring encircling cylindrical part of pharynx, located 208.3 ± 23.2 (189.0–234.0) μm from anterior end. Anterior slender part of pharynx usually coiled in its posterior region. Basal bulb long and cylindrical, 129.9 ± 14.8 (112.0–153.0) μm long or approximately one-fourth of neck length, and 21.0 ± 1.6 (19.0–23.0) μm in diameter. Glandularium 116.3 ± 6.9 (109.0–125.0) μm long. Normal arrangement of pharyngeal glands (Chen et al., 1997; Loof & Chen, 1999): nuclei of DN and SVN glands situated at 14.6% ± 2.8 (12.9–17.8%) and 47.8% ± 0.9 (47.3–48.9%) of the distance from anterior end of pharyngeal bulb, respectively. Dorsal gland nucleus slightly larger than nuclei of two SVN (4.0–5.0 vs 3.0–4.0 μm in diameter). Cardia conoid-rounded, 16.3 ± 1.5 (15.0–18.0) μm long. Reproductive system with both genital branches equally developed, ranging between 391–615 μm long, (G1) 6.9 % ± 1.1 (5.7–8.9), (G2) 6.8% ± 1.0 (5.8–8.8%) of body length, respectively. Vulva in form of a transverse slit, located about mid-body, vagina perpendicular to body axis, extending to approximately 2/3 of corresponding body width, surrounded by well-developed muscles. Uterus filled with sperm cells in most female specimens observed; well-developed sphincter between uterus and oviduct, usually containing numerous sperm cells too. Prerectum variable in length, 560 ± 164 (440–839) μm long, and rectum 37.7 ± 2.3 (35.0–39.0) μm long, anus a small rounded slit. Tail short, dorsally convex and terminus bluntly rounded to almost hemispherical, bearing between two and three pairs of caudal pores.

**Male.** Less frequent than female (1:2 ratio). Morphologically similar to female except for genital system and posterior region considerably curved ventrally. Tail convex conoid, ventrally concave with broad blunt terminus, a deep depression posterior to anus and the thickened outer cuticular layer. Male genital tract diorchic with testis opposed, containing multiple rows of spermatogonia in the germinal zone. Spicules arcuate, robust, 1.5–2.1 times longer than tail length, lateral guiding pieces more or less straight. One pair of adanal supplements preceded by a row of 14–19 ventromedian supplements.

**Juveniles.** All four juvenile stages (first-, second-, third- and fourth-stage) were distinctly separated by morphological characters, such as body length, length of replacement and functional odontostyle (Robbins et al., 1996). Juveniles similar to adults apart from developed reproductive system.
Table 2  Morphometrics of *Longidorus iliturgiensis* sp. nov. from black alder at Andújar (Jaén, Spain). Measurements are in micrometres (μm) and in the form: mean ± standard deviation (range).

| Characters/ratios | Holotype | Paratype |
|------------------|----------|----------|
|                  | Females  | Males    | J1  | J2  | J3  | J4  |
| n                | 18       | 12       | 2   | 5   | 6   | 4   |
| L                | 4.1      | 4.6 ± 0.37 | (4.1–6.1) | 1.7 ± 0.27 | 2.7 ± 0.19 | 3.4 ± 0.29 |
| a (body length/maximum body width) | 156.3 ± 12.9 | 159.0 ± 18.3 | (138.6–179.4) | 81.5 ± 4.7 | 112.3 ± 11.5 | 145.4 ± 7.0 |
| b (body length/pharyngeal length) | 16.2 ± 2.0 | 15.7 ± 1.5 | (13.5–19.9) | 9.1 ± 1.5 | 11.3 ± 0.8 | 12.3 ± 2.4 |
| c (body length/tail length) | 106.8 ± 16.4 | 103.6 ± 10.5 | (87.4–139.6) | 38.1 ± 5.4 | 58.8 ± 4.5 | 74.7 ± 7.6 |
| c’ (tail length/body width at anus) | 2.2 ± 0.3 | 2.0 ± 0.1 | (1.8–2.6) | 3.2 ± 0.1 | 2.7 ± 0.2 | 2.6 ± 0.2 |
| V or T ((distance from anterior end to vulva or male gonad length/body length) × 100) | 48.7 ± 1.7 | 29.2 ± 7.0 | (46.0–52.0) | – | – | – |
| Odontostyle length | 61.7 ± 2.9 | 62.8 ± 2.4 | (57.0–69.0) | 44.5 ± 1.1 | 52.0 ± 2.3 | 53.4 ± 2.1 |
| Replacement odontostyle length | – | – | – | (45.0–46.5) | 49.5 ± 0.9 | 56.8 ± 2.2 | 60.9 ± 2.0 |
| Odontophore length | 38.0 ± 4.8 | 34.2 ± 4.5 | (29.5–47.5) | 26.5 ± 0.28 | 27.3 ± 2.8 | 33.6 ± 3.2 |

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| Character                     | Mean | Standard Deviation | Range          | Mean | Standard Deviation | Range          | Mean | Standard Deviation | Range          |
|-------------------------------|------|--------------------|----------------|------|--------------------|----------------|------|--------------------|----------------|
| Total stylet length           | 99.0 | 99.7 ± 5.6         | (92.0–110.0)   | 97.0 | 5.5                | (87.0–107.5)   | –    | –                  | –              |
| Lip region width              | 8.5  | 9.6 ± 0.5          | (8.5–10.5)     | 9.3  | 0.5                | (7.0, 7.5)     | 7.8  | 0.6                | (7.0–8.5)     |
| Oral aperture guiding-ring    | 23.0 | 23.2 ± 1.0         | (21.5–24.5)    | 23.0 | 1.3                | (15.0, 15.5)   | 17.3 | 1.4                | (16.0–19.0)   |
| Tail length                   | 42.5 | 45.7 ± 5.4         | (36.0–55.0)    | 44.2 | 2.7                | (40.5, 42.5)   | 45.0 | 1.6                | (43.0–46.5)   |
| Spicules                      | –    | –                  | –              | 33.5 | 1.9                | (30.5–37.0)    | –    | –                  | –              |
| Lateral accessory piece       | –    | –                  | –              | 9.9  | 1.2                | (8.0–12.0)     | –    | –                  | –              |
| J (hyaline tail region length)| 10.0 | 9.0 ± 1.0          | (7.0–10.0)     | 9.4  | 1.4                | (4.0, 4.5)     | 6.3  | 0.3                | (6.0–6.5)     |
|                              |      |                    |                |      |                    |                |      |                    |                |

DIVERSITY OF NEEDLE NEMATODES (LONIDORUS) IN SPAIN
Figure 8  Line drawings of Longidorus pacensis sp. nov. (A) Female neck region. (B) and (C) Female lip regions. (D) and (E) Female tails. (F) Male tail. (G) First-stage juvenile tail
**Figure 9** Light micrographs of *Longidorus pacensis* sp. nov. (A)–(G) Anterior regions. (H) Vulval region. (I)–(K) Female tails. (L) Detail of sperm cells. (M) and (N) Male tails. (O)–(R) First-, second-, third-, and fourth-stage juvenile (J1–J4) tails, respectively. Abbreviations: a = anus; af = amphidial fovea; gr = guiding ring; sp = spicules; spl = ventromedian supplements; v = vulva. Scale bars = 20 μm
### Table 3

Morphometrics of *Longidorus pacensis* sp. nov. from fallow at Badajoz (Badajoz, Spain). Measurements are in micrometres (μm) and in the form: mean ± standard deviation (range).

| Characters/ratios                      | Holotype | Paratype |
|----------------------------------------|----------|----------|
|                                        |          | Females  | Males | J1  | J2  | J3  | J4  |
| n                                      | 1        | 13       | 6     | 4   | 3   | 4   | 5   |
| L                                      | 6.9      | 7.3 ± 0.51 | 7.2 ± 0.39 | 2.2 ± 0.86 | 3.2 ± 0.54 | 4.4 ± 0.18 | 6.0 ± 0.45 |
|                                        |          | (6.7–8.3) | (6.6–7.7) | (2.09–2.27) | (2.67–3.74) | (4.20–4.61) | (5.48–6.51) |
| a (body length/maximum body width)     | 104.1    | 102.4 ± 5.7 | 107.7 ± 11.6 | 101.4 ± 9.2 | 102.3 ± 6.1 | 92.6 ± 14.8 | 99.3 ± 1.6 |
|                                        |          | (91.8–112.8) | (85.6–116.2) | (88.7–109.7) | (96.9–108.9) | (74.4–106.5) | (82.5–109.9) |
| b (body length/pharyngeal length)      | 12.1     | 15.2 ± 1.9 | 15.2 ± 1.9 | 9.3 ± 1.4 | 10.7 ± 3.5 | 9.5 ± 1.7 | 12.3 ± 1.3 |
|                                        |          | (12.1–18.1) | (12.7–16.7) | (7.3–10.6) | (8.1–14.6) | (7.8–11.9) | (10.8–13.7) |
| c (body length/tail length)            | 170.2    | 184.1 ± 16.3 | 178.0 ± 11.6 | 53.2 ± 2.5 | 79.5 ± 17.7 | 95.3 ± 14.9 | 141.4 ± 14.0 |
|                                        |          | (155.9–212.9) | (164.8–195.2) | (50.2–55.9) | (63.5–98.5) | (79.3–115.3) | (123.2–154.9) |
| c’ (tail length/body width at anus)    | 0.9      | 0.8 ± 0.1 | 1.0 ± 0.1 | 2.5 ± 0.1 | 1.6 ± 0.2 | 1.2 ± 0.1 | 1.0 ± 0.1 |
|                                        |          | (0.7–0.9) | (0.9–1.1) | (2.4–2.6) | (1.3–1.7) | (1.1–1.3) | (0.9–1.1) |
| V or T ((distance from anterior end to vulva or male gonad length/body length) × 100) | 50 | 49.5 ± 0.7 | 54.7 ± 6.5 | – | – | – | – |
|                                        |          | (48.0–50.0) | (47.6–63.2) | – | – | – | – |
| Odontostyle length                     | 123.0    | 122.4 ± 4.0 | 119.4 ± 4.9 | 72.3 ± 0.5 | 83.7 ± 3.2 | 95.4 ± 2.1 | 107.1 ± 3.6 |
|                                        |          | (115.0–129.0) | (114.0–125.0) | (72.0–73.0) | (80.0–86.0) | (93.0–98.0) | (101.0–110.0) |
| Replacement odontostyle length         | –        | –         | –         | 80.9 ± 3.3 | 92.3 ± 6.1 | 113.0 ± 1.6 | 122.8 ± 2.2 |
|                                        |          |            |            | (77.0–85.0) | (87.0–99.0) | (111.0–115.0) | (121.0–126.0) |
| Odontophage length                     | 64.0     | 64.3 ± 3.4 | 55.4 ± 9.6 | 36.1 ± 1.4 | 42.8 ± 3.6 | 47.4 ± 1.4 | 55.3 ± 1.6 |
|                                        |          | (57.0–68.0) | (48.0–69.0) | (34.0–37.0) | (40.5–47.0) | (46.0–49.0) | (54.0–58.0) |
| Total stylet length                    | 187.0    | 186.7 ± 6.7 | 174.8 ± 14.1 | – | – | – | – |
|                                        |          | (175.0–197.0) | (163.0–193.0) | – | – | – | – |
| Trait                        | Mean | Standard Deviation | Range 1 | Range 2 | Range 3 | Range 4 |
|-----------------------------|------|--------------------|---------|---------|---------|---------|
| Lip region width            | 12.0 | 11.7 ± 0.7         | (11.0–12.5) | (10.5–12.5) | (5.0–6.5) | (7.0–8.0) | (8.5–10.0) | (9.5–11.5) |
| Oral aperture guiding-ring  | 36.5 | 38.2 ± 1.5         | (36.5–40.5) | (36.0–40.5) | (23.0–24.0) | (25.5–28.0) | (28.0–32.0) | (35.5–36.5) |
| Tail length                 | 41.0 | 40.1 ± 4.6         | (32.5–50.0) | (36.5–43.0) | (39.0–43.5) | (38.0–42.0) | (40.0–53.0) | (41.0–44.5) |
| Spicules                    | –    | –                  | –       | –       | –       | –       | –       | –       |
| Lateral accessory piece     | –    | 12.8 ± 1.5         | (10.0–14.0) | (10.0–14.0) | –       | –       | –       | –       |
| J (hyaline tail region length) | 20.5 | 19.3 ± 2.4         | (16.5–24.0) | (14.5–22.0) | (10.0–11.5) | (12.5–17.0) | (12.5–16.5) | (14.5–18.0) |
shorter body length, tail shape and presence of replacement odontostyle (fig. 7b). Tail becoming progressively shorter and stouter in each moult (fig. 9 and table 4). J1s characterised by a bluntly rounded to cylindrical tail with a c’ ratio 2.4–2.6 (table 3). J2–J4 tails dorsally convex conoid and terminus bluntly rounded compared to that of female in shape (fig. 9 and table 4).

Remarks. Morphologically and according to the updated polytomous key by Chen et al. (1997) and the supplement by Loof & Chen (1999), the specific matrix code for this species is A4(5)-B1-C3-D1-E3-F4-G1-H1-I2–J1–K1. Based on sorting of matrix codes A (length of odontostyle), C (distance of guiding-ring from anterior body end), D (lip region shape), F (body length), and H (tail shape) (fig. 9, table 3), L. pacensis sp. nov. is closely related to L. baeticus, and L. fasciatus, from which it can be differentiated by a combination of several characters discussed below. From L. baeticus, it can be mainly differentiated by a higher c’ ratio in females (0.7–0.9 vs. 0.5–0.7), a higher c’ ratio in J1 (2.4–2.6 vs 1.7–2.0), a slightly higher a ratio (av. 103.3 (91.8–112.8) vs av. 94.8 (73.4–106.3)), a slightly longer female tail (av. 40.1 (32.5–50.0) vs av. 37.1 (30.0–43.0 μm)), slightly lower c ratio (av. 184.1 (155.9–212.9) vs av. 207.5 (180.0–286.2)) and shorter spicules (62.0–79.0 vs 80.0–95.0 μm) (Gutiérrez-Gutiérrez et al., 2013).

From a morphological and morphometrical point of view, this new species is almost indistinguishable from L. fasciatus and, therefore, should be considered a cryptic species, since all morphometric characters are within the range of the original description and those from Gutiérrez-Gutiérrez et al. (2013). Nevertheless, it can be clearly separated by specific 28S rRNA, ITS1 rRNA and CoxI sequences. Morphologically L. pacensis sp. nov. is also close to L. andalusicus Gutiérrez-Gutiérrez, Cantalapiedra-Navarrete, Montes-Borrego, Palomares-Rius and Castillo, 2013, but the new species can be clearly differentiated by a longer body (6.7–8.3 vs 3.7–5.1 mm), and longer odontostyle (115.0–129.0 vs 78.0–86.0 μm) (Gutiérrez-Gutiérrez et al., 2013). In addition, L. pacensis sp. nov. is molecularly related to L. macrodorus Archidona-Yuste, Navas-Cortés, Cantalapiedra-Navarrete, Palomares-Rius and Castillo, 2016a, but it can be differentiated by important characters, such as the shorter body and odontostyle length (6.3–8.3 vs. 8.3–10.1 mm, 115.0–129.0 vs. 183.0–210.0 μm, respectively) (Archidona-Yuste et al., 2016a).

Morphology and Morphometry of other Longidorus Species

Remarks. Morphological and morphometric data as well as DNA sequences were provided for L. africanus, L. carpetanensis, L. nevesi, L. cf. olegi, and L. pini. For these species, below brief descriptions and comparisons with previous records are provided.

Longidorus africanus Merny, 1966 (fig. 10, table 4)

Remarks. The Spanish population of this species was detected in the rhizosphere of lemon trees in Álora, Málaga, southern Spain (table 4). This population was characterised by a lip region broadly rounded, separated from the rest of the body contour by a very slight depression; amphidial fovea slightly bilobed posteriorly; female tail dorsally convex-conoid with a broadly rounded terminus; male specimens not found. Morphology and morphometrics of our specimens agree closely with other studied populations from South Africa (Jacobs & Heyns, 1987), Sudan (Zeidan & Coomans, 1991), Portugal (Bravo & Roca, 1995), Egypt (Lamberti et al., 1996), and Tunisia (Guesmi-Mzoughi et al., 2017). This specimen is the first report of this species in Spain and confirms its wide distribution in the Mediterranean Basin. According to the polytomous key by Chen et al. (1997), the supplement by Loof & Chen (1999), and additional codes (Peneva et al., 2013; Archidona et al., 2016), this species has the following code: A32-B1-C2-D2-E2-F2-G23-H23-I12–J1–K6.

Longidorus carpetanensis Arias, Andrés & Navas, 1986 (fig. 11, table 4)
Figure 10  Light micrographs of Longidorus africanus Merny, 1966. (A) female anterior region. (B) female lip region. (C) vulval region. (D)–(E) female tails. Abbreviations: a = anus; gr = guiding ring; v = vulva. Scale bars = 20 μm

Figure 11  Light micrographs of Longidorus carpetanensis Arias et al., 1986 from Puebla de Sanabria, Zamora (A)–(F), and topotypes from Navalmorral, Avila (G)–(L). A–C, G and H, female anterior regions. D, I, female tails. E and F, J and L, male tail with detail of spicules. Abbreviations: a = anus; gr = guiding ring; spl = ventromedian supplements. Scale bars = 20 μm
Table 4  Morphometrics of Longidorus africanus and Longidorus carpetanensis studied from Spain. Measurements are in micrometres (μm) and in the form: mean standard deviation (range)

| Localities                      | L. africanus | L. carpetanensis |
|---------------------------------|--------------|-----------------|
| Locality                        | Females     | Male            | Females     | Male            |
| Álora (Málaga, Spain)           | 7            | 4               | 1           | 5               | 1               |
| Na valmor de la Sierra (Ávila, Spain) Topotypes | 4.7 ± 0.47 | 4.9             | 5.1 ± 0.64 | 5.42            |
| Navalmoral de la Sierra (Ávila, Spain) Topotypes | (4.25–5.36) | (4.19–5.93)     |             |                 |
| Puebla de Sanabria (Zamora, Spain) | 108.7 ± 9.5 | 126.9 ± 12.9   | 137.6       | 140.9 ± 12.2   | 146.5           |
| (97.7–122.0)                    | (109.0–139.3)|                |             |                 |
| b (body length/pharyngeal length) | 10.9 ± 1.4 | 14.0 ± 2.2   | 13.1        | 16.8 ± 2.2     | 17.2            |
| (9.1–13.1)                      | (11.6–16.8)  |                |             |                 |
| c (body length/tail length)     | 100.5 ± 9.6 | 91.5 ± 5.9    | 97.7        | 91.1 ± 11.5    | 94.3            |
| (85.9–111.8)                    | (85.0–96.6)  |                |             |                 |
| c’ (tail length/body width at anus) | 1.5 ± 0.1 | 1.9 ± 0.2    | 1.8         | 2.3 ± 0.1      | 2.4             |
| (1.4–1.6)                      | (1.8–2.1)    |                |             |                 |
| V or T ((distance from anterior end to vulva or male gonad length/body length) × 100) | 46.9 ± 1.2 | 48.3 ± 1.3    | –            | 47.4 ± 2.3      | –               |
| (46.0–49.0)                    | (46.5–49.5)  |                |             |                 |
| Odontostyle length              | 83.5 ± 3.0  | 62.6 ± 1.4    | 64.0        | 63.6 ± 1.8     | 58.5            |
| (79.0–87.0)                    | (61.0–64.0)  |                |             |                 |
| Odontophore length              | 48.3 ± 4.2  | 36.3 ± 3.5    | 33.0        | 42.0 ± 1.8     | 47              |
| (42.0–54.0)                    | (33.0–40.0)  |                |             |                 |
| Total stylet length             | 132.4 ± 5.3 | 98.9 ± 3.5    | 97.0        | 105.5 ± 3.4    | 105.5           |
| (121.0–141.0)                  | (95.0–102.5) |                |             |                 |
| Lip region width                | 10.6 ± 0.7  | 10.0 ± 0.4    | 10.0        | 9.6 ± 0.3      | 9.5             |
| (10.0–11.5)                    | (9.5–10.5)   |                |             |                 |
| Oral aperture-guiding ring      | 28.4 ± 0.9  | 25.6 ± 1.8    | 26.5        | 24.5 ± 1.4     | 27.5            |
| (27.5–29.5)                    | (23.5–27.5)  |                |             |                 |
| Tail length                     | 40.8 ± 3.0  | 49.6 ± 4.9    | 50.0        | 55.5 ± 2.0     | 57.5            |
| (37.5–46.0)                    | (43.5–55.5)  |                |             |                 |
| Spicules                        | –            | –              | 42.5        | –               | 40              |
| Lateral accessory piece         | –            | –              | 11.5        | –               | 11.0            |
| J (hyaline tail region length)  | 10.4 ± 1.1  | 9.9 ± 1.5     | 9.5         | 10.1 ± 1.3     | 11.5            |
| (8.5–11.5)                     | (8.5–12.0)   |                |             |                 |

Remarks. Longidorus carpetanensis was originally described from around roots of common broom (Cytisus scoparius L.), in Navalmoral, Avila Province, Spain. Subsequently, Bravo & Lemos (1997) reported it in the rhizosphere of cereals and peach trees from Constância and Abrantes, province of Ribatejo, Portugal. A Longidorus population resembling this species was detected in the rhizosphere...
of common oak, at Puebla de Sanabria, Zamora Province, Spain, which prompted us to study this population and to characterise molecularly the topotype specimens in order to confirm its identification.

The population of Puebla de Sanabria agrees closely with original description (Arias et al., 1986) in morphology and morphometry (fig. 11 and table 4). This study expands its distribution to another province in north-western Spain. According to the polytomous key by Chen et al. (1997), the supplement by Loof & Chen (1999), and additional codes (Peneva et al., 2013; Archidona et al., 2016), this species has the following code: A12-B12-C2-D4-E3-F2-G2-H56-I2–J1–K?

Longidorus nevesi Macara, 1985 (fig. 12, table 5)

Remarks. The Spanish population of this species is characterised by a long body, ventrally curved in an open C when killed by heat, lip region conoid and continuous with body contour. Amphidial fovea bilobed, slightly asymmetrical, odontostyle long and robust, approximately 2 times longer than odontophore. Female tail short, bluntly conoid with rounded terminus and c’ < 1.0. Males frequent with robust spicules, ventrally arcuate approximately 100 μm long. The morphology and morphometrics of this population closely agree with the original description (Macara, 1985). Apart from the original description, this species has been reported also from forests in Portugal (Macara, 1994; Bravo & Lemos, 1997), and to our knowledge, this is the first report from Spain. This new report confirms the hypothesis that this species is an Iberian endemic (Gutiérrez-Gutiérrez et al., 2016). According to the polytomous key by Chen et al. (1997), the supplement by Loof & Chen, (1999), and additional codes (Peneva et al., 2013; Archidona et al., 2016), this species has the following code: A56-B34-C34-D1-E3-F35-G12-H1-I2–J1–K3.

Longidorus cf. olegi Kankina & Metlitskaya, 1983 (figs. 13 and 14, table 6)

Remarks. This species was described by Kankina & Metlitskaya (1983) from the rhizosphere of raspberry at the experimental farm of the Rososhanski fruit-berry experimental station in the

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**Figure 12** Light micrographs of Longidorus nevesi Macara 1985. (A) female anterior region. (B) female lip region. (C) vulval region. (D) female tail. (E)–(F) male tail with detail of spicules. Abbreviations: a = anus; gr = guiding ring; spl = ventromedian supplements; V = vulva. Scale bars = 20 μm.
**Table 5** Morphometrics of *Longidorus nevesi* and *L. pini* studied from Spain. Measurements are in micrometres (μm) and in the form: mean standard deviation (range)

| Characters/ratios | L. nevesi | L. pini |
|------------------|-----------|---------|
|                  | Santa Olalla (Toledo, Spain) | Nava de Francia (Salamanca, Spain) |
|                  | Female | Male | Female | Male |
| n                | 5 | 3 | 2 | 1 |
| L                | 8.0 ± 10.95 (7.4–9.9) | 8.6 ± 0.64 (8.1–9.3) | (130.0, 148.8) | 120.8 |
| a (body length/maximum body width) | 88.4 ± 6.1 (80.0–97.0) | 92.0 ± 8.0 (87.1–101.3) | (12.4, 12.7) | 15.5 |
| b (body length/pharyngeal length) | 12.6 ± 1.4 (11.3–14.0) | 14.1 ± 0.6 (13.4–14.5) | (90.2, 95.5) | 81.6 |
| c (body length/tail length) | 166.0 ± 28.2 (143.0–201.8) | 162.5 ± 4.2 (158.0–166.4) | (2.1, 2.3) | 2.0 |
| c’ (tail length/body width at anus) | 0.8 ± 0.1 (0.7–0.9) | 0.9 ± 0.0 (0.9–1.0) | (47.0, 48.0) | – |
| V or T ((distance from anterior end to vulva or male gonad length/body length) × 100) | 48.9 ± 1.9 (47.0–51.0) | – | (47.0, 48.0) | – |
| Odontostyle length | 134.1 ± 3.4 (130.0–139.0) | 134.0 ± 3.3 (131.0–137.5) | (79.0, 80.0) | 80.5 |
| Odontophore length | 71.0 ± 7.9 (63.0–81.5) | 79.0 ± 3.6 (75.0–82.0) | (46.0, 48.5) | 45 |
| Total stylet length | 205.1 ± 6.0 (198.5–211.5) | 213.0 ± 6.1 (206.0–217.5) | (125.0, 129.5) | 125.5 |
| Lip region width | 15.7 ± 0.6 (15.0–16.5) | 15.7 ± 1.0 (14.5–16.5) | (9.5, 10.5) | 10 |
| Oral aperture-guiding ring | 45.0 ± 2.1 (42.0–47.5) | 45.3 ± 0.6 (45.0–46.0) | (28.5, 30.0) | 31.5 |
| Tail length | 50.5 ± 2.2 (47.5–52.5) | 53.0 ± 3.3 (49.5–56.0) | (60.0, 66.5) | 54 |
| Spicules | – | 96.8 ± 3.5 (93.5–100.5) | – | 39.5 |
| Lateral accessory piece | – | 23.0 ± 2.2 (21.5–25.5) | – | 8.5 |
| J (hyaline tail region length) | 19.7 ± 2.0 (17.5–21.5) | 16.5 ± 0.5 (16.0–17.0) | (8.5, 10.0) | 10 |
DIVERSITY OF NEEDLE NEMATODES (*LONGIDORUS*) IN SPAIN

**Figure 13** Light micrographs of *Longidorus cf. olegi* Kankina & Metlitskaya, 1983. (A)–(B) female anterior region. (C)–(F) female lip regions. (G) detail of basal bulb. (H) vulval region. (I)–(L) female tails. (M) and (N), male tail with detail of spicules. (O)–(R) First-, second-, third-, and fourth-stage juvenile (J1–J4) tails, respectively. Abbreviations: a = anus; af = amphidial fovea; gr = guiding ring. Scale bars = 20 μm.
Figure 14   Relationship of body length to length of functional and replacement odontostyle (Ost and rOst, respectively) length in all developmental stages from first-stage juveniles (J1) to mature females of *Longidorus cf. olegi* Kankina & Metlitskaya, 1983

Rossoshanski region of Voronezh Province (Russia), based only on two females and three males; there is no other report on it. The Spanish population of *Longidorus* collected from the rhizosphere of Portuguese oak (*Quercus faginea* Lam.), at Arroyo Frío, Jaén province, Spain showed morphological and morphometric characteristics resembling this species, which prompted us to study this population, characterising morphologically females and males, as well as providing descriptions for the first time of first-, second-, third- and fourth-stage juveniles, including a new molecular characterisation by D2–D3, ITS1, and partial 18S sequences (supplementary fig. S1 and table 6). Given the low number of specimens used in the original description of this species, a detailed characterisation of both morphology and morphometrics was provided in the present study. However, the taxonomic assignment is here given as *L. cf. olegi* because of the few specimens used in the original species description. For this reason, we consider this species as *L. cf. olegi* until topotypes of this species can clarify the similarity, or not, with the Spanish population.

The morphology and morphometrics of the Spanish population from Portuguese oak at Arroyo Frío, Jaén Province, agree closely with those of the original description by Kankina & Metlitskaya (1983) (table 6), except for a slightly shorter odontostyle (av. 104.5 (96.0–115.0) μm vs. 112.0–116.0 μm); slightly longer distance from anterior end to guiding ring (34.4 (32.0–37.0) vs. 32.0 μm); slightly higher V ratio (54.0 (51.5–57.5) vs. 52.6 (51.3–54.0); and spicules (81.0 (80.0–82.0) μm vs. 66.0 (62.4–73.2) μm). These differences further expand the intraspecific variation in this species that can be a consequence of the few specimens measured in the original description by Kankina & Metlitskaya (1983). The population examined by us represents the first report of the species in a country outside of Russia. According to the polytomous key by Chen et al. (1997), the supplement by Loof & Chen (1999), and additional codes (Peneva et al., 2013; Archidona et al., 2016), this species has the following code (codes in parentheses are exceptions): A4(3)-B2(3)-C4-D1-E2-F4-G2-H1-I2-J1–K6.

Description. Female. Body cylindrical, tapering towards anterior end, usually assuming an open C-shape when heat relaxed. Cuticle 3.0–4.5 μm at mid-body, and 11.5–15.5 μm at tail tip, and marked by very fine superficial transverse striae mainly in
tail region. Lip region rounded, continuous with body contour. Amphidial fovea pocket-shaped; deeply bilobed and slightly asymmetrical. Odontostyle moderately long and narrow, 1.9 ± 0.4 (1.5–2.6) times as long as odontophore, straight or slightly arcuate; odontophore weakly developed, with rather weak basal swellings. Pharynx consisting of an anterior slender narrow part, extending to a terminal pharyngeal bulb, well demarcated anteriorly and cylindrical, 134.9 ± 8.7 (120.0–148.0) μm long, occupying approximately 25% of total pharyngeal length, and 31.1 ± 4.6 (24.5–31.0) μm wide. Glandularium 117.4 ± 7.3 (106.0–128.0) μm long. Normal arrangement of pharyngeal glands (Chen et al., 1997; Loof and Chen, 1999): DN and SVN pharyngeal gland located at 34.5 ± 4.1 (26.9–39.6), 57.6 ± 2.9 (53.4–62.2) % of distance from anterior end of pharyngeal bulb, respectively. Dorsal gland nuclei slightly larger than nuclei of two SVN (3.5–5.0 vs 3.0–4.5 μm in diam.). Cardia well-developed, hemispherical to conoid, 16.3 ± 6.7 (12.0–24.0) μm long. Reproductive system with both genital branches equally developed, 8.3 ± 1.1 (7.0–10.2), 8.2 ± 1.3 (5.8–9.8) % of body length, respectively. Vulva in form of a transverse slit, located slightly posterior to mid-body, vagina perpendicular to body axis, 28.1 ± 2.7 (24.0–32.0) μm long, or 30–45% of corresponding body width, surrounded by well-developed muscles. Uterus and oviduct of about equal length, usually with sperm cells in the female specimens examined. Prerectum very long, 1,235.2 ± 175.7 (1,055–1,545) μm, and rectum 0.8 ± 0.1 (0.7–1.0) times as long as anal body diameter, anus a small rounded slit. Tail relatively short, convex conoid to bluntly conoid, with rounded terminus, bearing two or three pairs of caudal pores.

**Male.** Very rare, only two male specimens were found (1:7 ratio). Morphologically similar to female except for genital system and posterior region slightly curved ventrally. Tail convex conoid, ventrally slightly concave with broad blunt terminus and thickened outer cuticular layer. Male genital tract diorchic with testis opposed, containing multiple rows of spermatogonia in the germinal zone. Spicules arcuate, robust, 1.6–1.8 times longer than tail length, lateral guiding pieces more or less straight. One pair of adanal supplements preceded by a row of 13–14 ventromedian supplements.

**Juveniles.** All four juvenile stages (J1–J4) distinctly separated by differences in body length, odontostyle and replacement odontostyle length (Robbins et al., 1996) were detected (figs. 13 and 14). Morphologically, juveniles resemble adults except for the smaller size and undeveloped reproductive system. J1s were characterised by a subdigitate tail with a rounded tip and a c' ratio 2.4–2.6 (table 6). J2–J4 tails dorsally convex conoid and terminus bluntly rounded comparable to that of female in shape (fig. 13 and table 6).

**Longidorus pini** Andrés & Arias, 1988 (fig. 15, table 5)

**Remarks.** Longidorus pini was originally described from around roots of Scots pine (Pinus sylvestris L.) and Pyrenean oak (Quercus pyrenaica Willd.) at San Martin del Pimpollar, Avila Province, and *Juncus* sp. in Capileira, Granada Province, Spain (Andrés & Arias, 1988). A *Longidorus* population resembling this species was detected in the rhizosphere of Pyrenean oak, at Nava de Francia, Salamanca Province, Spain, which prompted us to study this population in order to characterise it molecularly and compare it with paratype specimens, thus supporting identification. The population from Nava de Francia was characterised by a medium body size, lip region offset and slightly expanded, amphidial fovea symmetrically bilobed, and female tail conical dorsally convex, ventrally concave. One male specimen was found, representing the first report for this species and showing a similar morphology than that of females, except for the genital system and posterior region being slightly curved ventrally. Spicules arcuate, robust, 0.7 times shorter than tail length, lateral guiding pieces more or less straight. One pair of...
Table 6  Morphometrics of *Longidorus cf. olegi* Kankina and Metlitskaya, 1983 from Portuguese oak at Arroyo Frío (Jaén, Spain). Measurements are in micrometres (μm) and in the form: mean standard deviation (range)

| Characters/ratios | Arroyo Frío (Jaén, Spain) | Paratypes (Kankina and Metlitskaya, 1983) |
|-------------------|---------------------------|------------------------------------------|
|                   | Females       | Males       | J1       | J2       | J3       | J4       | Females       | Males       |
| n                  | 14            | 2           | 6        | 5        | 6        | 6        | 2            | 3           |
| L                  | 7.5 ± 0.66    | (6.1–8.7)   | 1.44 ± 0.40 | 2.6 ± 0.86 | 3.5 ± 0.30 | 5.4 ± 0.50 | 8.41 (7.85, 8.98) | 7.38 (6.80–8.17) |
| a (body length/maximum body width) | 97.0 ± 13.2 | (69.8–123.4) | 49.6 ± 0.9 | 65.5 ± 5.4 | 69.7 ± 6.3 | 87.6 ± 11.8 | 113.5 (107.9, 119.2) | 111.3 (107.0–118.6) |
| b (body length/pharyngeal length) | 14.8 ± 2.0 | (10.2–17.4) | 5.4 ± 0.5 | 8.0 ± 1.2 | 9.9 ± 1.4 | 10.8 ± 1.3 | 16.8 (15.1, 18.6) | 16.7 (13.6–21.6) |
| c (body length/tail length) | 173.6 ± 21.5 | (132.9–205.1) | 30.9 ± 6.4 | 62.3 ± 3.2 | 79.8 ± 14.5 | 121.6 ± 16.9 | 170.4 (163.6, 177.3) | 144.4 (142.9–145.4) |
| c´ (tail length/body width at anus) | 0.8 ± 0.1 | (0.7–1.0) | 2.2 ± 0.2 | 1.5 ± 0.1 | 1.1 ± 0.1 | 0.9 ± 0.1 | 0.87 (0.86, 0.88) | 0.9 (0.9–1.0) |
| V or T (distance from anterior end to vulva or male gonad length/body length x 100) | 54.0 ± 1.5 | (27.2, 45.7) | –        | –        | –        | –        | 52.6 (51.3, 54.0) | 38.4 (32.8–42.7) |
| Odontostyle length | 104.5 ± 5.6 | (96.0–115.0) | 58.2 ± 1.2 | 65.4 ± 2.8 | 79.0 ± 4.2 | 90.3 ± 2.0 | 112,116 | – |
| Replacement odontostyle length | – | – | 66.9 ± 4.0 | 77.3 ± 2.4 | 90.5 ± 7.4 | 102.7 ± 3.5 | – | – |
| Odontophore length | 55.7 ± 9.9 | (44.0–73.0) | 34.2 ± 9.0 | 46.9 ± 3.0 | 47.0 ± 5.7 | 58.4 ± 7.0 | 50.4, 52.2 | – |
| Metric                          | Mean ± SD | Range          | InTab | Range          | 95% CI | InTab |
|--------------------------------|-----------|----------------|-------|----------------|--------|-------|
| Total stylet length            | 156.2 ± 16.7 | (149.5, 158.0) |       | (149.5, 158.0) | 165.9  | (163.2, 168.6) | 164.5  |
| Lip region width               | 15.1 ± 1.2  | (14.5, 16.5)   | 8.4 ± 0.4 | (8.0–9.0) | 9.4 ± 0.5 | (8.5–10.0) | 12.3 ± 1.5 | (10.5–14.0) | 12.7 ± 0.7 | (11.5–13.5) | –               | –               |
| Oral aperture-guiding ring     | 31.4 ± 1.4  | (34.0, 35.0)   | 20.8 ± 0.9 | (19.5–22.0) | 24.2 ± 0.8 | (23.5–25.0) | 26.8 ± 1.4 | (25.5–28.5) | 30.8 ± 2.7 | (27.5–34.0) | 32.5            | –               |
| Tail length                    | 43.4 ± 4.3  | (44.0, 50.0)   | 48.0 ± 6.9 | (36.0–52.5) | 42.0 ± 2.1 | (39.0–44.0) | 45.1 ± 5.8 | (37.5–52.0) | 44.4 ± 4.0 | (40.0–49.0) | 48.0, 57.0       | –               |
| Spicules                       | –          | (80.0, 82.0)   | –     | –              | –      | –      | –      | –      | 66.2          | (62.4–73.2)    |
| Lateral accessory piece        | –          | (20.5, 22.0)   | –     | –              | –      | –      | –      | –      | –             | –               |
| J (hyaline tail region length) | 12.9 ± 1.1  | (12.0, 13.5)   | 12.8 ± 0.9 | (12.0–13.5) | 7.7 ± 1.3 | (6.5–9.5) | 9.5 ± 2.5 | (6.5–11.5) | 10.6 ± 1.2 | (9.0–12.0)  | –               | –               |
adanal supplements preceded by a row of 7 ventromedian supplements (fig. 15). The population of Nava de Francia agree with the original description and examined paratypes (Andrés & Arias, 1988) in morphology and morphometry (fig. 15 and table 5), except for a slightly longer body (5.7–6.0 mm vs. 4.0–5.7 mm), longer odontostyle (79.0–80.0 μm vs. 65–74 μm), and shorter female tail length (60.0–66.5 μm vs. 57.0–75.0 μm). These differences should be regarded as geographical intraspecific variation. In addition, this study expands its distribution to another province in north-western Spain. According to the polytomous key by Chen et al. (1997), the supplement by Loof & Chen (1999), and additional codes (Peneva et al., 2013; Archidona et al., 2016), this species has the following code (codes in parentheses are exceptions): A2-B1-C2-D3-E2-F3-G3-H6-I2-J?–K?.

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